Transfer of resistance traits from carrot into tobacco by asymmetric somatic hybridization: Regeneration of fertile plants

(protoplast fusion/methotrexate resistance/dihydrofolate reductase/chromosome elimination)

DENES DUDITS^{*†}, ESZTER MAROY^{*}, TUNDE PRAZNOVSZKY^{*}, ZOLTAN OLAH^{*}, JANOS GYORGYEY^{*}, AND RINO CELLA[‡]

^{*}Institute of Genetics, Biological Research Center, Hungarian Academy of Sciences, 6701 Szeged, Hungary; and [‡]Dipartimento di Genetica e Microbiologia, Università di Pavia, 27100 Pavia, Italy

Communicated by Georg Melchers, August 11, 1987 (received for review May 15, 1987)

ABSTRACT Transfer of methotrexate and 5-methyltryptophan resistance from carrot (*Daucus carota*) to tobacco (*Nicotiana tabacum*) was achieved by fusion between leaf mesophyll protoplasts of tobacco and irradiated cell culture protoplasts of carrot. Some of the regenerated somatic hybrids exhibited normal tobacco morphology with coexpression and independent segregation of the transferred resistance markers. Chromosomal instability resulted in aneuploid somatic hybrids with significantly lower chromosome number than predicted by simple addition of parental chromosome number. The methotrexate resistance phenotype was correlated with the expression of carrot-specific dihydrofolate reductase as judged by isozyme and immunological characteristics of the enzyme. The genomic construct of these somatic hybrids made the transmission of the resistance character into the next sexual generation possible.

Gene flow between incompatible species is strongly restricted by evolutionary boundaries in sexual crossings. The use of somatic cells as targets in genetic manipulation experiments, based on DNA transformation or cell hybridization, has opened new horizons for combining diverse genes from unrelated species. If the gene of interest has been cloned, advanced transformation systems may provide the most efficient way of introducing it into a foreign host genome (1, 2). When isolated genes are lacking, asymmetric hybridization mediated by protoplast fusion offers an alternative approach for alien gene transfer. The asymmetric nature of nuclear genomes in cell hybrids originates from spontaneous or induced genome instability, with preferential loss of chromosomes belonging to one of the parental species. Enforced chromosome elimination can be a prerequisite of hybrid plant production in several wide fusion combinations in which somatic incompatibility prohibits hybrid development (3–5). The transient coexistence of two diverse genomes in hybrid cells may provide the opportunity for recombination of chromosomes or a few genes from the eliminated partner. To test this hypothesis, experimental methods are needed to control chromosome elimination from fusion products. Irradiation-induced genomic fragmentation was successfully used to produce asymmetric nuclear hybrids (6–8) and to transfer cytoplasmic organelles (9, 10). Irradiating donor protoplast populations before fusion results in intergeneric somatic hybrids that have retained only one or two chromosomes and few phenotypic traits from the irradiated partner (6, 7). Since these asymmetric hybrids failed to produce progenies, sexual transmission of new characters could not be analyzed. Recently, irradiated barley protoplasts were fused with nitrate reductase-deficient tobacco

protoplasts (11). In some of the resulting nitrate reductase-positive tobacco plants, barley enzyme was detected with immunological methods. Because of leakiness of selection techniques, based on a single recessive and unstable mutation, the authors did not conclude that cell fusion-mediated gene transfer had occurred. At present, available experimental data are insufficient to explore the potential of asymmetric cell fusion for widening genetic variability.

In this paper we describe a fusion system that allowed the monitoring of two dominantly acting selectable markers in tobacco + carrot fusion products. Molecular evidence confirming the expression of carrot-specific dihydrofolate reductase (DHFR; EC 1.5.1.3) in regenerated hybrid plants with normal tobacco morphology is presented. Furthermore, sexual progenies are analyzed to test the inheritance of transferred genetic markers.

MATERIALS AND METHODS

Plant Material and Culture Conditions. *Nicotiana tabacum* (Solanaceae) [cv. Petit Havana SR1 (12)] plantlets were propagated aseptically by cuttings on MS-P medium (13). Suspension cultures of carrot cell line H47 were maintained on MS medium (14) supplemented with 2.2 mM 2,4-dichlorophenoxyacetic acid, 10 μ M methotrexate (MTX), and 0.1 mM 5-methyltryptophan [Trp(5Me)]. Combination of the two resistance markers was previously achieved by protoplast fusion between a MTX-resistant cell line, TX3 (unpublished), and a Trp(5Me)-resistant cell line isolated by Sung (15). The dominant nature of these two resistance markers was shown by expression of MTX and Trp(5Me) resistance in fusion products.

Protoplast Isolation, Fusion, and Culture. SR1 tobacco leaf protoplasts were isolated as described by Marton (16). Protoplasts of carrot [*Daucus carota* (Umbelliferae)] (H47 line) were isolated from cell suspension cultures incubated with enzyme solution (17). After digestion, cell cultures were filtered through a 44- μ m stainless steel sieve, and the resulting protoplasts were exposed to various doses of γ rays, using a ⁶⁰Co source that yielded 0.06 gray (Gy)/sec at 4.5 cm from the source. Fusion experiments were carried out with carrot protoplasts that absorbed 53, 107, or 166 Gy of irradiation in enzyme solution. Protoplasts were washed in a glucose solution (17) and mixed in a 1:1 ratio at a density of 10⁵ cells per ml. Fusion was induced with polyethylene glycol, combined with high-calcium and high-pH washing (18) in the presence of dimethyl sulfoxide (19). Both treated and control parental protoplasts were cultured in K75 medium (20). Frequency of heteroplasmic fusion was 3–7%.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: MTX, methotrexate; Trp(5Me), 5-methyltryptophan; DHFR, dihydrofolate reductase; NICA, tobacco + carrot somatic hybrids.

[†]To whom reprint requests should be addressed.

One month after fusion colonies were plated on K75 medium supplemented with 1 μ M MTX. The survival rate of irradiated carrot protoplasts was determined under the same culture conditions. Outgrowing tobacco calli with MTX resistance were isolated from fusion material and transferred into K75 differentiation medium with benzyladenine at 1 mg/liter and naphthaleneacetic acid at 0.1 mg/liter. During shoot initiation selected tissues were not exposed to MTX. After regeneration plantlets were propagated from nodal cuttings. Expression of MTX resistance was tested both in shoot cultures and in primary calli initiated from regenerants. Trp(5Me) resistance was tested only in callus tissues. Chromosome counts were carried out as published previously (21).

Protein and Enzyme Analyses. Young shoots with leaves or callus tissues were homogenized, at 1 g (fresh weight) per ml, in extraction buffer containing 50 mM potassium phosphate at pH 7.2, 1 mM phenylmethylsulfonyl fluoride, 0.1% 2-mercaptoethanol, and 10% (vol/vol) glycerol. Crude homogenate was centrifuged at $21,000 \times g$ for 20 min. Protamine sulfate (1%, wt/vol) was added to the supernatant at 7% (vol/vol). After centrifugation at $21,000 \times g$ for 20 min, the resulting supernatant was fractionated with ammonium sulfate. The fraction precipitating at 30–60% saturation was collected and dialyzed against 400 vol of extraction buffer. Protein content was determined as described by Bradford (22).

DHFR isozymes were analyzed by slab polyacrylamide gradient (5–15%) gel electrophoresis at 4°C in Tris/glycine, pH 8.3, at 20 mA per gel. After electrophoresis gels were washed twice with 100 mM potassium phosphate, pH 7.2, then incubated at 37°C for 25 min in a reaction mixture with 100 mM potassium phosphate at pH 7.2, 1.12 mM dihydrofolic acid, 0.36 mM β -NADPH (sodium salt), 0.72 mM 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide. Under denaturing conditions, proteins were separated by using 5–20% polyacrylamide gradient gels according to Laemmli (23). Electrophoretic transfer to nitrocellulose filters and immunoblotting were performed according to Towbin *et al.* (24), using 10 mM imidazole/30 mM glycylglycine/0.1% NaDodSO₄/20% (vol/vol) methanol, pH 7.2–7.4 (25). Prior to antibody treatment, nitrocellulose filters were incubated in 2% ovalbumin in phosphate-buffered saline (PBS; 136 mM NaCl/2 mM KH₂PO₄/8 mM Na₂HPO₄/2.6 mM KCl) overnight to reduce nonspecific binding. Bound antiserum to carrot DHFR (26) was detected with peroxidase-conjugated swine antiserum to rabbit serum diluted 1:250 in PBS.

RESULTS

Selection and Morphological Characterization of Somatic Hybrids. The choice of tobacco as recipient partner was based on high sensitivity of tobacco tissues to MTX and Trp(5Me). At 10 nM MTX tobacco protoplasts did not form colonies, and at 0.1 mM Trp(5Me) callus growth was inhibited. Significant differences in tobacco and carrot callus morphology simplified identification of parental tissues in mixed cultures. Donor carrot cells (H47) carried two dominant resistance markers, the ability to grow in the presence of 0.1 mM MTX and 0.5 mM Trp(5Me). Carrot protoplasts were exposed to γ rays at various dosages to induce preferential loss of carrot nuclear material from the fusion products. Irradiation significantly reduced survival of carrot protoplasts, as shown by colony formation expressed as percent of nonirradiated control: 53 Gy, 18%; 107 Gy, 2%; 166 Gy, 0.5%. Nonirradiated control and polyethylene glycol-treated cultures were maintained in complex rich K75 medium. After 1 month of nonselective growth, small colonies were plated on selective medium (1 μ M MTX). MTX-

resistant colonies, with white and compact callus morphology similar to tobacco calli, were isolated and further characterized. From four independent fusion experiments, with various combinations of radiation treatment, 37 MTX-resistant tobacco type colonies (designated NICA) were selected and transferred onto regeneration medium lacking MTX.

The morphology of regenerants from NICA-1 was very similar to that of SR1 tobacco plants (Fig. 1A). When grown in soil under greenhouse conditions, NICA plants were somewhat shorter and flowered earlier than SR1 parental control tobacco plants. Flowers were male sterile. In addition to NICA plants with normal tobacco morphology, narrow-leaf regenerants were also found (Fig. 1B). Plants with shorter internodes have developed from NICA-4 and NICA-35 tissues. Despite morphological deviations, most redifferentiated NICA plants exhibited almost normal tobacco phenotype.

Chromosome Number in Regenerants. The allotetraploid tobacco parent, SR1 genotype, had $2n = 48$ chromosomes. The average chromosome number in cell suspension culture of carrot H47 line was 24, with variations between 20 and 30. As shown in Table 1, the number of chromosomes in NICA plants ranged between 38 and 68. Four months after fusion NICA-1 callus showed 42–46 chromosomes. All the regenerants from this callus had chromosome numbers lower than 48. Several NICA-1 plants had variable chromosome numbers in root tips. Cytological characterization of shoot-forming NICA-2 revealed 52–56 chromosomes. After a prolonged culture period regenerants appeared with higher chromosome numbers (68). NICA-3 callus with shoot differentiation initially exhibited 52 chromosomes. The NICA-305 regenerant from the same callus had 44–46 chromosomes after propagation of tissue material. Plants with 38–44 chromosomes were also regenerated from NICA-4.

FIG. 1. Morphological characteristics of regenerated tobacco + carrot somatic hybrids grown under greenhouse conditions. (A) Comparison of regenerants from NICA-1 MTX-resistant callus with SR1 parent. (B) Narrow leaf shape of regenerant from NICA-4. SR1, tobacco; N, NICA-401; C, carrot.

Table 1. Expression of resistance markers in regenerants from various fusion products

Selected lines*	Regenerant identification no.†	Resistance phenotype‡			Chromosome number
		0.5 μ M MTX	1 μ M MTX	0.1 mM Trp(5Me)	
NICA-1 (53 Gy)	Callus	—	+	—	42–46
	101	0	0	0	—
	102-2	0	0	+	41–46
	102-3	+	0	+	40–46
	104-D	0	0	+	46
	105-2	+	+	0	42
	105-4	+	+	0	—
	105-5	+	0	+	42–46
	105-6	+	+	+	42–44
NICA-2 (107 Gy)	Shoots	—	+	—	52–56
	201-1	—	0	+	68
	201-2	+	+	0	—
NICA-3 (53 Gy)	Shoots	—	+	—	52
	303	+	0	+	—
	304	—	0	0	—
	305	+	+	0	44–46
NICA-4 (166 Gy)	401	+	0	+	40–46
	404	—	0	0	—
	405	+	+	0	38–40

*Dosage of γ irradiation used to treat carrot protoplasts is given in parentheses.

†Identification numbers indicate independent regenerants from selected NICA calli.

‡Resistance test: +, callus formation in the presence of inhibitor; 0, no growth response; —, not tested.

In the present fusion combinations, differences in size and morphology between parental chromosomes have not made a reliable identification of individual chromosomes possible. Chromosome numbers reflected a continuous change in genomic structure of NICA plants during maintenance *in vitro*. NICA plants in general tended to lose chromosomes, relative to additive numbers of parental chromosomes. This was also a characteristic of NICA-3, which initially had a chromosome number higher than 48.

Expression and Somatic Segregation of Carrot-Specific Resistance Markers. MTX resistance was used to select tobacco + carrot somatic hybrids, and Trp(5Me) resistance served as a nonselected marker. Expression of MTX resistance was retested in both shoot and primary callus cultures. It can be seen in Fig. 2A that nodal cuttings from SR1 control plants did not root on medium containing 0.01 or 0.1 μ M MTX and died in the presence of 1 μ M MTX. Both NICA regenerants formed roots and grew on the same medium. NICA-105-6 plants developed new shoots on 1 μ M MTX. For analysis of Trp(5Me) resistance, callus tissues were initiated in its presence. Fig. 2B compares differential expression of Trp(5Me) resistance of two NICA lines with the SR1 parental control. Table 1 shows that among the various regenerants from the same NICA tissue, plants expressing MTX, or Trp(5Me), or both of these resistances developed, in addition to sensitive plants. Regeneration of MTX-sensitive plants from a MTX-resistant callus reflected possible genomic instability of fusion products. Independent segregation of the two markers was observed in somatic tissues of all NICA lines analyzed, regardless of γ irradiation dosage.

Detection of Carrot Dihydrofolate Reductase in Hybrid Plants. MTX is a strong and specific inhibitor for DHFR in higher plants (27). In carrot parental cells the high level of MTX resistance is a consequence of decreased enzyme sensitivity to the drug and increased specific activity (unpublished preliminary results). Hybrid selection was based on the MTX-resistance phenotype. Therefore characteriza-

FIG. 2. Expression of carrot-specific resistance markers in somatic hybrids and their progenies. (A) Test of the hybrid plants for resistance against MTX at the following concentrations: column 1, 0; column 2, 0.01 μ M; column 3, 0.1 μ M; column 4, 1 μ M. Top row, NICA-105-7; middle row, SR1 tobacco; bottom row, NICA-105-6. (B) Trp(5Me) sensitivity of hybrid and tobacco tissues. Tube 1, SR1 tobacco with 0.1 mM Trp(5Me); tube 2, SR1 tobacco with 0.25 mM Trp(5Me); tube 3, NICA-102-2 with 0.1 mM Trp(5Me); tube 4, NICA-102-2 with 0.25 mM Trp(5Me); tube 5, NICA-104 with 0.1 mM Trp(5Me); tube 6, NICA-104 with 0.25 mM Trp(5Me). (C) Growth of seedlings from seeds of NICA-105 plants backcrossed with SR1 tobacco, on medium with 0.1 μ M MTX.

tion of DHFR in regenerated hybrids may provide information about specific gene transfer.

One approach to identification of parental DHFR is the comparison of isozymes separated by nondenaturing PAGE and stained for enzyme activity. As shown in Fig. 3A, the patterns of isozymes in cell extracts from parental species are different. The prominent carrot band is absent from tobacco. Detection of this isoenzyme in NICA tissues suggests a combination of parental DHFRs. Among the fast-migrating isozymes is a minor band that was not detected in tobacco or carrot. Analyses of DHFR isozymes from various regenerants revealed that MTX-resistant regenerants (NICA-102-3) had a new isozyme pattern, while MTX-sensitive regenerants (NICA-102-2) exhibited patterns identical to tobacco (data not shown).

DHFR from NICA tissues was further characterized by using antiserum against purified carrot DHFR (28). After NaDodSO₄/PAGE and immunoblotting of carrot cell extracts, carrot DHFR antiserum recognized a main band at 58 kDa and a minor band at 22 kDa (Fig. 3B). Carrot antiserum cross-reacted with a 24-kDa protein from tobacco extracts. Two NICA regenerants were analyzed with carrot DHFR

FIG. 3. Characterization of DHFR in somatic hybrids and parental species. (A) Separation of DHFR isozymes with nondenaturing PAGE and staining for DHFR activity in H47 carrot (lane 1); NICA-1 (lane 2); NICA-3 (lane 3); NICA-2 (lane 4), and SR1 tobacco (lane 5). (B) NaDodSO₄/PAGE of proteins from MTX-resistant NICA-105-6 calli grown on 1 μ M MTX (lane 1), NICA-105-6 plants (lane 2), SR1 tobacco (lane 3), MTX-sensitive NICA-102-2 (lane 4), and carrot (lane 5). DHFR proteins were visualized with antiserum to carrot DHFR after blotting. Molecular masses in kDa are indicated on the right.

antiserum. NICA-105-6 is a MTX-resistant hybrid, while NICA-102-2 has lost MTX resistance during culture under nonselective conditions. Cell extracts prepared from NICA-105-6 calli grown in the presence of MTX (1 μ M) or from a plant grown in the absence of MTX reacted with carrot DHFR antiserum. Carrot (58-kDa), tobacco (24-kDa), and unique (86-kDa) proteins were visualized in extracts from NICA-105-6. MTX-sensitive NICA-102-2 retained the 24-kDa band and is indistinguishable from the tobacco parent. Carrot DHFR antiserum revealed an apparent correlation between the presence of 58- and 86-kDa proteins and MTX resistance. This antiserum was previously shown to react with purified carrot 58-kDa DHFR (26). Synthesis of DHFR in MTX-resistant hybrids, with molecular weight and antigenic properties similar to those of carrot DHFR, is a further indication of carrot-specific enzyme expression in tobacco.

Progeny Analysis. Somatic hybrid plants regenerated from NICA-1 flowered under greenhouse conditions. Formation of short filaments and abnormal anthers prevented self-pollination; therefore, these plants were fertilized with SR1 pollen. The resulting seeds were germinated and tested for MTX resistance. As shown in Fig. 2C, seedlings segregated toward resistant and sensitive phenotypes. Sensitive plantlets bleached and growth was strongly inhibited in the presence of 0.1 μ M MTX. Resistant green plants grew continuously. Resistance was also retested in callus cultures, where primary inocula formed callus on medium containing 1 μ M MTX. Out of 99 seedlings from NICA-105, 36 were resistant

and 63 were sensitive to MTX. The deviation from the expected 1:1 ratio for a single dominant trait could originate from meiotic abnormalities caused by aneuploidy.

The observed segregation of progenies showed the nuclear determination and sexual transmission of MTX resistance.

DISCUSSION

In the described experimental system somatic hybrid plants with tobacco morphology and MTX resistance phenotype have been produced by fusion between tobacco leaf and irradiated carrot protoplasts. Fusion origin of selected clones and regenerants was reconfirmed by expression of Trp(5Me) resistance as a nonselected marker. Coexpression and frequent independent segregation of MTX and Trp(5Me) dominant traits are consistent with the hybrid nature of NICA plants and make spontaneous mutation an unlikely source of MTX resistance. The presence of carrot-specific DHFR isozymes and antigenic determinants in MTX-resistant regenerants supports the conclusion that somatic cell hybridization has resulted in gene transfer between two unrelated plant species—i.e., tobacco and carrot.

Carrot + tobacco cell hybrids that could not be regenerated have previously been selected by amino acid analog resistance complementation (29). Among other reasons, the additive chromosome number in these fusion products could be responsible for the nonmorphogenic behavior of such hybrid cell lines. In the present studies carrot protoplasts were irradiated to induce chromosomal instability in somatic hybrids. Cytological analysis of selected tissues and various regenerants confirmed the asymmetric nature of hybrid genomes. Chromosome numbers in NICA hybrids were significantly lower than the sum of parental chromosomes. Since the degree of chromosome loss and irradiation dosage were not correlated, factors in addition to irradiation are suggested for induction of somatic segregation. Similar to other phylogenetically distant hybridizations (21, 30, 31), chromosome loss in NICA regenerants could be a consequence of incompatible reaction in fused somatic cells. Considering the 38–46 chromosomes in NICA tissues, we have to postulate the elimination of some tobacco chromosomes during selection and regeneration. Regeneration of tobacco plants with 40–46 chromosomes was also observed after intraspecific *N. tabacum* fusion with one irradiated partner (P. Medgyesy, personal communication). This finding suggests that formation of aneuploids from protoplasts of amphidiploid species could be a nonspecific effect of *in vitro* techniques. However, in production of NICA plants the significance of long-term exposure to MTX at a high concentration cannot be excluded. The MTX-induced chromosome loss was revealed by cytological data indicating reduction of chromosome number and induction of aneuploidy in a MTX-resistant carrot cell line (32).

Limitations in identification of individual chromosomes did not allow determination of the actual contribution of fusion partners to the hybrid genome. In addition to preferential loss of carrot chromosomes, chromosomal translocations and substitutions could also influence the genomic constitution of NICA plants. An alternative interpretation of observed chromosome numbers could be that NICA plant lines did not carry whole carrot chromosomes. Considering the expression of carrot-specific functions, the integration of small chromosome fragments could be suggested as a cytological mechanism in generating NICA genotypes. If this is the case, somatic segregation of resistance markers could relate to the loss of tobacco chromosomes with carrot genes. Under the applied conditions, the specific effect of MTX should also be analyzed as a possible inducer of recombination or karyological changes. In experiments with mammalian cells MTX-directed thymidylate stress was a strong

initiator of recombination for the introduced foreign gene (33). Accumulation of DNA strand breaks was also detected in MTX-treated cells (34). A variety of chromosomal aberrations was characteristic for MTX-resistant cells, as discussed by Schimke *et al.* (35). Before predicting a similar mode of action in plant cells, further experiments are needed to determine the possible role of MTX-induced recombination in cell hybridization and in transformation of higher plants.

At present we do not know the molecular and cytological mechanisms that produced asymmetric hybrids after tobacco and carrot protoplast fusion. The important feature of the resulting hybrids is that their genetic constitution has made possible the regeneration of normal plants capable of producing sexual progenies. These hybrids are different from the previous intergeneric somatic hybrids, which were hampered with serious morphological abnormalities (21, 36) or sterility (37). In the present experiments segregation of MTX-resistant seedlings among sexual progenies has provided evidence for transfer of an introduced foreign trait into the next generation. Considering seed production from these somatic hybrids sharing characters from two phylogenetically diverse plant species, the asymmetric cell hybridization method is of great potential in practical plant breeding. These studies on tobacco + carrot fusion products indicate that selection of plant species, radiation-induced genomic instability, and specific MTX effects can be key factors in generation of these hybrid plants. Their significance should be analyzed with other fusion experiments. Phenotypic characteristics and chromosome number of NICA somatic hybrids make these plants a unique experimental material for isolation of carrot-specific DNA sequences related to the studied resistance markers.

We thank Dr. Gyula Hadlaczky for his valuable suggestions, Dr. Marvin A. Smith for critical reading of the manuscript, Mrs. Eva Torok and Mrs. Gizella Nagy for their excellent technical assistance, and Mr. Bela Dusha for photographic work. These studies were supported by the Hungarian National Council of Technical Development.

- Goldsbrough, P. B., Gelvin, S. B. & Larkins, B. A. (1986) *Mol. Gen. Genet.* **202**, 374–381.
- Nagy, F., Kay, S. A., Boutry, M., Hsu, M. Y. & Chua, N. H. (1986) *EMBO J.* **5**, 1119–1124.
- Dudits, D., Hadlaczky, Gy., Lazar, G. & Haydu, Z. (1980) in *Plant Cell Cultures: Results and Perspectives*, eds. Sala, F., Parisi, B., Cella, R. & Ciferri, O. (Elsevier, Amsterdam), pp. 207–214.
- Harms, C. T. (1983) *Q. Rev. Biol.* **58**, 325–353.
- Dudits, D. & Praznovszky, T. (1985) in *Biotechnology in Plant Science*, eds. Zaitlin, M., Day, P. & Hollaender, A. (Academic, Orlando, FL), pp. 115–127.
- Dudits, D., Fejer, O., Hadlaczky, Gy., Koncz, Cs., Lazar, G. B. & Horvath, G. (1980) *Mol. Gen. Genet.* **179**, 283–288.
- Gupta, P. P., Shaw, D. S., Gupta, M. & Schieder, O. (1984) *Mol. Gen. Genet.* **197**, 30–35.
- Itoh, K. & Futsuhara, I. (1983) *Jpn. J. Genet.* **58**, 545–553.
- Aviv, D. & Galun, E. (1980) *Theor. Appl. Genet.* **58**, 121–127.
- Menczel, L., Galiba, G., Nagy, F. & Maliga, P. (1982) *Genetics* **100**, 487–495.
- Somers, D. A., Narayanan, K. R., Kleinhofs, A., Cooper-Bland, S. & Cocking, E. C. (1986) *Mol. Gen. Genet.* **204**, 296–301.
- Maliga, P., Sz-Breznovits, A. & Marton, L. (1973) *Nature (London) New Biol.* **244**, 29–30.
- Maliga, P. (1984) in *Cell Culture and Somatic Cell Genetics of Plants*, ed. Vasil, I. K. (Academic, Orlando, FL), pp. 552–562.
- Murashige, T. & Skoog, F. (1962) *Physiol. Plant.* **15**, 437–497.
- Sung, Z. R. (1979) *Planta* **145**, 339–345.
- Marton, L. (1984) in *Cell Culture and Somatic Cell Genetics of Plants*, ed. Vasil, I. K. (Academic, Orlando, FL), pp. 514–521.
- Dudits, D. (1984) in *Cell Culture and Somatic Cell Genetics of Plants*, ed. Vasil, I. K. (Academic, Orlando, FL), pp. 391–397.
- Kao, K. N., Constabel, F., Michayluk, M. R. & Gamborg, O. L. (1974) *Planta* **120**, 215–227.
- Menczel, L., Nagy, F., Kiss, Z. S. & Maliga, P. (1981) *Theor. Appl. Genet.* **59**, 191–195.
- Kao, K. N. & Michayluk, M. R. (1975) *Planta* **126**, 105–118.
- Dudits, D., Hadlaczky, Gy., Bajszar, Gy., Koncz, Cs., Lazar, G. & Horvath, G. (1979) *Plant Sci. Lett.* **15**, 101–112.
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
- Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
- Hadlaczky, Gy., Went, M. & Ringertz, N. R. (1986) *Exp. Cell Res.* **167**, 1–15.
- Albani, D., Parisi, B., Carbonera, D., Etteri, L., Maestri, E. & Parisi, B. (1987) *J. Plant Physiol.* **127**, 135–146.
- Cella, R., Crosti, P., Nielsen, E. & Parisi, B. (1983) *J. Exp. Bot.* **34**, 1183–95.
- Albani, D., Parisi, B., Carbonera, D. & Cella, R. (1985) *Plant Mol. Biol.* **5**, 363–372.
- Hauptmann, P., Kumar, P. & Widholm, J. (1983) in *Protoplasts 1983*, eds. Potrykus, I., Harms, C. T., Hinnen, A., Hutter, R., King, P. J. & Shillito, R. D. (Birkhauser, Basel), pp. 92–93.
- Kao, K. N. (1977) *Mol. Gen. Genet.* **150**, 225–230.
- Binding, H. & Nehls, R. (1978) *Mol. Gen. Genet.* **164**, 137–143.
- Cella, R., Albani, D., Biasini, M. G., Carbonera, D. & Parisi, B. (1984) *J. Exp. Bot.* **35**, 1390–1397.
- Ayusawa, D., Koyama, H., Shimizu, K., Kaneda, S., Takeishi, K. & Seno, T. (1986) *Mol. Cell. Biol.* **6**, 3463–3469.
- Li, J. C. & Kaminskis, R. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 5694–5698.
- Schimke, R. T., Sherwood, S. W., Hill, A. B. & Johnston, R. N. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 2157–2161.
- Gleba, Y. Y. & Hoffmann, F. (1980) *Planta* **149**, 112–117.
- Melchers, G., Sacristán, M. D. & Holder, A. A. (1978) *Carlsberg Res. Commun.* **43**, 203–218.