Theor Appl Genet (1986) 72, 59–65

Regeneration of somatic hybrid plants formed between *Lycopersicon esculentum* and *Solanum rickii*

M. A. O'Connell [1] **and M. R. Hanson** [2]

[1] Plant Genetic Engineering Laboratory and Department of Horticulture, New Mexico State University, Las Cruces, NM 88003, USA
[2] Section of Genetics and Development, Bradfield Hall, Cornell University, Ithaca, NY 14853, USA

Received August 5, 1985; Accepted November 14, 1985
Communicated by P. Maliga

Summary. A single somatic hybrid callus clone was identified following the fusion of *Lycopersicon esculentum* protoplasts and *Solanum rickii* suspension culture protoplasts. The hybrid nature of the callus and the plants regenerating from it was determined by assaying phosphoglucomutase-2 isozyme expression. The chloroplast genome present in four somatic hybrid plants was characterized by probing digests of total DNA with nick translated *L. esculentum* chloroplast DNA(cpDNA). All four somatic hybrid plants had inherited *S. rickii* cpDNA. Two clones of plant mitochondrial DNA (mtDNA), soybean 18S and 5S rDNA and maize cytochrome oxidase subunit II were used to characterize the mtDNA present in total DNA digests of four somatic hybrid plants. In both cases, the somatic hybrid plants had inherited most but not all of the *S. rickii* specific fragments, but none of the *L. esculentum* specific fragments.

Key words: Tomato – Somatic hybrid – Organelle genome – Regeneration – Protoplast fusion

Introduction

Somatic hybridization is a method by which a number of novel plants have been engineered (Maliga et al. 1978; Melchers et al. 1978; Gleba and Hoffmann 1980; Pelletier et al. 1983; Shepard et al. 1983). This procedure of fusing protoplasts of somatic cells from different species allows one to produce novel genotypes which can not be produced by sexual hybridization. In addition to nuclear hybridization, protoplast fusion also generates hybrid cytoplasms, which after a number of cell divisions usually sort out to a stably inherited genotype. The somatic hybrid plant usually has one or the other parental chlorplast genome, (Chen et al. 1977; Belliard et al. 1978; Pelletier et al. 1983; Bonnett and Glimelius 1983), and a mitochondrial genome which contains portions of both parental genomes, (Belliard et al. 1979; Nagy et al. 1981; Galun et al. 1982; Boeshore et al. 1983; Hanson 1983; Chetrit et al. 1985).

Sexual hybridization between *L. esculentum* and wild relatives in the *Lycopersicon* and *Solanum* genera has been used to introgress several characters into the cultivated tomato (Rick 1982). Somatic hybridization offers the possibility of increasing the number of wild relatives which can be used as sources of germplasm since this procedure sidesteps the sexual incompatibility barriers present between many members of the *Lycopersicon* and *Solanum* genera (Rick 1979). In this report we describe the regeneration of somatic hybrid plants formed by fusion of *L. esculentum* protoplasts with *S. rickii* protoplasts. *S. rickii,* like *L. esculentum,* has 12 chromosomes, yellow corollas, lacks tubers, has pinnately divided leaves but is incompatible with *L. esculentum* (Rick 1979). *S. rickii* is compatible with *S. lycopersicoides,* a species which can cross with *L. esculentum* (Rick 1979). Because of the relatedness of *S. rickii* and *L. esculentum,* there was a reasonable prospect of producing a viable plant by protoplast fusion which could not be constructed using traditional breeding approaches.

Materials and methods

Plant material

Solanum rickii, LA 1974, seeds were generously supplied by C. Rick, Tomato Genetics Stock Center, University of Calif., Davis. *Lycopersicon esculentum* cultivar 'San Marzano' was from Burpee Seed Co.

Protoplast isolation, fusion and culture

Protoplasts were prepared from either leaves of 'San Marzano' or from suspension culture of *S. rickii* as described by O'Connell and Hanson (1985). *S. rickii* suspension cultures were maintained on UM1a medium (Uchimaya and Murashige 1974) with the casein hydrolysate concentration reduced to 0.5 g/l. The fusion procedure of Medgyesy et al. (1980) was modified to reduce the polyethylene glycol concentration and reduce the rate of dilution of the fusogen (O'Connell and Hanson 1985). Protoplasts were cultured in liquid media with frequent dilutions as described by O'Connell and Hanson (1985).

DNA isolation, organelle DNA probes

Total DNA was isolated from frozen lyophilized leaf tissue essentially as described by Murray and Thompson (1980). Only one cesium chloride gradient centrifugation step was utilized and rather than dialyzing, the DNA was precipitated after the addition of four volumes of sterile distilled water. Chloroplast DNA (cpDNA) was prepared as described by Saltz and Beckmann (1981). The clone of 18S and 5S soybean rDNA (pSB2-2, 1.8 kb EcoR1-Sal1 soybean fragment in pBR322) was a gift of Peter H. Morgens and has been described (Morgens et al. 1984). The clone of maize cytochrome oxidase subunit II (pZmE1, 2.4 kb EcoR1 maize fragment in pBR322) was a gift of T. Fox and also has been described (Fox and Leaver 1981). DNA to be used as a probe was nick translated with α-^{32}P-dCTP (New England Nuclear) as described by Rigby et al. (1977).

DNA restriction, southern transfer and hybridization

Four µg of DNA were digested for 4 h at 37 °C in 30 µl with either Nru 1 (New England Biolabs) in 100 mM NaCl, 50 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol or Bgl 1 (New England Biolabs) in 50 mM NaCl, 10 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol. Reactions were stopped with the addition of 5 µl 1% SDS, 0.125 M EDTA, 50% glycerol, 0.1% bromophenol blue and electrophoresed on 1% agarose gels, 40 mM Tris, 1 mM EDTA, 5 mM Na Acetate pH 7.75. Samples were electrophoresed at 55 mAmperes for 18 h. The gels were washed for 1 h in 1.5 M NaCl, 0.5 M NaOH, and then for 1 h in 3 M NaCl, 0.5 M Tris pH 7.0. The DNA was transferred to Genescreen® with 1X SSC (Southern 1975). The dried blots were washed with 5X Denhardt's solution (Denhart 1976), 6X SSC, total volume of 20 ml for 18 h at 65 °C. The blots were hybridized with α^{32}P-dCTP labeled probe in 0.5% SDS, 0.2 mg/ml salmon sperm DNA 6X SSC, 5X Denhardt's, total volume 10 ml for 18 h at 65 °C. The blots were then washed for 1 h with 20 ml 5X Denhardt's solution 6X SSC, 0.5% SDS, followed by two washes of 500 ml 0.4X SSC for 1 h each at 65 °C. The blots were mounted and exposed to X-ray film at –70 °C.

Starch gel analysis of phosphoglucomutase

Approximately 1 cm² of leaf was ground up using a blunt object in 0.12 M reduced glutathione adjusted to pH 7.6 with 1 M Tris. Samples were kept on ice during processing, the sample was absorbed into paper wicks and electrophoresed on starch gels as described by Tanksley (1979 a). Gels were stained for phosphoglucomutase activity as described by Tanksley (1979 b).

Chromosome counts

Root tips were collected from shoot cultures, the tips were incubated overnight at 4 °C in water saturated with 1-bromonaphthalene. The next morning the tips were fixed with 5 N HCl, 15 min, rinsed with water, stained with acetocarmine, 1 min, and squashed.

Results

Culture of fusion products

L. esculentum cv 'San Marzano' leaf mesophyll protoplast (1×10^5) were fused with the same number of protoplasts prepared from a suspension culture of *Solanum rickii.* Following the polyethylene glycol mediated fusion, the protoplasts were cultured with 1 ml JSC1 2.5 medium in 35 mm dishes in the dark. Two days, 9 days, and 15 days later the culture was fed with 2 ml, 2 ml, and 7 ml, respectively, with a 1 : 1 mixture of JSC1 2.5 and JSR media. Seventeen days after plating, the liquid cultures were dispensed onto JSC-12 solid media, 5 ml/90 mm plate and cultured in dim light, 12 h day. After one more month on solid media, the microcalli were individually transferred to fresh JSC-12. Approximately two months after initial plating of the protoplasts, shoots were first observed on some of the calli. At this time the calli were transferred to TR-1 media and cultured under bright lights. One month later the shoots were moved to root inducing media, N13 and after a month on N13 or 5 months since the initial protoplast plating, the plantlets were potted in vermiculite and moved to environmental growth chambers.

Identification of somatic hybrid plants

Of approximately 100 calli which were selected and transferred to JSC-12 media, 30 continued to grow well and turn green. All but one of these green calli developed shoots rapidly. These regenerating calli were scored as *S. rickii* plantlets on the basis of their appearance and their phosphoglucomutase isozyme patterns (data not shown). The green but nonregenerating callus was subcultured on shoot inducing media, TR-1, for several months, and 11 months after the initial protoplast plating this callus also developed shoots. The genotype of this callus and the first shoot which regenerated from it were determined.

In Fig. 1, the phosphoglucomutase isozymes present in this callus and a leaf from its regenerated shoot are compared with the isozyme pattern of the fusion partners *L. esculentum* and *S. rickii.* Both the callus and the shoot derived from it have both parental forms of phosphoglucomutase. Analysis of the phosphoglucomutase patterns in 10 other shoots which regenerated

Table 1. Chromosome counts in root tip preparations from regenerated somatic hybrid plants

Plant	Chromosome count
S. rickii	24, 24
'San Marzano'	24, 26
Somatic hybrid no. 2	44, 48
3	45, 48, 48
4	64, 61, 64
7	64, 63, 60
9	49, 52, 51
27	130, 71, 67

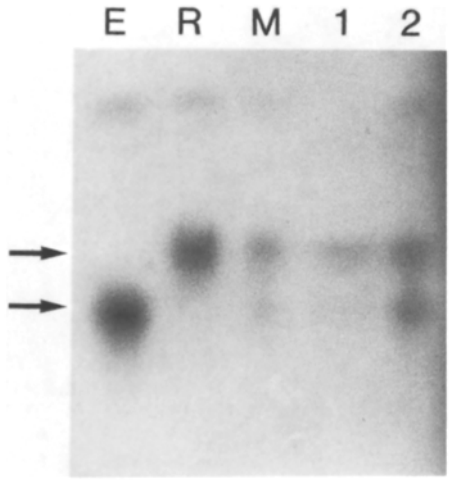


Fig. 1. Starch gel analysis of phosphoglucomutase activities. *Lane E* contains an extract from *L. esculentum* leaf, *R* contains an extract from *S. rickii* leaf, *M* contains a mixture of extracts from *S. rickii* and *L. esculentum. Lane 1* contains an extract from callus from the fusion culture, and *2* contains an extract from the shoot regenerating from the callus tested in *lane 1.* The species-specific forms of phosphoglucomutase-2 are indicated by *arrows.* Phosphoglucomutase-1, invariant between these two species, is visible at the *top* of the gel, the anodal front

from subcultures of this original callus all had both parental forms of this isozyme. All the plants regenerated from this callus gave the isozyme pattern predicted for a somatic hybrid. The growth habits and leaf shapes of the subclone regenerants were varied but intermediate with respect to the parents. Figure 2 shows some examples of the leaf shapes of the regenerants. The number of chromosomes in several of these regenerants was determined (Fig. 3), and these counts were listed in Table 1. Somatic hybrids no. 2, 3 and 9 have the expected tetraploid counts (4n=48). Somatic hybrids no. 4, 7 and 27 have greater than tetraploid counts, approaching hexaploidy.

Organelle genomes in somatic hybrid plants

Total cellular DNA was isolated from lyophilized samples of somatic hybrid shoots. After restriction with either Nru 1 or Bgl 1 endonucleases, electrophoresis in agarose gels and transfer to blots, the samples were probed with either ^{32}P-labelled chlorplast DNA (Fig. 4) or cloned fragments of plant mitochondrial DNA (Figs. 5 and 6). When cut with Bgl 1, *L. esculentum* cpDNA has two fragments which are not present in *S. rickii* cpDNA digests, 9.1 and 3.5 kb (Fig. 4). *S. rickii* also has a 10.5 kb Bgl 1 fragment not found in *L. esculentum* digests (Fig. 4). Using these species-specific marker fragments, it was possible to identify the cpDNA present in the somatic hybrid plants. Lanes 1–4 in Fig. 4 contain DNA from four somatic hybrid shoots and all four shoots have *S. rickii* cpDNA.

The mtDNA present in the somatic hybrid plants was probed by the soybean clone encoding 18S and 5S mitochondrial ribosomal RNAs and the maize clone encoding cytochrome oxidase subunit II. Figure 5 shows the fragments containing homology to the ri-

Fig. 2. Comparison of leaf shapes in regenerants. A *S. rickii* leaf is shown on the *left,* and a partially expanded *L. esculentum* leaf is shown on the *right.* Leaves taken from three regenerants, no. 4, 9, and 10, are shown in the *center,* no. 4 *top,* no. 9 *middle,* and no. 10 *bottom*

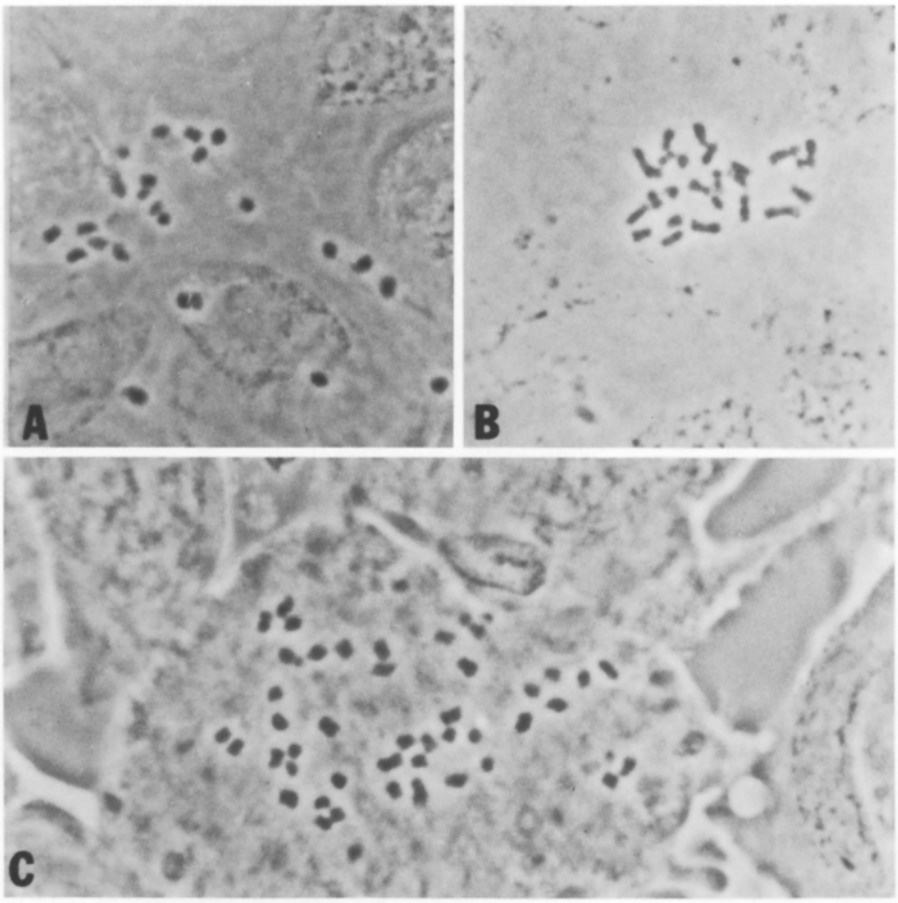
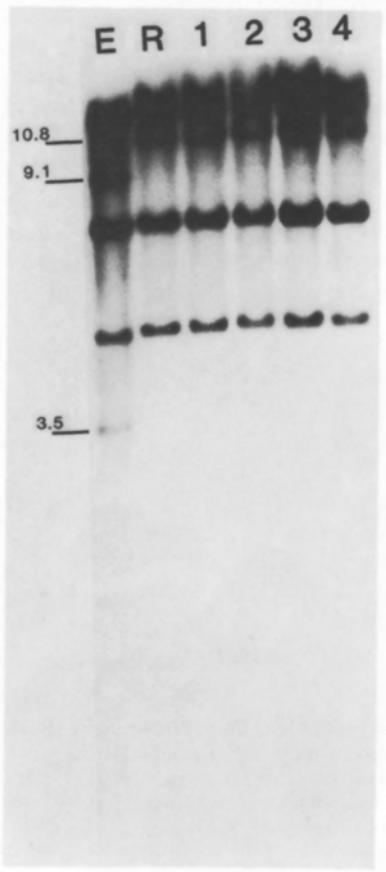


Fig. 3A–C. Chromosome spreads in root tip cells. **A** *S. rickii;* **B** *L. esculentum* cv. 'San Marzano'; **C** Somatic hybrid no. 2 (×2,000 fold)



bosomal genes after the DNA has been restricted with Nru 1. *L. esculentum* mtDNA contains 15.2 and 12.6 kb fragments which are not present in *S. rickii* mtDNA. *S. rickii* mtDNA contain 16.0, 14.4, 4.3 and 2.5 kb fragments which are not found in digests of *L. esculentum* mtDNA. The four somatic hybrid plants analyzed contain the 16.0, 4.3 and 2.5 kb fragments specific for *S. rickii* but not the 14.4 kb fragment. They do not contain any detectable quantities of the *L. esculentum* specific fragments. The four somatic hybrids have noticeably lower amounts of the 4.3 and 2.5 kb fragments, relative to the intensities of these fragments in the *S. rickii* lane. Figure 6 shows the fragments containing homology to the cytochrome oxidase subunit II gene after digestion with Bgl 1. *L. esculentum* mtDNA contains 9.7 and 3.9 kb fragments which are not found in digests of *S. rickii* mtDNA. *S. rickii* mtDNA con-

Fig. 4. Identification of chloroplast genomes present in somatic hybrid plants. *Lane E* contains DNA from *L. esculentum, R* contains DNA from *S. rickii. Lanes 1–4* contain DNA from the somatic hybrid plants no. 4, 5, 6, and 7, respectively. All lanes contain 4 μg DNA restricted with Bgl 1. The blot of the agarose gel was probed with ^{32}P labeled *L. esculentum* cpDNA. Restricted lambda DNA was used as size markers. The sized band indicate species-specific fragments

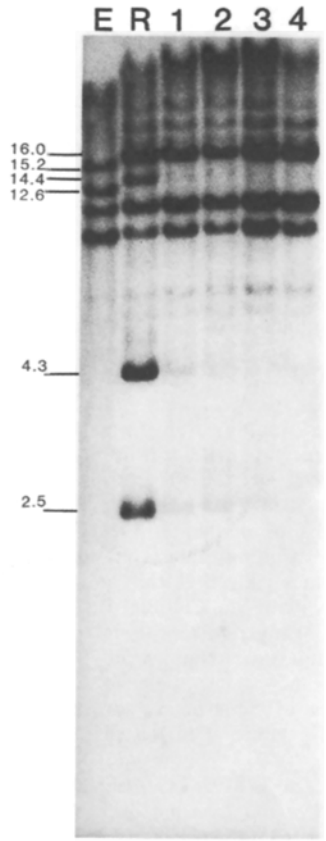


Fig. 5. Identification of mitochondrial ribosomal RNA genes in somatic hybrid plants. *Lane E* contains DNA from *L. esculentum, R* contains DNA from *S. rickii. Lanes 1–4* contain DNA from somatic hybrid plants no. 4, 5, 6, and 7, respectively. All lanes contain 4 μg DNA restricted with Nru 1. The blot of the agarose gel was probed with ^{32}P labeled soybean clone containing 18S and 5S mitochondrial rDNA. Restricted lambda DNA was used as size markers. The sized bands indicate species-specific fragments

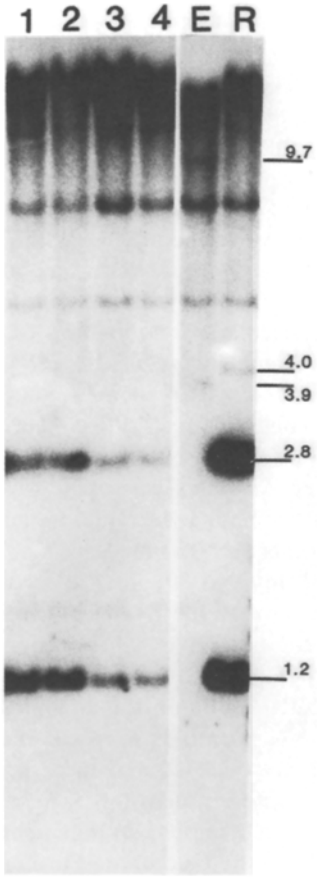


Fig. 6. Identification of mitochondrial cytochrome oxidase subunit II genes in somatic hybrid plants. *Lane E* contains DNA from *L. esculentum, R* from *S. rickii. Lanes 1–4* contain DNA from somatic hybrid plants no. 4, 5, 6, and 7, respectively. All lanes contain 4 μg DNA restricted with Bgl 1. The blot of the agarose gel was probed with ^{32}P labeled clone containing the maize mitochondrial cytochrome oxidase subunit II gene. Restricted lambda DNA was used as size markers. The sized bands indicate species-specific fragments

tains 4.0, 2.8, and 1.2 kb fragments which are not found in *L. esculentum* digests. The four somatic hybrid plants tested in lanes 1–4 contain the 2.8 and 1.2 kb fragments specific for *S. rickii* mtDNA, but no detectable levels of the *L. esculentum* specific bands or the 4.0 kb *S. rickii* fragment.

Discussion

Using polyethyelene glycol as a fusogen, we were able to produce intergeneric somatic hybrid plants. *Solanum rickii* and *Lycopersicon esculentum* when fused were still able to produce organized structures and regenerate into plants. All of the somatic hybrid plants arose from a single callus clone and are assumed to be the result of a single protoplast fusion event. The plants, however, differ from one another with regard to leaf shape (Fig. 2) and ploidy (Table 1). It is not clear yet whether the leaf shape differences are the result of a gene dosage effect, as we have observed with *L. esculentum* × *L. pennellii* regenerants (O'Connell and Hanson 1984), the loss of chromosomes or regions of chromosomes from either parent, or alteration in gene expression.

While the somatic hybrid callus was chimeric with regard to the ploidy of the plants regenerated from it, all four plants tested had inherited *S. rickii* cpDNA (Fig. 4) and had similar mtDNA patterns, (Figs. 5 and 6). We saw no evidence of cpDNA recombinations in these somatic hybrids. While intramolecular recombination of cpDNA occurs within a chloroplast (Kolodner

and Tewari 1979; Palmer and Thompson 1981), it has only recently been reported to occur between different parental cpDNA molecules following protoplast fusion, and then it is a very rare event (Medgyesy et al. 1985). The mitochondrial genome in *S. rickii* and *L. esculentum* is greater than 300 kb (McClean and Hanson 1985), and only a fraction of this genome has been characterized using the mtDNA specific probes. Within the limited regions of mtDNA examined, there was no evidence of mtDNA recombination in these somatic hybrids. Inter- and intra-molecular recombination occurs in plant mtDNA (Palmer and Shields, 1984; Lonsdale 1984), and is routinely observed in somatic hybrid and cybrid plants (Belliard et al. 1979; Nagy et al. 1981; Boeshore et al. 1983; Hanson 1983; Chetrit et al. 1985). However, when *Solanum tuberosum* was fused with *L. esculentum,* the somatic hybrid plants had only potato mtDNA (Shepard et al. 1983).

While no non-parental mtDNA fragments were detected using heterologous probes, the *L. esculentum–S. rickii* somatic hybrids did not have hybridization patterns identical with either parent. Overall, the ribosomal and cytochrome oxidase subunit II homologies in the somatic hybrid mtDNA resembled *S. rickii,* but several specific bands were not detected and the relative amounts of bands were different than the *S. rickii* pattern. These results suggest that some rearrangement of the somatic hybrid mtDNA had occured. Further characterization of the somatic hybrids' mitochondrial genome will be required to determine whether any DNA regions derived from the *L. esculentum* parent are present.

Acknowledgements. We are grateful to Linda Hosticka for her excellent technical assistance. This research was supported by Agrigenetics Research Associates. This work was done while the authors were in the Biology Department, University of Virginia.

References

Belliard G, Pelletier G, Vedel F, Quetier F (1978) Morphological characteristics and chloroplast DNA distribution in different cytoplasmic parasexual hybrids of *Nicotiana tabacum.* Mol Gen Genet 165:231–237

Belliard G, Vedel F, Pelletier G (1979) Mitochondrial recombination in cytoplasmic hybrids of *Nictotiana tabacum* by protoplast fusion. Nature 218:401–403

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

Bonnet HT, Glimelius K (1983) Somatic hybridization in *Nicotiana:* Behavior of organelles after fusion of protoplasts from male-fertile and male-sterile cultivars. Theor Appl Genet 65:213–217

Chen K, Wildman SG, Smith HH (1977) Chlorplast DNA distribution in parasexual hybrids as shown by polypeptide composition of fraction 1 protein. Proc Natl Acad Sci USA 74:5109–5112

Chetrit 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

Denhardt D (1976) A membrane filter technique for the detection of complementary DNA. Biochem Biophys Res Commun 23:641–646

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 U, 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

Gleba Yu Yu, Hoffman F (1980) "Arabidobrassica": a novel plant obtained by protoplast fusion. Planta 149:112–117

Hanson MR (1983) Stability variation and recombination in plant mitochondrial genomes via cell culture and somatic hybridization. Oxford Surv Plant Mol Cell Biol 1:33–52

Kolodner R, Tewari KK (1979) Inverted repeats in chlorplast DNA from higher plants. Proc Natl Acad Sci USA 76:41–45

Lonsdale DM (1984) A review of the structure and organization of the mitochondrial genome of higher plants. Plant Mol Biol 3:201–206

Maliga P, Kiss Z, Nagy AH, Lazar G (1978) Genetic instability in somatic hybrids of *Nicotiana tabacum* and *Nicotiana knightiana.* Mol Gen Genet 163:145–151

Medgyesy P, Menczel L, Maliga P (1980) The use of cytoplasmic streptomycin resistance: chloroplast transfer from *Nicotiana tabacum* into *Nicotiana sylvesis,* and isolation of their somatic hybrids. Mol Gen Genet 179:693–698

Medgyesy P, Fejes E, Maliga P (1985) Interspecific chloroplast recombination in a *Nicotiana* somatic hybrid. Proc Natl Acad Sci USA (in press)

Melchers G, Sacristan MD, Holder AA (1978) Somatic hybrid plants of potato and tomato regenerated from fused protoplasts. Carlsberg Res Commun 43:203–218

McClean PE, Hanson MR (1985) Mitochondrial DNA sequence divergence among *Lycopersicon* and related *Solanum* species. Genetics (in press)

Morgens PH, Grabau EA, Gesteland F (1984) A novel soybean mitochondrial transcript resulting from a DNA rearrangement involving the 5S rRNA gene. Nucleic Acids Res 12:5665–5684

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

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

O'Connell MA, Hanson MR (1984) Somaclonal variation in *Lycopersicon* regenerates. Plant Physiol 75:98

O'Connell MA, Hanson MR (1985) Somatic hybridization between *Lycopersicon esculentum* and *Lycopersicon pennellii.* Theor Appl Genet 70:1–12

Palmer JD, Shields CR (1984) Tripartite structure of the *Brassica campestris* mitochondrial genome. Nature 307: 437–440

Palmer JD, Thompson WF (1981) Rearrangements in the chloroplast genomes of mung bean and pea. Proc Natl Acad Sci USA 78:5533–5537

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

Rick CM (1979) Biosystematic studies in *Lycopersicon* and closely related species of *Solanum*. In: Hawkes JG, Lester RN, Skelding AD (eds) The biology and taxonomy of the Solanaceae. Academic Press, London, pp 667–677

Rick CM (1982) Thee potential of exotic germplasm for tomato improvement. In: Vasil IK, Scowcroft WR, Frey KJ (eds) Plant improvement and somatic cell genetics. Academic Press, New York, pp 1–28

Rigby P, Dieckmann M, Rhodes C, Berg P (1977) Labelling deoxyribonucleic acid to high specific activity in vitro by nick tanslation with DNA polymerase I. J Mol Biol 113: 237–251

Saltz Y, Beckmann J (1981) Chloroplast DNA preparation from *Petunia* and *Nicotiana*. Plant Mol Biol Newslett 2:73

Shepard JF, Bidney D, Barsby T, Kemble R (1983) Genetic transfer in plants through interspecific protoplast fusion. Science 219:683–688

Southern, EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Tanksley SD (1979a) An efficient and economical design for starch gel electrophoresis. Rep Tomato Genet Coop 29: 37–39

Tanksley SD (1979b) Linkage, chromosomal association and expression of *Adh-1* and *Pgm-2* in tomato. Biochem Genet 17:1159–1167

Uchimaya H, Murashige T (1974) Evolution of parameters in the isolation of viable protoplasts from cultured tobacco cells. Plant Physiol 54:936–944