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Plant protoplasts as genetic tool: selectable markers for developmental studies

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ABSTRACT Protoplasts have usually been presented as a methodological tool. Even as such, they make possible an impressive array of applications in plant biology. Here we report on the use of protoplast-derived selectable markers in the study of several disturbed genetic systems with obvious effects on plant development: (1) auxotrophic mutants and the control of amino acid biosynthesis and transport in vegetative and reproductive tissues; (2) introgression of alien genetic information across phylogenetic boundaries by protoplast fusion, a consequence of controlled dedifferentiation-redifferentiation processes and attenuated incompatibility reactions in cultured cells; (3) expression (in)stability of foreign genes in transgenic plants during successive meiotic generations and in crosses between independent transformants.

KEY WORDS: *protoplasts, selectable markers, amino acid auxotrophs, asymmetric somatic hybrids, gene transfer and stability*

Introduction

Plant protoplasts have been in constant use for more than two decades, and have become one of the most versatile analytical tools in plant biology. They can be isolated in large quantities from a variety of tissues or organs. Freed from the cellulosic cell walls, the plasma membrane becomes accessible for investigation or purification. The relatively homogeneous population of individualized wall-less cells can be subjected to various experimental treatments. Depending upon the species and culture conditions, the protoplasts can divide mitotically and proliferate clonally. Eventually they can be induced to differentiate into shoots, roots or embryos and produce a complete plant.

These characteristics of plant protoplasts make them the material of choice for the following applications:

(1) Isolation of valuable biochemical mutants, with emphasis on recessive metabolic mutations that are difficult or impossible to obtain in mutagenized seed populations (Jacobs *et al.*, 1985, 1987; Cammaerts *et al.*, 1989).

(2) Introduction of foreign DNA into host genomes: (a) via protoplast transformation, probably the most universal method of gene transfer (Gharti-Chhetri *et al.*, 1992) and (b) via protoplast fusion, a tool to «illegitimately» transfer nuclear and/or organellar genetic information beyond phylogenetic boundaries (Negrutiu *et al.*, 1989).

(3) Transient gene expression experiments with considerable implications in (a) elucidating tissue-specific regulation and *cis*- and *trans*-regulatory interactions among foreign and/or endogenous genes, (b) analyzing translational processes independent of transcriptional events. Examples from the animal field concern the elucidation of the regulatory cascades of genes controlling pattern formation in the embryonic development of *Drosophila* (Belote, 1989), while in plants gene regulation in the photosynthetic pathway (Sheen, 1990) or seed storage proteins (Hilson *et al.*, 1990) have been reported.

(4) Molecular cytogenetics, with particular emphasis on *in situ* hybridization for gene localization on metaphase chromosomes. Protoplasting techniques from root meristems contributed to the development of reproducible and sensitive methods of gene mapping in plants (Mouras *et al.*, 1987; Mouras, 1991), one example being the identification in *Agrobacterium*-induced tumor cells of «marker» chromosomes containing T-DNA sequences (Mouras and Negrutiu, 1989).

(5) Flow-cytometry experiments, comprising cell-cycle analysis and chromosome sorting (Brown *et al.*, 1991; Veuskens *et al.*, 1992). This opens up the possibility of constructing chromosome-specific

Abbreviations used in this paper: BudR, 5-bromodeoxyuridine; R_1, plants obtained by selfing; F_1, plants obtained by back-crossing a mutant or an asymmetric hybrid with the wild type; F_2, plants obtained by selfing F_1 plants; BC_2, plants obtained by back-crossing again an asymmetric hybrid with a diploid wild type.

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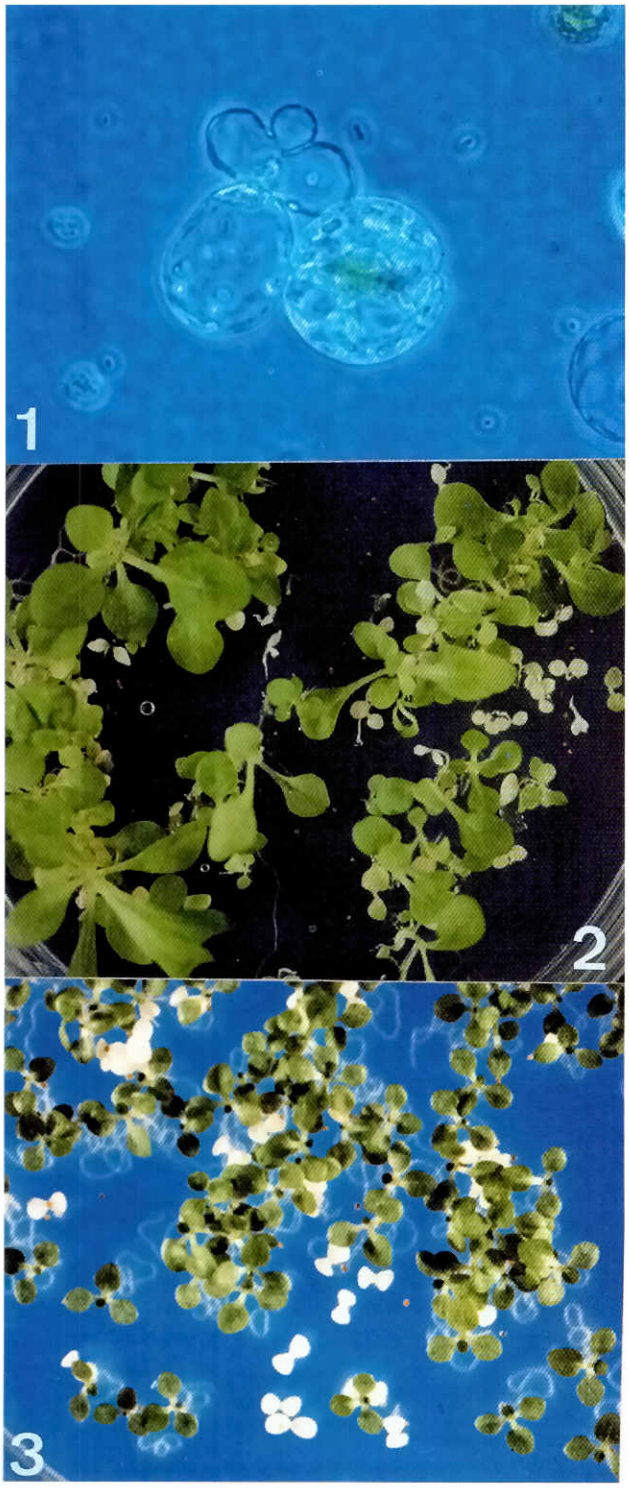


Plate I. Two selectable markers produced in protoplast culture. (1) *Protoplasts after 48 hours in culture: a dividing protoplast showing an unequal division and a budding protoplast.* **(2)** *Kanamycin resistant and sensitive progeny in a transgenic* N. plumbaginifolia *plant indicating high level of expression and monogenic inheritance (cf. Gharti-Chhetri* et al., *1990).* **(3)** *A nitrate reductase-deficient mutant isolated in a haploid protoplast culture of* N. plumbaginifolia. *The mutation segregates as a single recessive locus in the F_2 generation (cf. Negrutiu* et al., *1983).*

libraries or performing transfer experiments with purified chromosomes.

(6) Cloning of large DNA inserts (megabase cloning) in DNA preparations from agarose-embedded protoplasts associated with DNA analysis and long-range mapping via pulsed-field gel electrophoresis (Ferretti *et al.*, 1991; Siedler and Graner, 1991).

Protoplasts as a tool for studying developmental processes

Some of the above-mentioned applications have already been explored for a better understanding of developmental aspects of plant biology. For example, in a model system such as *Nicotiana plumbaginifolia* (Plate I/1), protoplasts have been used to select for fully defective mutants in the various components of the nitrate reductase (NR) complex, an enzyme of particular significance in plants (Caboche and Rouze, 1991). Another rare class of mutants, namely that of auxotrophs defective in defined steps of amino acid biosynthesis, has been produced exclusively from haploid protoplast cultures. In both classes, regenerated mutant plants made possible a genetic evaluation of various complementation groups and an assessment of inheritance pattern for these traits. Our results invalidated, for example, speculations on a constitutive inability of NR mutant lines to differentiate into complete plants (Marton *et al.*, 1982). Furthermore, protoplast technology made possible a directed transfer of genetic information among species via:

*organelle transfer, such as mitochondria, with as consequence genetic imbalance and associated cytoplasmic male sterility in cybrid plants (Pelletier, 1986).

*chromosome transfer across phylogenetic gradients, establishing parallels between sexual and somatic hybridization and incompatibility, meiotic and somatic recombination etc. (Negrutiu *et al.*, 1989).

*transfer of cloned genes, both selectable and non- selectable, with as major concern the understanding of expression behavior of foreign DNA in a new genomic environment (Gharti-Chhetri *et al.*, 1992). Some of these matters are discussed below.

A choice of selectable markers

The genetic approach we have taken involves the isolation and exploitation of selectable markers isolated in populations of protoplasts capable of sustained division and regeneration of fertile plants.

We shall concentrate on biochemical markers, obtained by mutation or transformation. Most of the marker traits have been isolated by positive selection, that is, supplementation of the culture medium by inhibitory combinations of amino acids, amino acid analogues, purine or pyrimidine base analogues, antibiotics, herbicides, heavy metals or phytotoxins or application of stress factors such as salt, chilling or drought. Two main categories of biochemical markers can be distinguished.

Dominant markers, which confer to the cell resistance against a wide range of antimetabolites. Most of them have been isolated as biochemical mutants resulting from a mutagenesis-selection procedure (Jacobs *et al.*, 1987). However, transformants in which selectable markers are cloned genes can also be included (Plate I/2; Negrutiu *et al.*, 1990). Preference was usually given to two widely used bacterial genes, namely the neomycin phosphotransferase type II (npt) gene from Tn5 and the hygromycin phosphotransferase gene (*hpt*). Both code for enzymes which detoxify aminoglycoside

TABLE 1

LIST OF SELECTABLE MARKERS USED IN VARIOUS GENETIC STUDIES MENTIONED IN THIS PAPER

Origin	Marker phenotype	Biochemical alteration	Genetical basis	Utilization
Mutagenesis-selection	Resistance to chlorate	Defective nitrate reductase	Monogenic recessive	Selection of somatic hybrids; assessment of asymmetrization
Mutagenesis-screening	Amino acid requiring such as histidine$^-$ methionine$^-$	Defective IAP amino transferase ß-cystathionase	Recessive	Metabolic complementation gene rescue experiment genetic transmission
Transformation	Resistance to kanamycine	Neomycin phospho-transferase	Foreign gene (*npt, hpt*) dominant	Selection of somatic hybrids; foreign DNA stability and transmission

compounds, such as kanamycin and hygromycin, basically absent from plant cells.

Recessive markers require the use of cell cultures derived from haploid plants and generally result from loss of activity in enzymes involved in metabolic pathways. Some of them have been isolated via a positive selection procedure, as exemplified by nitrate-reductase (NR^-) and alcohol dehydrogenase (ADH^-) deficiencies. In the case of NR^- mutants, they exhibit chlorate resistance and inability to use nitrate as the sole source of nitrogen (Plate I/3; Negrutiu *et al.*, 1983; Muller and Mendel, 1989). Auxotrophic mutants represent a category of biochemical mutants isolated by means of non-selective procedures or enrichment schemes (Jacobs *et al.*, 1987). Amino acid auxotrophs are specially considered in a section of this paper. Worth mentioning here is the successful attempt to demonstrate genetic complementation in a heterologous plant-yeast system (Colau *et al.*, 1987). The ILV-I gene from yeast was transferred to an isoleucine deficient (ile^-) mutant of *N. plumbaginifolia* and shown to restore the corresponding defective function in the plant cells.

Table 1 describes the main characteristics of selectable markers reported in this paper as useful tools in genetic studies.

Amino acid auxotrophs from protoplasts of *Nicotiana plumbaginifolia:* biased transmission of the auxotrophic trait to the progeny

Auxotrophic mutants are nutritionally deficient mutants which cannot grow on a basal medium due to a block in a biosynthetic pathway. They should only grow on a minimal medium to which the essential metabolite—an amino acid, a vitamin or a nucleic acid—is adjoined.

There are several reasons for the scarcity of plant auxotrophs. As recessive mutations, the screening for auxotrophs should be best done in haploids which are not easily available; the lack of viability of a true auxotrophic plant is surely an obstacle; genetic redundancy involving more than one isozyme for a defined step in a biosynthetic pathway may obscure the effect of a single mutation.

However, successful attempts have been reported in the last few years; in the case of amino acid auxotrophy, non-selective, total isolation methods as well as enrichment methods were developed with cultured plant cells. Haploid protoplasts appeared to be the most efficient source for isolating this class of auxotrophs (Gebhardt *et al.*, 1981; Sidorov *et al.*, 1981; Sidorov and Maliga, 1982; Shimamoto and King, 1983).

In our laboratory, 13 amino acid-deficient lines were obtained from UV-irradiated protoplasts of haploid *Nicotiana plumbaginifolia* following incubation with BUdR and recovery on complete medium (Negrutiu *et al.*, 1985). Most of the lines were characterized by precursor feeding tests, enzyme assays and protoplast fusion-complementation experiments (see Table 2).

In the case of the histidine-requiring types, three lines were first isolated and characterized by a very low activity of the enzyme imidazole acetol phosphate (IAP) aminotransferase which controls the second step in histidine biosynthesis. Complementation by protoplast fusion between these three cell lines could not be established, indicating that they belong to the same group of auxotrophs (His_1^-).

A second histidine auxotrophic line was isolated using a protoplast culture system in which non-dividing cells were separated from dividing ones by centrifugation on sucrose. Somatic hybridization between this histidine requiring type and lines deficient in IAP aminotransferase resulted in complementation, indicating the existence of a second mutation (His_2^-) in the pathway.

The methionine-requiring auxotroph lacks ß-cystathionase activity. The biochemical lesions in six other amino acid-requiring auxotrophic lines have been thoroughly investigated by means of feeding experiments with ^{14}C, unlabeled substrates and enzyme analysis. (Wallsgrove *et al.*, 1986). Three of the lines required isoleucine for growth, were unable to synthesise 2-oxobutyrate *in vivo* and have no detectable threonine dehydratase activity *in vitro*. The other three lines requiring isoleucine + valine accumulated radioactive dihydroxymethylvalerate when fed with ^{14}C-L-threonine and contained no detectable dihydroxyacid dehydratase, the third enzyme of the common pathway of the branched-chain amino acids, leucine, valine and isoleucine.

TABLE 2

AUXOTROPHIC MUTANTS IN *NICOTIANA PLUMAGINIFOLIA*

Properties	Types Ileu	Met$^-$	Ileu$^-$+Val$^-$	His_1^-	His_2^-
Requirement	ileu 0.3mM	met 0.3mM	ileu+val 0.3mM	his 0.1mM	his 0.1mM
Growth response on precursors (+)	2-oxo butyrate	homo-cysteine	-	histidinol (P)	non-tested
Defective enzyme	threonine dehydratase	ß-crysta-thionase	dihydroxyacid dehydratase	IAP amino-transferase	non-tested
Fusion comple-mentation	with Met_1^-	with Ileu His_1^-	non-tested	with His_2^- with $Tryp^-$ with NR^-	with His_1^-
Plant phenotype	rosette no shoots	dwarf narrow leaves	abnormal rosette no shoots	dwarf plant	dwarf plant
Sexual transmission	no generative organs	+	no generative organs	+	+
Pollen fertility	-	++	-	+++	++++

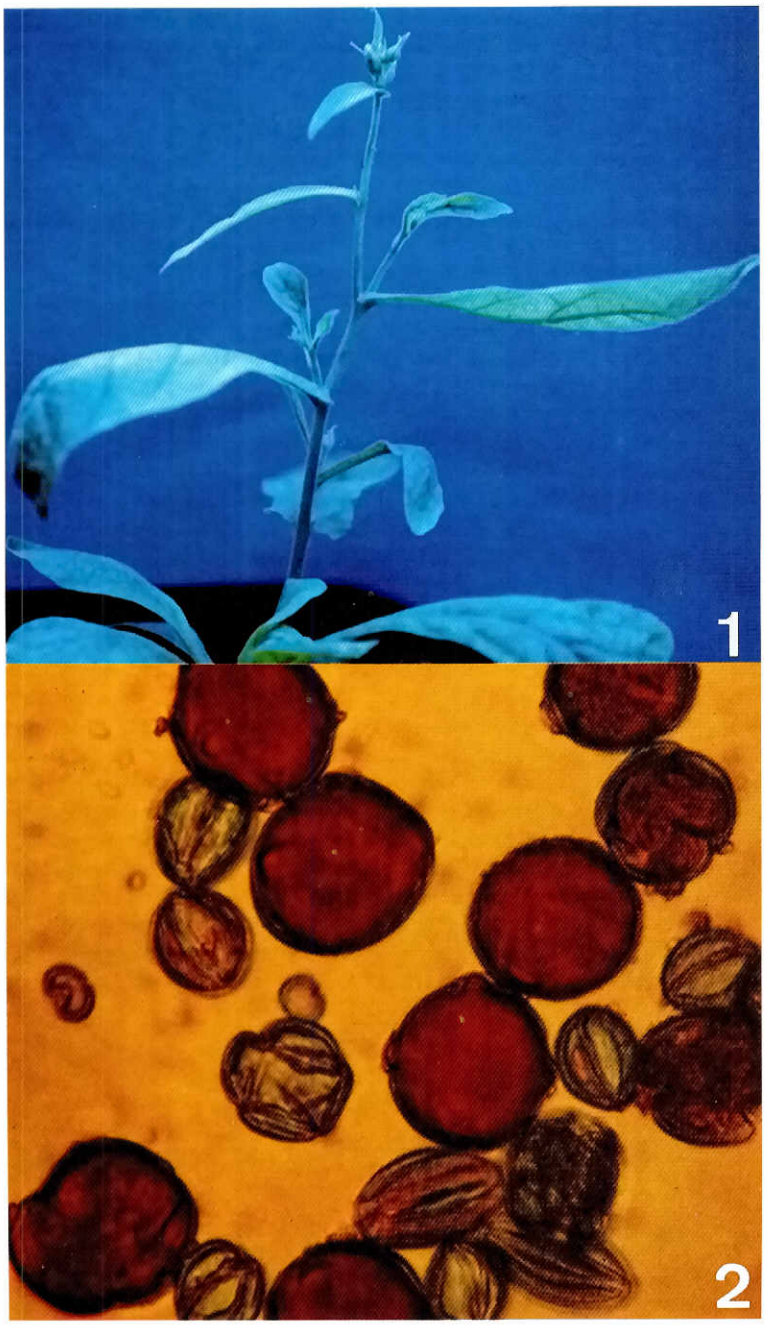


Plate II. The methionine deficient mutant Met$^-_1$ (10.1-9) of *N. plumbaginifolia* (1) *The homozygous recessive regenerant upon transfer to the greenhouse. Terminal inflorescence and axillary shoots on a dwarf-type plant (height± 12 cm).* **(2)** *Pollen from mature flowers of the above plant showing a complete gradient of sterility to fertility. Alexander's stain was used.*

Among these amino acid auxotrophs, only the histidine and methionine-requiring types (His$^-_1$, His$^-_2$, Met$^-$) were regenerated into fertile plants (Plate II and III). Their development strictly depends on histidine or methionine supplementation, either by spraying or by root feeding in a hydroponic system. The sexual transmission of the auxotrophic trait was analyzed in detail in progeny obtained from selfed regenerants and after crosses with the wild type.

Expression of amino acid auxotrophy in the progeny of homozygous recessive mutants

R_1 seeds obtained by selfing His$^-_1$, His$^-_2$ and Met$^-_1$ regenerants germinate, open their cotyledons but show no further leaf or root development on non-supplemented medium. The seeds sown or seedlings transferred on histidine or methionine supplemented medium produced normal plantlets. An example is shown in Plate III/1, 2 and 5. Thus, the auxotrophy is transmitted as a homozygous trait to the progeny of first generation mutant plants.

Fluctuation in segregation rates of the auxotrophic trait in F_2 generation according to the amino acid supplementation

F_1 plants were obtained by back-crossing the three mutant types. They exhibited complete wild type phenotypes able to grow on a non-supplemented medium. When F_2 seeds were sown on minimal medium, the observed segregations indicated a marked

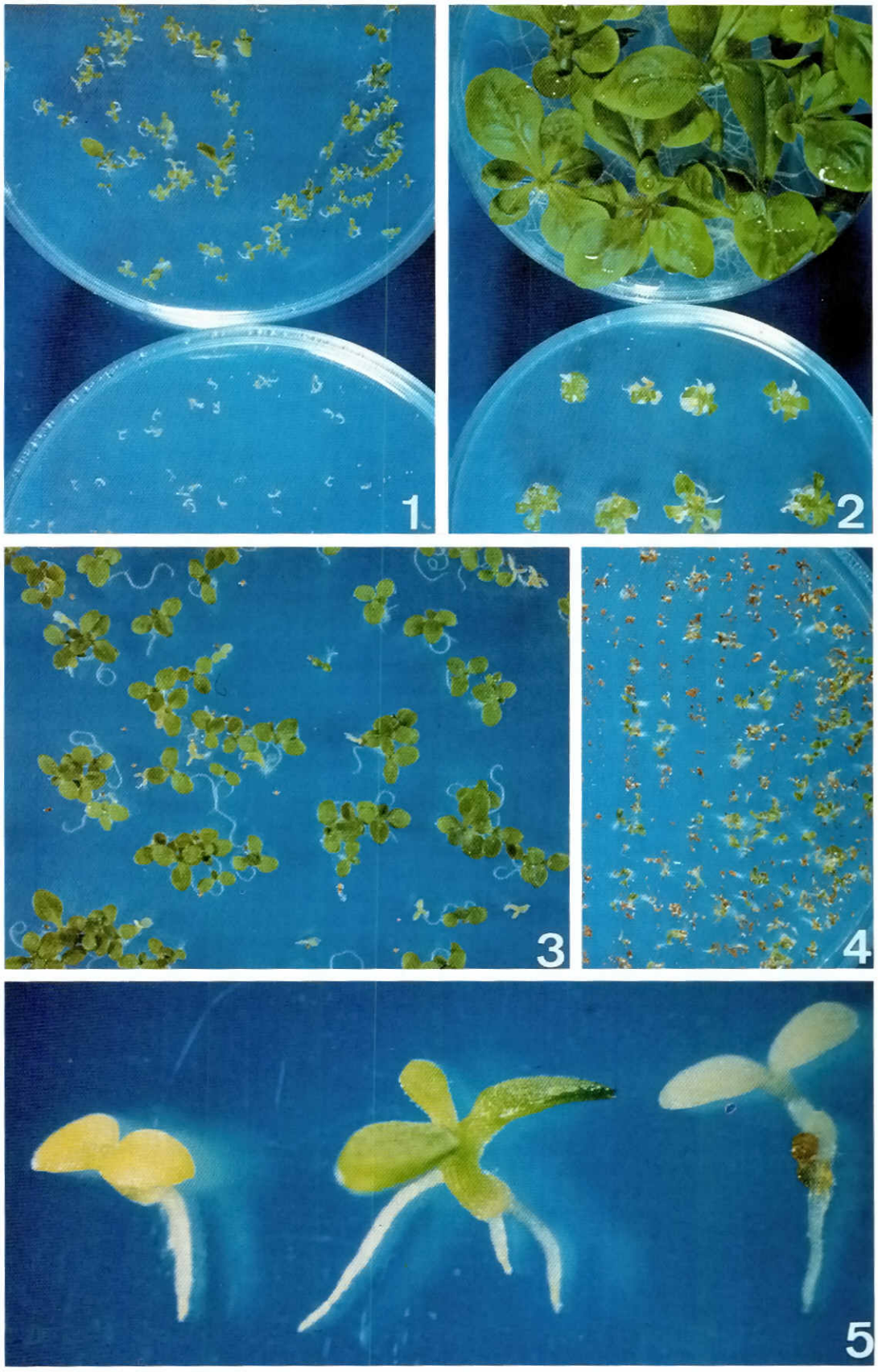


Plate III. Main characteristics of His^-_1 and His^-_2 auxotrophs in segregation tests. **(1)** *Seedlings from His^-_2 seeds obtained by selfing a homozygous mutant on minimal (bottom) and histidine supplemented medium (top).* **(2)** *Homozygous His^-_2 mutants: showing normal growth on histidine-supplemented medium (top) but stop growing when transferred to minimal medium (below).* **(3)** *His^-_2 mutant in the F_2 generation, showing three-week-old seedlings segregating on minimal medium.* **(4)** *A large number of His^-_2 seedlings grown after embryo rescue.* **(5)** *Three-week-old His^-_1 R_1 seedlings; from left to right: His^-_1 seedling on minimal medium; His^-_1 seedling on histidine-supplemented medium; an albino mutation as a comparative control.*

TABLE 3

SEGREGATION FOR AUXOTROPHY IN F_2 GENERATION FROM F_1 PLANTS SUBMITTED TO TWO TYPES OF TREATMENT: SPRAYING WITH AMINO ACID OR NOT (1.5mM) AND BY ADDITION OR NOT OF AMINO ACID (0.1 mM)ON EMBRYO RESCUE MEDIUM

Spraying	+	-	+	-	
Embryo rescue with histidine or methionine in the medium	+	+	-	-	
F_2 progeny from selfed F_1 back-cross	+/- (ratio)	+/- (ratio)	+/- (ratio)	+/- (ratio)	Theoretical ratio for a recessive trait
His^-_1xWT	1101/132 (8:1)	308/18 (37:1)	412/0 -	219/0 -	3:1
His^-_2xWT	612/31 (20:1)	3243/102 (32:1)	7074/794 (9:1)	8448/34 (249:1)	3:1
Met^-_1xWT	937/65 (14:1)	1983/103 (19:1)	3685/6 (614:1)	4017/16 (251:1)	3:1

Segregation ratios were evaluated on minimal medium: (+) growing, (-) not growing.

underrepresentation of His^-_2 and Met^- classes and even a total lack of His^-_1 auxotrophs (Table 3, Plate III).

Therefore, F_1 plants were supplemented with the corresponding amino acid, by spraying histidine or methionine and/or by culturing dissected embryos obtained from *in vitro* pollinated flowers on a medium supplemented with the required amino acid (embryo rescue, Plate III/4).

The results in Table 3 show that for His^-_1, segregation rates of respectively 11% and 6% could only be obtained by embryo rescue on a histidine-containing medium associated or not with histidine spraying on F_1 plants. For His^-_2 the segregation of the auxotrophic trait was increased significantly by spraying, while embryo rescue did not improve the amount of His^-_2 auxotrophs in F_2 compared to the spraying treatment above. For the Met^-_1 trait, the class of auxotrophic segregants increased to 5% when embryos were rescued on methionine-supplemented medium and to 6.5% when the same treatment was combined with the spraying of the F_1 plant.

In conclusion, these results show that a continuous supply of the corresponding amino acid is necessary for the growth, development and seed production of the histidine and methionine-less mutants. Both traits are sexually transmitted upon selfing and the seeds obtained from both homozygote mutants are totally dependent on histidine or methionine supplementation for their growth. The way the auxotrophic trait is transmitted to progeny after back-crossing is rather different. Histidine spraying during the growth of the F_1 plants was sufficient to markedly increase the amount of His^-_2 auxotrophs in F_2 progeny. For His^-_1 and Met^-_1 mutants, embryo rescue on an amino acid supplemented medium was required to recover the auxotrophic trait in a substantial ratio in F_2 generation.

Our results suggest that it is imperative to ensure availability and translocation of the required amino acid at the time of zygote formation and throughout the development of the embryo. This explains why auxotrophic progeny do not segregate out in F_2 generation unless an external amino acid supply is provided to the heterozygous F_1 plants. This also explains previous failure in obtaining amino acid auxotrophs in M_2 progeny of mutagenized seeds. In one case, tryptophan auxotrophic mutants were obtained by M2 seed screening and the Mendelian 3:1 segregation in F_2 showed that the nutritional deficiency was inherited as a simple monogenic trait (Last and Fink, 1988).

Our data do not allow us to conclude that the his^- and met^- traits are single gene mutations, but raise questions on the translocation process of amino acids from leaves to seeds, on their interconversion in reproductive tissues and availability to the zygote and embryo.

Chromosome transfer: asymmetric hybridization, phylogenetic gradients and expression constraints

Symmetric and asymmetric hybrids refer to the nuclear constitution of fusion products: symmetric hybrids consist of complete sets of chromosomes from the two parents, while asymmetric ones undergo a preferential or unidirectional elimination of chromosomes belonging to one of the fusion partners. Highly asymmetric hybrids are defined as containing less than 10% of the chromosomes from the donor partner.

The method of choice in producing asymmetric somatic hybrids is the donor-recipient technique in which recipient protoplasts are fused with irradiated (sub- or lethal doses) donor protoplasts.

Following irradiation of the donor partner, the degree and speed of asymmetrization depends on several factors, among which the most relevant are the phylogenetic distance (i.e., the extent of various incompatibility processes), chromosome number, morphology and size, cell cycle duration and degree of chromatin condensation, differentiation status of the cells to be fused, sequence of *in vitro* culture steps before morphogenesis is induced, presence or

TABLE 4

EFFECT OF THE SELECTION PRESSURE ON THE EXPRESSION OF MARKER GENES IN FUSION PRODUCTS BETWEEN *N. PLUMBAGINIFOLIA* (R) AND *N. TABACUM* (D)

Selection pressure	Number of tested	Number of regenerating	Recipient type regenerants			Aberrant regenerants		
			NR^+KM^R	NR^+KM^S	NR^-KM^R	NR^+KM^R	NR^+KM^S	NR^-KM^R
NO_3^-	377	181	7 (3.8)	11 (6.0)	0	37 (20.5)	126 (69.6)	0
Km	184	70	1 (1.4)	0	13 (18.6)	23 (32.9)	0	33 (47.1)
NO_3^-Km	57	14	5 (35.7)	0	0	9 (64.3)	0	0

The recipient R is a NR^-/cnx type mutant, the donor (D) being a Km^R SR_1 tobacco. Percentages are given in brackets.

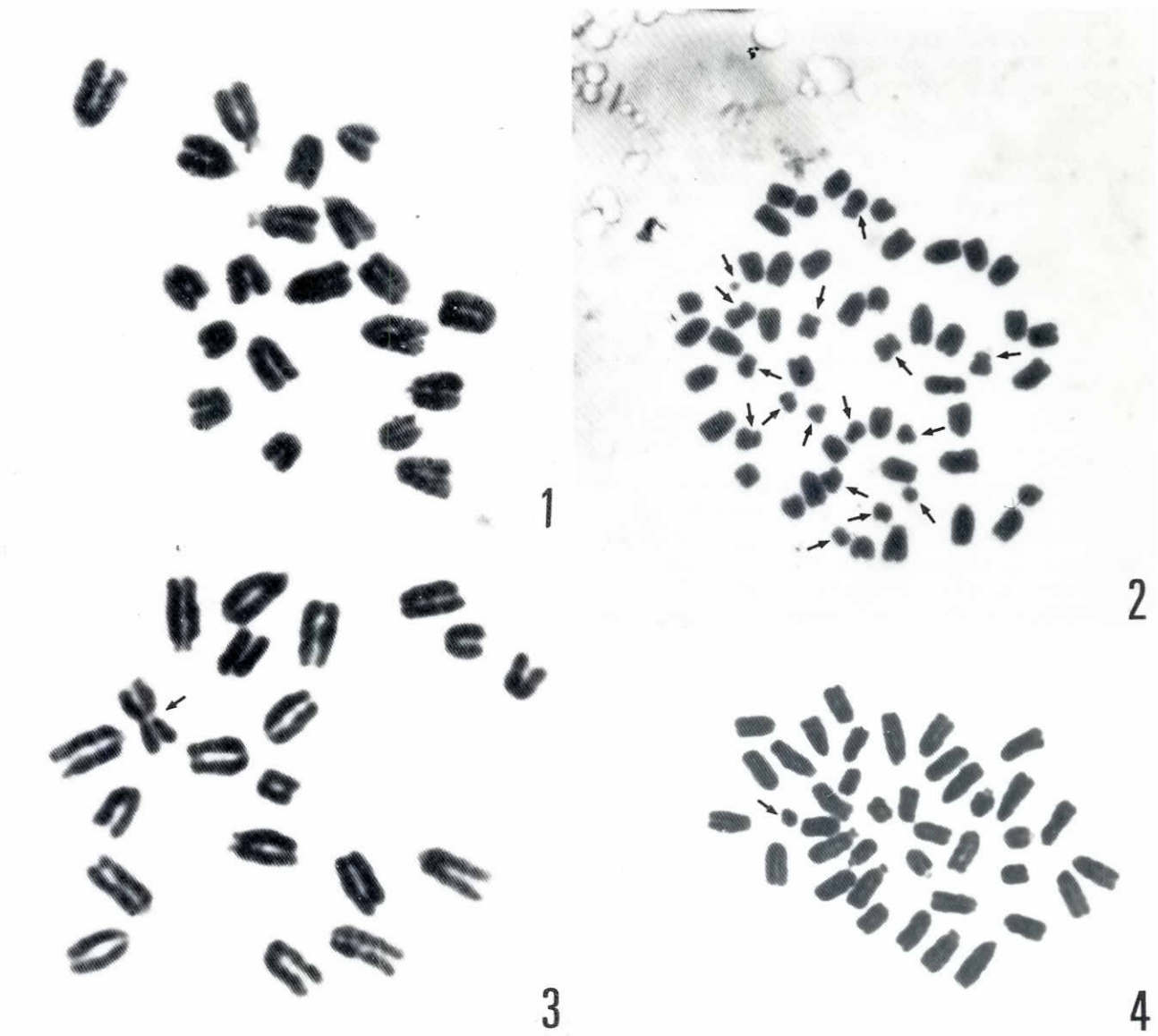


Plate IV. Degree of asymmetrization in fusion products between *N. plumbaginifolia* (recipient) and *N. sylvestris* or *Petunia hybrida* (donors). Chromosome preparations are from R_0 plants (primary regenerants) which have not passed meiosis. (1) *A control metaphase plate showing the chromosome morphology (all telocentrics) of* N. plumbaginifolia, *the recipient fusion partner (2n=20).* **(2)** *An asymmetric fusion product* N. plumbaginifolia + N. sylvestris *(2n= 24) with numerous fragmented donor chromosomes present (arrowed).* **(3 and 4)** *Two highly asymmetric lines in the combination* N. plumbaginifolia + P. hybrida *(2n= 14), one with a metacentric chromosome (hybrid PI) (3), the other with a minichromosome (4) (arrowed).*

absence of selection pressure and alternance of somatic (such as embryo rescue) and sexual cycles of regenerated fusion products (Negrutiu *et al.*, 1989).

One of the major issues in somatic hybridization is gene introgression via legitimate or illegitimate recombination. Any attempts to control such processes have to take into account the fact that the fate of parental genomes in fusion products in culture is first of all dependent on the dynamics of chromosome distribution in time and space within the heterokaryon: chromosomes are known to have specific organizational relationships with the nuclear membrane and between them. During the very first division cycles the separation pattern of the two genomes is «sectorial», changing with time into a «radial» pattern (Gleba *et al.*, 1987). It is obvious that the sectorial pattern during the first mitotic divisions is a major handicap in facilitating mechanical opportunities for recombination. This is of particular importance in cases where irradiation is being used to direct elimination of donor chromosomes: chromosome fragments, which are the major source of recombination material, are eliminated during the first mitotic divisions. Actually the system selects for fragmented chromosomes with functional centromeres

TABLE 5

EVOLUTION OF CHROMOSOME NUMBER FROM RECIPIENT AND DONOR PARTNERS IN *N. PLUMBAGINIFOLIA* + *A. BELLADONNA* FUSION PRODUCTS IN F_1 VERSUS BC_2 GENERATION

Sexual generation	Number of chromosomes per metaphase	Number of *N. plumbaginifolia* chromosomes	Number of *A. belladonna* (mini)chromosomes
F_1 (7 plants)	29.7 - 31	28-29	1.7-2.1
BC_2 (6 plants)	23.3-24.1	21.5-22	1-1.1

Figures represent range of variation as established in at least 6 distinct lines. 45 metaphase plates were analyzed per progeny

(Plate IV) . Their subsequent elimination depends on factors such as phylogenetic distance, type of selection pressures applied (metabolic and/or morphogenetic) and opportunity to perform backcrosses (passage through meiosis). The specific role of such parameters is detailed in Table 4, 5 and 6. The results show that:

(1) The irradiation determines the direction of elimination but not the extent of elimination within the donor genome. This suggests that the number of induced breaks is relatively small and that the recipient cells have the ability to efficiently rescue the damaged chromosomes by cross-acting repair mechanisms. The extent of asymmetrization in fusion products increases with the phylogenetic distance, the transfer of limited amounts of genetic information to the recipient species being more rapidly achieved in wider cross combinations (cf. Plate IV).

In most intrageneric combinations, a limited elimination of donor chromosomes seems to be the rule. The degree of asymmetrization was by far more important in intergeneric hybrids or, as reported by others, in interfamily combinations (Dudits *et al.*, 1979). Within this general frame, the outcome of a novel combination is unpredictable, strongly depending on additional parameters such as chromosome number in the donor, chromosome morphology (telomeric versus metacentric) in the recipient, duration of defined cell cycle stages and premature chromatin condensation, etc.

(2) Selectable marker genes that act as early as possible after fusion (e.g., resistance to kanamycin) in combination with *in vitro* culture and regeneration conditions specific to recipient partner undoubtedly favor the production of highly asymmetric hybrids. Donor marker genes used for fusion product selection appeared to be relatively stably expressed under selection conditions, while non-selectable genes, such as isozymes or opine genes exhibited a highly variable level of expression (Gleba and Sytnik, 1984; Famelaer *et al.*, 1989). Interestingly, in many asymmetric hybrids under investigation, changes in the copy number of rDNA from both parents were shown to occur rather independently of other (isozymes, RFLP) markers (Miesfeld and Arnheim, 1984; Pijnacker and Ferwerda, 1987; Gleba *et al.*, 1988; Moore and Sink, 1988). The physical presence of other alien genes may not necessarily result in appropriate expression, both because one of the adverse side-effects of irradiation is inactivation of non-selected genes by mutation, and because correct expression of these genes may be altered in the context of the recipient genome.

(3) The *in vitro* culture passage is perceived as an environment where developmental controls and incompatibility barriers are, at least temporarily, attenuated or absent. As a matter of fact, fusion products from a variety of wide species combinations usually proliferate actively at callus stage but fail to develop further. Furthermore, the ability of certain fusion combinations or products to regenerate, reveals built-in selection pressures that accentuate the asymmetrization process. On the other hand, an improved regeneration response is observed at higher levels of asymmetrization, this being true in several species combinations tested.

(4) Gene introgression, via legitimate or illegitimate recombination, in irradiation-induced asymmetric hybrids remains an extremely rare event, probably because the opportunities for intergenomic exchanges are rather limited and unpredictable. Since in most cases entire or fragmented donor chromosomes are retained, a Mendelian genetic transmission of donor traits is the exception rather than the rule (Table 6). It was only in a combination of somatic fusion, successive backcrossing associated with embryo rescue and ploidy reduction, that we were able to ensure an appropriate level of genomic balance and Mendelian inheritance in the intergeneric combination *N. plumbaginifolia* (2n= 20) + *Atropa belladonna* (2n= 72) (Table 5, Gleba *et al.*, 1988). In the interspecific combination *N. plumbaginifolia* + *N. sylvestris* (2n= 24), the genetic transmission could be improved from 4 to 17% with an increase in the degree of asymmetrization (Famelaer *et al.*, 1989). In both cases, the passage through meiosis was the critical factor, indispensable for both transfer of large blocks of genes and for establishing an appropriate balance in fusion products.

Ideal cases are two intergeneric combinations, namely *N. plumbaginifolia* + *Petunia hybrida* (2n= 14) and *N. plumbaginifolia* + *Lycopersicum esculentum* (2n= 24) in which rapid and extensive asymmetrization was associated with efficient production of fertile regenerants, Mendelian transmission of the selectable marker gene and evidence of illegitimate recombination events (Hinnisdaels *et al.*, 1991).

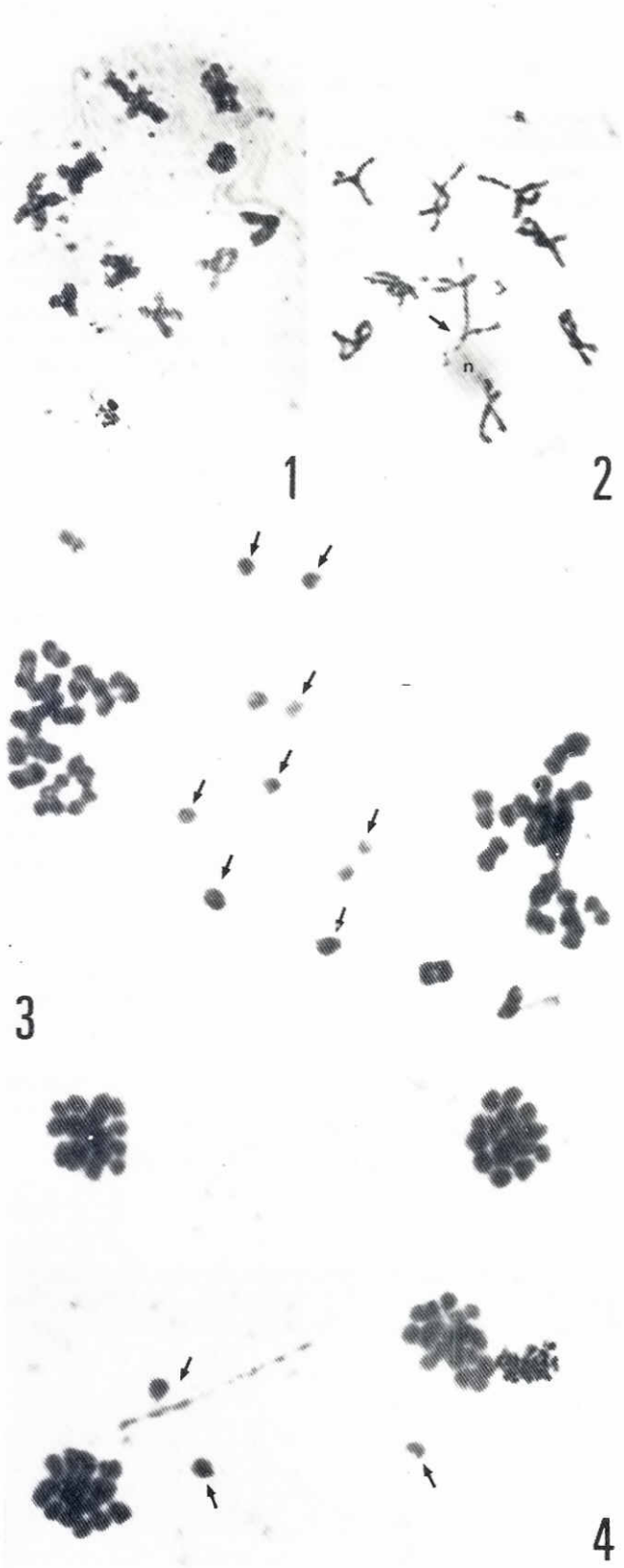
In the case of *N. plumbaginifolia* + *P. hybrida*, selection of hybrids relied on the presence of kanamycin resistance in the irradiated

TABLE 6

EFFECT OF PHYLOGENETIC GRADIENT AND TYPE OF SELECTION PRESSURE ON THE EXTENT OF HYBRID ASYMMETRIZATION AND ABILITY TO TRANSMIT THE MARKER TRAIT TO PROGENY

Recipient + donor according to a phylogenetic gradient	Selectable marker	Hybrids	Number of cases: Chromosome analysis	Highly asymmetric	Genetic transmission
NP (nia26)+NS	NR/NO_3^-	261	23	0*	9 (3%)
NP (cnx20)+NS	NR/NO_3^-	213	23	4%	36 (17%)
NP (nia26)+NT	NR/NO_3^-	25	25	0	20 (80%)
NP (ncx20)+AB	NR/NO_3^-	64	17	12%	1** (1.5%)
NP+PH	npt/Km^R	26	14	86%	22 (92%)
NP+LE	npt/Km^R	29	14	100%	27 (93%)

*14% (1/7) highly asymmetric hybrids in R_1 generation; **Embryo rescue; NP, *Nicotiana plumbaginifolia;* AB, *Atropa belladonna;* NS, *N. sylvestris;* NT, *N. tabacum;* PH, *Petunia hybrida;* LE, *Lycopersicon esculentum.*



donor species *(Petunia)*. All shoots that have been regenerated resembled the recipient partner *Nicotiana plumbaginifolia*. The presence of the kanamycin gene in hybrid regenerants has been shown at the biochemical level (by enzyme assay) and also by Southern hybridization on genomic DNA. The gene segregated in a Mendelian fashion with some slight deviations in few lines (Table 6). Ninety-two percent of *N. plumbaginifolia* + *P. hybrida* hybrids followed Mendelian segregation, 80% showing monogenic and 12% exhibiting digenic or more pattern of transmission. Genetic analysis of *N. plumbaginifolia* + *L. esculentum* hybrids also followed similar trends: 88% of the hybrids segregated for monogenic and 5% of them had digenic or multiple gene inheritance. Most of the lines analyzed only contained *Nicotiana* type chromosomes. In some lines we were able to demonstrate the presence of a few donor chromosome fragments (Plate V). In yet another line one metacentric chromosome could be identified besides a nearly complete *Nicotiana* complement (19 chromosomes) (hybrid P_1) (Plate IV/3).

Chromosome pairing at meiosis and *in situ* hybridization made it possible to demonstrate the occurrence of illegitimate recombination between recipient and donor chromosomes. Indeed, in metaphase I or at diakinesis, one arm of the metacentric chromosome paired with one chromosome of the recipient *N. plumbaginifolia* to form a heteromorphous bivalent (Plate V, 1 and 2). Moreover, this abnormal bivalent was closely related with the nucleolus, indicating the presence of nucleolar organizers in this bivalent. As we previously demonstrated that in *N. plumbaginifolia* the nucleolar organizers are present on chromosome 9 (Mouras *et al.*, 1986), the heteromorphous bivalent resulted from the translocation of a *Petunia* chromosome fragment to one chromosome 9 of *N. plumbaginifolia*. The presence of rDNA on chromosome 9 and on one arm of the metacentric chromosome has been demonstrated by *in situ* hybridization. *In situ* hybridization experiments using labeled total genomic DNA of *Petunia* as a probe, in competition with unlabeled total genomic DNA of *Nicotiana*, further demonstrated that one arm of the metacentric chromosome hybridized with the *Petunia* probe.

Direct gene transfer—from expression instability to genomic disorders

Integrative transformation of foreign genes and their fate and stability in successive generations determine the applicability of direct gene transfer. In *Nicotiana plumbaginifolia*, a true diploid species, one to 10 copies of foreign DNA usually integrate into the plant genome with simple to complex patterns of integration. In general, the passage through meiosis (comparing R_0 and R_1) will not

Plate V. Analysis of meiotic chromosome pairing in asymmetric somatic hybrids between *N. plumbaginifolia* and kanamycin resistant *P. hybrida*. **(1)** *Diploid* N. plumbaginifolia *showing 10 bivalents at metaphase I.* **(2)** *Hybrid PI, having a metacentric chromosome in the complement (see Plate IV/3). At diplotene a heteromorphic bivalent is observed being always associated with the nucleolus.* **(3 and 4)** *Different* N. plumbaginifolia + Petunia *asymmetric hybrids showing late migrating chromosome fragments (arrowed) at anaphase I and II respectively. The bivalents migrated already towards the cell poles.*

TABLE 7

INSTABILITY PROFILE OF *npt* GENE EXPRESSION IN CROSSES BETWEEN INDEPENDENT TRANSFORMANTS ACCORDING TO THE NUMBER OF INSERTION LOCI

Specification	Cross categories: Monogenic x Monogenic	Monogenic x Digenic	Monogenic x Trigenic	Digenic x Digenic	Trigenic x Trigenic
Most expected segregation profile	Monogenic	Digenic	Trigenic	Digenic	Trigenic
	Observed segregation profiles				
as expected	62	78	0	57	0
more loci expressed	10	4	0	0	0
less expressed loci	15	14	53	43	100
deviating segregations	13	3	37	0	0
Total level of instability	38	21	100	43	100

Values are expressed as % of the number of analyzed cases (a total of approx. 200 transformants). Observed segregation ratios were compared to expected ones for each analyzed combination. Constructs used are as reported in Negrutiu *et al.* (1990) and Gharti-Chhetri *et al.* (1992).

affect the overall integration profile. There is no direct correlation between copy number of inserted DNA and the number of independently segregating loci. Genetic analysis showed that in more than 50% of the cases DNA inserted at two or more loci in the genome (Gharti-Chhetri *et al.*, 1990, 1992).

The constructs used in the integrative transformation experiments reported here contain the CaMV-derived expression cassettes 19S or 35S that control *npt* (kanamycin resistance) or *hpt* (hygromycin resistance) (Negrutiu *et al.*, 1990). The transformants retained for further analysis were screened at the R_0/R_1 generation for their average to high levels of kanamycin resistance (Plate I/2). Deviations from the initial level of *npt* expression or segregation pattern during successive meiotic generations are considered as instability and/or inactivation events.

From a large sample of analyzed monogenic transformants, we concluded that the loss of foreign gene expression occurred both in successive selfing generations (up to 20% of the lines in F_3 and F_4) and in crosses between F_1 transformants (25%) or more (Table 7 and Gharti-Chhetri *et al.*, 1992).

Physical loss of the inserted gene was less than 1% among approximately 150 analyzed cases. Instead, loss of gene expression resulted from inactivation processes, such as methylation (Cherdshewasart, 1991) or extensive rearrangements of *npt* sequences, such cases probably being associated with co-suppression (Jorgensen, 1990).

Of particular relevance are the results obtained in crosses between independent transformed lines. The chosen transformants exhibited good seed setting in selfed generations indicating a stabilized genomic context. The results showed that the level of instability increased with the increase in number of insertion loci, reaching 100% whenever a trigenic partner was involved. (Table 7).

Crosses between monogenic transformants produced evidence of frequent recombination events, since as much as 10% of the analyzed cases exhibited an increase in the number of expressing loci. In crosses between digenics or whenever one of the parents was a trigenic transformant, instability was exclusively due to inactivation events. The results indicated that the new genomic context produced in crosses among independent transformants generated genetic instability at high rates. This was further confirmed in a series of diallel crosses among digenic or trigenic transformants (Table 8). In such crosses, embryo or seed abortion occurred at particularly high frequencies indicating extensive genomic imbalance in particular combinations of otherwise stabilized transgenomes (compare seed setting in selfing versus diallel crosses). A possible explanation is that insertion sites behave as areas of «genomic disorders» when combined in one individual and in a given conformation («position» effect). The above facts recall the other type or genomic disorders described in somatic hybridization experiments and demonstrate that such disorders and expression inconsistencies can be produced even in the presence of relatively reduced amounts of foreign genetic information.

Concluding remarks

Auxotrophic mutants have been an invaluable tool for the development of microbial and mammalian cell genetics, as well as selectable markers in the elucidation of biochemical pathways and their regulation.

Amino acid biosynthesis is a major event in the primary and secondary metabolism of plants. It turned out that amino acid auxotrophic mutants were difficult to isolate, as such mutations have developmental implications: the homozygous recessive mutants are dwarfs and the analysis of genetic transmission to progeny shows non-Mendelian, fluctuating segregation ratios. This can be explained by an irregular diffusion and translocation to the reproductive tissues of exogenously supplied amino acids. Genetic evidence of this sort is shedding new light on complex patterns of interaction and distinct developmental constraints in vegetative and embryonic tissues.

Data accumulated in sexual and somatic hybrids from both plants and animals share important similarities in a number of essential processes, such as chromosome elimination, pairing and recombination. In plants, as far as the obtention of highly asymmetric hybrids between phylogenetically unrelated species is concerned, the protoplast technology may represent the method of choice. Selectable and/or detectable nuclear and cytoplasmic

TABLE 8

SEED SETTING IN SELFED VERSUS DIALLEL CROSS COMBINATIONS IN TRANSFORMANTS WITH DEFINED *npt* GENE INSERTION PROFILES: MONO-, DI- AND TRIGENIC TYPE OF INHERITANCE

	Inheritance profile: Monogenics (*npt*+*hpt*)		Monogenics *npt*		Digenics *npt*		Trigenics *npt*	
Specification	+	-	+	-	+	-	+	-
selfing	100	0	96	4	100	0	57	43
diallel crosses	100	0	100	0	28.5	71.5	9.5	90.5

Successful seed setting is classified (+), lack of seed setting is classified (-). Figures represent % of the total cases analyzed (at least 7 transformants per profile, i.e., 49 combinations). Crosses between *npt* (Km^R) and *hpt* (Hy^R) monogenic transformants are presented as an additional control.

markers have played an important role in understanding and controlling asymmetrization processes in somatic hybridization experiments between phylogenetically related and unrelated species.

Functional asymmetric hybrids are restricted to interspecific, some intergeneric and few interfamily combinations, with evidence that induced somatic asymmetric hybrids can reach more rapid meiotic balance or cover a wider range of species combinations than their symmetric or sexual counterparts. Screening for recipient-type phenotypes involves identification of individuals that undergo important unidirectional elimination of donor material. As centromere retention is the major event with respect to both intra- and intergenomic recombinations in heterokaryons produced by fusion, retention of asynthenic gene combinations must also be poor.

The association of irradiation with fusion, *in vitro* culture, selection, regeneration, and passage through meiosis represents a more versatile experimental tool of gene introgression compared to the available breeding methods.

Transfer to plant cells of cloned genes under the control of plant expression signals is considered the simplest and most refined technique to genetically modify plants. We show that in a true diploid species, *N. plumbaginifolia*, the *npt* gene is incorporated in one to ten copies per genome at one or several insertion sites. The results indicated that the plant cells do «recognize» such limited amounts of foreign genetic information during several successive meiotic generations, its expression being modulated by a variety of processes: physical loss, inactivation with or without rearrangements, via methylation, co-suppression, etc. Such phenomena occur with high frequency and can result, once multiple insertion loci are combined in one transgenic plant, in genomic imbalance and disorders, such as embryo lethality.

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