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Review

Advances in somatic cell genetics of higher plants – the protoplast approach in basic studies on mutagenesis and isolation of biochemical mutants

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Summary. Selection strategies developed in microbial genetics were successfully extrapolated to in vitro cell culture systems of higher plants and are having a major impact in the elucidation of regulatory mechanisms of basic cellular processes in eukaryotes. Although an increasing number and wide spectrum of biochemical variants have been isolated in such cell culture systems, their routine selection, characterization, and manipulation have not yet been achieved. Methodological limitations are considered to be one of the major reasons. Suspension or callus cultures, so extensively employed during the last decade in mutation-selection experiments and so useful in demonstrating the potentialities of in vitro screening techniques in obtaining various biochemical markers, have inherent drawbacks which limit in our opinion their further contribution in this field. Protoplast cultures represent an ideal tool for mutation and selection experiments. It is the purpose of this review to show how, due to recent methodological advances in the manipulation of some model protoplast culture systems, essential aspects of mutagenesis and selection of biochemical mutants can be reconsidered. These systems are simple and efficient, and lend themselves to statistical interpretation. Genetic analysis of selected variants should help us to understand and define better the new set of problems and concepts revealed by the somatic cell genetics of higher plants; combined with biochemical analyses it should elucidate the basic relationship between control of biological processes at cellular and whole organism level.

Key words: Plant protoplasts – Mutagenesis – Mutant selection – Regeneration – Expression – Genetic analysis – Somatic cell genetics

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"Since the first isolated haploid cells were successfully cultured, the dream of most plant biologists was to achieve the elegance and sophistication that now characterize the most refined prokaryotic experimental genetic systems, and to apply them to a wide variety of basic problems specific to higher plants."

Melchers and Bergmann 1958

I Introduction

Once the culture and regeneration of plant cells had been achieved, the selection and characterization of biochemical mutants became a major objective in the

field of plant sciences. It was optimistically expected that the methodology of microbial genetics could be rapidly extended to plant genetics and to the breeding of important crops with new properties (Carlson 1973 a; Carlson and Polacco 1975; Bottino 1975). Progress has been hampered by a number of factors including a lack of understanding of the methods required to induce and select valuable mutants (Miflin 1973; Maliga 1976, 1978; Zryd 1978; Widholm 1978; Parke and Carlson 1979). Because of this lack of basic knowledge considerable effort has been devoted to finding model plant cell culture systems where such methodology could be established.

So far, most of the experimental data on mutation and selection have been obtained with cell suspension cultures. They have definitely demonstrated the potential of in vitro screening techniques with plant cell systems. However, such cultures are not ideal for mutagenesis and selection studies as they show cell aggregation, chromosome instability, and low regenerative capacity. The isolation of recessive mutants appeared extremely difficult, even with such highly performing suspension culture systems as tobacco or carrot (Carlson 1973 a; Widholm 1981). Such drawbacks limit, in our opinion, the future contribution of established cell cultures in this field. Recently, protoplast cultures from several species of Solanaceae and embryogenic suspensions of carrot have been successfully used in studies on mutagenesis and/or selection for various biochemical mutants. Their merits essentially consist in the ability to produce large enough numbers of haploid or diploid cells per manipulation (thus achieving a high enough degree of genetic resolution when screening for mutants), and to regenerate fertile plants from variant cultures (by reducing the time interval between initiation of the cell culture and regeneration step). Consequently, it became possible to analyze the expression of altered phenotypes induced and selected at cellular level along various stages of differentiation and follow their transmission to progeny. This is a crucial problem when trying to understand developmental processes in eukaryotic systems.

Appropriate and efficient lines of investigation in this area of plant research can be brought about by integrating recent results from protoplast work with some substantial data accumulated in established cell culture and whole-plant systems during the last two decades. In reviewing these aspects, the following points will be stressed:

1) the contribution of various methodological innovations in setting up reliable protoplast experimental systems; it will be further shown that, thanks to these improvements, essential aspects of mutagenesis and selection of biochemical mutants can be reconsidered;

2) the stability of expression of a selected trait in cultured cells and regenerated plants; some of the parameters that are involved in the variant cell selection – plant regeneration cycle will be discussed;

3) the biochemical and genetic characterization of selected variants; an insight into the differential regulation of various metabolic functions and its possible implications in selection strategies will be given.

II Methodological advances in the culture of plant protoplasts – towards routine selection of biochemical mutants

A culture system of isolated, synchronous, and actively dividing cells is a prerequisite for quantitative studies on mutagenesis as well as for efficient selection of biochemical mutants. Such an experimental system is provided by several species of *Solanaceae,* namely tobacco, *Nicotiana sylvestris, N. plumbaginifolia, Petunia hybrida, Datura innoxia* and *Hyoscamus muticus.* In addition, promising results have recently been reported with certain varieties of potato (Binding et al. 1978; Sheppard 1980; Thomas 1981) and tomato (Zapata et al. 1981). *N. sylvestris* and *N. plumbaginifolia,* two wild species of *Nicotiana,* are believed to be true diploids and are the object of extensive investigation in our laboratories. They will be often referred to as examples.

1 Isolation and culture of protoplasts

Culture conditions for greenhouse and in vitro propagated protoplast mother plants have been worked out: success in protoplast culture appears to depend essentially on the physiological state of the initial material. This state can be controlled through factors such as light cycle and light intensity, composition of nutrient solution or culture medium, age and position of leaves on the plant. Despite some differences in experimental protocols, simplified standardized procedures for isolation and culture of haploid and diploid protoplasts of the above mentioned species have been reported (Durand et al. 1973; Binding 1974; Schieder 1975; Bourgin et al. 1979; Durand 1979; Lörz et al. 1979; Negrutiu and Mousseau 1980; Negrutiu 1981).

Axenic shoot cultures grown under defined conditions represent a suitable source of protoplasts whenever large numbers ($> 10^8$) of dividing protoplasts are required. They also ensure reproducible high plating efficiencies of such protoplast cultures (Binding 1975; Caboche 1980; Negrutiu and Mousseau 1981; Negrutiu 1981). In the case of tobacco and *N. plumbaginifolia,* greenhouse-grown plants represent a suitable source of protoplasts as well (Bourgin et al. 1979; Negrutiu 1981). By their intrinsic nature mesophyll protoplasts have a high degree of genetic homogeneity: in certain species such as *H. muticus* (Shimamoto, personal communication) or *N. glutinosa* (Galbraith personal communication) freshly isolated protoplasts are enriched in 4C DNA containing protoplasts, while in tobacco, *N. sylvestris* or *N. plumbaginifolia* (mature leaves mainly), 90% or more are in Go (G1 arrest) (Galbraith

Table 1. Sequence and timing of protoplast manipulation for selection against S-aminoethylcysteine – resistance in *N. sylvestris* and *N. plumbaginifolia* as developed in our laboratory

Sequence	Timing	
	N. sylvestris	*N. plumbaginifolia*
Protoplast isolation and mutagen treatment	0–24 (72) h	0–24 (48) h
Segregation – expression of the mutation trait (3–4 division cycles)	12–14 days	8–10 days
Dilution – selection (regeneration possible for *N. sylvestris*)	+3–4 weeks	+2–3 weeks
Confirmation – regeneration (one step) or confirmation at the level of regenerated variants	+4–5 weeks	–
Confirmation + regeneration (two steps)	–	+6–8 (10) weeks
Multiplication of regenerated variant plants	+3 weeks [8–16 weeks]	+3 weeks [8–16 weeks]
– tests for resistance – biochemical analyses – cloning → greenhouse (selfing, back-crosses)	+12–16 weeks	+6–8 weeks
colonies / regeneration → tests for resistance		
Total time required to obtain F_1 or selfed progeny[a]	24 (29) weeks	18 (23) weeks

[a] A similar selection scheme applied to *Arabidopsis thaliana* M_2 seeds requires 10 (14) weeks

et al. 1981; Magnien and Devreux 1980; Magnien et al. 1980b). In addition, protoplasts of *N. plumbaginifolia* can withstand repeated manipulation or incubation at high cell densities, and recover their division capacity after treatments that block cell division (starvation, sublethal concentrations of drugs, cold treatments).

Plant regeneration in protoplast-derived cultures of several *Solanaceous* species is rather efficient. Various *Nicotiana* species exhibit high morphogenic potential during the first subcultures (Bourgin et al. 1979). Among them, *N. plumbaginifolia* has a particularly short "life-cycle" (from test-tube cuttings to seeds) of 6–8 weeks, as shown in Table 1.

2 Plating of protoplasts at low densities and selection in protoplast cultures

Establishment of simple chemically defined media for growth at low cell densities of protoplast-derived cells of tobacco (Caboche 1980; also see Kao and Michayluk 1975; Gleba 1978) represented a breakthrough in the manipulation of plant protoplasts. Similar methods have been successfully adapted for mesophyll protoplasts of other species, namely *N. sylvestris, N. plumbaginifolia,* and *Petunia* cv. 'Mitchell', as well as for tobacco protoplasts of cell suspension origin (Magnien et al. 1980b; Negrutiu and Muller 1981; Negrutiu 1981; Muller et al. 1983): such protoplast cultures exhibited active growth and high plating efficiencies (≧ 50%) when diluted to low densities a few days after initiation of the culture. Simple expeditious techniques are thus available and open up new powerful research possibilities with such protoplast systems. As with bacterial systems, the estimation of cell survival following dilution is highly accurate and reproducible. Consequently, routine tests can be set up and used to study various culture parameters, mutagenic agent effects, or to perform rapid cloning etc.

Such dilution media appeared to be very suitable for selection purposes (Caboche and Muller 1980; Negrutiu and Muller 1981; Negrutiu 1981; Negrutiu et al. 1983a). The time required to see resistant, presumptive mutant colonies has been considerably reduced: 20–25 days, and 30–40 days after protoplast isolation for *N. plumbaginifolia* and *N. sylvestris,* respectively. The control of several screening parameters (such as expression time, optimal density at selection, composition of selection medium) has also been achieved by simulation and reconstruction experiments of mutant selection (Bourgin et al. 1980a; Negrutiu and Muller 1981; Pental et al. 1982; for work with suspension cultures see Zrijd 1976; Horsch and Jones 1980a, b; King et al. 1980).

These technical advances mean that protoplast cultures of both species are useful as model experimental systems in studies on biological effects of mutagens (Magnien and Devreux 1980; Magnien et al. 1980a, b, 1981) and mutagenesis. Various resistant or auxotrophic mutants have been recently selected in protoplast cultures of *N. sylvestris, N. plumbaginifolia,* and *Hyoscyamus muticus* (see V.2 and Table 2; Maliga et al. 1982). The whole sequence of selection steps – isolation, regeneration, and genetic analysis of selected variants – has been evaluated in our laboratory (Table 1).

Table 2. Expression at plant level and/or inheritance of variant phenotypes ("nuclear" type only) selected in in vitro culture systems

Selection scheme	Species, culture system, ploidy, mutagen treatment, selection type, variants	Mutant phenotype	Expression at the level of				Mode of inheritance	Reference
			Regenerated plant	Derivative culture	Progeny	Derivative culture		
A Established cell cultures								
Lysine + threonine	*Zea mays;* cc; 2n; nm; GIC; 2 var.	op (+)	E	E	E (1 var.)	–	mgD	Hibberd and Green 1982
	D. carota; sc/ec; 2n; MNNG; IC; 1 var.	op (+)	E	E	ftr	–	ftr	Cattoir et al. 1983
5-methyltryptophan	*D. carota;* sc; 2n; nm; IC; 1 var.	no uptake	nE	E	–	–	?	Widholm 1974
	N. tabacum; sc; 2n; ?; IC; 1 var.	op (+)	nE	E	–	–	?	Widholm 1980
	D. innoxia; sc; n; EMS; IC; 4 var.	op (+)	E	E	ftr	–	ftr	Ranch et al. 1983
p-fluorophenylalanine	*N. tabacum;* Su/Su; sc; 2n; nm; 3 var.	ftr	E	E	ftr	–	ftr	Flick et al. 1981
S-aminoethylcysteine	*A. thaliana;* sc; 2n; EMS; IC; 3 var.	no incorporation	nt	E	–	–	?	Negrutiu et al. 1978
	Oryza sativa; cc; n; nm; IC; 1 var.	various modific.	nt	nt	nE only seeds were tested	–	ftr	Shaeffer and Sharpe 1981
Isonicotinic acid hydraz.	*N. tabacum;* sc; n; UV; IC; 10 var.	(+)	E	E	nE/E (5 out of 10 var.)	E	D	Berlyn 1980; Zelitch and Berlyn 1982
Glycine hydroxamate	*N. tabacum;* sc; n; UV; IC; 35 var.	increased free pool	nE	E	nE (not in all var.)	E	ftr	Lawyer et al. 1980
Ethionine	*M. sativa;* sc; 2n; EMS; IC; 1 var.	op?	nt	E	nt	–	?	Reish et al. 1981
Chlorate	*N. tabacum;* sc; n; NU; IC; 1 var.	(+)	E	nt	E	E	dgR	Pental et al. 1982
BUdR	*N. tabacum;* cc; n; nm; IC; 1 var.	unknown	nt	E	nt	E	mgD	Marton and Maligna 1975
FUdR	*N. tabacum;* sc; 2n; nm; IC; 2 var.	unknown	nt	E	–	–	?	Chaleff and Keil 1982
Aminopterin	*D. innoxia;* sc; n; nm; GIC; 2 var.	unknown	E	nt	nt	–	?	Mastrangello and Smith 1977
Picloram	*N. tabacum;* sc; 2n; ?; IC; 4 var.	unknown	nt	E	E	nt	mgD or mgSD	Chaleff 1981
Carboxine	*N. tabacum;* sc; n; ?; IC; 1 var.	unknown	nt	nt	nE	E	mgD	Polacco cited by Chaleff 1981
Ph. infestans toxin	*S. tuberosum;* cc; n; nm; IC; 10 var.	unknown	E	E	nt	–	?	Behnke 1980
Hydroxyurea	*N. tabacum;* sc; 2n; nm; IC; 7 var.	unknown	nE	E	nE	E	mgD	Chaleff and Keil 1982

Table 2 (continued)

Selection scheme	Species, culture system, ploidy, mutagen treatment, selection type, variants	Mutant phenotype	Expression at the level of				Mode of inheritance	Reference
			Regenerated plant	Derivative culture	Progeny	Derivative culture		
Methothrexate	*N. tabacum;* sc; n; nm; IC; 5 var.	unknown	nE	E	nE	E	mgSD	Chaleff and Keil 1982
Streptomycin	*N. sylvestris;* cc; n; nm; IC; 1 var.	unknown	nt	E	E	nt	mgR	Maliga 1981
Glycerol utilization	*N. tabacum;* sc; 2n; nm; IC; 1 var.	unknown	nt	E	nt	E	mgD	Chaleff 1981
B Protoplast cultures								
Lysine + threonine	*N. tabacum;* n; UV; IC; 2 var.	op	E	–	E	–	mgD	Bourgin et al. 1980 b
	N. sylvestris; 2n; UV; IC; 2 var/2 classes	op	E	E	E	E	mgD	Negrutiu, unpublished
Valine	*N. tabacum;* n; UV; IC; 7 var./2 classes	no uptake ftr	E	E	E	E	dgR mgD/mgSD	Bourgin et al. 1980 b and personal communication
Methionine sulfoximine	*N. tabacum;* n; ?; IC; 3 var./2 classes	op ?	E	–	E	–	mgD dgR	Carlson 1973 b
S-aminoethyl-cysteine	*N. sylvestris;* 2n; UV; IC; 2 var.	op (+)	E	E	E	E	mgD	Negrutiu et al. 1981, 1983 b
	N. plumbaginifolia; n; UV; IC; 1 var.	no uptake	–	E	E	E	mgR	Negrutiu et al. 1983 a and unpublished
Isopropyl-phenylcarb.	*N. tabacum;* n; EMS; IC; 1 var.	unknown	nE	nt	nE	nt	?	Aviv and Galun 1977 a
Chlorate	*N. plumbaginifolia;* n; nm; IC; 30 var.	(+)	E	E	E	E	mgR	Negrutiu et al. 1983 a
Temperature sensitive	*H. muticus;* n; NG; 1 var.	ftr	E	nt	ftr	–	ftr	King et al. 1981
Nicotineamide requiring	*H. muticus;* n; NG; 1 var.	ftr	E	nt	ftr	–	ftr	King et al. 1981
Isoleucine requiring	*N. plumbaginifolia;* n; gamma; 1 var.	(+)	E	–	ftr	–	R	Sidorov et al. 1981
Histidine requiring	*H. muticus;* n; NG; 4 var.	ftr	E	nt	ftr	–	R	Shimamoto and King 1983
	N. plumbaginifolia; n; UV; 2 var.	ftr	E	E	ftr	–	ftr	Negrutiu 1983

Abbreviations: Culture system: cc, ec, and sc – callus, embryoid, and suspension cultures, respectively. *Mutagen treatment:* nm – not mutagenized; EMS – ethane methane sulphonate; MNNG – n-methyl N′-nitro N-Nitrosoguanidine; NU – N-ethyl-N-Nitrosourea. *Selection type:* GIC – gradually increasing concentrations; IC – inhibitory concentrations; var. – number of variants. *Mutant phenotype:* op – overproduction; (+) – evidence for specific alterations in the regulatory properties of a control enzyme. *Expression:* E – expressed; nE – not expressed; nt – not tested; ftr – further tests required. *Inheritance:* D – dominant; SD – semidominant; R – recessive; mg – monogenic; dg – digenic

The availability of a "reliable" plant cell culture system means that various parameters of the mutation – selection sequence now can be examined in some detail.

III Mutagen treatment

1 Cell suspensions versus protoplast cultures

Cell suspensions or callus cultures were initially used to investigate various biological effects of mutagens on plant cells. Studies on mutagenesis per se were developed only recently (Sung 1976; Christianson and Chiscon 1978; Werry and Stoffelsen 1979, 1980; Colijn et al. 1979; Weber and Lark 1980; Horsch and Jones 1980a). They are discussed in Section IV.I. In their review on radiation biology of cultured plant cells, Howland and Hart (1977) made a thorough analysis of the effects of UV and ionizing radiations at molecular, chromosomal, physiological, and cellular level. Criteria employed in evaluating radiation sensitivity (i.e. damage criteria) of cultured cells were critically assessed – it was emphasized that a lack of actively proliferating true single-cell systems was a major limitation in quantitative radiation dosimetry and analysis of survival. Several other drawbacks appeared inherent to such cell suspensions and obviously reduce their further contribution to this field: they consist of cell clusters which dissociate at irregular rates, are rather difficult to synchronize, and have variable chromosome numbers and karyotype. In addition, cell suspensions can have a previous mutation history (Skirvin 1978; Larkin and Scowcroft 1981; Howard-Flanders 1981). The number of mutant subclones in a cell suspension can vary with these and other factors, and makes comparison and interpretation of data obtained with various sources of cell suspensions very difficult.

Most of the drawbacks encountered with cell suspensions can be avoided with protoplast systems as those described in Chapter II. Thus the mutagenic treatment can be applied to a highly synchronized population of isolated, genetically homogeneous cells. The capacity of cells to divide (the most sensitive and significant parameter of genotoxic effect – see below) can be easily estimated due to the clonal growth of plant protoplasts and used as an accurate measure in quantitative mutagenesis (and thus avoiding weight-increase measurements and staining for viability, so commonly described with suspension cultures). The size of colonies, degree of greening, regeneration rates can also be taken into account in the estimation of genotoxic effects (Section 2 below). An early dilution should overcome problems such as interference of temporary proliferation of irradiated cells so often encountered in mutation experiments (Howland and Hart 1977), density dependence phenomena (Raveh et al. 1973; Cella and Galun 1980), or radiation induced feeding effects (Skirvin 1978; Magnien and Devreux 1980).

Recent advances in protoplast technology contributed to a better evaluation of both genotoxic (i.e. genetic effects from mutagen treatment) and mutagenic (i.e. mutations only) effects of various mutagen agents. Measurable damage criteria, such as changes in DNA content, replicative DNA or repair DNA synthesis, and replicative death (i.e. damage to division capacity) were assessed in protoplast cultures of tobacco, *N. sylvestris,* and *N. plumbaginifolia* (Gleba et al. 1978; Gleba and Gleba 1978b; Durand 1979; Magnien and Devreux 1980; Magnien et al. 1980a; Caboche and Muller 1980; Magnien et al. 1981; Negrutiu 1981; Sala et al. 1982; Magnien et al. 1982; Vunsh et al. 1982). It was shown that (1) changes in radiosensitivity occur during cell cycling; (2) biphasic dose-response curves were obtained only when plating efficiency of cultured protoplasts is low; (3) isolated protoplasts were more sensitive to mutagens than callus cells but less sensitive than mesophyll cells; (4) chemical mutagens were more toxic to protoplasts than physical mutagen agents; (5) haploid cells were of greater sensitivity than diploid ones; (6) the extent of cytological heterogeneity in freshly isolated protoplast populations varied with species, genotypes, developmental age of axenic leaves, culture substrate. Various mutagens, namely N-methyl-N-nitro-N-nitrosoguanidine (review article by Gichner and Veleminsky (1982)), X-, gamma-, and UV rays were used to induce resistance to valine (Caboche and Muller 1980; Vunsch et al. 1982) or to chlorate (Marton et al. 1982; Negrutiu et al. 1983a) in protoplast cultures; such studies demonstrate the reliability of the protoplast approach in studies on mutagenesis and are discussed in Section IV.I.

2 Time of application, killing – and dose rate

Most usually freshly isolated protoplasts were subjected to mutagen treatment as the treatment can be easily fitted into the washing step. It has already been mentioned in Section II.1 that such protoplasts are synchronized to a large extent in Go in certain species. In the case of *N. plumbaginifolia,* leaf protoplasts have been reported to enter S and G2 phases starting with the 20th hour of culture, reaching a maximum after 36 h (Magnien et al. 1980a). Similar conclusions can be drawn from data on tobacco protoplasts (Galbraith et al. 1981). Irradiation of haploid protoplasts one day after isolation can still be used to induce non-chimeric recessive mutants, as shown with various auxotrophic phenotypes isolated in *H. muticus* (Gebhardt et al. 1981; Shimamoto and King 1983) and *N. plumbaginifolia* (Negrutiu et al. 1983a). To avoid excessive manipulation of chemically mutagenized protoplasts, permanent incubation at lower mutagen concentrations

can be attempted (Marton et al. 1982). A different approach consisted in replacing irradiation of isolated protoplasts by that of buds giving rise to protoplast mother plants (Caboche and Muller 1980).

One of the major determinants of radiosensitivity was shown to be the cell cycle stage (Howland and Hart 1977). Choice of time of application, dose, and dose rate are essential to induce high rates of mutation. It is known from animal cells that higher frequencies of mutation are obtained in cells undergoing rapid proliferation as compared to resting cells or cells synthesizing DNA at lower rates; this was correlated with an enhanced postreplication "error-prone" repair of DNA lesions in the first group of cells (Howland and Hart 1977 and references therein). Studies of this sort can at present be envisaged with plant protoplasts provided that mutagenesis is applied during the first division cycle while selecting for a dominant marker.

Most of the experiments on mutagenesis indicate that the frequency of variants increases with increasing mutagen dose (Sung 1976; Werry and Stoffelsen 1979, 1980; Weber and Lark 1980; for animal cells see Asquith et al. 1978). The general tendency is therefore to work at killing rates greater than 90, 95, or even 99%. High killing rates were also employed when screening for biochemical mutants (Caboche and Muller 1980; Sidorov and Maliga 1982; Marton et al. 1982; Shimamoto and King 1983). It is our opinion that the choice of optimum mutagen doses for mutant screening should consider not only the frequency of mutation but also take into account the occurrence, extent, and effect of secondary (unselected) lesions on differentiation (ability to regenerate plants etc.). As a matter of fact Eapen (1976) has shown that in haploid tobacco cell suspensions regeneration of shoot buds from gamma-irradiated cells was completely inhibited already at doses above LD_{50}, while the same phenomenon with UV-irradiated cells only occurred at a survival rate of 15%. Using protoplast-derived cells, Caboche and Muller (1980) have shown that, at high killing rates ($\geqq$ 90%), the proportion of colonies able to regenerate buds decreased proportionally with the dose of mutagen. Use of high doses of mutagen in the protoplast culture of *N. plumbaginifolia* (Sidorov and Maliga 1982) resulted in abberant regenerants or lack of morphogenic response in selected lines or their fusion products. In addition, at high doses of mutagen, growth of surviving colonies is slowed and very irregular and sectorial growth within the same colony is frequently observed. This probably means that secondary deleterious mutations and altered morphogenic capacity occur and also means that such cell populations no longer divide synchronously and actively. The above discussion suggests that experiments on weak dosage and/or various dose rates are to be extended.

3 Calculation of mutation frequency

The frequency of mutant isolation is claimed to be higher in protoplast cultures than in cell suspensions (Maliga 1980). However, two observations appear necessary: (1) only in a very few systems, namely suspension cultures of soybean and *Haplopappus gracilis,* and protoplast-derived cells of tobacco, have the mutation – selection conditions been worked out in any detail (Werry and Stoffelsen 1979; Weber and Lark 1980; Caboche and Muller 1980; Horsch and Jones 1980a, b); (2) the calculation of mutation frequency or mutation rate (as defined by Chaleff 1981) with plant cell systems has not yet received a generally accepted solution. As a matter of fact, it is not always clear how the reported frequencies were calculated.

Most frequently, mutation frequencies are expressed on the basis of "per colony-forming unit" calculations; similar methods are used with animal cell cultures. Correction coefficients for the occurrence of aggregates of various sizes in cell suspensions have occasionally been established (Howland and Hart 1977; Werry and Stoffelsen 1980; Murphy 1982). Analysis tests for occurrence and distribution of mutants conceived for bacterial systems were recently recommended for plant cell cultures (Malmberg 1981; Murphy 1982). Thus in the Po method of Lea and Coulson (1949), statistical calculations are made of the distribution numbers of mutants in a culture of bacteria in which the number of mutants increases on account both of new mutations and of division of old mutants. The question is whether this method or similar ones can be applied with enough accuracy to suspension cultures (see III.1), and whether it does not introduce unnecessary variables in the case of protoplast systems.

Such protoplast cultures consist of isolated cells at mutagenesis which give rise during several successive divisions to non-dissociating cell colonies (i.e. clones) (see above Galbraith et al. 1981). The average number of cells in such aggregates can be determined (Magnien and Devreux 1980; Magnien et al. 1981). Preexisting mutations most probably do not exceed the rate of spontaneously arising ones. All these characteristics of protoplast systems such as tobacco, *N. sylvestris, N. plumbaginifolia,* enable an accurate calculation of mutation frequencies and mutation rates: total numbers of mutagenized protoplasts (Tm), survival rates in control (S) and mutagenized (Sm) plates should be determined and used to establish killing curves and mutation frequency (expressed on a "per cell exposed", "per cell plated" or "per surviving cell" basis respectively). Survival rates of protoplast-derived cells at dilution – selection (II.2) were shown to averige 50% and depend less on cell density than on the number on cells per colony (Caboche 1980; Negrutiu and Muller

1981). Therefore, calculated mutation frequencies can vary with the length of expression time (IV.2) and are expected to be lower than real ones as we are not yet able to rescue every potential mutant. The number of isolated variants (V) in a given selection experiment should be confirmed (Vc) by at least a second passage under selective conditions. The ratio Vc/V should inform us on the accuracy of various screening procedures and selection conditions. Mutant frequencies should further be expressed on a "per (relative) unit of mutagen dose" basis. Such figures lend themselves to interpretation as a function of high, medium, or low killing rates etc.

IV Selection and expression of the selected trait at the cell level

1 Selection schemes for experiments in mutagenesis and mutant screening

Once essential aspects of the cell culture system have been solved it is important to devise appropriate selection procedures and use them to optimise mutagenesis and to search for various biochemical mutants. These two objectives are largely interdependent, but the criteria they should fulfill are not necessarily identical.

Selection for biochemical mutants in plant cell cultures takes advantage of the fact that a wide range of screening schemes can be efficiently applied in such systems. Once isolated, the variants should undergo detailed biochemical and genetic analysis which in turn might facilitate to discriminate between genetic and epigenetic changes. A detailed discussion of these aspects follows in Section V.2.

On the other hand, the choice of appropriate screening procedures for the study of mutagenesis per se has to meet more restrictive criteria. The marker phenotype should result from genetic changes and preferentially be of "nuclear" type (see below). The spontaneous rate of mutation for the chosen trait should not be too low ($< 10^{-6}$ or $5 \cdot 10^{-6}$) or it will be too difficult to isolate. With few exceptions, the number of isolated and characterized variants among the various classes of mutants is rather low, so the published mutant frequencies should be considered with caution (Section III.3). The selection conditions should be strictly controlled in order to avoid false positives. Such false positives have been frequently reported in variants resistant to isonicotinic acid hydrazyde, isopropyl N-phenylcarbamate, p-fluorophenylalanine, valine, lysine + threonine, kanamycin, D-hydroxylysine (Aviv and Galun 1977a; Berlyn 1980; Bourgin et al. 1980b; Maliga et al. 1980; Horsch and Jones 1980b; Flick et al. 1981). Accurate studies on mutagenesis require monitoring the screening conditions (composition of the culture medium, segregation time of the mutation event, strength of the selection pressure), ploidy level and cell cycle stage of the cell population. These aspects are discussed below.

Two different approaches, selective and non-selective, can be used in studies on mutagenesis.

Selective assays for the isolation of defined classes of mutants. Usually resistance to various metabolites and other compounds supplied in toxic concentrations was employed. Among them and of particular interest: amino acids, alone or in combinations, and amino acid analogs. Data from selection experiments with suspension and protoplast cultures revealed that the resistance was of the "nuclear" type and resulted from modifications at the level of various cellular mechanisms (Table 2; review articles by Maliga 1978, 1980; Chaleff 1981). Valine and lysine + threonine might be of interest as more universal markers in mutation studies. Valine has already been proposed (Bourgin 1978b; Bourgin et al. 1980a) and used (Caboche and Muller 1980; Vunsch et al. 1982) as such a marker. Vunsch et al. (1982) have established the following mutation frequency for valine-resistance in protoplast culture: haploid *N. sylvestris* > haploid *N. tabacum* > diploid *N. sylvestris*. However, the major limitation in the valine system apparently comes from the low proportion (17%) of resistant cell lines resulting from heritable changes as judged from expression of valine-resistance in progenies of regenerated variant plants. The fact that valine-resistance can result from alterations in more than one gene (permease, modified feedback control – Bourgin, personal communication) should also be mentioned. Resistance to lysine + threonine is characterized in several species by a common phenotype (overproduction of threonine – Table 2); it should therefore be of interest as a model-marker in mutagenesis.

Resistance to amino acid analogs, while extremely interesting as genetic markers, show up at rather low frequencies ($> 10^{-7}$, Maliga 1976; Gleba and Gleba 1978a; Christianson and Chiscon 1980; Negrutiu and Muller 1981) and appear therefore less suitable in studies on mutagenesis.

In the case of nucleic acid base analogs the frequency of resistant variants appears somewhat higher than with amino acid analogs (Maliga 1976; Dulieu personal communication). Horsch and Jones (1980a) have found that the resistance to 6-azauracil is a more reliable indicator in mutagenesis than 8-azaguanine (and than D-hydroxylysine). However, the mechanism(s) of resistance has not been clearly established.

Resistance to antibiotics has been shown to occur at relatively high frequencies and predominantly be of the "cytoplasmic" type (Maliga et al. 1980). Chlorophyll deficient and pigmentation mutants have also been reported (review articles: Schieder 1978; Maliga 1980; Chaleff 1981). Screening for such markers can bring important preliminary information on the efficiency of various mutagen treatments but there are reasons to believe that they can not be used as model-systems to study mutagenesis in cell culture systems: (1) the screening conditions are often not stringent enough

(Schieder 1976; Maliga et al. 1980; Sidorov and Maliga 1982); (2) cytoplasmic traits are often under double genetic control (nuclear and cytoplasmic); in addition, estimating the number of genes responsable for a mutant phenotype with maternal inheritance is a complex matter; (3) organelle segregation is continuous and non-random; (4) chlorophyll content varies with colony size, composition of the culture medium (nitrogen source, sugar, and hormone content – Dalton and Street 1977; Negrutiu, unpublished results), light and temperature of incubation. Furthermore, sectorial greening occurs within cell colonies and this increases with the strength of mutagen treatment. Chlorophyll and pigment deficient mutants express rather late in relation to initiation of cultures.

Other selection systems have been proposed for studies on mutagenesis. Maltose as a unique carbon source was used as a marker by Weber and Lark (1980). However the nature of this process is yet to be determined. The resistance to cycloheximide was described by Sung (1976) and Werry and Stoffelsen (1979, 1980); the resistance was considered to result mainly from epigenetic events (Maliga 1980).

Most of the above markers suffer from the disadvantage that the molecular basis of the resistance is as yet unknown. On the other hand, resistance to chlorate could become an excellent marker-system in mutagenesis. Several mechanisms may account for the resistance to the toxic effect of chlorate (Murphy and Imbrie 1981). The spontaneous frequency of mutant isolation in haploid protoplasts of *N. plumbaginifolia* was estimated by us at about 10^{-6}. The possibility to specifically select for only one particular mechanism of resistance could be envisaged: for example, using haploid cells, supplying an appropriate source of nitrogen, correctly monitoring expression time and strength of selection pressure one could be able to preferentially isolate nitrate reductase-less mutants (Negrutiu et al. 1983a). In contrast to the valine system, the selection conditions are rather stringent and, in addition, such mutants respond to a double screening: resistance to chlorate and inability to use nitrate as sole source of nitrogen. Last but not least, recovery of backmutants and other kinds of revertants in this experimental system provides further opportunities for more sophisticated studies on mutagenesis and molecular genetics.

Detection of mutants with non-selective systems. Selection studies such as those described above concern a very limited number of loci. The biochemical approach would involve induction of modifications in the protein and/or isozyme pattern at an early stage of colony formation following the mutagen treatment of plant protoplasts. Isozymes can be used for non-selective screening of mutants, provided that many enzymes are checked simultaneously (Siciliano et al. 1975). Isozyme variants can be induced by mutagen treatments; their genetic control is generally relatively simple and in many instances genes controlling isozymes are codominantly expressed. Such mutants should be detected on gels as electrophoretic shifts of specific enzymes and proteins. Clear visualization of several hundred proteins with two dimensional polyacrylamide gel electrophoresis allowed recently a more accurate determination of mutation rates in both somatic and germ lines of human cells (Neel 1983). Since mutant genes can be detected in a heterozygote due to the codominance, we have here a situation in which a high ploidy level should increase the probability of detection of such electrophoretic changes. Another major advantage of this system is that one is dealing with phenotypes reflecting a modification at gene level detectable early after the induction of the mutation.

2 Expression of mutations at cell culture level

Mutants can be selected once the mutations become expressed in the cell. Knowledge of the expression time, so important to optimize selection schemes, is rather fragmentary in plant cell systems. Experience with animal cells showed that selection is highly efficient if applied after 2–4 cell divisions following mutagenesis (Howard-Flanders 1981). In plant cell cultures rather variable intervals before selection pressure is applied have been reported: from a few hours to days, or even weeks. It is not clear whether expression time should be given in time units or cell doublings. The selection of the correct expression time will involve knowledge of the cell cycle stage and number of cells per colony at mutagenesis, as well as the number of cell divisions occurring in the presumptive mutant before selection is applied. The expression time should depend on the process responsible for the establishment of the mutation, and on the nature of the phenotype produced (altered permease, biosynthetic enzyme etc.). In this respect, recovery following mutagenesis must allow sufficient time for segregation of mutational events and growth of any variant to a colony size compatible with the expression of a given trait (i.e. increase of the pool of the modified enzyme before effective expression can occur, dilution of the wild type gene product, switch-on of the given function). For example, the expression of resistance was a function of the colony size in two different types of valine resistant mutants selected in tobacco (Bourgin et al. 1980b). Horsch and Jones (1980a) showed that resistance to D-hydroxylysine required longer recovery periods ($\geqq 35$ cells per colony) than resistance to azauracil (1–5 cells per colony) (Glimelius et al. 1978; Yamada 1978; Negrutiu and Muller 1981; Murphy and Imbrie 1981). Furthermore, screening for auxotrophs implies that exhaustion of endogenous stocks of metabolites takes place before the selective killing of wild type cells can be accomplished. The duration of the starvation period might vary with a given group of, or a particular compound (Li et al. 1967). In more general terms, expression at a different colony size following the mutagen treatment could be one of the possible means to distinguish between

various types of mutants, or between genetic and epigenetic changes.

Other factors that can influence the time required before the expression of a defined mutation occurs are the screening procedures employed (use of borderline drug concentrations, gradual increase of the selection pressure etc.), and the relative ploidy or gene redundancy level. As a rule expression time should be at least 1–2 doublings in protoplast cultures and longer periods might be required.

V Regeneration of variants selected in cell culture and expression of the selected trait at the plant level

Regeneration of variants selected in cell culture makes it possible to trace and establish the relationship between various molecular and developmental processes and to analyse genetically the induced variation by classical test-crosses. The discussion will concentrate on mutants isolated in cell culture that are stably expressed and/or transmitted sexually in the regenerated plant.

1 Regeneration and cloning of selected variants

Fertile plants have only occasionally been obtained from variants selected in established cell cultures (Table 2). Protoplast systems should avoid many of the drawbacks related to regeneration of variant lines. Routine procedures for plant regeneration have been established in several protoplast systems for primary callus cultures. Three major factors intervene and interact when regenerating variant cultures: the mutagen treatment, the type of gene affected by mutation and revealed during selection (i.e. the selected trait), and the in vitro passage.

Mutagenesis per se can influence the morphogenic capacity of cell cultures. Certain aspects have been discussed earlier (Section III.2). Our own experience with regenerated variants (approximately 70 were studied in various selection experiments) in *N. sylvestris* and *N. plumbaginifolia* shows that such variants responded very differently to the induction of morphogenesis; the regenerated variants showed phenotypes ranging from normal to "teratoma"-like structures. On the other hand, multiple plants derived from the same selected callus usually produced morphologically identical plants.

The selected trait. Problems can arise in the regeneration of variant cultures if the induced mutation affects the morphogenic process directly or disrupts a regulative pathway which is involved in morphogenesis. For example, in a 5-methyltryptophan-resistant variant the altered regulation of tryptophan synthesis interfered with IAA metabolism, resulting in suppression of embryogenesis (Sung et al. 1979; also see Ranch et al. 1983). In other cases, p-fluorophenylalanine (Flick et al. 1981) and isopropyl N-phenylcarbamate (Aviv and Galun 1977a) resistance were shown to preclude regeneration in tobacco variants. The production of "suicide" concentrations of phenolics, frequently associated with p-fluorophenylalanine resistance (Gathercole and Street 1978; Berlin 1980) could provide an explanation of a failure of regeneration in certain circumstances. Ethionine- and FUdR-resistance were associated with unusually high frequencies of morphological abnormalities in regenerated alfalfa or tobacco plants respectively (Reisch and Bingham 1981; Chaleff and Keil 1982). Among such mutants some might correspond to what Parke and Carlson (1979) described as "developmentally sensitive".

The in vitro passage. Mutant selection usually necessitates additional steps (confirmation, cloning, establishment of defined nutritional requirements) and therefore delays regeneration in variant cultures by prolonging the in vitro stage. One consequence may be an increased nonspecific variability due to the in vitro culture phase (review by Larkin and Scowcroft 1981). By their genetic constitution, true diploids such as *N. sylvestris* and *N. plumbaginifolia* may be more susceptible than polyploid species such as tobacco both to the action of factors generating somaclonal variation and to the mutagen treatment per se (Section III.2 and Negrutiu et al. 1983a). Secondly, it may alter the sequence of stimuli controlling morphogenic responses as established in primary callus cultures (Negrutiu 1978). In the case of resistant mutants the ideal situation would be to combine the confirmation step with regeneration while selection pressure is still being applied. This is not always possible as cell cultures and regenerated shoots may exhibit a different sensitivity to selection agents.

Optimized mutation – selection conditions combined with an early regeneration of selected variants into fertile plants are expected to reduce to a minimum the time required to obtain backcrossed or selfed progenies of the variants. In tobacco this interval was estimated from 2–3 years (Maliga 1980) to 9–18 months (Bourgin 1978a). An example based on results obtained in our laboratory with protoplast cultures of *N. sylvestris* and *N. plumbaginifolia* is presented in Table 1 where the time to F1 was approximately 6 months. The timing of various steps is likely to close to the theoretical limits of these two protoplast systems. Selection schemes using microspore cultures may further reduce the time required to obtain mutated progeny. The comparison with *Arabidopsis thaliana* "whole plant"-system indicates the limits of plant experimental systems when compaired to microbial ones.

Cloning of selected variants. The problem will be briefly evoked here. It is important to note that an in vitro regenerated plant may derive from more than one initial cell (Thran Than Van et al. 1974; Sree Ramulu et al. 1976). Formation of both normal and mutant plants from a given variant suggests that it was chimeral (for example, glycerol-utilizing callus cultures of tobacco – Chaleff 1981). The design of the selection scheme can be critical in determining whether chimeric cell "lines" will be recovered (Chaleff 1981): the degree of cross-feeding and protection of wild type cells by resistant cells inside the colony may vary from one selection scheme to another.

From the practical point of review four situations can be considered when a selected variant is regenerated: 1) all the regenerants express the selected trait; 2) none of them do so, but the selected character can be recovered (a) or not (b) by reinitiating cell cultures from the regenerated plants (Sung and Jaques 1980; Maliga et al. 1980; Chaleff 1981); 3) both "expressing" and "non-expressing" plants are regenerated (Widholm 1974; Berlyn 1980; Maliga et al. 1980; Chaleff 1981). If expression is obtained following regeneration (situation 1, 2a and 3, we recommend that cloning is done from regenerated variant plants.

Cloning of isolated variants can facilitate their genetic and biochemical analysis, as discussed by Carlson and Widholm (1978) in their work on 5-methyltryptophan-resistant strains of potato. In addition, protoplast isolation in view of cloning might also represent a substitute to replica plating, an unsolved problem in plant cell cultures.

2 Designing selection schemes – implications in the expression of altered phenotypes at plant level

Approximately 75 reports on selection of biochemical variants from plant cell culture systems have been surveyed by us. Only those variants presented in Table 2 (mutations of the "nuclear" type, most belonging to the class of amino acid and amino acid analog-resistants) have been analysed for expression at regenerated plant level. It is significant that more than three-quaters of them date from the last four years. Several reports have been recently published on the isolation of various auxotrophic lines in tobacco, *D. innoxia, N. plumbaginifolia,* and *H. muticus* (Pental et al. 1982; King et al. 1980; Sidorov et al. 1981; Strauss et al. 1981; Gebhardt et al. 1981; Shimamoto and King 1983; Negrutiu 1983), some of which have been regenerated into plants. The fact that an increasing number and wider spectrum of auxotrophs are being isolated is essential as it invalidates at least some of the assertions developed by Li et al. (1967), Redei (1975), Aviv and Galun (1977b) concerning the possible drawbacks in isolating auxotrophs in green plants.

It should be noticed that most of the reported variants selected in mesophyll protoplast cultures have been regenerated into fertile plants, while only 17% (i.e. 12 variants, 8 of which in tobacco) of those selected from suspension or callus cultures were able to produce such fertile regenerants. This indicates that protoplast systems are a valuable material in such mutation – selection experiments.

Screening for mutants in plant cell cultures has opened new lines of investigation in eukaryotic systems. They represent a rather unique opportunity to study the relationship between regulatory processes at cellular and whole plant level. This is highly relevant to fundamental and applied problems as well. The available experimental data enable us to outline the following picture.

The screening process. Similar selection schemes can produce different variants in different plant species, as shown in bacteria (Umbarger 1971). Different cellular mechanisms within a particular species might be implicated in the resistance to a single toxic compound. This has been demonstrated by Bode and Birnbaum (1981) in the fungus *Hansenula henricii* where four classes of 5-methyltryptophan-resistant mutants were isolated: permeation, feedback insensitive, increased specific activity of anthranilate synthetase, and derepressed anthranilate synthetase mutants. In tobacco, 31 variants resistant to p-fluorophenylalanine were classified by Berlin (1980) as permeation variants, overproducers, and variants exhibiting increased activity of phenylalanine ammonia-lyase.

Several factors have to be considered when screening for a particular mutant phenotype in a given plant species: the affinity of the selection agent for various intracellular targets, the strength of the selection pressure, the ploidy level and tissue-origin in the cells, the cell culture system employed, the composition of the culture medium etc. (Negrutiu et al. 1983a, b). By monitoring these factors one should be able to specifically select for a particular phenotype.

The specificity of selection. It is critical to determine if plant cells preferentially develop mechanisms of resistance at the site(s) of maximal analog toxicity. Several examples in Table 2 demonstrate that such a specificity of selection usually operates. However, less specific changes may also occur. In such cases ("non-specific" selection), the screening procedure reveals the modification of more general molecular mechanisms than those expected from a specific response to the selection pressure (such as changes in Km properties of certain enzymes, transethylation processes). Alternatively, the same altered phenotype can be obtained using different selection schemes. Lack or very low affinity of the

selection agent towards one of the possible intracellular targets (i.e. false-feedback inhibition versus incorporation) can also result in a non-specific response to selection in the case of mutations occurring in that particular mechanisms.

Two examples will illustrate this notion. In glycine hydroxamate-resistant variants of tobacco (Lawyer et al. 1981) the resistance was due neither to a modified rate of uptake nor to degradation of the toxic compound, nor to a modification of the glycine decarboxylase, the enzyme specifically inhibited by glycine hydroxamate which might a priori be the expected site of alteration. Instead, the mechanism of resistance was believed to lie in the accumulation of higher levels of all free amino acids in the resistant lines. Alteration of a common (general) permease for amino acids is another example. The properties of valine-resistant recessive mutants in tobacco (Bourgin et al. 1980a and personal communication) appear to fit such an interpretation.

Another source of variation is revealed trough selection in cell cultures: cell-variants[1] should be identified under rather diverse screening conditions. For example, Matthews et al. (1980) isolated an S-aminoethylcysteine-resistant line in carrot cell suspensions which showed a rather complex pattern of modifications in the activity of pathway-specific enzymes and in uptake properties; it was believed to result from a process of cell-variant selection (also see Phillips et al. 1981). Certain conditions (the cell culture system employed, origin of explant, plant species) may favour the occurrence of cell-variants.

Expression at plant level. Two observations have to be taken into account. Firstly, selection of mutants in cell culture is presently limited to those functions (i.e. basic cellular functions, primary metabolism) that are effectively expressed in vitro (Parke and Carlson 1979; Chaleff 1981). Secondly, more and more evidence is available (Chaleff 1983) that cultured cells do not express the same number and spectrum of genes as the whole plant and certain genes which are expressed in cultured cells may not be active in the plant. Somehow this was to be expected: a "whole plant" consists already of complex patterns of differential gene expression among various organs, tissues.

The problem of expression at such different levels as a mature plant and an isolated cell is therefore very critical for the exploitation of the variation obtained by selection and appears to be complex. Expression at plant level of phenotypes selected in cell culture may preferencially occur 1) in variants exhibiting "specific" changes (see above), 2) when the changes concern basic metabolic processes, or 3) when similar regulation patterns opperate in both plant and derivative tissue cultures. Several examples in Table 2 illustrate these points. Occurrence of similar regulation patterns in cell culture and plant tissues with subsequent expression of modifications of such control mechanisms in variant plants or their progeny is better documented in reports on lysine + threonine-resistant mutants in maize (Galbraith et al. cited by Chaleff 1981) and carrot (Cattoir et al. 1983).

On the other hand, a differential regulation of control mechanisms in plants and derivative cell cultures has also been established (Matthews and Widholm 1978). This could result in differential or lack of expression of a selected trait in regenerated variant plants. Several examples in Table 2 show that the modified trait was expressed only in derivative cell cultures of regenerated variant plants but not in seedlings or plants themselves (i.e. resistance to 5-methyltryptophan, isonicotinic acid hydrazide, hydroxyurea, carboxine or methothrexate, and glycerol-utilization). For most of them the regulation mechanisms involved are yet to be established. However, in the tobacco variant resistant to 5-methyltryptophan it was shown that leaves of the regenerated resistant variant did not express the altered feedback resistant form of the anthranilate synthetase, which in turn was present in derivative cell cultures from several variant plants. On the contrary, another tryptophan overproducer isolated in cell culture of *D. innoxia* expressed an altered form of anthranilate synthetase in both variant cultured cells and regenerants (Ranch et al. 1983).

In the case of variants resistant to hydroxyurea and carboxine, and those utilizing glycerol it appeared that, despite lack of expression at plant level (repressed genes), the selected trait was transmitted sexually as shown by segregation tests in derivative callus cultures of progeny plants. Two more aspects will be evoked here. Firstly, it has to be emphasized that differential regulation patterns can operate among plant organs and result in "organ-specificity" of expression for a selected phenotype. So far this was established in an uptake mutant resistant to S-aminoethylcysteine in barley (Bright et al. 1979; also see Zelitch and Berlyn 1982). A systematic checkup of various mutants selected in plants can provide further evidence in this respect. Secondly, the occurrence of cell-variants (see above) actually reflects the existance of such differential regulation patterns among plant organs and tissues. Cell-variants, by their intrinsic nature, are expected to recover the wild type condition through regeneration and, contrary to induced genetic changes, not to be transmitted through seeds.

Genetics versus epigenetics. The above discussion implicitely refers to the genetic-epigenetic problem. From the small

1 Note the difference between variants, lines, or somaclones (Maliga 1976; Larkin and Scowcroft 1981), and cell-variants (preexisting, tissue-specific variation)

amount of genetic data available from in vitro genetic experiments it was stated that only 5–8% of the variants exhibited a meiotically transmissible trait (Christianson and Chiscon 1979 with reference to Carlson 1970 and 1973b, and Radin and Carlson 1978). Somewhat similar results were reported by Vunsch et al. (1982) in valine-resistant lines of tobacco and *N. sylvestris*. However, much more experimental data are required before conclusive statements could be made (also see Negrutiu et al. 1983a, b).

Steady developments in the somatic cell genetics of higher plants provided further evidence of how extremely complex and confusing the epigenetic-genetic controversy can appear (Parke and Carlson 1979; Chaleff 1981; also see Chu 1974; Larkin and Scowcroft 1981). Firstly, epigenetic changes may show high stability in their phenotypic expression (Chaleff 1981). Secondly, expression at plant level of a variant trait and transmissibility through a sexual cycle are not necessarily linked (see above). It has to be mentioned that even the criterion of transmissibility through a sexual cycle to progeny plants, considered to be the best way of distinguishing between genetic and epigenetic events, was challenged by Meins et al. (1981) when speaking about "inducible" genetic changes in cytikinin production by cultured cells. Furthermore, besides "classical" genetic changes (an altered phenotype resulting from a change in nucleotide sequence), we also have to take into account "mutation like" events such as gene amplification, occurrence of transposable elements etc. (Filner 1980; Larkin and Scowcroft 1981; Yamaya and Filner 1981) which have to be classified as genetic events but are not always stably inherited in a Mendelian fashion.

The significance of all these results and their implications in selection strategies are manyfold, but ultimately depend on the objectives to be reached. The molecular basis of the differential expression of regulatory processes at cellular and whole plant level are rather poorly understood at present. It has been suggested that alternate (Li et al. 1967; Parke and Carlson 1979), or dominant (Thorpe 1978) pathways might operate and be responsible for switching on or off the regulatory loops at different stages of differentiation, or modify the relative proportion among the elements of such regulatory mechanisms. However, we are ignorant of where and how such alternate pathways work. Further isolation and characterization of altered phenotypes by means of cell culture techniques should help us understand the developmental implications of these functions, the possible interactions between various developmental processes (developmental interlock), as well as the part epigenetic changes play in the regulation of various basic and developmental processes. The use of appropriate methodological tools is of primary importance in this respect: it was the purpose of this review to assess the potential of protoplast systems based on recent results in mutagenesis and selection of biochemical mutants. As a further step, establishment of more elaborated correlative criteria between cellular and developmental functions is expected to impulse the contribution of somatic cell genetics to both basic knowledge and plant breeding.

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