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Some theoretical and practical possibilities of plant genetic manipulation using protoplasts

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Protoplasts capable of division and plant regeneration are now available for a large number of vegetable, oil and forage crops. However, routine hybrid production is not possible due to methodological limitations in selection and culture of hybrid cells. Recent improvement in techniques are the use of a double mutant as a universal hybridizer and the use of fluorescence activated cell sorter to recover hybrid cells. Interest is also centered on limited gene transfer by protoplast fusion. We propose a model of generating triploid plants by somatic cell fusion to transfer limited genomic information from an alien plant to a crop plant. Somatic hybridization has some novel features but, in practice and conception, it is an extension of the methods of sexual hybridization. By contrast, genetic transformation is a radically different approach to plant genetic manipulation. The success of this approach will depend upon how readily genotype can be related to phenotype in a tangible way so as to ascertain what biochemical and developmental activity is controlled or modulated by a DNA sequence.

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Intervention seems to be the obsession in modern biology. However, intervention has two facets: one is to understand systems and phenomena and the other is experimental manipulations towards a desired applied goal. COCKING (1960) isolated protoplasts from root tips of tomato for obtaining uniform populations of cells for physiological and biochemical studies. Once such protoplasts were available, it was found that they could be induced to divide and regenerate plants, that they could be fused with protoplasts from other species and could also be induced to take up foreign DNA. Numerous somatic hybrids have been obtained by means of several types of selection techniques, and genetic transformation of higher plants has been obtained by infecting plant protoplasts with *Agrobacterium* carrying prokaryotic drug resistance genes. Recently, a spate of books and review articles has appeared on what has been accomplished and what could be accomplished with somatic cells of higher plants (DAVEY and KUMAR 1983; GLEBA and EVANS 1983; HARMS 1983—to cite a few).

In this review we will confine ourselves to the following three areas.

1) Protoplasts capable of division and plant regeneration are now available for a large number of species. Although there are still difficulties with regenerating plants from protoplasts of cereals and grain legumes, a totipotent response can be elicited from the somatic cells of a large number of vegetable, oil and forage crops. Numerous cases have been identified where somatic cell hybridization could be of use in practical breeding (COCKING 1983; SHEPARD et al. 1983). However, in such crops routine hybrid production is not possible due to methodological limitations on selection and culture of hybrid cells. We will discuss the use of a double mutant, which can be used as a universal hybridizer. We will point out the limitations of the double mutant approach and discuss the possibilities of manual and automated isolation of a large number of hybrid cells of wild-type parents to synthesize somatic hybrids.

2) An interesting possibility with somatic cell hybridization is to have limited gene transfer from an alien species into a crop species. This can be achieved by X- or γ-irradiation of protoplasts of the alien parent before fusions with crop plant protoplasts. We will discuss our research in this area along with the published work. We further propose a model of generating triploid plants by somatic cell

fusion to transfer a limited number of genes from an alien plant to a crop plant.

3) Somatic cell hybridization is an extension of sexual hybridization. It overcomes sexual incompatibility barriers to hybridization and creates a novel cytoplasmic mix, as organelles of both parents come together in a common cytoplasmic milieu after protoplast fusion. However, there is very little control of what happens to the hybrid genomes. Thus each somatic combination will have to be empirically tested for crossability, vigour, etc. This approach is akin to the approach breeders have used in conventional breeding. The genetic transformation approach to plant genetic manipulation is conceptually different. It relies on establishing genotype-phenotype relationships and isolation of the desired gene sequences. We argue that establishing genotype-phenotype relationships would be critical for genetic transformation for applied purposes.

Double mutant as a universal hybridizer

Previously, mutants have been hybridized with other mutants to recover hybrids by genetic complementation (MELCHERS and LABIB 1974) or hybridized with species which have constraints of limited growth (POWER et al. 1980) or lack of regeneration (MALIGA et al. 1977) in certain nutrient media. Such selection methods require the development of new mutants, or markers based on differential growth responses, for each combination of species. We proposed an alternative approach which involves combining an auxotrophic mutation and a dominant resistant mutation in a species to produce a double mutant (PENTAL et al. 1982a). Such a double mutant can be hybridized with any wild type species eliminating the necessity for any other selectable marker (Fig. 1). We produced such a double mutant by sexually crossing streptomycin resistant *Nicotiana tabacum* SR1 (♀) with nitrate reductase deficient *N. tabacum nia*-130 (♂) (HAMILL et al. 1983). In *N. tabacum* $NR^{-}SR^{+}$, nitrate reductase deficiency is due to a recessive mutation at two loci in the nuclear genome, and streptomycin resistance is due to a mutation in the chloroplast genome.

We tested our proposal on the use of a double mutant by fusing leaf mesophyll protoplasts of *N. tabacum* $NR^{-}SR^{+}$ with cell suspension protoplasts of wild type *N. rustica* and selected a large number of green cell colonies in the selection medium from which plants were regenerated. Since the selection was for nitrate reductase proficiency and streptomycin resistance, we expected plants regenerated from the selected colonies either to have the nuclear genome of both parents and chloroplasts of *N. tabacum*, or to have the nuclear genome of *N. rustica* and chloroplasts of *N. tabacum* (Fig. 1). In *N. tabacum* and *N. rustica* fusion we did not find a combination of the latter type.

We have further used *N. tabacum* $NR^{-}SR^{+}$ for fusions with *Petunia hybrida*. *N. tabacum* and *P. hybrida* show strong pre- and post-zygotic incompatibility (ZENKTELER and MELCHERS 1978). As *N. tabacum* and *P. hybrida* protoplasts were used in the initial experiments on demonstrating totipotency of somatic cells, there has been considerable historic interest in bringing the two genomes together by somatic cell fusion. We find that hybrid callus can be selected by using the double mutant selection procedure, but the hybrid genome is unstable and over a period of time there is loss of *N. tabacum* and *P. hybrida* genes. This has been inferred indirectly by studying isozyme profiles of hybrid callus over a period of one year.

A double mutant of *N. tabacum* could be constructed by combining nitrate reductase deficiency with a nuclear-encoded resistance marker, e.g. valine resistance. Valine resistant mutants of *N. tabacum* have been isolated by BOURGIN (1978). In fusion studies with such a mutant, hybrid colonies could be selected for valine resistance and nitrate reductase proficiency to produce hybrids with the cytoplasm of either parent.

Can the double mutant approach be used for species other than *N. tabacum*?

As a negative selection marker, nitrate reductase deficiency appears to be useful, as several such auxotrophs have been reported in higher plants, e.g. *Hyoscyamus muticus* (STRAUSS et al. 1981), *N. plumbaginifolia* (MARTON et al. 1982; NEGRUTIU et al. 1983), *N. tabacum* (MÜLLER 1983). Other auxotrophs have been isolated (SIDOROV et al. 1981). Most of the auxotrophic cell lines are available as non-regenerating cell lines or abnormal shoots. Only in a few cases has an analysis of the mutants been done by studying their transmission genetics (c.f. NEGRUTIU et al. 1984). A similar situation exists for mutants carrying positive selection markers, e.g. amino acid analogue resistance in *N. sylvestris* (WHITE and VASIL 1979) and *Daucus carota* (WIDHOLM 1978), lincomycin resistance in *N. plum-*

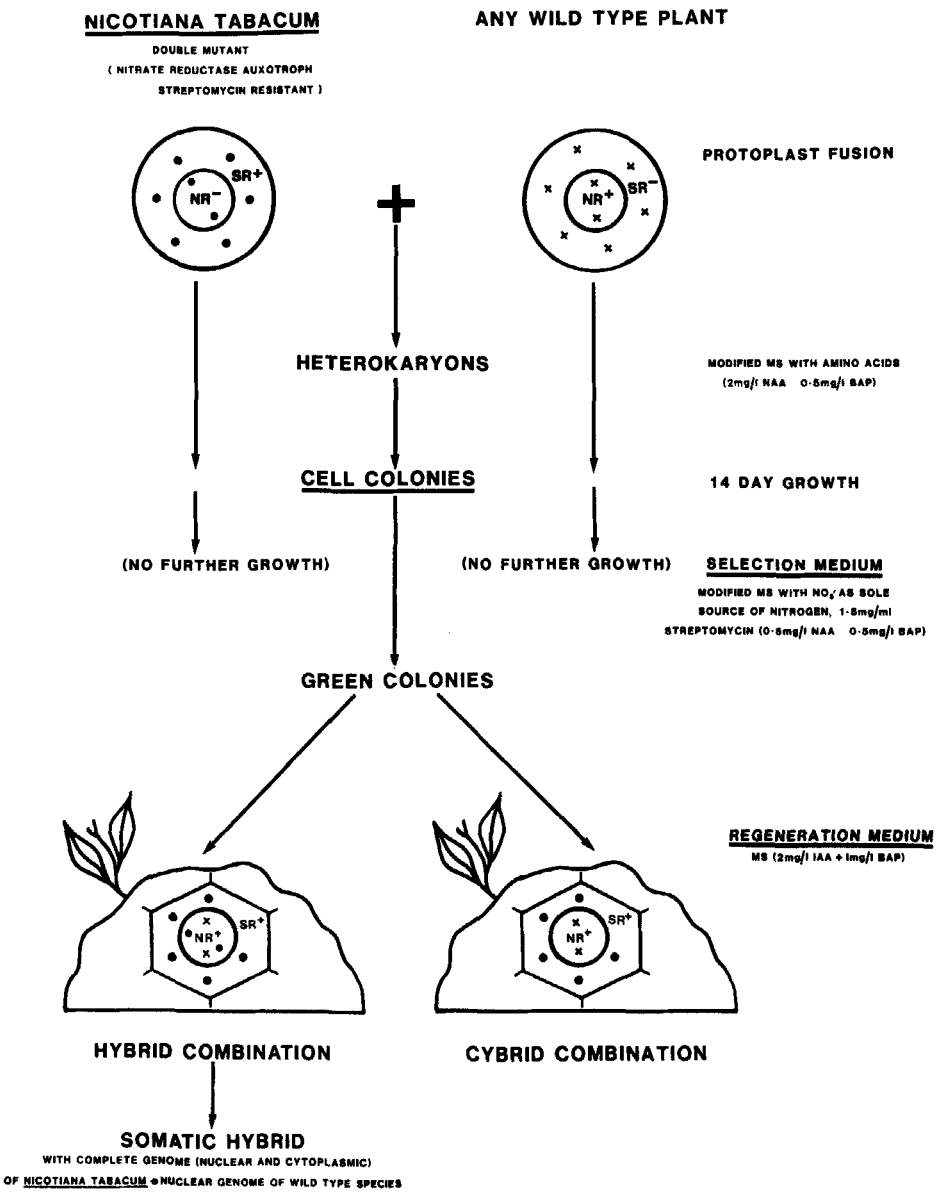

Fig. 1. Use of a double mutant of *Nicotiana tabacum* (tobacco) for somatic hybridization.

baginifolia (Cseplo and Maliga 1982) and valine resistance in *N. tabacum* (Bourgin 1978). Cell lines, on prolonged culturing, develop abnormal chromosome structure and numbers (Murata and Orton 1983) and lose their regeneration capability. The shortcomings of using such cell lines for somatic hybridization are obvious.

The problems of using a double mutant cell line for somatic hybridization are well illustrated by the work of Lo Schiaro et al. (1983). They produced a *D. carota* cell line which is resistant to 8-azaguanine and α-amanitin. Cells resistant to 8-azaguanine are impaired in hypoxanthine phosphoribosyl transferase and cannot grow on a medium containing hypoxanthin, aminopterin and thymidine. This served as an auxotrophic marker, while resistance

to α-amanitin served as a resistance marker. The double mutant line was fused with wild type *D. carota* cells and cell colonies were recovered from the selection medium. No plants were regenerated and no biochemical analysis of hybrid colonies was reported. While the report confirms the utility of a double mutant approach, the cell line used was incapable of plant regeneration, possibly had chromosomal abnormalities and hence its utility in studying genomic compatibility of *D. carota* with other species and in plant breeding is minimal. For effective use in a somatic hybridisation programme, a double mutant should have normal chromosome number and full fertility.

Provided one has the time and patience and the crop plant of interest is going to be central to a substantial somatic cell hybridization programme, it would be worthwhile to develop a double mutant approach. Crop plants like *Solanum tuberosum* and *Brassica campestris* are suited for this approach as in both species monoploids are available for inducing recessive mutants and protoplasts of these species can be induced to divide and regenerate plants.

Somatic hybrids by manual isolation of heterokaryons

A selection method of general applicability is to isolate heterokaryons after fusion and to culture them. We described a simple method for manually isolating heterokaryons after fusion (PATNAIK et al. 1982). Viable heterokaryons can be isolated using a micromanipulator with a capillary pipette. Heterokaryons of fusions between *N. tabacum* leaf mesophyll and *N. rustica* cell suspension protoplasts were successfully isolated and were placed in a nurse culture of albino *P. hybrida* (HAMILL et al. 1984a). Six somatic hybrids were produced from 30 heterokaryons cultured. The isolation of heterokaryons of *N. tabacum* and *N. rustica* is comparable in method to the isolation and culture of *Atropa belladonna* and *N. chinensis* heterokaryons (GLEBA et al. 1982). In this case also one type of parental protoplast was isolated from leaf mesophyll cells and the other from suspension cells. The heterokaryons were isolated by a hand-held pipette.

HEIN et al. (1983) have produced hybrid calli by isolation of heterokaryons of *N. paniculata* and nitrate reductase deficient *N. tabacum cnx*, and *N. sylvestris* and *N. tabacum cnx* using a fixed micropipette. In this case fusion frequency was around 3 %, which is comparable to fusion frequencies observed between *N. rustica* and *N. tabacum*. Isolated heterokaryons were cultured in a nurse culture of *N. tabacum cnx* parent. On a selection medium containing nitrate as the sole source of nitrogen, *N. tabacum cnx* colonies failed to grow, but the hybrid calli proliferated. As an auxotroph line of *N. tabacum cnx* was used both as a fusion parent and as a nurse culture, and a selection medium was employed, the isolation of heterokaryons did not need to be stringent. The half selection method of HEIN et al. (1983) is limited in its need of an auxotrophic line and as discussed in the previous section such cell lines usually have abnormal chromosome numbers.

In our opinion, there are two major shortcomings in producing somatic hybrids by the micropipette isolation method: 1) only a limited number of heterokaryons can be isolated; and 2) one needs to develop media for the growth of very small populations (1–30 cells) of cells, or else a nurse culture has to be employed. In the latter case, lack of growth of hybrid cells due to genomic incompatibility cannot be monitored.

Two promising developments could alleviate the above problems.

1) One way is to use the method of electrofusion (ZIMMERMAN and SCHEURICH 1981) of plant protoplasts. If significantly higher frequencies (10–20 %) can be reproducibly generated in comparison to less than 4 % obtained (in our experience) by chemical fusion, it may not be necessary to isolate heterokaryons. A population of protoplasts could be grown after fusion and plants could be regenerated from a significant sample to identify hybrids. However, the crucial question is—do electrofused protoplasts give rise to viable heterokaryons which can produce somatic hybrids? It has been shown that yeast spheroplasts can be fused to produce viable hybrids (HALFMANN et al. 1983). However, the fusion frequencies are comparable to those produced by chemical fusion. Thus it is vital to assess electrofusion for the production of viable hybrid cells. It is also important to devise equipment for fusion of large populations of protoplasts.

2) Even with low (1–2 %) fusion frequency, it may be possible to isolate heterokaryons from the parent protoplasts by means of a fluorescence activated cell sorter. Protoplasts of both leaf mesophyll and cell suspension origin can be sorted, and have been shown to remain viable after passage through a cell sorting machine (HARKINS and GALBRAITH 1984). However, as yet, the actual sorting of large numbers of heterokaryons by such instruments has not been demonstrated. This could be possible with minor modifications of the available commercial cell sorting machines.

Limited gene transfer by use of irradiated protoplasts

The synthesis of allotetraploids by protoplast fusion will be most useful for crops which are used for their vegetative parts, e.g. forage legumes and fiber crops. While a number of natural and artificially produced allotetraploids have vegetative vigour, they have lower sexual fecundity compared to their parents (DE WET 1980). In a number of situations the challenge is to transfer limited amount of genomic information from an alien plant to a crop plant. This would maintain all the important agronomic traits of the crop parent and add to it some of the desirable traits of the alien plant, without sacrificing sexual fecundity.

In the allotetraploids, during meiosis, there could be crossing over between homeologous chromosomes leading to introgression of alien genes into the crop plant. If such alloploids could be backcrossed to the crop parent, it may be possible to recover the crop plant with introgressed alien genes in a backcrossing programme. Alternatively, it would be possible to make partial somatic hybrids combining the complete nuclear genome of a parent with a few genes, chromosomes, or organelles of an alien plant. The development of methods capable of producing partial hybrids is an important area of study.

One method of limited gene transfer is to fuse γ or X-irradiated protoplasts of an alien plant with protoplasts of a crop plant. The transfer of the cytoplasmic genome in *N. tabacum*, by fusion of X-irradiated protoplasts of the donor (alien) variety with non-irradiated protoplasts of the recipient (crop) variety, has been demonstrated by ZELCER et al. (1978). We have used this method to transfer streptomycin resistance (the trait is encoded by the chloroplast genome) from *N. tabacum* SR1 into the nuclear background of nitrate reductase deficient *N. tabacum nia*-130 (HAMILL et al. 1984b). The sexually produced double mutant (discussed under double mutants) was early flowering with a small flowering height and weak growth habit. All these traits are attributed to nuclear genes from *N. tabacum* SR1, a dwarf early flowering variety. The *nia*-130 mutant was isolated from *N. tabacum* cv. Gatersleben, a large, sturdy and late flowering variety. Ideally, it is desirable to have a *nia*-130 nuclear genome combined with the chloroplast genome of SR1. Such an alloplasmic line has been produced by fusing γ- irradiated protoplasts of SR1 with *nia*-130 protoplasts, thus alleviating the necessity of doing backcrosses with the sexually produced double mutant. Production of alloplasmic lines or cybrids where organelle genomes have recombined is of importance to introduce traits like herbicide resistance and cytoplasmic male sterility from alien species.

Fusions have been made between diploid protoplasts of albino *D. carota* and irradiated diploid protoplasts of *Aegopodium hortense* to reduce genomic input from *A. hortense* into the hybrid cells (DUDITS et al. 1980). Green colonies selected in culture had the isozyme profile of *D. carota*, but also had some *A. hortense* specific isozymes. One of the regenerated plants had an extra chromosome above the normal diploid number of *D. carota*. It is not clear whether the regenerated plant had any *A. hortense* specific isozymes. In a similar study GUPTA et al. (1983) fused nitrate reductase deficient *N. tabacum cnx* protoplasts with irradiated protoplasts of *Physalis minima* and *D. innoxia*. Cell colonies were selected for their ability to grow on a medium with nitrate as the sole source of nitrogen. As the *cnx* line used had lost its regeneration capacity, no plants were recovered. Selected lines were proficient in nitrate reductase, but did not show any biochemical markers specific of irradiated parents. These results are intriguing because only the nitrate reductase gene and nothing else is transferred. It is possible that the transfer of other genes could not be demonstrated due to lack of methodology to characterise any *D. innoxia* or *P. minima* specific proteins in the nitrate reductase proficient colonies.

In our experiments, γ-irradiated protoplasts of *N. glutinosa* have been fused with leaf mesophyll protoplasts of nitrate reductase deficient *N. tabacum nia*-130 (COOPER-BLAND et al. unpublished work; PENTAL et al. 1982b). Cell colonies selected for their ability to grow in a medium containing nitrate as the sole source of nitrogen have regenerated plants. These plants show isozymes characteristic of *N. tabacum*, but also carry some of the isozyme bands characteristic of *N. glutinosa*. A profile of the genomic information from *N. glutinosa* in the selected plants is being worked out by 2-dimensional protein electrophoresis.

Limited gene transfer by synthesis of triploids

Transfer of limited nuclear genome information from an alien species into a crop species is achieved in conventional breeding by backcrossing. Although widely applied this approach has some shortcomings.

1) Strong linkages—often the gene(s) controlling the desirable trait are strongly linked with genes which have undesirable effects. Even after long

periods of backcrossing this linkage group is inherited as a unit.
2) Long periods of backcrossing—a number of generations of backcrossing are needed to restore the genome of the crop parent.
3) Infertility of F_1—in crosses between distantly related species, the F_1 hybrids may produce non-functional gametes due to lack of adequate pairing. Even if some introgression can occur, no viable plants can be produced to assess the possibility of limited gene transfer that could occur.
4) Postzygotic incompatibility—in numerous cases traits of agronomic interest are present in the alien species which is sexually incompatible with the crop species.

We suggest that an effetive way of limited gene transfer from an alien species to a crop species is by synthesizing triploids between the two by somatic cell fusion (Fig. 2). This procedure will solve problem No. 4 and alleviate problems 2 and 3. Problem 1 can be effectively solved only by gene cloning and genetic transformation methods. Diploid protoplasts of crop species can be fused with haploid protoplasts of an alien species. Synthesis of triploids should be possible with all the crop species whose protoplasts can be induced to divide and regenerate plants. Haploid protoplasts can be isolated from pollen mother cells at the tetrad stage (Bhojwani and Cocking 1972). Although there is no report of haploid protoplasts dividing, it should not be limiting for fusions because hybrids can be produced by fusing a dividing and a non-dividing protoplast system (Medgyesy et al. 1980). Haploid protoplasts could also be isolated from haploid plants produced by anther culture (Negrutiu et al. 1984). In the triploid cells, introgression could occur by somatic crossing over, by transpositions, but most probably would occur at meiosis. As the triploid plants undergo meiosis, there will be trivalent formation if sufficient homology exists between the homeologous chromosomes. This will result in introgression of alien genes. The limiting factor in such a model will be the extent of homology between the crop plant chromosomes and the alien plant chromosomes. Most of the unpaired alien genome will be lost. The progeny plants will need backcrosses for restoring the crop plant genome as would be needed for sexual hybrids or allopolyploid somatic hybrids.

In theory, gene transfer by synthesis of triploids would be superior to bringing about limited nuclear gene transfer by use of irradiated protoplasts. Irradiations cause chromosome breakages and gene mutations. Chromosome breakages could lead to preferential elimination of chromosomes of the alien parent, before they have the possibility of pairing with homeologous chromosomes at meiosis in the generative phase. There may also be deleterious mutations at the desired loci due to irradiation. The triploid model, presented here, can be tested by the double mutant selection approach. As the methodology for wild type fusions improves, triploids can be generated by the improved methods of higher fusion or heterokaryon enrichment using a cell sorter.

What is the relation of somatic cell hybridization to sexual hybridization?

Some of the unique features of somatic hybridization are quite clear. To summarise these:
1) somatic hybridization can bring together genomes of sexually incompatible species;
2) by fusion, one can synthesise allopolyploids in which heterozygosity and resulting hybrid vigour have become fixed in one step; and
3) in sexual reproduction organelles are usually inherited uniparentally, whereas somatic cell fusion creates a novel situation by making a cytoplasmic mix of the two parents.

This mix can generate novel cytoplasmic-nuclear relationships (Gleba and Evans 1983). In sexual hybridization genomes of chloroplast and mitochondria are inherited together, but by protoplast fusion hybrids can be produced which combine the chloroplast genome of one parent with the mitochondrial genome of the other (Aviv et al. 1984). The mitochondrial genomes of the two parents have been shown to recombine to give true cybrids (Belliard et al. 1979). There is no conclusive report of recombinant chloroplast genome in somatic hybrids.

Somatic cell hybridization also has its own peculiar problems. Cells in culture tend to drift in their chromosome number and also accumulate mutations. In analysing a somatic hybrid with abnormal chromosome number, it would be hard to discern whether abnormalities are due to genomic incompatibility or due to drift in chromosome numbers and structure. To be useful for practical breeding, a somatic hybrid must have some fertility. We have produced seventeen hybrid plants of *N. tabacum* and *N. rustica* and only three of these have some fertility (Hamill et al. unpublished work). The more spectacular hybrids synthesized between sexually incompatible species *Petunia parodii* and *P. parviflora* (Power et al. 1980), *Lycopersicon esculentum*

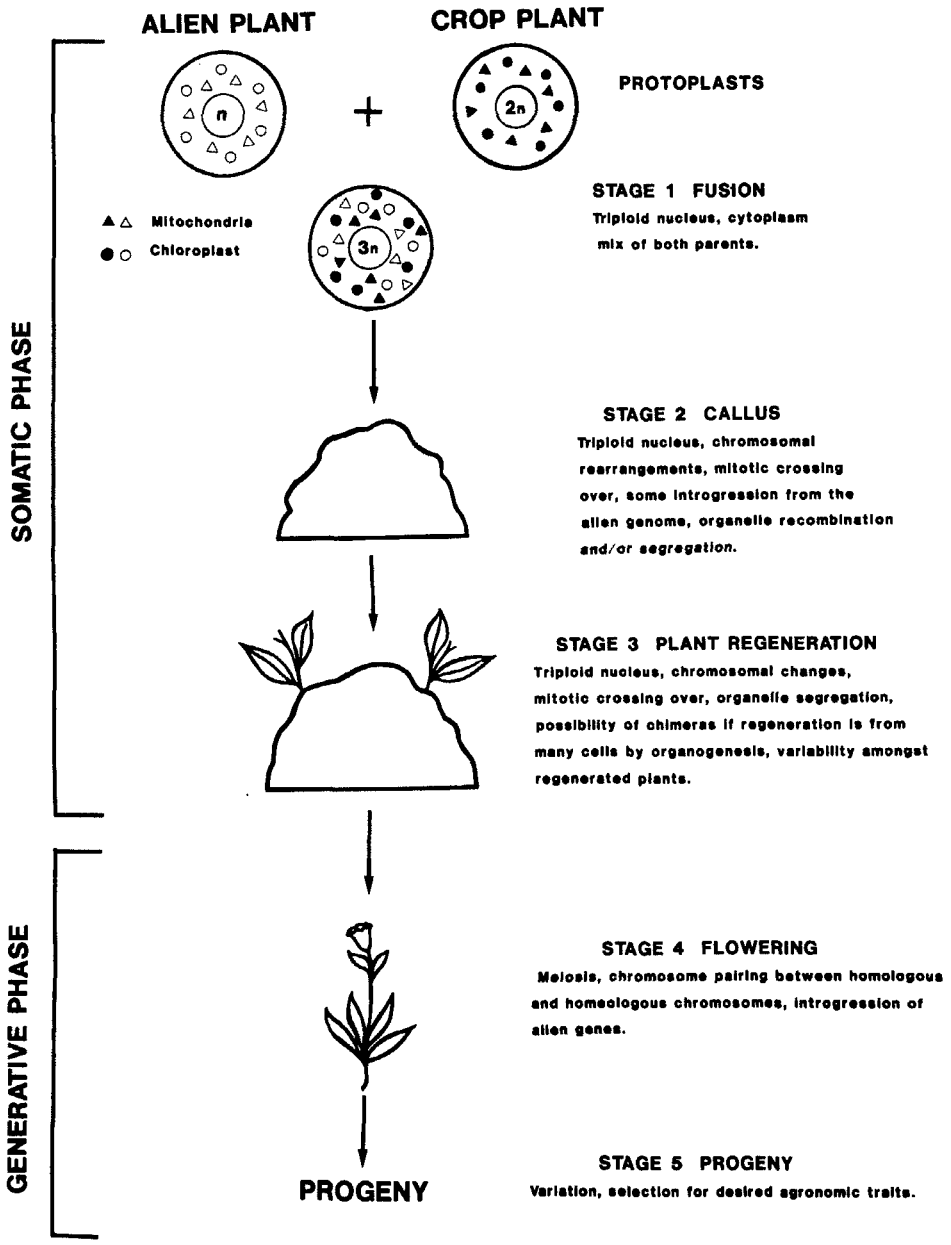

Fig. 2. A model explaining production of triploids by somatic cell fusion for limited gene transfer from an alien plant to a crop plant.

and *Solanum tuberosum* (MELCHERS et al. 1978) have limited practical utility as they are infertile on selfing and crossing. Whether one synthesizes an allopolyploid, a partial hybrid by using irradiated protoplasts or a triploid, fertility of the hybrid is vital for carrying on the lineage. The generative phase would bring about introgression of alien genes into the crop plant genome. The generative phase is cleansing and stabilizing while the somatic phase is prone to undesirable variability.

In a recent review SYBENGA (1983) has broadly distinguished two approaches to plant genetic manipulation:
1) in vitro/molecular/somatic; and
2) plant level/generative.
This categorization is arbitrary. Firstly, sexual fertility is vital to bring about stable introgression of alien genes in the progeny. Secondly, somatic hybridization, in practice and in concept, is an extension of the methods of sexual hybridization. Once methodological improvements are made in culture and selection of hybrid cells, somatic cell hybridization will fall in the domain of general breeding programmes.

Somatic hybridization and genetic transformation are conceptually different approaches

Somatic hybridization and genetic transformation are two radically different approaches to manipulation of plant genomes. In somatic and sexual hybridization one is aware that phenotype is related to genotype, but the reliance is on selecting for a stable phenotype with desirable traits. Of course, predictions are made on the outcome of breeding programmes, but these are based on 'gene-in-there' approach. In contrast, successful genetic manipulation by recombinant DNA technology is dependent upon relating genotype to phenotype in a tangible way so as to ascertain what biochemical and developmental activity is controlled or modulated by a DNA sequence. The success of this approach will depend upon how readily phenotype can be related to genotype, and how readily the desirable gene sequence can be isolated from the plant genome and cloned. Genetic transformation thus is a 'gene-in-hand' approach. The genetic methods used in microorganisms for relating genes with aspects of development have been discussed by BOTSTEIN and MAURER (1982). Such genetic analysis is dependent on the ability to isolate a series of mutants for a particular biochemical or developmental pathway. However, the conventional mutagenesis approach for isolating auxotrophs in higher plants has serious limitations. The problems in relating phenotype to genotype by mutant isolation in higher plants have been discussed (MALMBERG et al. 1980), but very few solutions have been proposed. NEGRUTIU et al. (1984) have reviewed the subject recently and argued that certain refinements like using protoplasts for mutagenesis instead of cells will help isolation of mutants. However, there is no suggestion on how to solve the problem of selecting for mutant phenotypes and also how to regenerate plants from mutant lines as they tend to drift in chromosome numbers in culture. For example, in only two cases have nitrate reductase auxotrophic plants been isolated from single cells; in the rest of the cases only lines or aberrant plants incapable of sexual reproduction have been recovered (NEGRUTIU et al. 1984).

It is possible that, in future, a transposon mutation system will be developed in higher plants. Using an expression vector system which has a strong random integration capability in the plant genome, mutants could be isolated. Such a mutant-inducing system will also help in isolation of the gene sequences into which the transposon has integrated. In the past few years there has been remarkable success in developing vectors for genetic transformation of higher plants. The vectors use a prokaryotic drug resistance gene controlled by the nopaline synthase promotor sequence from *Agrobacterium tumefaciens* (BEVAN et al. 1983; FRALEY et al. 1983; HERRERA ESTRELLA et al. 1983). Chimeric genes have been introduced into plant cells using the infectivity of *Agrobacterium*. Such expression vectors have been used to transform *N. plumbaginifolia* protoplasts to kanamycin resistance and plants carrying drug resistance genes have been regenerated (HORSCH et al. 1983). The expression vectors could be modified further by including plant transposable elements to develop mutation inducing transformation systems. However, the problems of the large genome size of plants, of selection for the mutant phenotype and of regenerating mutant plants would still remain.

A systematic approach to dissection of plant genotype-phenotype relationship may take a long time to materialize or may be just impossible due to some of the properties of the higher plant genome. An interesting discussion along this line is that of FREELING (1984). Meanwhile, cloning of DNA sequences which transcribe high levels of mRNA or encode for large amounts of a protein will continue to be carried out. These systems and some of the viralplant and *Agrobacterium*-plant systems will provide sequences which could be used for efficient transcription of structural genes to be introduced into plant cells. Much of the applied possibilities in recombinant DNA come from introducing prokaryotic genes into higher plants by developed expression vectors. Mutants could be isolated in suitable prokaryotes towards stress-causing agents in higher plants, e.g. toxins, herbicides, salinity, etc. Interesting work in this direction has been the isolation of *Salmonella typhimurium* mutants resistant to the herbicide glyphosate (COMAI et al. 1983). The genotype-phenotype relationship can be established

much more readily in prokaryotes and desirable sequences could be cloned. Such sequences could be mobilized into higher plants, at least dicotyledons, which can be infected with *Agrobacterium* carrying transforming genes.

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