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Progress in cellular engineering of plants: biochemical and genetic assessment of selectable markers from cultured cells

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Summary

Recent availability of stable and well characterized selectable markers and ability to combine alien genomes parasexually have contributed to the development of molecular biology in higher plants, including gene expression and genetic manipulation.

Several types of biochemical mutants (resistant to inhibitory concentrations of aminoacid(s) or aminoacid analogs as well as deficient for enzyme activity) have recently been isolated and characterized biochemically and genetically. Among them, mutants with alterations in the nitrogen and aminoacid metabolism, or in the activity of alcohol dehydrogenases are being used in the development of more efficient techniques of gene transfer.

The manipulation of whole genomes by sexual or somatic cell fusion offers new potential in this field, but refinement of transfer techniques is desirable. The new set of selectable markers obtained through advanced cellular technology, as well as our ability to regenerate plants from manipulated cell lines are expected to play a major role in cellular engineering.

Introduction

Genetic engineering of higher organisms has made rapid progress in the last few years. Construction of suitable vectors carrying a desirable gene makes possible efficient uptake, integration, and expression of defined foreign genetic information in receptor cells. Further progress implies a parallel development in cellular engineering – a technology based on somatic cell genetics and cell culture techniques – which is concerned with problems such as isolation and characterisation of selectable markers, establishment of optimal conditions for expression of altered or foreign genes in cell culture (reconstruction experiments on mutant selection, isolation of revertants, metabolic complementation through fusion), as well as alien gene introgression by means of protoplast fusion, gametic gene transfer, or chromosome transplantation. This field is essential and complementary to the work devoted to obtain adequate vectors and isolate plant genes. In this respect, plant biology suffers from a poor or uneven understanding of molecular mechanisms underlying various cellular and developmental processes. Too often the powerful formal genetics that has been developed for a few plant species has overlooked the molecular bases of gene expression. Consequently, gene cloning and construction of genetic maps, depending on availability of molecular and selectable markers have seriously been hampered.

Our intention is to summarize the little that has been done in the domain of cellular engineering, and to assess the lot that remains to be accomplished.

General merits of cellular technology

The advent of *in vitro* cell culture and somatic cell genetics provided scientists with a new and

powerful technology and raised enthusiasm and expectation for the possibility of extrapolating methodology and basic knowledge from microbial and mammalian cell genetics to plant experimental systems. The possibility of obtaining plants from single cells under controlled culture parameters has changed our way of doing and thinking plant genetics and crop breeding, while new concepts are born.

Present limitations in cellular technology

It is still an elite technology for a restricted number of plant species, most of them having no economic use (Tables 1 and 2). Species for which extensive genetic knowledge is available (maize, tomato, Arabidopsis) are still recalcitrant to *in vitro* technology. Even in those species where genetically uniform cell populations can be cultured as true single, haploid or diploid, cells that can subsequently be regenerated into fertile plants, the *in vitro* passage per se produces genetic alterations (known as somaclonal variation (24)). The extent and the intrinsic causes of the process are hardly understood, its implications in cellular engineering being negative and positive at the same time. On the one hand, as cellular technology represents the substrate of most genetic manipulation devices, a stable genotype is desirable, mainly in those cases where genetic analysis remains either the unique or the least disputable proof in the interpretation of the results. On the other hand, the *in vitro* phase can have valuable consequences in alien gene introgression by facilitating the by-pass of incompatibility barriers, or by extending the exchange of genetic material.

Cellular engineering: objectives and perspectives

Biochemical markers are usually characterized by a simple genetic lesion and readily identifiable molecular basis. They allow the sorting-out of transformed cells at an early stage after gene transfer by means of appropriate selection procedures. The setting up of accurate selection procedures makes possible the extention of such methodoloy to new objectives such as the transfer of a few desirable genetic attributes by organelle or chromosome transfer, by protoplast fusion following inactivation of defined genetic components, together with its sexual counterpart, the so-called 'egg transformation'. Actually, a few convenient selectable markers are available. They allow testing of various proposed technologies of gene transfer and evaluation of the magnitude of the problems to be encountered. These aspects are described and discussed in this paper.

Isolation and characterization of biochemical mutants with potential use as selectable markers in genetic manipulation experiments

Almost 20 years of research in this field contributed to produce some of the originally expected results. Establishment of reliable cellular systems evolved in parallel with the search for accurate selection strategies and this in a context of insufficient knowledge of the biochemical bases of the studied processes, as well as incomplete understanding of the relationship between cellular and whole plant phenotypes. This resulted, as written by Meredith (29), 'in a glut of published reports of questionable value'. What was to a certain extent inevitable, should no longer be acceptable. Periodic reviews have given a comprehensive coverage of available data (26, 27, 49, 28, 7, 8, 29, 1, 22, 34). The main purpose of this paper consists in assessing the potential value of existing biochemical mutants in the development of genetic manipulation systems in higher plants.

Cellular experimental systems can efficiently be submitted to a wide range of selection agents or selection procedures. Selectable marker phenotypes have been obtained in the following categories:

1. *Resistant types*, isolated by selecting for resistance against aminoacid(s) or aminoacid analogs, antibiotics, pesticides, toxins, or culture filtrates of plant pathogens, analogs of nucleic acid bases or of precursors, inhibitors of protein synthesis, phytohormones, heavy metals, salts. Several of them are potentially of economic value.

2. *Deficient types*, characterized by nutritional deficiency (i.e. nitrate reductase-less and various auxotrophs for aminoacids, vitamins, or nucleic acid bases), lack of enzyme activity (alcohol dehydrogenase null mutants), or conditional lethality (temperature sensitivity).

3. *Ti transformed cells*, obtained by transforming whole plants or protoplasts with *Agrobacterium tumefaciens* or Ti plasmids, followed by screening

for regenerants which still express opine genes. Once stabilized by meiosis, such markers should be considered in the same way as group 1 or 2 above.

Different underlying mechanisms can be responsible for such marker phenotypes: three major classes of variants have been identified: (1) cell-variants (i.e. pre-existing tissue specific variation due to differential regulation patterns of gene expression); (2) epigenetic variants (i.e. reflecting changes in gene expression); (3) mutants (by definition the only ones to exhibit heritable changes). Our presentation concerns this last category only (for epigenetic traits and cell-variants see (38, 7, 34)). From the many altered phenotypes reported, little or inconsistent information on the biochemical nature of the variation is available. Only a small minority was examined for expression of the selected trait in regenerated plants, either because plant regeneration from many cellular systems and plant species is not feasible, or because the ability to regenerate plants could be altered or even prevented by the selection agent employed or by the mutation re-

Table 1. Selectable markers from mutant plants with potential use in genetic cellular engineering: phenotypic, biochemical, and genetic features.

Marker phenotype	Altered function	Inht.	Expression		Species
			Regn.	Prog.	
I. RESISTANCE MUTANTS					
	a. Aminoacid metabolism				
Lysine + threonine	Thr overprod.; altered aspartate kinase (°)	mgD or mgSD	E	E	Maize, barley, tobacco
Valine	No uptake or not established	dgR or mgD	E/nE	E/nE	Tobacco, *N. sylvestris*
5-methyltryptophan	No uptake or altered anthranilate synthetase	? or mgD	nE/E	nE/E	Carrot, tobacco, *Datura innoxia*
	b. Nucleic acid bases metabolism				
BUdR, FUdR, methotrexate, hydroxyurea	Not established	mgD or mgSD	nE	nE	Tobacco
	c. Pathogen toxins or toxic culture filtrates				
Helminthosporium m., Phytophthora inf., Alternaria sp, *Pseudomonas* sp, *Phoma* sp	Not established	?/mt	E	E or ?	Maize, potato, tobacco, rape
	d. Antibiotics				
Streptomycin, lincomycin	Not established	mgR or mt	E	E	Tobacco, *N. sylvestris*
	e. Herbicides				
Picloram, carboxine, bentazon, phenmediphan	Not established	mgD or mgSD or mgR	E/nE	E/nE	Tobacco
	f. Photorespiration pathway				
Isonicotinic ac. hydrazide, gly hydroxamate, 1% CO_2 atmosph.	Defects in various enzymes associated with the pathway where assayed	D/? or mgR	E/nE	E/nE	Tobacco, *A. thaliana*
II. DEFICIENT (RESISTANT) MUTANTS					
Chlorate	Defective nitrate reductase or xanthine dehydrogenase	dgR or mgR	E	E	Tobacco, *N. plumbaginifolia*, barley
Allyl alcohol	Defective alcohol dehydrogenases	mgR	–	E	Maize
III. DEFICIENT MUTANTS					
Aminoacid requir.	*his⁻, ile⁻, trp⁻*, defective biosynthetic enzymes(°)	R	E	?	*Hyoscyamus muticus, N. plumbaginifolia*
Nicotineamide requir.	Not established	R	E	?	*H. muticus*
Temp. sensitive	Not established	R	E	?	*H. muticus*

Abbreviations: Inht. = inheritance; Regn. = regenerants; Prog. = progeny; (°) = where assayed; mg = monogenic; dg = digenic; D = dominant; SD = semidominant; R = recessive; mt = maternal; ? = not reported; E = expressed; nE = not expressed; BUdR and FUdR = 5-bromo and 5-fluorodeoxyuridine.

Synthetic description of the listed mutants and references in (7, 22, 34, 28, 2, 15, 3, 45, 47).

vealed by particular selection agents (34). Furthermore, even less cases are known where the mode of inheritance of the selected trait was analysed. However, from the available data it became obvious that expression at plant level of a variant trait and transmissibility through a sexual cycle are not necessarily linked. This is particularly true (Table 1) in the case of nucleic acid base analog-resistant lines.

Tables 1 and 2 summarize data on those available biochemical markers that have been examined for stability and expression at cellular and plant level, and eventually analyzed biochemically and genetically. They belong to 10 plant species. In terms of genetic manipulation objectives the list should be further restricted to those species that provide efficient and reproducible protoplast culture systems, i.e. several *Solanaceae* and carrot. Choice among them of most appropriate biochemical mutants for genetic manipulation experiments depends mainly on our ability to identify the biochemical lesion, to regenerate plants from the mutant lines, and on the availability of cloned genes. Last but not least, one should be able to control various environmental and cellular parameters so that adequate expression and recovery of a transformation event can occur.

Keeping in mind the above prerequisites we will describe below several biochemical mutants isolated in our laboratory (Table 2), as well as some attempts to use such selectable markers in genetic manipulation experiments.

Selectable markers from protoplast cultures, embryogenic cell suspensions, and embryos: biochemical and genetic properties

The following plant species are considered: *Nicotiana sylvestris, N. plumbaginifolia, Daucus carota, Arabidopsis thaliana* and *Hordeum sativum*. Plant material, mutagen treatment, and selection procedures were chosen in such a way that obtention of mutant plants be technically ensured. Protoplast cultures of *Nicotiana* sp. regenerate readily (34, 20), while embryogenic cell suspensions were used in the case of carrot, and M2 seeds or dissected embryos for *A. thaliana* and barley, respectively. Selection at embryo level presents additional guarantees in ob-

Table 2. Phenotypic, biochemical, and genetic characterization of biochemical mutants isolated in this laboratory from protoplast cultures, embryogenic cell suspensions, and cultured embryos. The number of isolated mutants is given in brackets.

Marker phenotype	Altered function	Inht.	Expression		Species
			Regn.	Prog.	
I. RESISTANT MUTANTS					
Lysine + threonine	Thr overproduction; altered	mgD	E	E	*N. sylvestris* (2)
	aspartate kinase	?	E	?	Carrot (1)
	where assayed,	mgSD	–	E	Barley (3)
	no uptake, or thr or lysine	dgR	–	E	*A. thaliana* (80)
	overproduction	or [?]			
S-aminoethylcysteine	Lys overproduction,				
	altered DHPS,	mgD	E	E	*N. sylvestris* (2)
	no uptake or not established	mgR D/[?]	–	E	*A. thaliana* (48)
II. DEFICIENT (RESISTANT) MUTANTS					
Chlorate	Defective nitrate reductase or				
	xanthine dehydrogenase	mgR	E	E	*N. plumbaginifolia* (30)
Allyl alcohol	Defective alcohol dehydrogenase	mgR	–	E	*A. thaliana* (11)
III. DEFICIENT MUTANTS					
Aminoacid requir.	*his*$^-$, *ile*$^-$, *trp*$^-$, *met*$^-$, *ile*$^-$ + *val*$^-$, *leu*$^-$;				
	defective enzymes where assayed	R	E	?	*N. plumbaginifolia* (13)
IV. Ti TRANSFORMED PLANTS					
Opine (+), tumor growth	Opine biosynthesis and opine				
	synthases	D	E	E	*N. sylvestris* (7)

Abbreviations: same as in Table 1. [?] = isolated as homozygotes; crosses are required to establish the mode of inheritance; DHPS = dihydrodipicolinate synthase.

taining mutant plants by implicitly being associated with counterselection against developmentally altered phenotypes. With the exception of chlorate-resistant mutants all the others were isolated after mutagenesis with UV, ethylmethanesulphonate (EMS), N methyl-N'nitro-N-nitrosoguanidine (MNNG), or sodium azide. In positive selection experiments inhibitory concentration of lysine plus threonine, lysine, S-aminoethylcysteine (a lysine analog), chlorate, allyl alcohol were applied to select for resistance. Aminoacid requiring lines were isolated through negative selection based on the use of 5-bromodeoxyuridine (BUdR).

Resistance against S-aminoethylcysteine (AEC)

Selection in protoplast cultures of diploid *N. sylvestris* resulted in the isolation of two lysine overproducer lines exhibiting an altered dihydrodipicolinate synthase (DHPS), the key enzyme in the biosynthesis of lysine (32). Lysine overproduction (10–20 fold), resistance to AEC (25–50 fold), and the feedback insensitive form of DHPS were also expressed in plants regenerated from the variant cultures. Genetic analysis indicated that the mutation conferring the above marker trait was inherited as a single dominant nuclear gene. The frequency of mutation is rather low (2×10^{-7}). Aneuploidy and secondary alteration (male sterility, albinism, loss of apical dominance) were observed in the regenerants and represented temporary handicaps in the full assessment of eventual developmental effects of the mutation.

Additional AEC-resistant mutants were isolated from protoplast of haploid *N. plumbaginifolia* and from M2 seeds of *A. thaliana* (cf. Table 2).

Resistance to lysine + threonine

A similar selection procedure as in the case of AEC was applied to protoplast cultures of diploid *N. sylvestris* and resulted in the isolation of several cell lines showing various degree of resistance to inhibitory concentrations of lysine + threonine. Again, the frequency of mutation was low, i.e. 10^{-7}. Two highly resistant lines were obtained as regenerated plants. In both cases the trait was inherited as a single dominant nuclear gene, one of the mutants overproducing threonine (6–8 fold). Contrary to similar mutants reported in other plant species (Tables 1 and 2), the mutant exhibited no altered feedback sensitivity of the lysine-sensitive aspartate kinase (AK) isozyme. Two other key-enzymes in the pathway, homoserine dehydrogenase and DHPS, were unaffected in both specific activity and allosteric inhibition pattern. Homozygous resistant plants are being analysed for threonine accumulation and assayed for AK isoenzymes.

More lysine + threonine resistant mutants were collected from three other species, namely carrot, barley, and *A. thaliana*. In carrot the selection pressure was applied to a suspension of embryoids derived from a MNNG-treated cell culture. The obtained mutant (6) was accumulating threonine (8 fold) and isoleucine (3 fold). The AK activity partially lost its sensitivity to lysine. In barley, three lysine + threonine-resistant mutants accumulating threonine were obtained from 60 000 embryos dissected from M2 seeds (5). In the RLT8 mutant, isolated as homozygous, the resistance and overproduction were correlated with a decrease in sensitivity to lysine of AK activity, and were inherited as a single semidominant gene. The laboratory plant *A. thaliana*, easily amenable to selection by direct seeding M2 seeds on petri dishes containing the selection agents, yielded large numbers of lysine + threonine-resistant mutants (80), among which two major types were identified: threonine or lysine overproducers (5).

Chlorate-resistant, nitrate reductase-less mutants

Protoplast-derived colonies of haploid *N. plumbaginifolia* were selected for chlorate-resistance. From 80 confirmed resistant lines 50 were regenerated into plants, and 30 characterized biochemically and, most of them, genetically as well (33). Ninety percent were found to have no detectable nitrate reductase (NR) activity, the others being leaky phenotypes. Both *nia* (apoenzyme defective) and *cnx* (cofactor defective) type of mutants were identified, with complementation groups in the cofactor but not in the apoenzyme function. Genetic evidence demonstrated that chlorate-resistance and NR deficiency were inherited as a single recessive gene in both *nia* and *cnx* mutants. The spontaneous and induced frequency of mutation were 10^{-6} and 10^{-5}, respectively. Their reversion mutation rates were rather low, from 10^{-7} to 10^{-6} and were shown to vary significantly among various *nia* mutants.

That NR deficiency is an excellent selectable marker was further confirmed through experiments of the DNA-mediated gene transfer type. Protoplasts from *nia* mutants with low reversion frequency were incubated in the presence of polycations (for example, PEG 6000, 12.5% w/v) with high molecular weight DNA extracted from donor wild type cells (10–40 μg/ml; 10^6 protoplast/ml; 30–90 min). The average size of the DNA was estimated after agarose gel electrophoresis at approx. 40 kb. Corrected phenotypes, i.e. colonies growing on selection medium, were recovered at frequencies ten times superior to the corresponding spontaneous reversion rates. They were of the same order of magnitude as those reported with animal cells, i.e. 10^{-6}–10^{-5} (40, 44). All 23 selected lines were further confirmed to stably express the recovered NR function. Most of the lines were regenerated into plants and made the object of biochemical and genetic examinations. Greenhouse plants from several lines were tested for *in vivo* NR activity: they exhibited lower specific activities than control plants, but similar to those obtained in revertant mutants (i.e. 35 to 88%).

Aminoacid requiring lines

Enrichment selection and recovery of surviving clones on complete mixtures of aminoacids enabled us to isolate 13 auxotrophs from UV exposed and nonmutagenized populations of haploid *N. plumbaginifolia* protoplasts (31). This negative selection which was only working under defined experimental conditions (i.e. starvation time, length of incubation with BUdR, BUdR concentration, concentration of supplied aminoacids, etc.), appeared to be relatively efficient in terms of yield and spectrum of auxotrophs: *his-*, *met-*, *ile-*, *leu-*, *val* + *ile-*, and *trp-*. Plants were regenerated for each type, the *leu-* excepted. All the lines were stable with respect to their requirement for more than one year in culture, and so were the regenerants. Precursor feeding, enzyme analysis, and/or fusion with biochemically characterized auxotrophs were used to identify the biochemical lesion. So far the obtained data indicate that both *ile-* are threonine deaminase defective, the *leu-* lines being 2-OH-3-carboxyisocaproate-isomerase or -dehydrogenase defective; the *his-* lines are histidinol phosphate transaminase defective; all *val+ile-* are valine transaminase defective, while *met-* line is β-cysthathionase defective.

Protoplasts isolated from auxotrophic plants were used to select for revertants. The best studied thus far are the *his-* auxotrophs: both spontaneous and induced revertants were obtained, at reversion frequencies of 2×10^{-7} and 4.8×10^{-6} respectively. Fusions between NR- (*nia* type) and *his-* or *trp-*, *his-* + *ile-*, *met-*+*ile-* demonstrated metabolic complementation and support the recessive nature of the mutations. Regenerated auxotrophs, as well as regenerants from revertants and complementing fusion products are being used to analyse genetically the various auxotrophic mutants.

Alcohol dehydrogenase (ADH) null mutants

The isolation of ADH null mutants is based on the ability of ADH to oxidize allyl alcohol to a toxic aldehyde, acroleine. *Arabidopsis* M2 seeds obtained from M1 plants mutagenized by EMS were incubated for 2 h with 35–50 mM allyl alcohol and sown on perlite moistened with a mineral medium. Green seedlings were selected and their resistance confirmed in M3 progenies. Callus induced from M3 seeds was analysed for ADH activity: 11 null types were obtained and their genetic analysis indicated that the absence of ADH activity was due to a null allele of the previously identified ADH-1 locus (10). The frequency of mutation was rather high: 3.7×10^{-5}. Among the defective mutants, the type R002, in which no gene product could be identified, was further used to define selection conditions for transformants recovery both at seed and cell suspension level. This is based on the ability of ADH-containing individuals to survive anaerobic treatment or to detoxify acetaldehyde. Thus, mutant seeds treated anaerobically in a nitrogen atmosphere (40–48 h) were no longer able to germinate, while wild type seeds were but slightly affected by the same treatment. Cell suspensions from the null ADH activity mutant R002 did not grow in the presence of 0.05% acetaldehyde, while wild type cells maintained a 50% increase in fresh weight as compared to controls. In a reconstruction experiment where wild type cells and R002 cells were mixed in a 1:10 ratio and cultured in the presence of 0.05% acetaldehyde, all surviving colonies tested were positive for ADH activity.

The same selection principle was applied to protoplast-derived cultures from haploid *N. plumbaginifolia* plants. ADH isoenzyme pattern of the cultures indicated the presence of two distinct

genes. Two selection procedures were devised: (1) incubation of late log phase cultures with allyl alcohol (0.08–0.26 μM) for 12 to 24 h, followed by wash out of the compound and dilution of the cultures; (2) exposure of visible microcolonies (4–5 weeks old) growing on top of agar plates to allyl alcoholic vapours (same concentration as above). More than 275 escapes were screened for ADH activity by spot-testing and all appeared to be positive. Since the selection conditions were rather severe, and 2.5×10^7 protoplast-derived colonies from UV-exposed cultures had already undergone allyl alcohol selection, it can be concluded that isolation of double mutants may require the screening of even larger populations of cells.

Opine synthesising N. sylvestris *plants*

Tumor induction was obtained on diploid and haploid plants with wild type plasmids (pTiB6S3, pTiT37), as well as mutant ones (pGV2100, pGV-2206). From such tumor lines it was possible to obtain regenerants characterized by the presence of nopaline or octopine according to the original *Agrobacterium* strain. Some of these plants were self-pollinated or crossed with the wild type. Genetic analysis of the progenies showed that the opine markers were inherited in a Mendelian fashion. A plant obtained after infection with pGV2206 exhibited a 3:1 segregation ration for octopine positive versus octopine negative, and a 1:1 ratio in the cross with the wild type. In the case of a pTiT37 tumor line, the nopaline synthase gene appeared to be stably inserted in at least two different sites of the plant genome on the basis of the observed segregation ratios. From crosses between octopine and nopaline plants it was concluded that the traits were inherited independently.

Preliminary data on DNA hybridization indicated that complete T-DNA was present in the regenerated plants obtained from pTiT37 lines. Such analysis is now conducted at the level of F2 progenies.

Appropriate selectable markers for genetic manipulation of higher plants

Among the listed selectable gene markers with defined molecular lesions, best candidates in our opinion are the following.

1. *Resistance dominant markers*, with emphasis on several aminoacid analog-resistant mutants. None of the genes coding for the characterized altered products (aspartate kinase, anthranilate synthetase, dihydrolipicolinate synthetase) have been cloned thus far. Simulation and reconstruction experiments of mutant selection using resistance markers have shown that the counterselection conditions need to be highly standardized in order to obtain full expression of the mutation and reproducibility of the procedure (34).

2. *Resistance recessive markers (deficient mutants)*, such as ADH° and NR– mutants. In the case of ADH system, the gene has been cloned in maize recently (39), and provides an ideal opportunity in vector design with a dominant selection for ADH+ transformants among cells deficient for ADH activity. Unfortunately, the only ADH° available mutants are in maize and *A. thaliana*, both recalcitrant species to protoplast technoloy. An opposite situation exists for the NR system: while deficient mutants are available in appropriate receptor species, the gene(s) has not yet been cloned in plants.

3. *Recessive deficient markers,* with auxotrophs for several aminoacids as best candidates. Again, no cloned wild type genes are available in plants, but DNA-mediated gene transfer and construction of chimeric genes containing yeast or bacterial genes which depend upon efficient promotors, such as opine synthase promotor (*nos*), can certainly be envisaged for aminoacid auxotroph and NR-less mutants. These markers revert at very low rates and allow a convenient identification of transformed cells by simply omitting the required aminoacid(s) from the culture medium.

4. *Opine synthesizing genes.* Both octopine and nopaline synthase genes have been sequenced and promotor and terminator sequences identified (see ref. this conference). They should be considered as very useful markers as they are not 'home-made' genes. Relatively laborious to screen, they provide very accurate probes for DNA hybridization assays. In the case of octopine+ plants, the tissues were reported (9) to be more tolerant to lysine and arginine analogs, which should speed up the screening in its preliminary steps at least.

Plant genes which have been reported until now (such as phaseoline, prolamines, leghemoglobin, subunits of ribulose-biphosphatecarboxylase, and with the exception of ADH) are either difficult to screen or are expected not to be expressed under

cell culture conditions. Identification of transformants of this sort implies the use of vectors containing an additionally inserted selectable (resistant) gene allowing the cotransfer of the nonselectable gene.

In conclusion, several convenient selectable markers have become available, and their use in cellular engineering (protoplast fusion with irradiated partners, 'egg transformation') has already started. Progress having been made in isolation and sorting-out of plant chromosomes (46, 23), uptake or microinjection of individual or fragmented chromosomes will soon be attempted. In terms of vector design, availability of new transformation markers – cloned genes present on the vector which enables convenient identification of transformed cells – will strongly impulse the development of mechanical gene introduction by microinjection or polycation-mediated uptake of DNA by protoplasts.

Alien gene introgression (AGI) – new tools for an old objective

Making possible the transfer of a limited defined amount of genetic information into a desired genetic background is the legitimate objective of wide-crossing breeding. To illustrate that AGI is a realistic challenge to cellular engineering, reference is made to the elegant and by now historical experiment of Sears (41), in which leaf rust resistance from *Aegilops umbellulata* was transferred through insertion of a chromosome fragment containing the resistance trait in a wheat receptor chromosome by combining wide crossing, irradiation, and selection methods. Two novel experimental systems compete for AGI: 'egg transformation' and fusion of protoplasts with an irradiated partner (designated rather arbitrarily by us as 'γ-fusion', for the sake of abbreviation). In both systems the donor genome is delivered into the receptor cell subsequent to chemical or radiation inactivation, which is followed by unidirectional elimination of the donor genome with eventual retention of certain nuclear encoded traits.

Egg transformation (gametic gene transfer), makes use of irradiated pollen in crosses within and between species, and has been claimed to offer rapid means of transferring specific, desirable genes (35–37). The resulting plants resemble the female parent in all but a few characters or markers traits, similar to those carried by the pollen partner. Classical markers, such as aleurone colour, flower colour, kernel shape, leaf tip colour, as well as plant height, self-incompatibility genes, and isoenzymes, have been used in such experiments. The available evidence suggests that distinction should be made between experiments performed at sublethal or lethal radiation dosis (37). In the first case, the products – resulting from conventional fertilization – were partial hybrids. On the other hand, lethal irradiation would result in 'pseudo-fertilisation'; parthenogenetic development or double fertilization are required to recover seed progeny exhibiting but few traits from the irradiated pollen. This last system is certainly the most attractive in terms of AGI, but limited experimental data are available at present (37, 35). Several underlying mechanisms, merely hypothetical, have been suggested (35, 43, 48, 50): (1) repair of damaged segments of paternal chromosomes by segments from maternal chromosomes (legitimate somatic recombination, gene conversion), (2) large scale inactivation of paternal genome, (3) mutational damage, and (4) gametophytic selection.

γ-fusion, with particular emphasis on remote hybridization, provides further opportunities of transgressing existing barriers of sexual incompatibility. Protoplast fusion can bring diverse genomes together with possibility of AGI provided that somatic gene exchange between the two genomes occurs simultaneously with chromosome elimination of one of the partners (18), both features being eventually facilitated by irradiation and/or *in vitro* culture phase.

Thus, correction of an albino phenotype and of a molybdenum-cofactor deficiency (*cnx*) in the nitrate reductase function were reported in carrot following fusion with x-irradiated protoplasts of parsley (12) and in tobacco after fusion with x-irradiated protoplasts of *Physalis* and *Datura* (17). Other specific gene products of the donor partners (i.e. several isoenzymes) were also shown to be expressed in the receptor cells. It is interesting to note that similar results were obtained without inactivation of one fusion partner by irradiation in another series of intergeneric fusions between carrot (albino) + *Aegopodium podagraria* (wild type) (11) and *Hyoscyamus muticus* (nicotinamide requiring) + tobacco (NR deficient) (21). The authors concluded in favour of stable integration of a small sample of the donor DNA into the host genome. Whether

irradiation is superfluous in remote hybridization by protoplast fusion remains an open question (also see below and Table 3). The results of Hauptmann *et al.* (19) are illustrative in this respect. Thus, somatic cell hybrids between carrot and tobacco cell lines were selected by amino acid analog resistance complementation. They possessed both carrot and tobacco chromosomes, but double resistance was gradually lost although a continuous selection pressure was maintained throughout the culture period. It can be argued that specific selection pressure(s) per se, applied on a preexisting genomic unbalance where plant regeneration is no longer feasible, and in the absence of unilateral loss of genetic information, is not sufficient to ensure maintenance of a desired trait and to favour gene introgression.

The claim and stake of most of the above described results being the transfer of plant genes, more evidence is necessary to justify it and to demonstrate that the correcting gene has been integrated in one of the host chromosomes. As in the case of 'egg transformation' the implicated mechanisms are not clearly established. Both 'egg transformation' and γ-fusion are very attractive experimental tools, being technically relatively simple and unexpensive, and making possible direct delivery of the desired gene into the receptor nucleus. Speaking of gene transfer on the basis of available data is somewhat presumptious at present, as there is no clear evidence and control over the fate of the introduced genetic information in the sexual or somatic hybrid cells.

A comparison between γ-fusion and 'egg transformation' with respect to their merits and limitations is presented in Table 3. The superiority of novel techniques over conventional methods in

Table 3. Established and hypothetical features of γ-fusion and 'egg transformation' with respect to radiation effects, incompatibility reactions, genetic consequences of the *in vitro* culture phase, specific selection pressures, and cell cycle stage in view of alien gene introgression (AGI).

1. RADIATION EFFECTS (complete mitotic inactivation of the donor genome)
- Accelerated loss of donor chromosomes (25), weakened or impaired initial gross incompatibility reactions, disfunctional DNA repair mechanisms in the donor cell coupled with recombination events directed by the DNA repair enzymatic pool of the host cells;
- Higher doses in the case of 'egg transformation' ($\leqslant$ 500 krad) as compared to γ-fusion ($\sim$50 krad).

2. THE INCOMPATIBILITY REACTIONS (mainly of zygotic or graft type)
- Generate profound genetic rearrangements, together with extensive and progressive loss of chromosomes (18); the loss is not necessarily uniparental, and is under genetic control and non-random (14);
- Fusion of somatic cells makes possible to combine or manipulate a greater genetic complexity, while sexual incompatibility barriers are avoided.

3. THE *IN VITRO* CULTURE PHASE AND PLANT REGENERATION
- Generates at relatively high rates continuous alterations of the genetic material, both gross and cryptic (24, 16). Mitotic gene conversion and DNA transposition are believed to represent major features of the process (42, 13);
- By modulating the dedifferentiation (callus) *vs.* redifferentiation (regenerants) phases, somatic incompatibility can be weakened or altered, while providing more opportunities for recombination or mutation-like events;
- The use of established cell lines as fusion partners is a widespread practice, and represents a source of preexisting variation, generating in its turn even more genetic variation (18, 19);
- Plant regeneration can impose developmental constraints upon cultured cells and therefore reduces genomic unbalance or karyotypic heterogeneity. It may act as a complex selection pressure and stabilize AGI events in their evolution.

4. APPLICATION OF SPECIFIC SELECTION PRESSURES
- Use of selectable markers means that there is permanent control over the fate of the traits to be transferred, and an eventual guarantee that they are preferentially maintained against most of the superfluous genetic donor-material;
- γ-fusion: large cell populations can be subjected to early and permanent biochemical and environmental constraints;
- 'Egg transformation': less amenable to screening for selectable markers; early developmental constraints combined with selection pressures (after seed formation) could favour the recovery of more accurate AGI events.

5. THE CELL CYCLE STAGE
- γ-fusion: protoplasts can be synchronized with or without blocking cell wall synthesis (30); use of Go cells may favour enhanced recombination exchanges during heterokaryon formation from Go to S phase;
- 'Egg transformation': pollen is synchronized in G2; produced subchromatid breaks are essential in terms of chromosome pairing, recombination, and repair (37).

AGI has to be demonstrated in terms of: (1) increasing the range of genetic complexity that can be manipulated; (2) making possible a more systematic unidirectional elimination of chromosomes; (3) providing a higher potential for intergenomic somatic recombination. Among the various factors listed in Table 3, several are expected to interact in various ways and thus modulate each other's effects. They are briefly discussed below.

In the case of 'egg transformation' the joint effects of irradiation and incompatibility can mainly be modulated through radiation dosage control, which in turn may determine the extent, rate, and unidirectionality of chromosome elimination. By means of appropriate selection pressures one can eventually select for products having integrated the desired trait early enough, so that transmission through the germ line can be ensured. The available data is restricted to intra- and in few cases to interspecific combinations; it appears desirable to extend such experiments to wider crosses.

With respect to γ-fusion, there are so many interacting factors implicated in this process that we are far from monitoring the sequence of events from fusion of partner protoplasts to newly engineered plants. Radiation effects associated with incompatibility reactions and genetic consequences of the *in vitro* culture phase may contribute to enhance the illegitimate recombination of genetic information. The control over the *in vitro* phase is still a wish, as the response of cells in culture is species and even explant dependent, while the methodologies are non-standardized and mostly empirical. For both 'egg transformation' and γ-fusion it may be necessary to screen among a large number of products in order to identify an uncertain but desired event. Use of specific selection pressures would represent a permanent control over the fate of transferred traits, and an eventual guarantee that the trait is preferentially retained. All other experimental parameters should be directed towards enhanced recombination events and elimination of redundant chromatin. Regeneration induction, which often occurs under culture conditions that are species or explant-specific, may represent – when combined with specific selection pressures – an efficient screening step in favour of host-like phenotypes containing but few donor genetic attributes.

Selectable markers in AGI experiments – a strategy and some preliminary data

Refinement of methodologies in view of AGI was envisaged within the context of considerations discussed in the previous section. Selectable markers, coupled with the ability to regenerate plants from genetically manipulated cells, represent in this respect a necessary option. Both resistant dominant and deficient recessive mutants are being used, particular attention being paid to regulatory requirements and expression constraints inherent to each genetic marker. Most of the donor partners used possess additional marker traits.

In the case of egg transformation, the accent is placed on the use of lethal doses of radiation (between 50 and 400 krad) in order to avoid formation of partial hybrids (37). The extension of this technique to wider crosses makes possible to evaluate the contribution of incompatibility mechanisms in AGI. We concentrated our efforts on the lysine + threonine-resistant mutants, which overproduce threonine and are available in several plant species (cf. Table 2). The marker is fully expressed in germinating seeds of mutant plants.

Detailed results are available so far for the intraspecific (*N. sylvestris* × *N. sylvestris* RLT) combination. We were able to show that the viability and *in situ* growth of pollen grains were affected by heavy irradiation at various extents, but not irreversibly damaged. At various intervals (0–3 days) after the initial pollination with the γ-donor pollen a second pollination with pollen from the host species was performed in order to obtain seeds. 70 000 to 135 000 T1 seeds were tested per experimental variant (dose level versus time interval between the first and second pollination) by germinating the seeds on inhibitory concentration of lysine (2 mM) + threonine (1 mM). From a total of 380 000 T1 seeds examined no resistant phenotypes analogous to the mutant type could be identified. However, 310 weakly resistant individuals were followed into T2 generation. Again, plantlets with low levels of resistance were recovered in a few cases. The segregation ratios for resistance did not fit the expected 3 resistant: 1 sensitive Mendelian segregation ratio; instead, 1:1.4 and 1:3 values were obtained. In addition, among the 310 tested T1 plants, one aneuploid was recovered from the 50 krad treatment, and 3 other plants with very low seed

setting capacity in the 50 and 100 krad treatments. A morphologically abnormal plant was also observed.

The fact that we were unable to recover the desired dominant resistant marker among very large numbers of T1 seeds submitted to appropriate selection pressure suggested to us that the marker trait was not fully, if at all, integrated and/or expressed under the experimental conditions tested. Thus, the advantage of using dominant selectable markers was not obvious in this case. If integration-expression of a selectable marker gene does not occur in T1 seedlings, later stages or next generations must be used for detection. Under such circumstances, the frequency of integration-expression has to be rather high (10^{-2}–10^{-3}), otherwise the method is of little, if any, experimental value.

The other observed phenomena can probably be best interpreted in terms of mutagenic effects of the irradiated DNA.

With respect to γ-fusion, a comparison with the 'egg transformation' approach was carried out by using identical selectable markers and the same plant species as fusion partners. Protoplast from lysine + threonine and AEC-resistant *N. sylvestris* mutants were employed as donors after heavy γ-irradiation (60–100 krad) in fusion with wild type protoplast of *N. sylvestris* and *N. plumbaginifolia*. Under the designed experimental conditions the survival of the protoplast population was extremely low (0–1%), a consequence of the joint effects of drastic irradiation conditions and fusion stress. No resistant γ-fusion products were recovered so far, and lower doses are being tested at present while extending the range of donor species to lysine + threonine-resistant mutants of carrot and barley.

The role played by incompatibility mechanisms in AGI was further assessed in another series of experiments by progressively reducing the phylogenetic relatedness between the receptor partner (NR- and *his*- mutants) and a series of donor species. To avoid undesirable genetic variability, partners with balanced, euploid genomic conditions (i.e. leaf protoplasts) were preferentially chosen in order to be able to regenerate plants from the manipulated cell lines. Combination of heavy radiation effects and selection pressure which was applied starting with the first 2–4 division cycles of a fusion product were studied as a possible means of forcing gene transfer events at an early stage after fusion.

Thus, a NR- *nia* type from *N. plumbaginifolia* was fused with γ-irradiated protoplasts of *N. sylvestris* (albino), *D. innoxia* (5-methyltryptophan-resistant), and sugarbeet (habituated line). The following doses were used: 5, 10, 20, and 30 krad, fusion with non-irradiated donor partner serving as controls. The results demonstrated that corrected phenotypes occur at all doses and in all cell combinations tested at frequencies ranging from 4×10^{-4} to 2×10^{-5}, which is much above the spontaneous reversion frequencies established for the two marker genes. In general, colonies recovered from the combination *nia* + sugarbeet were unstable, even though a continuous selection pressure was maintained. Regenerants were obtained from most of the combinations, and 30 such clones are presently analysed caryologically, biochemically, and genetically. In the combination *nia* + *N. sylvestris*, several lines were unable to regenerate, some others produced abnormal regenerants, and a few exhibited an apparently normal hybrid phenotype. Preliminary data suggest that within the range of doses tested the correction of the NR deficiency is mainly due to transfer of whole donor chromosomes (also see (48)).

Final remarks

Recent availability of stable and well characterized selectable markers and ability to combine alien genomes parasexually have contributed to the development of molecular biology in higher plants, including gene expression and genetic manipulation.

The manipulation of whole genomes by cell fusion is expected to offer new potential in the transfer of single-to-multiple DNA segments, but refinement of transfer techniques per se and by utilizing smaller sizes of genomes (minicells, chromosomes) is desirable. This is the only way cellular engineering can pretend to become a credible alternative to vector-mediated gene transfer. Among these techniques, 'egg transformation' is at present the accessible solution in view of AGI for many plant species for which cellular technology is not yet feasible.

Gene transfer implies by definition the integration of donor DNA into the host genome. Present results in cellular engineering of higher plants do

not allow us to establish whether gene transfer has taken place under the experimental conditions reported. More refined molecular probes are required to demonstrate that transfer of single-copy genes or of small samples of DNA can be achieved. This is an essential point, as it should help us to distinguish gene transfer from other possible mechanisms by which DNA can mediate alterations in genotype or phenotype.

Unambiguous demonstration of transformation of higher plants with foreign genes has only been achieved with recombinant Ti plasmids. As the significance to gene expression of precise DNA constructs is now beginning to be understood in part because of cell transformation methodology, the eventual failure with cellular-mediated transfer systems could be more easily apprehended. In the case of 'egg transformation', a possible approach in assessing the occurrence and rate of gene transfer would be the use as donor genes of marker genes that are not 'home-made', such as opine synthesizing ones, available by now in several plant species. Similarly, an appropriate experimental system in γ-fusion would make use of deficient nutritional mutants (defective enzyme) as receptor and of a mutant altered in the regulatory activity of that particular enzyme as donor.

Confidence in the success of plant cellular engineering still relies on results obtained with mammalian cells, in which case it has been demonstrated that transfer can occur through cell fusion, as well as by DNA and chromosome-mediated transfer techniques (44). It was equally shown that huge portions of foreign genetic material can be processed and stabilized by cells *in vivo*, that donor genetic information does undergo recombination prior to integration, and that eukaryotic genomes have an increased potential for nonhomologous recombinational events (40). Last but not least, the level of foreign gene activity in the progeny of transformed animals may be enhanced, unchanged, diminished, or abolished (4).

The accumulation of molecular, selectable and identifiable markers in the rapidly developing somatic cell genetics of higher plants, as well as our ability to regenerate plants from genetically manipulated cells represent two winning cards for the near future.

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