Theor Appl Genet (1989) 78:547–552

Transmission of organelles in triploid hybrids produced by gametosomatic fusions of two *Nicotiana* species

D. Pental, A.K. Pradhan and A. Mukhopadhyay

Tata Energy Research Institute, 90 Jor Bagh, New Delhi – 110003, India

Received March 28, 1989; Accepted April 12, 1989
Communicated by G. Wenzel

Summary. Gametosomatic hybrids produced by the fusion of microspore protoplasts of *Nicotiana tabacum* Km^+Sr^+ with somatic cell protoplasts of *N. rustica* were analysed for their organelle composition. For the analysis of mitochondrial (mt)DNA, species-specific patterns were generated by Southern hybridization of restriction endonuclease digests of total DNA and mtDNA with four DNA probes of mitochondrial origin: cytochrome oxidase subunit I, cytochrome oxidase subunit II, 26s rDNA and 5s–18s rDNA. Of the 22 hybrids analyzed, some had parental-type pattern for some probes and novel-type for the others, indicating interaction between mtDNA of the two parent species. For chloroplast (cp)DNA analysis, species-specific patterns were generated by Southern hybridization of restriction endonuclease digests of total DNA with large subunits of ribulose bisphosphate carboxylase and cpDNA as probes. All the hybrids had *N. rustica*-specific patterns. Hybrids were not resistant to streptomycin, a trait encoded by the chloroplast genome of *N. tabacum*. In gametosomatic fusions of the two *Nicotiana* species, mitochondria but not the chloroplasts are transmitted from the parent contributing microspore protoplasts.

Key words: Microspore protoplasts – Protoplast fusion – Gametosomatic hybrids – Organelle transmission – *Nicotiana* species

Introduction

Triploid hybrids (n + 2n) synthesized by sexual crosses (O'Mara 1940; Savitsky 1975; Rick et al. 1986) and used for the transfer of alien genes to crop plants can also be produced by the fusion of microspore protoplasts (n) with somatic cell protoplasts (2n) (Pental and Cocking 1985; Pirrie and Power 1986; Pental et al. 1988). Such gametosomatic hybridization would not be limited by pre- and post-zygotic sexual incompatibilities. In addition, gametosomatic hybrids may have novel mitochondrial/chloroplast/nuclear combinations, as has been reported for somatic hybrids (Belliard et al. 1979; Pelletier et al. 1983; Rothenberg et al. 1985), provided organelles can be transmitted from the species contributing the microspore protoplasts.

During microsporogenesis and pollen development, radical changes have been observed in the organization of the organelles (Dickinson and Heslop-Harrison 1977; Bird et al. 1983; Sangwan and Sangwan-Norreel 1987). Organelles have been observed to be highly dedifferentiated at the early tetrad stage in a number of plant species belonging to diverse families of Angiosperms, e.g. Liliaceae, Solanaceae, Gramineae (Dickinson and Heslop-Harrison 1977; Sangwan and Sangwan-Norreel 1987).

Are the microspore protoplasts, isolated at the early tetrad stage, capable of contributing their organelle genome to the hybrids produced by gametosomatic fusions? We have tried to answer this question by studying the transmission of organelles in triploid hybrids which were produced by the fusion of *N. tabacum* Km^+Sr^+ microspore protoplasts, isolated at the early tetrad stage, with somatic cell protoplasts of WT *N. rustica* (Pental et al. 1988). *N. tabacum* Km^+Sr^+ plants carry in their nuclear genome a chimaeric gene, combining *nos* promoter with a structural gene encoding NeoII enzyme from Tn5, and conferring resistance to kanamycin (Hain et al. 1985). Resistance to streptomycin is due to a mutation in the chloroplast genome (Maliga et al. 1973). The selection of hybrids relied upon the inability of microspore protoplasts to divide (Bhojwani and Cocking 1972; Pirrie and Power 1986; Pental et al. 1988) and lack

of resistance to kanamycin in the protoplasts isolated from cell suspensions of *N. rustica*. We report that, at least in this combination of species, mitochondria but not the chloroplasts are transmitted from the microspore protoplasts to the hybrids.

Materials and methods

Methods of protoplast culture and production of gametosomatic hybrids of *N. tabacum* Km^+Sr^+ and *N. rustica* have been reported earlier by Pental et al. (1988). Hybrid plants were maintained in vitro on MS (Murashige and Skoog 1962) medium by propagation of axillary buds. Hybrids were code-named as follows: e.g. 11.4 B.1 – 11 is the number of the fusion experiment, 4 is the number of the selection dish in fusion no. 11, B is the number of the colony from dish 11.4 and 1 is the number of the plant regenerated from colony 11.4B. Callus was induced from excised leaf pieces of *N. tabacum* and of hybrid plants on MS medium containing 2 mg/l NAA (naphthalene acetic acid) and 0.5 mg/l BAP (6-benzyl aminopurine). Total DNA was isolated from callus tissues of *N. tabacum* and the hybrids by the method of Murray and Thompson (1980). For *N. rustica*, total DNA was isolated from cell suspensions which were originally used for protoplast isolation and fusion. Chloroplast DNA was isolated from pot-grown plants of *N. tabacum* Km^+Sr^+ following the method of Hanson et al. (1986). Mitochondrial DNA was isolated from callus tissue following the method of Kemble (1987) and was further purified by centrifugation in cesium chloride. DNA was digested with various restriction endonucleases following the conditions recommended by the manufacturers. Electrophoresis was performed in the horizontal mode in 0.8% or 1% agarose gels. Nick translation of various DNA probes and Southern hybridization were carried out as described by Evans et al. (1983). Hybridized blots were washed twice in 2 × SSC (65 °C), twice in 2 × SSC with 0.1% SDS (65 °C), followed by a wash in 0.1 × SSC at room temperature.

Results

Transmission of chloroplast genome

Leaf tissues of the two parents and 96 confirmed gametosomatic hybrids that were recovered on a selection medium containing kanamycin were tested for resistance to streptomycin on MS with 0.5 mg/l BAP, 0.1 mg/l NAA and 1.5 mg/l streptomycin sulfate. On this medium, *N. tabacum* Km^+Sr^+ produced green nodular callus, while *N. rustica* and tissues of hybrid plants turned pale yellow. Thus, none of the hybrids inherited the trait of streptomycin resistance from the chloroplast genome of *N. tabacum* parent.

Species-specific patterns were generated by Southern hybridization of total DNA isolated from the callus tissue of the two parent species, digested with various restriction endonucleases, with ^{32}P-labelled large subunit of ribulose bisphosphate carboxylase (Gatenby et al. 1981) and with cpDNA of *N. tabacum* Km^+Sr^+. In ClaI digests hybridized to ribulose bisphosphate carboxylase probe, a 1.9-kb band was specific to *N. tabacum* and a 1.6-kb band was specific to *N. rustica* (Fig. 1). All 22 hybrids listed in Table 1 (only 5 are shown in Fig. 1) had *N. rustica* type pattern. In analysis using cpDNA as a probe, a 5.1-kb band in EcoRI digests, specific to *N. tabacum*, was not present in any of the hybrids. A 4.8-kb band in HaeIII digests, specific to *N. rustica*, was present in DNA digests of all 22 hybrids that were tested.

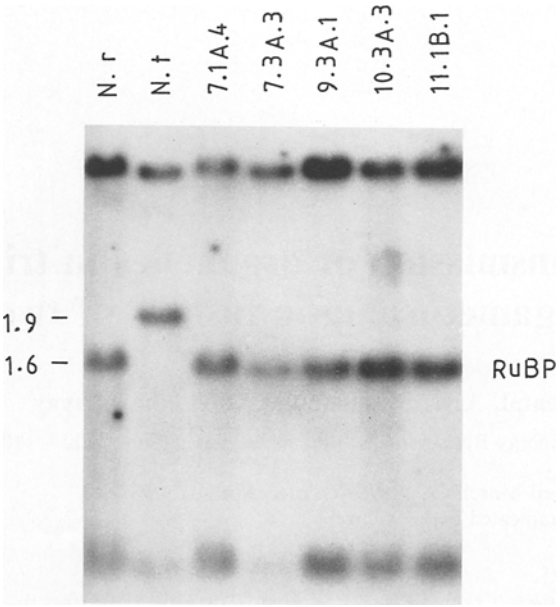

Fig. 1. Chloroplast genome analysis by Southern blotting of the two parental species *N. tabacum* (N.t). *N. rustica* (N.r) and five hybrids. Restriction endonuclease used is ClaI and probe is large subunit of ribulose bisphosphate carboxylase. Fragment size in kb is given on the left-hand side of the Figure

Transmission of mitochondrial genome

The composition of the mitochondrial genome of the gametosomatic hybrids was studied by Southern hybridization of specific mtDNA probes with restriction endonuclease digests of total cellular DNA or mtDNA. Initially, species-specific patterns were generated by hybridization of four different probes, namely cytochrome oxidase subunit I (COI) (Isaac et al. 1985), cytochrome oxidase subunit II (COII) (Fox and Leaver 1981), 26s rDNA (Dale et al. 1984) and 5s–18s rDNA (Chao et al. 1983), with restriction endonuclease digests of total DNA isolated from the two parent species. The following restriction endonucleases and probe combinations were found to give species-specific hybridization patterns: ClaI and PstI digests with COI, BamHI and BglI digests with COII, BamHI and EcoRI digests with 26s rDNA, PstI and SalI digests with 5s–18s rDNA. Patterns for ClaI digests hybridized to COI, BglI digests hybridized to COII, BamHI digests hybridized to 26s rDNA and SalI digests to 5s–18s rDNA, are shown in Fig. 2.

Table 1. Types of mtDNA patterns for four different probes in 22 *Nicotiana tabacum* and *N. rustica* hybrids. N.r stands for *N. rustica*-type pattern and N.t for *N. tabacum*. N1, N2, etc. are novel types of patterns: N1 pattern has one novel band, N2 has one novel band and N.r specific bands, N3 has one novel band and both N.r and N.t specific bands, N4 has two novel bands and N.t specific bands, N5 has N.t specific bands and faint hybridization for N.r specific bands

Hybrid \ R.E.: / Probe:	ClaI COI	BglI COII	BamHI 26s rDNA	SalI 5s–18s rDNA
4.1 A.1	N.r	N.r	N.r	N.r
7.1 A.7	N.r	N.r	N4	N1
7.3 A.3	N.r	N2	N4	N.t
7.4 A.2	N.r	N1	N.r	N.r
9.3 A.1	N.r	N.r	N.r	N.r
10.1 D.4	N.r	N.r	N.r	N.r
10.1 G.1	N.r	N1	N.r	N.r+N.t
10.2 C.4	N.r	N.r	N.r	N.r
10.3 A.3	N.r	N2	N.r	N.t
10.6 A.2	N.r	N.r	N.r	N.r
10.9 C.3	N.r	N.r	N4	N.r
10.10B.1	N.r	N.r	N.r	N.r
11.1 B.1	N.r	N1	N1	N.t
11.3 A.7	N.r	N1	N.r	N.r
11.4 B.1	N.r	N1	N.r	N.r
11.5 A.4	N.r	N.r	N.r	N.r
11.7 A.4	N.r	N.r	N.r	N.r
11.8 A.2	N.r	N2	N.r	N.r
11.10B.1	N.r	N1	N4	N.t
11.12C.2	N.r	N1	N3	N5
11.14A.1	N.r	N1	N1	N.t
12.2 B.1	N.r	N1	N1	N.t
No. of different types	N.r 22	N.r 10 N1 9 N2 3	N.r 14 N1=3 N3=1 N4=4	N.r 13 N.t 6 N.r+N.t 1 N1 1 N5 1

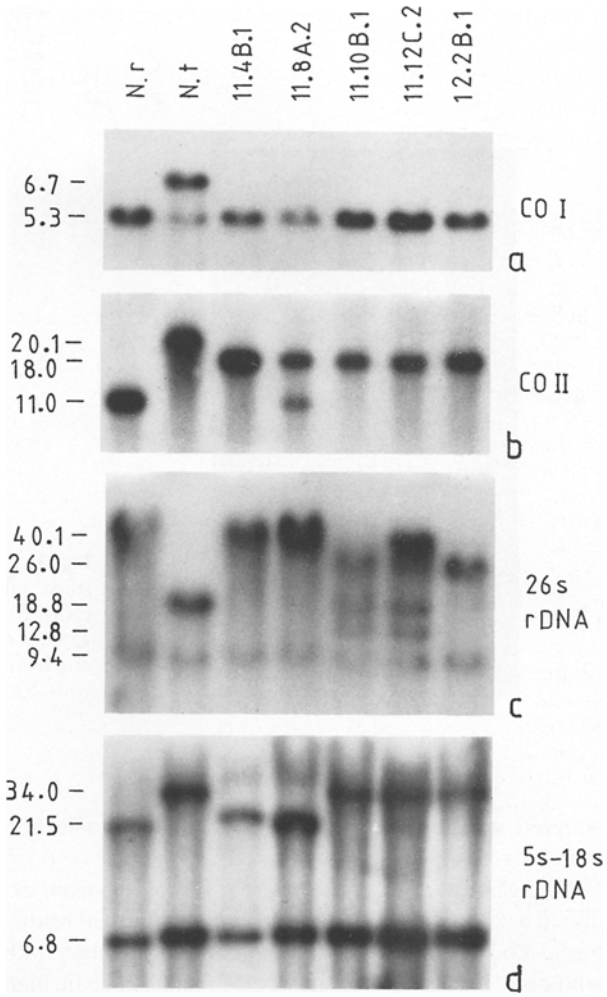

Fig. 2a–d. Mitochondrial genome analysis of the parental species: *N. tabacum* (N.t), *N. rustica* (N.r) and five hybrids. Total DNA was digested with four different restriction endonucleases and hybridized to four different mitochondrial DNA probes by Southern hybridization. The probes used are given on the right-hand side of the Figure. Fragment size in kb is given on the left-hand side. Hybrid number is given at the top of **a**. Numbering of hybrids is explained in the text. The ordering of parents and hybrids is the same in **a**, **b**, **c** and **d**. **a** Restriction endonuclease ClaI, probe COI. **b** Restriction endonuclease BglI, probe COII. **c** Restriction endonuclease BamHI, probe 26s rDNA. **d** Restriction endonuclease SalI, probe 5s–18s rDNA

As some of the mtDNA sequences can have strong homology with DNA sequences present in chloroplast and nuclear genomes (reviewed by Schuster and Brennicke 1988), species-specific patterns generated by hybridization of total DNA were checked for their mitochondrial specificity, by using mtDNA isolated from the callus tissue of *N. tabacum* and cell suspensions of *N. rustica*. For COI and COII probes, the hybridization patterns for mtDNA were similar to that of total DNA shown in Fig. 2a and b. The 26s rDNA probe hybridized with BamHI digest of total DNA of *N. tabacum* and *N. rustica* gave species-specific bands at 18.8 kb and 40.1 kb, respectively, and an additional 9.4-kb band common to both the parents (Fig. 2c). However, in the mtDNA digests with BamHI, no hybridization was observed for the 9.4-kb common band (Fig. 3a and b). Consequently, this band was disregarded in the hybrid analysis. The 5s-18s rDNA probe showed strong hybridization with a 21.5-kb band in *N. rustica* and a 34-kb band in *N. tabacum* in both the mtDNA and total DNA digests made with SalI (Fig. 2d). However, in total DNA digests, some additional bands were observed above the 34-kb band in both the parents. These bands were disregarded in hybrid analysis.

Southern hybridization patterns of the hybrids for each one of the probes were either like one of the two parents or were novel (Fig. 2). The hybridization patterns for four of the restriction endonucleases and four probes have been summarized in Table 1. All the hybrids

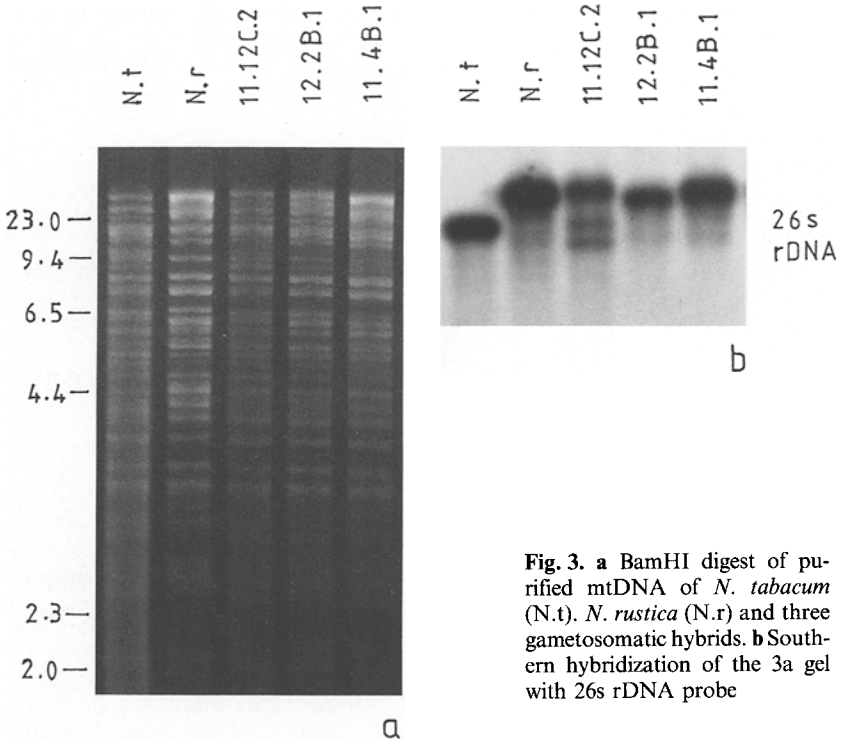

Fig. 3. a BamHI digest of purified mtDNA of *N. tabacum* (N.t). *N. rustica* (N.r) and three gametosomatic hybrids. **b** Southern hybridization of the 3a gel with 26s rDNA probe

analyzed were regenerated from independent selection events.

In digests of ClaI probed with COI, *N. tabacum* exhibited a conspicuous 6.7-kb band and a faintly hybridizing 5.3-kb band, while *N. rustica* showed a strong 5.3-kb band only. All 22 hybrids showed *N. rustica*-specific high intensity band at 5.3 kb (Fig. 2a). This was further confirmed by PstI digest of total DNA hybridized to COI. In this analysis, *N. rustica* showed the presence of prominent 21-kb and 8.8-kb bands, whereas *N. tabacum* had a prominent 13.5-kb band and a faint 21-kb band. None of the analyzed hybrids had the 13.5-kb *N. tabacum*-specific band.

For DNA digested with BglI and probed with COII, *N. rustica* showed an 11-kb band and *N. tabacum* showed a 20.1-kb band. The hybrids either had *N. rustica*-type pattern or two types of novel patterns (Fig. 2b): N1 resembled neither of the two parents (e.g. 11.4B.1) and N2 had an 11-kb *N. rustica*-specific band and another 18-kb band characteristic of the N1 pattern (e.g. 11.8A.2). None of the hybrids had an *N. tabacum*-type pattern (Table 1). BamHI digests probed with COII confirmed the results given in Table 1.

In the BamHI digests probed with 26s rDNA (Figs. 2c and 3a and b), hybrids either had an *N. rustica*-type pattern (e.g. 11.4B.1) or three types of novel patterns: N1 had a novel 26-kb band (e.g. 12.2B.1), N3 had parent-specific bands and a novel 12.8-kb band (e.g. 11.12C.2), N4 had two novel bands at 26 kb and 12.8 kb and an 18.8-kb *N. tabacum*-specific band (e.g. 11.10B.1). Frequency distribution of different types is given in Table 1. None of the hybrids had a strictly *N. tabacum* type of pattern. The nature of the novel patterns observed with 26s rDNA probe hybridized to total DNA was confirmed by the hybridization of mtDNA of three of the hybrids with this probe (compare Figs. 2c and 3a and b).

In the SalI digests probed with the 5s–18s rDNA sequence (Fig. 2d), hybrids showed patterns which were either like *N. rustica* (e.g. 11.4B.4) or *N. tabacum* (12.2B.1). One of the hybrids had a pattern that was essentially a sum of the patterns of the two parents (not shown). In another variation (N5), strong hybridization was observed for the 34-kb band and weak hybridization was observed for the 21.5-kb band (e.g. 11.12C.2).

All the Southern blots were overexposed to look for some faint hybridization in the two parents, which may correspond with the novel patterns of hybridization observed in the hybrids. However, no such bands were detected.

Discussion

Our observations of the inheritance of drug resistance trait and species-specific DNA pattern analysis shows that there is no transmission of chloroplast genome from the *N. tabacum* parent which contributed the microspore protoplasts to the gametosomatic fusions. In earlier work on somatic cell fusions of *N. tabacum* and *N. rustica*,

plants were produced which either carried the *N. tabacum* or *N. rustica* chloroplast genome (Iwai et al. 1980; Douglas et al. 1981; Hamill et al. 1984). The lack of transmission of cpDNA from microspore protoplasts could be either due to the dedifferentiated state of plastids (Dickinson and Heslop-Harrison 1977; Sangwan and Sangwan-Norreel 1987) and/or due to stochastic reasons – that the number of plastids in microspores is too low as compared to the somatic cells. The debilitation of plastids during meiosis could be only developmental and not genetic, as haploid plants regenerated from *N. tabacum* microspores at the first pollen mitosis are green with functional plastid genome (Sunderland and Roberts 1977).

The following conclusions can be drawn from the results described for mtDNA analysis of the two parents and the hybrids: (1) Some of the hybrids have Southern hybridization pattern like one parent for one of the probes and a hybridization pattern like the other parent for another probe (e.g. 11.14A.1, 12.2B.1). This could come about only by genetic interaction between mitochondrial genomes of the two parental species. (2) There is a preponderance of *N. rustica*-specific patterns. Six of the 22 hybrids have Southern hybridization patterns for all four probes, similar to those of the *N. rustica* parent. None of the hybrids had a *N. tabacum*-specific pattern for all four probes.

The absence of hybrids with a *N. tabacum*-specific pattern for all four probes indicates the lack of transmission of a mitochondrial genome of *N. tabacum* on its own. This could be due to debilitation of mitochondria in the tetrad protoplasts and/or due to stochastic reasons. However, the mitochondria present in tetrad cells of *N. tabacum* must have the ability to fuse with mitochondria of somatic cells leading to the generation of recombinant types. As mtDNA is present in variable configurations and quantities (Dale 1981; Abott et al. 1985) in cells at different morphogenetic stages, this could influence the extent of recombination and eventual composition of genome in the hybrids. In this context, it would be interesting to compare the mtDNA composition of gametosomatic hybrids with somatic hybrids (Pental et al. 1984: Hamill et al. 1984) of the species used in this study.

For practical purposes, gametosoamtic hybrids would be useful for the transfer of alien nuclear and mitochondrial genes. As an example, gametosomatic hybrids could be used for the transfer of mitochondrial genes controlling cytoplasmic male sterility and nuclear genes controlling fertility restorer function in a concerted manner from an alien species to a crop species. Gametosomatic fusions may also have relevance for the crosses where it is undesirable to transfer an alien chloroplast genome (e.g. Pelletier et al. 1983) and where only a limited amount of information from the alien mitochondrial genome is to be transmitted to the crop parent.

Acknowledgements. Technical assistance was provided by B. S. Yadav. We thank Profs. C. J. Leaver and C. S. Levings III for mtDNA probes and Prof. J. Schell for seeds of transformed tobacco.

References

Abbott AG, O'Dell M, Flavell RB (1985) Quantitative variation in components of the maize mitochondrial genome between tissues and between plants with different male-sterile cytoplasms. Plant Mol Biol 4:233–240

Belliard G, Vedel F, Pelletier G (1979) Mitochondrial recombination in cytoplasmic hybrids of *Nicotiana tabacum* by protoplast fusion. Nature 281:401–403

Bhojwani SS, Cocking EC (1972) Isolation of protoplasts from pollen tetrads. Nature 239:29–30

Bird J, Porter EK, Dickinson HG (1983) Events in the cytoplasm during male meiosis in *Lilium*. J Cell Sci 59:27–42

Chao S, Sederoff RR, Levings CS III (1983) Partial sequence analysis of the 5s to 18s rRNA gene region of the maize mitochondrial genome. Plant Physiol 71:190–193

Dale RMK (1981) Sequence homology among different size classes of plant mtDNAs. Proc Natl Acad Sci USA 78:4453–4457

Dale RMK, Mendu N, Ginsburg H, Kridl JC (1984) Sequence analysis of the maize mitochondrial 26s rRNA gene and flanking regions. Plasmid 11:141–150

Dickinson HG, Heslop-Harrison (1977) Ribosome, membranes and organelles during meiosis in angiosperms. Philos Trans R Soc London 277:327–342

Douglas GC, Keller WA, Setterfield G (1981) Somatic hybridization between *Nicotiana rustica* and *N. tabacum* IV. Analysis of nuclear and chloroplast genome expression in somatic hybrids. Can J Bot 59:1509–1513

Evans IJ, James AM, Barnes SR (1983) Organization and evolution of repeated DNA sequences in closely related plant genomes. J Mol Biol 170:803–826

Fox TD, Leaver CJ (1981) The *Zea mays* mitochondrial gene coding cytochrome oxidase subunit II has an intervening sequence and does not contain TGA. Cell 26:315–323

Gatenby AA, Castleton JA, Saul MW (1981) Expression in *E. coli* of maize and wheat chloroplast genes for large subunit of ribulase bisphosphate carboxylase. Nature 291:117–121

Hain R, Stabel P, Czernilofsky AP, Steinbiss HH, Herrera-Estrella L, Schell J (1985) Uptake, integration, expression and genetic transmission of a selectable chimaeric gene by plant protoplasts. Mol Gen Genet 199:161–168

Hamill JD, Patnaik G, Pental D, Cocking EC (1984) The culture of manually isolated heterokaryons of *Nicotiana tabacum* and *N. rustica*. Proc Indian Acad Sci Sect B 93:317–327

Hanson MR, Boeshore ML, McClean PE, O'Connell MA, Nivison HT (1986) The isolation of mitochondria and mitochondrial DNA. In: Weissbach A, Weissbach H (eds) Methods in enzymology, vol 118. Plant molecular biology. Academic Press, New York, pp 437

Issac PG, Jones VP, Leaver CJ (1985) The maize cytochrome C oxidase subunit I gene: sequence, expression and rearrangement in cytoplasmic male sterile plants. EMBO J 4:1617–1623

Iwai S, Nago T, Nakata K, Kawashima N, Matsukyma S (1980) Expression of nuclear and chloroplastic genes coding for Fraction 1 protein in somatic hybrids of *N. tabacum*+*N. rustica*. Planta 147:414–417

Kemble RJ (1987) A rapid, single leaf, nucleic acid assay for determining the cytoplasmic organelle complement of rapeseed and related *Brassica* species. Theor Appl Genet 73:364–370

Maliga P, Breznovits AS, Marton L (1973) Streptomycin-resistant plants from callus culture of haploid tobacco. Nature 244:29–30

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321–4325

O'Mara JG (1940) Cytogenetic studies on Triticale. I. A method for determining the effects of individual *Secale* chromosome on *Triticum*. Genetics 25:401–408

Pelletier G, Primard C, Vedel F, Chetrit P, Remy R, Rousielle P, Renard M (1983) Intergeneric cytoplasmic hybridization in Cruciferae by protoplast fusion. Mol Gen Genet 191:244–250

Pental D, Cocking EC (1985) Some theoretical and practical possibilities of plant genetic manipulation using protoplasts. Hereditas 3:83–93

Pental D, Hamill JD, Cocking EC (1984) Somatic hybridization using a double mutant of *Nicotiana tabacum*. Heredity 53:79–83

Pental D, Mukhopadhyay A, Grover A, Pradhan AK (1988) A selection method for the synthesis of triploid hybrids by fusion of microspore protoplasts (n) with somatic cell protoplasts (2n). Theor Appl Genet 76:237–243

Pirrie A, Power JB (1986) The production of fertile, triploid somatic hybrid plants *Nicotiana glutinosa* (n) + *N. tabacum* (2n) via gametic:somatic protoplast fusion. Theor Appl Genet 72:48–52

Rick CM, DeVerna JW, Chetelat RT, Stevens MA (1986) Meiosis in sesquidiploid hybrids of *Lycopersicon esculentum* and *Solanum lycopersicoides*. Proc Natl Acad Sci (USA) 83:3580–3583

Rothenberg M, Boeshore ML, Hanson MR, Izhar S (1985) Intergenomic recombination of mitochondrial genomes in a somatic hybrid plant. Curr Genet 9:615–618

Sangwan RS, Sangwan-Norreel BS (1987) Ultrastructural cytology of plastids in pollen grains of certain androgenic and nonandrogenic plants. Protoplasma 138:11–22

Savitsky H (1975) Hybridization between *Beta vulgaris* and *B. procumbens* and transmission of nematode (*Heterodera schachtii*) resistance to sugarbeet. Can J Genet Cytol 17:197–209

Schuster W, Brennicke A (1988) Interorganeller sequence transfer: plant mitochondrial DNA is nuclear, is plastid, is mitochondrial. Plant Sci 54:1–10

Sunderland N, Roberts M (1977) New approach to pollen culture. Nature 270:236–238