

Rearrangements in the mitochondrial genome of somatic hybrid cell lines of *Pennisetum americanum* (L.) K. Schum. + *Panicum maximum* Jacq.

P. Ozias-Akins^{1,*}, D. R. Pring² and I. K. Vasil^{1, **}

¹ Department of Botany, ² USDA-ARS/Department of Plant Pathology; University of Florida, Gainesville, FL 32611, USA

Received October 30, 1986; Accepted February 6, 1987

Communicated by P. Maliga

Summary. Mitochondrial DNA from three somatic hybrid cell lines of *Pennisetum americanum* + *Panicum maximum* was compared with mitochondrial DNA of the parents. Gel electrophoresis of BamHI-restricted mitochondrial DNA indicated that extensive rearrangements had occurred in each of the three hybrid lines. The hybrid restriction patterns showed a combination of some bands from each parent plus novel fragments not present in either parent. Additional evidence for rearrangements was obtained by hybridization of eight DNA probes, carrying sequences of known coding regions, to Southern blots. Each of the somatic hybrids exhibited a partial combination of the parental mitochondrial genomes. These data suggest recombination or amplification of the mitochondrial genomes in the somatic hybrids.

Key words: Mitochondrial DNA – Somatic hybridization – *Pennisetum* – *Panicum*

Introduction

Mitochondrial recombination in somatic hybrids of higher plants was first suggested by Belliard et al. (1979). In this case two varieties of *Nicotiana tabacum* L. (var. Xanthi and var. Techne) with different cytoplasmas displayed distinctly different mitochondrial DNA (mtDNA) restriction patterns. Restriction digests of mtDNA from somatic hybrid plants revealed new

bands which could not be attributed to either parent. Subsequently, mitochondrial recombination has been proposed as the process responsible for altered mtDNA restriction patterns in other somatic hybrids or cybrids within various genera including *Nicotiana* (Nagy et al. 1981; Galun et al. 1982), *Petunia* (Boesshore et al. 1983), and *Daucus* (Matthews and Widholm 1985).

Most investigations of mtDNA rearrangements have dealt with solanaceous species and intrageneric somatic hybrids or cybrids. The only intergeneric cybrids analyzed thus far have been between the dicotyledonous plants, *Brassica napus* or *B. campestris* and *Raphanus sativus* (Cherit et al. 1985). As somatic hybrid calli within the Gramineae recently have been recovered (Ozias-Akins et al. 1986; Tabaeizadeh et al. 1986), it is possible now to determine the occurrence or extent of genetic recombination when mitochondria from two grass genera become mixed after protoplast fusion. This paper presents evidence for the presence of mtDNA rearrangements in three somatic hybrid cell lines of *Pennisetum americanum* + *Panicum maximum*.

Materials and methods

Cell lines

The culture background of the parental cell lines used and results of the somatic hybridization experiments have been published previously (Ozias-Akins et al. 1986). Somatic hybrid cell lines first were recovered and identified in December 1984 (SH 2 and SH 3) and February 1985 (SH 67). Somatic hybrid calli were subcultured at 4-week intervals on medium containing S-2-aminoethyl-L-cysteine (AEC) (300 mg/l) for 4–6 months before cell suspension cultures were initiated.

DNA isolation, restriction, and blotting

Mitochondrial DNA was isolated from suspension cultures 5 months after initiation by the procedure of McNay et al.

* Present address: Department of Horticulture, University of Georgia Coastal Plain Experiment Station, Tifton, GA 31793, USA

** To whom reprint requests should be addressed

(1984) (first isolation). After 5 additional months of growth in suspension culture with weekly subcultures on medium lacking AEC, mtDNA again was isolated (second isolation). DNA was restricted with BamHI according to supplier's recommendations and run on 0.8% agarose gels in 36 mM Tris base – 30 mM Na₂PO₄ · H₂O – 1 mM Na₂EDTA, pH 7.8 at 2 V/cm for 16 h. Gels were stained for 1 h with 0.4 µg/ml ethidium bromide in H₂O and photographed on a 302 nm UV transilluminator. Sandwich blots on nitrocellulose were prepared from gels after depurinating 10 min in 0.25 N HCl, denaturing 30 min in 0.6 M NaCl – 0.2 M NaOH, and neutralizing 30 min in 3 M NaCl – 94 mM Tris, pH 7.5. Molecular size markers were PstI plus HindIII-digested bacteriophage lambda DNA.

Nick translation and hybridization

Probes for selected mitochondrial coding sequences were labelled by nick translation as described by Rigby et al. (1977). Labelled DNA was separated from nucleotides on a Sephadex G-50 column prepared with a 1 ml disposable syringe. Probes included clones of maize mtDNA sequences coding for subunits 6 (atp6) (Dewey et al. 1985a), 9 (atp9) (Dewey et al. 1985b), and alpha (atp α) (Braun and Levings 1985) of F₁-F₀ ATPase; subunits I (COI) (Isaac et al. 1985) and II (COII) (Fox and Leaver 1981) of cytochrome c oxidase; 18s and 26s rDNA (Pring et al., unpublished); and sorghum chloroplast DNA coding for the large subunit of ribulose-1,5-bisphosphate carboxylase (rbcL) (Dang and Pring 1986). Clones of the F₁-F₀ ATPase subunits were provided by C.S. Levings III, and cytochrome c oxidase clones were provided by C.J. Leaver. Procedures for hybridization and washing of blots were carried out as described by McNay et al. (1984) except that the DNA was denatured by boiling for 10 min. Probed blots were autoradiographed on Kodak XAR-5 X-ray film with two intensifying screens at –80°C.

Results

Restricted mtDNA from the three somatic hybrid cell lines showed banding patterns which differed from one another as well as from both parents (Fig. 1). Combinations of bands unique to one or the other of the parents were seen in the somatic hybrids. In addition, novel bands which were not detected in either of the parental cell lines also were observed. A few differences could be detected in restriction patterns of mtDNA from the first and second isolations which were separated by about 5 months. No qualitative changes were observed in the parental lines over this period; however, the stoichiometry of at least one fragment of *P. americanum* was altered. In SH 3, one band of approximately 11 kb disappeared, and in SH 67, two bands (ca. 17 and 6 kb) disappeared.

More specific rearrangements could be identified after hybridization with nick-translated probes of known mitochondrial genes. These data were obtained only with mtDNA from the first isolation. Mitochondrial DNA fragments from the somatic hybrids which hybridized strongly with the eight probes have been tabulated (Table 1).

The 26s ribosomal gene probe hybridized to two *P. maximum* fragments, 8.9 and 6.4 kb in size, and one *P. americanum* fragment of about 22 kb (Fig. 2). None of the somatic hybrids contained the *P. maximum*

Table 1. Molecular sizes (kb) and probable parental origin of major hybridizing fragments in BamHI restricted mtDNA from the somatic hybrids. *Pm*: *Panicum maximum*; *Pa*: *Pennisetum americanum*

	26s	atp6	COI	18s	atp9	COII	rbcL	atp α
SH2	27.5	27.5	7.1 (<i>Pm</i>)	7.1 (<i>Pm</i>)	8.6	4.4 (<i>Pa</i>)	5.1	7.2 (<i>Pm</i>)
	23	23	5.0 (<i>Pm, Pa</i>)	5.0 (<i>Pm, Pa</i>)	8.2	1.6 (<i>Pm</i>)	3.3 (<i>Pm</i>)	5.6 (<i>Pa</i>)
		4.4 (<i>Pm</i>)			7.4 (<i>Pm</i>)		2.0 (<i>Pa</i>)	5.1
					4.5 (<i>Pa</i>)			0.9 (<i>Pm, Pa</i>)
					1.7 (<i>Pm</i>)			
SH3	27.5	27.5	7.1 (<i>Pm</i>)	7.1 (<i>Pm</i>)	7.4 (<i>Pm</i>)	4.4 (<i>Pa</i>)	5.1	7.2 (<i>Pm</i>)
	23	23			6.5	1.6 (<i>Pm</i>)	3.3 (<i>Pm</i>)	5.6 (<i>Pa</i>)
		2.6 (<i>Pm</i>)			4.5 (<i>Pa</i>)		2.0 (<i>Pa</i>)	5.1
					1.7 (<i>Pm</i>)			0.9 (<i>Pm, Pa</i>)
SH67	22 (<i>Pa</i>)	22 (<i>Pa</i>)	6.5 (<i>Pa</i>)	6.5 (<i>Pa</i>)	4.5 (<i>Pa</i>)	4.4 (<i>Pa</i>)	2.0 (<i>Pa</i>)	7.2 (<i>Pm</i>)
			5.0 (<i>Pm, Pa</i>)	5.0 (<i>Pm, Pa</i>)	1.7 (<i>Pm</i>)	3.6 (<i>Pa</i>)		5.6 (<i>Pa</i>)
						1.6 (<i>Pm</i>)		0.9 (<i>Pm, Pa</i>)
<i>Pm</i>	8.9	4.7	7.1	7.1	7.4	4.0	3.3	7.2
	6.4	4.4	5.0	5.0	1.7	1.6		0.9
		3.0						
		2.6						
<i>Pa</i>	22	22	6.5	6.5	4.5	4.4	2.0	5.6
			6.2	6.2	2.9	3.6		0.9
			5.0	5.0				

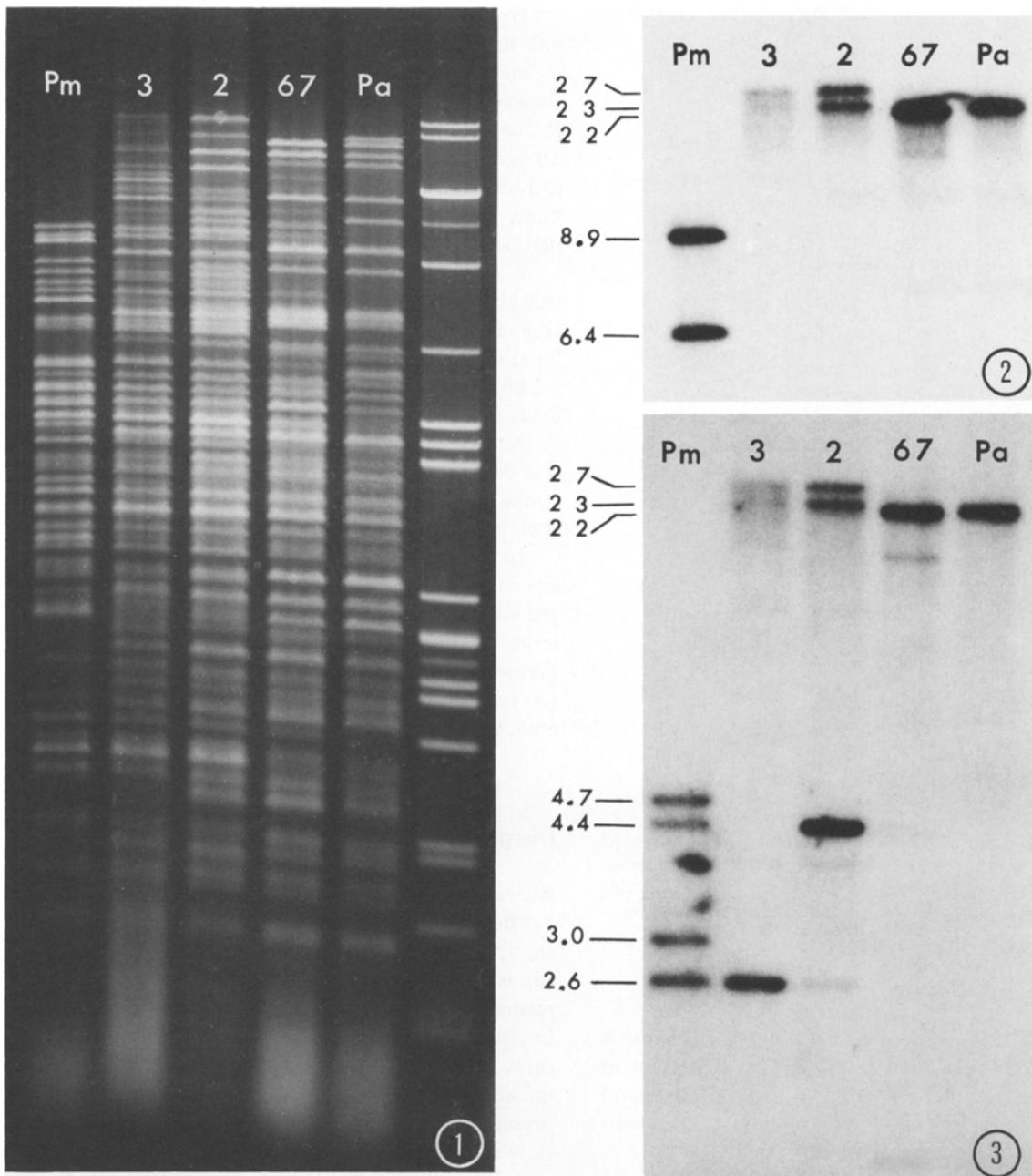


Fig. 1. Ethidium bromide stained gel of BamHI-restricted mitochondrial DNA from *Panicum maximum* (Pm), *Pennisetum americanum* (Pa), and somatic hybrid cell lines 2, 3, and 67

Figs. 2 and 3. Hybridization of various DNA probes to Southern blots of mitochondrial DNA from *Panicum maximum* (Pm), *Pennisetum americanum* (Pa), and somatic hybrid cell lines 2, 3, and 67. Numbers are kb values of hybridizing fragments. 2 26s ribosomal DNA; 3 F1-ATPase subunit 6

bands. However, hybridization of the probe to at least one (27.5 kb) and possibly a second (23 kb) novel high molecular weight band was observed in SH 2 and SH 3.

Probes for *atp6* hybridized to the same high molecular weight fragments in *P. americanum* and SH 2, 3, and 67 as did the 26s rDNA probe (Fig. 3). Additional bands in SH 3 (2.6 kb) and SH 2 (4.4 kb) apparently

originated from *P. maximum* in which four major bands were observed of 4.7, 4.4, 3.0, and 2.3 kb.

Fragments with strong homology to the COI gene probe and a probe for 18s ribosomal genes were of similar molecular weights. One fragment of about 5 kb, which was shared by both parents, was also present in SH 2 and 67 but only weakly detectable in SH 3. Two

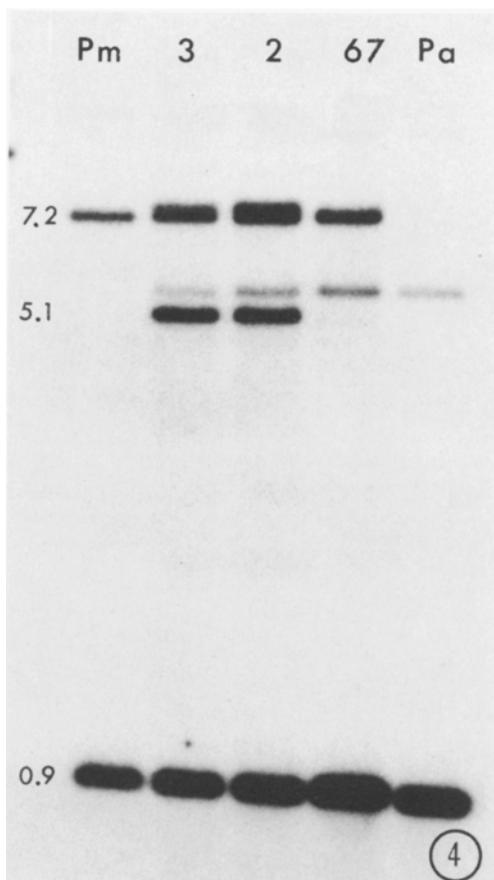


Fig. 4. Hybridization of F1-ATPase subunit alpha probe to Southern blots of mitochondrial DNA from *Panicum maximum* (*Pm*), *Pennisetum americanum* (*Pa*), and somatic hybrid cell lines 2, 3, and 67. Numbers are kb values of hybridizing fragments

bands (6.5, 6.2 kb) were unique to *P. americanum*. A unique *P. maximum* fragment (7.1 kb) was present in SH 2 and 3 but missing from 67. A 1.4 kb band hybridizing weakly to COI was common to *P. americanum* and all of the somatic hybrids.

Two unique bands with major homologies to the *atp9* probe were found in *P. maximum* (7.4, 1.7 kb) and *P. americanum* (4.5, 2.9 kb). All three hybrids contained the 1.7 kb hybridizing fragment from *P. maximum* and the 4.5 kb fragment from *P. americanum*. Somatic hybrids 2 and 3 also contained the 7.4 kb *P. maximum* band. Additionally, novel hybridizing bands were observed in SH 3 (6.5 kb) and SH 2 (8.6, 8.2 kb).

The COII probe hybridized to two unique bands in *P. maximum* (4.0, 1.6 kb) and two unique *P. americanum* bands (4.4, 3.6 kb). One *P. maximum* band and one *P. americanum* band were represented in SH 2 and 3. Somatic hybrid 67 contained both of these bands plus the other *P. americanum* band.

Hybridization to the *rbcL* probe was observed in one major band in *P. maximum* (3.3 kb) and one in *P. americanum* (2.0 kb). All of the somatic hybrids showed hybridization to the *P. americanum* band. The *P. maximum* band was present in only SH 2 and 3. These two hybrid lines also contained a common band (5.1 kb) not present in either of the parents but with major homology to the probe. All three hybrids exhibited novel weakly hybridizing bands.

A probe for *atpα* revealed one major fragment (0.9 kb) common to both parents and all three hybrids (Fig. 4). In addition, each parent contained one unique band with major homology to the probe (*P. maximum* – 7.2 kb; *P. americanum* – 5.6 kb). All three somatic hybrids contained both fragments unique to each parent. A distinct, strongly hybridizing novel band of 5.1 kb was detected in SH 2 and 3. A novel fragment of similar size in SH 2 and 3 also was observed to hybridize to the *rbcL* probe.

In only one instance (SH 67 probed with *atpα*) did any of the somatic hybrids show simple addition of parental bands. In no case did the hybridization patterns of the somatic hybrids exactly mimic either parent. Somatic hybrid specific fragments were observed to have major homologies to the 26s rDNA, *atp6*, *atp9*, *atpα*, and *rbcL* probes.

Discussion

Alterations in mtDNA restriction patterns of somatic hybrids appear to occur frequently. Quantitative changes or the disappearance of bands might be attributed, at least in part, to variation in intramolecular recombinational events. However, in many somatic hybrids, the magnitude of qualitative changes implicates intergenomic recombination rather than intramolecular variation. This is particularly evident in the present study where the three somatic hybrid cell lines showed extensive changes (most noticeable in high molecular weight fragments) when compared to each other and parental cell lines.

In tissue cultured cells of maize, stoichiometric changes have been observed in specific mtDNA fragments (McNay et al. 1984). More extensive rearrangements have been detected in T-cytoplasm maize plants regenerated from *Helminthosporium maydis* toxin-resistant cell lines selected through tissue culture. The toxin-resistant plants had mtDNA restriction patterns that were altered from the original T-cytoplasm mtDNA and varied from one regenerant to another (Gengenbach et al. 1981; Kemble et al. 1982). In contrast to the observations with maize, no mtDNA rearrangements were detected in antibiotic resistant *Nicotiana plumbaginifolia*.

baginifolia and *N. tabacum* protoplast and callus culture regenerants or intraspecific somatic hybrids of *N. plumbaginifolia*. Rearrangements were evident, however, in ten *N. plumbaginifolia* cybrids selected for *N. tabacum* chloroplasts (Nagy et al. 1983). These authors proposed that the *Nicotiana* mitochondrial genome may be more stable than the *Zea* genome, although this proposal was qualified with the observation that the maize plants studied were selected for a resistance trait residing in the mitochondria. Recombination of mtDNA and maintenance of certain populations of subgenomic circles may be dependent upon nuclear background and thus could be expected to vary from genotype to genotype. After several months of cell culture, some restriction fragments became undetectable in two of the somatic hybrid cell lines from the present study, but the only obvious change in one of the parents was quantitative. More subtle alterations might be observed with more extensive use of DNA probes. The changes which have occurred in the hybrids following extended culture could be a consequence of tissue culture-induced variation or continued rearrangement among the two combined parental genomes.

Although mitochondrial recombination was suggested as the explanation for novel restriction fragment patterns in *Nicotiana* somatic hybrids or cybrids, two processes were proposed by Boeshore et al. (1983) to account for the same phenomenon in *Petunia hybrida* + *P. parodii* somatic hybrids. They suggested that in addition to possible recombination, amplification of certain sequences not detectable in low copy number by ethidium bromide staining might occur. Direct evidence for recombination has come from subsequent analysis of a cosmid clone of *Petunia* somatic hybrid DNA (Rothenberg et al. 1985). The restriction site polymorphisms between the two parental species allowed assignment of specific restriction fragments within the clone to either of the parents.

The probes utilized in these experiments carry coding and flanking sequences, and thus may hybridize to non-coding fragments in the mtDNAs examined here. Both maize (Pring and Lonsdale, unpublished) and sorghum (Ferguson et al., unpublished) mtDNAs may display variation in hybridization to these probes depending on presence or absence of repeated sequences represented in the probes. *Pennisetum* and *Panicum* hybridization patterns also indicate presence of repeated sequences homologous to the probes. Homology to the probe carrying subunit 6 of ATPase in *Panicum* (Fig. 3) is suggestive of a recombinational active repeat, generating four fragments carrying homology to the probe. Most of the probes utilized hybridized to more than one fragment, indicating BamHI sites in regions of mtDNAs corresponding to the particular probe.

This study is significant since the first somatic hybrid cells of the Gramineae were analyzed for mtDNA rearrangements, previously reported only in dicotyledonous plants. A companion study with two somatic hybrid cell lines of *P. americanum* + *Saccharum officinarum* (Tabaeizadeh et al. 1987) has shown slightly different results. Mitochondrial DNA from one hybrid line (H2) totally resembled the *P. americanum* parent after restriction with PstI, BamHI, and XbaI endonucleases and hybridization with eight different cosmid clones. A second somatic hybrid cell line (H1) showed novel restriction fragments with PstI and after hybridization with several cosmid probes. It is not possible at this time to assign the primary cause of mtDNA variation in the *P. americanum* + *P. maximum* somatic hybrids to either of the phenomena discussed. Recently, the novel 5.1 kb BamHI restriction fragment which was identified from mtDNA of SH 3 with the *atpα* probe has been cloned (Ozias-Akins et al., unpublished). Approximately 90% of this clone is homologous with a portion of an 11 kb clone obtained from the *P. maximum* parent. It has yet to be determined if the 5.1 kb fragment resulted from recombination between *P. maximum* and *P. americanum* mtDNA.

Acknowledgements. Supported by funds provided to IKV by Monsanto Co. (St. Louis, MO), and by a co-funded project between the Gas Research Institute (Chicago, IL) and the Institute of Food and Agricultural Sciences, University of Florida. Florida Agricultural Experiment Station Journal Series No. 7848.

References

- Belliard G, Vedel F, Pelletier G (1979) Mitochondrial recombination in cytoplasmic hybrids of *Nicotiana tabacum* by protoplast fusion. *Nature (London)* 281:401–403
- Boeshore ML, Lifshitz I, Hanson MR, Izhar S (1983) Novel composition of mitochondrial genomes in *Petunia* somatic hybrids derived from cytoplasmic male sterile and fertile plants. *Mol Gen Genet* 190:459–467
- Braun CJ, Levings CS III (1985) Nucleotide sequence of the F1-ATPase subunit gene from maize mitochondria. *Plant Physiol* 79:571–577
- Cherit P, Mathieu C, Vedel F, Pelletier G, Primard C (1985) Mitochondrial DNA polymorphism induced by protoplast fusion in Cruciferae. *Theor Appl Genet* 69:361–366
- Dang LH, Pring DR (1986) A physical map of the sorghum chloroplast genome. *Plant Mol Biol* 6:119–123
- Dewey RE, Levings CS III, Timothy DH (1985a) Nucleotide sequence of ATPase subunit 6 gene of maize mitochondria. *Plant Physiol* 79:914–919
- Dewey RE, Schuster AM, Levings CS III, Timothy DH (1985b) Nucleotide sequence of FO-ATPase proteolipid (subunit 9) gene of maize mitochondria. *Proc Natl Acad Sci USA* 82:1015–1019
- Fox TD, Leaver CJ (1981) The *Zea mays* mitochondrial gene coding cytochrome oxidase subunit II has an intervening sequence and does not contain TGA codons. *Cell* 26:315–323

- Galun E, Arzee-Gonen P, Fluhr R, Edelman M, Aviv D (1982) Cytoplasmic hybridization in *Nicotiana*: mitochondrial DNA analysis in progenies resulting from fusion between protoplasts having different organelle constitutions. *Mol Gen Genet* 186:50–56
- Gengenbach BG, Connelly JA, Pring DR, Conde MF (1981) Mitochondrial DNA variation in maize plants regenerated during tissue culture selection. *Theor Appl Genet* 59: 161–167
- Isaac 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
- Kemble RJ, Flavell RB, Brettell RIS (1982) Mitochondrial DNA analyses of fertile and sterile maize plants derived from tissue culture with the Texas male sterile cytoplasm. *Theor Appl Genet* 62:213–217
- Matthews BF, Widholm JM (1985) Organelle DNA compositions and isoenzyme expression in an interspecific somatic hybrid of *Daucus*. *Mol Gen Genet* 198:371–376
- McNay JW, Chourey PS, Pring DR (1984) Molecular analysis of genomic stability of mitochondrial DNA in tissue cultured cells of maize. *Theor Appl Genet* 67:433–437
- Nagy F, Torok I, Maliga P (1981) Extensive rearrangements in the mitochondrial DNA in somatic hybrids of *Nicotiana tabacum* and *Nicotiana knightiana*. *Mol Gen Genet* 183: 437–439
- Nagy F, Lazar G, Menczel L, Maliga P (1983) A heteroplasmic state induced by protoplast fusion is a necessary condition for detecting rearrangements in *Nicotiana* mitochondrial DNA. *Theor Appl Genet* 66:203–207
- Ozias-Akins P, Ferl RJ, Vasil IK (1986) Somatic hybridization in the Gramineae: *Pennisetum americanum* + *Panicum maximum*. *Mol Gen Genet* 203:365–370
- Rigby PWJ, Dieckmann M, Rhodes C, Berg P (1977) Labelling deoxyribonucleic acid to high specific activity in vitro by nick translation with polymerase I. *J Mol Biol* 113:237–251
- Rothenberg M, Boesshore ML, Hanson MR, Izhar S (1985) Intergenomic recombination of mitochondrial genomes in a somatic hybrid plant. *Curr Genet* 9: 615–618
- Tabaeizadeh Z, Ferl RJ, Vasil IK (1986) Somatic hybridization in the Gramineae: *Pennisetum americanum* + *Saccharum officinarum*. *Proc Natl Acad Sci USA* 83:5616–5619
- Tabaeizadeh Z, Pring DR, Vasil IK (1987) Analysis of mitochondrial DNA from somatic hybrid cell lines of *Saccharum officinarum* (sugarcane) and *Pennisetum americanum* (pearl millet). *Plant Mol Biol* (in press)