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Somatic hybridization between *Lycopersicon esculentum* and *Lycopersicon pennellii*

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Summary. Selection and screening methods were devised which resulted in the identification of a number of somatic hybrid callus clones following fusion of *Lycopersicon esculentum* protoplasts and *L. pennellii* suspension culture protoplasts. Visual selection for callus morphology combined with a high fusion frequency and irradiation of one parental protoplast type (^{137}Cs source, 1.5 Krads) resulted in selection of a callus clone population containing a high proportion of somatic hybrids. Analysis of a dimeric isozyme for the presence of a heterodimeric form was found to be satisfactory for distinguishing parental-type calli, somatic hybrid calli, and mixed calli derived from both types of unfused parental cells. No somatic hybrid calli produced shoots, although the sexual hybrid between *L. esculentum* and *L. pennellii* regenerated well under the culture conditions employed. This result suggests that the non-regenerable growth habit of the *L. pennellii* suspension culture was dominant in the somatic hybrid. The culture conditions described here are suitable for obtaining regenerated plants from *L. esculentum* mesophyll protoplasts. *L. esculentum* protoplast calli from fusion cultures gave rise to shoots with *L. esculentum* phenotype at higher frequency than calli from control unfused *L. esculentum* mesophyll protoplast cultures. The use of probes for species-specific organelle DNA fragments allowed identification of organelle DNA restriction fragments in digests of total DNA from small samples of individual callus clones. The callus clones analyzed either carried predominantly one parental plastid DNA type or mixtures of both types. Use of a mitochondrial DNA (mtDNA) probe which distinguishes two parental mtDNA fragments revealed that the *L. pennellii*-specific fragment was present in all clones examined, but the *L. esculentum* fragment was absent or in low proportion.

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

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Introduction

The cultivated tomato, *Lycopersicon esculentum,* has not been as readily manipulated in cell cultures as other members of the family Solanaceae (Evans and Bravo 1983; Galun and Aviv 1983; Harms 1983). There have been three reports of the successful regeneration of tomato plants from protoplasts (Koblitz and Koblitz 1982; Morgan and Cocking 1982; Hanson 1982), and there have been two reports of regeneration of somatic hybrid plants formed by the fusion of protoplasts prepared from potato and tomato (Melchers et al. 1978; Shepard et al. 1983).

While sexual hybrids of most species carry only the maternal organelle genomes, somatic hybridization can result in transmission of either parental organelle genome or novel organelle genomes to the regenerated plants (Belliard et al. 1978, 1979; Boeshore et al. 1983; Galun et al. 1982; Hanson 1983; Menczel et al. 1981). *L. pennellii,* a green-fruited wild relative of the cultivated tomato, is sexually compatible with *L. esculentum* only when it serves as the pollen parent. The sexual hybrid between these two species grows very well in culture and fertile, healthy plants can easily be regenerated from protoplasts (O'Connell and Hanson 1984). We describe here fusion experiments designed to generate somatic hybrids between *L. esculentum* and

L. pennellii. Selection strategies based on callus morphology and growth inhibition due to irradiation of one fusion partner were utilized. Calli selected following protoplast fusion were analyzed with regard to a nuclear isozyme marker and organelle genome restriction fragment markers. This report also describes culture conditions suitable for regenerating tomato cultivars from mesophyll protoplasts.

Materials and methods

Plant material

Lycopersicon pennellii, LA716 collected in Atico Peru, was generously supplied by C. Rick; Tomato Genetics Stock Center, Univ. California, Davis. *Lycopersicon esculentum* cultivar 'UC82B' was a gift from A. Binns, Univ. Pennsylvania and 'Petoseed No. 46' was a gift from J. Watterson, Petoseed Co., Woodland, CA. All other cultivars were from commercial seed sources, Burpee, Herbst Bros. or Sun Seeds.

Protoplast isolation

Tomato (*Lycopersicon esculentum* cv. 'UC82B' or 'Petoseed No. 46') seeds were planted in vermiculite and germinated in an environmental chamber set for 12 h photoperiod, 1,500 lux, 22 °C day, 18 °C night at 60% relative humidity. Seedlings were watered daily with Nitsch watering solution, modified so that $(NH_4)_2SO_4$ was reduced to 0.1 mM (Nitsch 1965). When plants were 6–7 weeks old, one or two of the youngest fully expanded leaves were removed, the leaflets were cut off the petiole and surface sterilized in 0.2% SDS, 20% bleach (500 mls) for 15 min followed by three washes in sterile distilled water for 5, 5, and 10 min. The leaflets were then placed adaxial side down on 1/4X Nitsch watering solution (Nitsch 1965) and kept in the dark for 5 days at ambient temperature. Protoplasts were isolated and cultured based on the procedures described by Shepard (1981). The lower epidermis was peeled off using jeweler's forceps and the leaflets were placed abaxial side down on 0.3 M sucrose, 5 mM MES, 10 mg/l casein hydrolysate, 1 mM polyvinylpyrrolidine, 1/4X R salts (Table 1), 2 g/l Pectinol Ac (Rohm and Haas), 15 g/l Cellulysin (Calbiochem), pH 5.6 and digested in the dark at 25 °C on a gyratory shaker for 18 h. The protoplasts were collected by layering the digestion mixture over 2.5 mls 15% Percoll (Pharmacia) in JS Rinse (0.3 M sucrose, 1X R salts, 10 mg/l casein hydrolysate (ICN), pH 5.6) and centrifuging for 10 min at 500 g. The protoplasts that floated and those that collected at the Percoll-enzyme interface were carefully transferred to a fresh tube, washed with 7–10 volumes of JS Rinse, and spun for 10 min at 500 g. The protoplasts that floated on JS Rinse were collected, counted in a hemocytometer and diluted with W5 (Medgyesy et al. 1980) to the desired concentration.

Lycopersicon pennellii suspension cultures were maintained on UMlA (Uchimaya and Murashige 1974) with casein hydrolysate concentration lowered to 0.5 g/l, and transferred at weekly intervals. Five mls of packed cells were digested for 18 h with four volumes of 1/10X Nitsch salts, 10 mM MES, 0.5 M mannitol, 10 g/l cellulase RS, 5 g/l pectinol, 2.5 g/l Rhozyme HP150, 12.5 mg/l Fluorosceinisothiocyanate (FITC), pH 5.6 on a gyratory shaker in the dark at 25 °C. The suspension cell protoplasts were collected as described for mesophyll protoplasts. The protoplasts were adjusted to 1.6×10^6 protoplasts/ml and irradiated for 3 or 20 min in a Gammator, Isomedix Inc., ^{137}Cs source, 500 Rad/min.

Fusion protocol

The procedure of Medgyesy et al. (1980) was modified and used to fuse the *Lycopersicon* protoplasts. 0.3 ml (4×10^5 protoplasts) of a 1 : 1 or 1 : 10 mixture of the two protoplast stocks was transferred to 35 mm plastic petri dishes and the protoplasts were allowed to settle for 5 min. 0.15 ml of 30% polyethylene glycol, (MW 6,000, Koch-Light) 0.3 M glucose, 50 mM $CaCl_2$, was added slowly. After 15 min, 0.2 ml W10 (Medgyesy et al. 1980) was added. After 20 min, 1.5 mls culture media was added; 5 min later all the fluid was carefully removed from the dish and 1 ml of JSCl 2.5 media was added, the dishes were sealed with Parafilm® and cultured in the dark at 28 °C.

Protoplast culture

The cells were transferred from 35 mm dishes to 60 mm dishes four days after plating and diluted with 2 mls of a 1 : 1 mixture of JSCl 2.5 and JSR media (Table 1). Three days later, the culture was diluted again with 12 mls of a 1 : 1 mixture of JSCl 2.5 and JSR media and moved to a 100 mm dish. Five days later, 5 mls of culture were plated on JSC-12 media and grown until the calli were large enough (1–2 mm in diameter) to be independently transferred to fresh JSC-12 plates (Table 1). While on this medium, calli were illuminated with 1,500 lux for 12 h/day. After the calli had increased in size to 0.5 cm or larger and turned green (usually one month), they were transferred to TR-1, a shoot-inducing medium. Four weeks later, the green calli were transferred to fresh TR-1. When the shoots became large enough, they were cut off the callus and transferred to root-inducing medium, N13. After roots had formed, in 2–4 weeks, the plants were carefully removed from the sterile containers, potted in vermiculite and kept in plastic bags in an environmental chamber for a week. The bags were opened for 3–4 days, and finally the acclimatized plants were repotted and moved to the greenhouse. This sequence of events is documented in Fig. 1.

DNA isolation

Total DNA was isolated from frozen lyophilized callus or 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 adding four volumes of sterile distilled water. Chloroplast DNA prepared from *L. esculentum* was a gift of E. Clark (University of Virginia). The pUC8 clones of mitochondrial DNA isolated from *L. pennellii* will be described elsewhere (McClean and Hanson, submitted). DNA to be used as a probe was nick translated with α^{32}P-CTP (New England Nuclear) as described by Rigby et al. (1977).

Restriction endonuclease digestions

Four µg of DNA were digested for 2–4 h at 37 °C in 30 µl with either Sst I (BRL) in 50 mM Tris-HCL pH 8.0, 10. mM $MgCl_2$, 50 mM NaCl or Sma I (BRL) in 15 mM Tris-HCl pH 8.0, 6 mM $MgCl_2$ 15 mM KCl. Reactions were stopped with the addition of 5 µl 1% SDS, 0.125 M EDTA, 50% glycerol, 0.1% bromophenol blue, incubated at 65 °C for 5 min and immediately electrophoresed on 1% agarose gel, 40 mM Tris, 1 mM EDTA, 5 mM Na Acetate pH 7.75. Samples were electrophoresed at 55 mAmperes for 18 h. Gels were stained with ethidium bromide for 1 h and photographed under UV. 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 (Den-

Table 1. Media composition

	JSCL 2.5	JSR[a]	JSC-12	TR 1	N 13
Macroelements (g/l)					
KNO_3	7.6	1.9	1.9	1.9	0.41
$CaCl_2$–$2H_2O$	1.76	0.44	0.44	0.44	–
$MgSO_4$–$7H_2O$	1.48	0.37	0.37	0.37	0.55
KH_2PO_4	0.68	0.17	0.17	0.17	0.14
NH_4Cl	–	0.028	0.11	–	–
$Ca(NO_3)$–$4H_2O$	–	–	–	–	0.96
$(NH_4)_2SO_4$	–	–	–	–	0.014
KCl	–	–	–	–	0.0027
NH_4NO_3	–	–	–	1.65	–
Microelements (mg/l)					
Na_2ethylenediaminetetra-acetic acid	18.5	18.5	18.5	15	15
$FeSO_4$–$7H_2O$	13.9	13.9	13.9	11	11
H_3BO_3	3.1	3.1	3.1	6	6
$MnCl_2$–$4H_2O$	9.9	9.9	9.9	–	–
$ZnSO_4$–$7H_2O$	4.6	4.6	4.6	4	4
KI	0.42	0.42	0.42	0.8	0.8
Na_2MoO_4–$2H_2O$	0.13	0.13	0.13	0.25	0.25
$CuSO_4$–$5H_2O$	0.013	0.013	0.013	0.025	0.025
$CoSO_4$–$7H_2O$	0.015	0.015	0.015	–	–
$MnSO_4$–H_2O	–	–	–	18	18
$CoCl_2$–$6H_2O$	–	–	–	0.025	0.025
Organics (mg/l)					
Thiamine	0.05	0.5	0.5	5	2
Glycine	2	2	2	–	–
Nicotinic acid	5	5	5	3	0.5
Pyridoxine	–	–	–	0.5	–
Folic acid	0.5	0.5	0.5	–	–
Biotin	0.05	0.05	0.05	–	–
Casein hydrolysate	50	100	100	–	–
Myo-inositol	4,400	–	100	100	1,000
Adenine-SO_4	–	–	40	–	–
Sugars (g/l)					
Sucrose	68	34	4.7	–	5
Mannitol	4.4	8.8	109.2	–	–
Sorbitol	4.4	–	–	–	–
Xylitol	3.8	–	–	–	–
Glucose	–	–	–	30	–
Other (mg/l)					
6-Benzyladenine	0.4	0.2	–	–	–
Zeatin	–	–	2	1	–
2,4-Dichlorophenoxy-acetic acid	0.6	–	–	–	–
Indole-3-acetic acid-phenylalanine	–	–	0.1	0.1[7]	–
2-(N-morphilino)ethane sulfonic acid	–	–	970	–	–
Phytagar	–	–	7,000	7,000	7,000
pH	5.6	5.6	5.8	6.0	6.0

[a] 1×R salts is a stock solution of the macro and micro elements in JSR

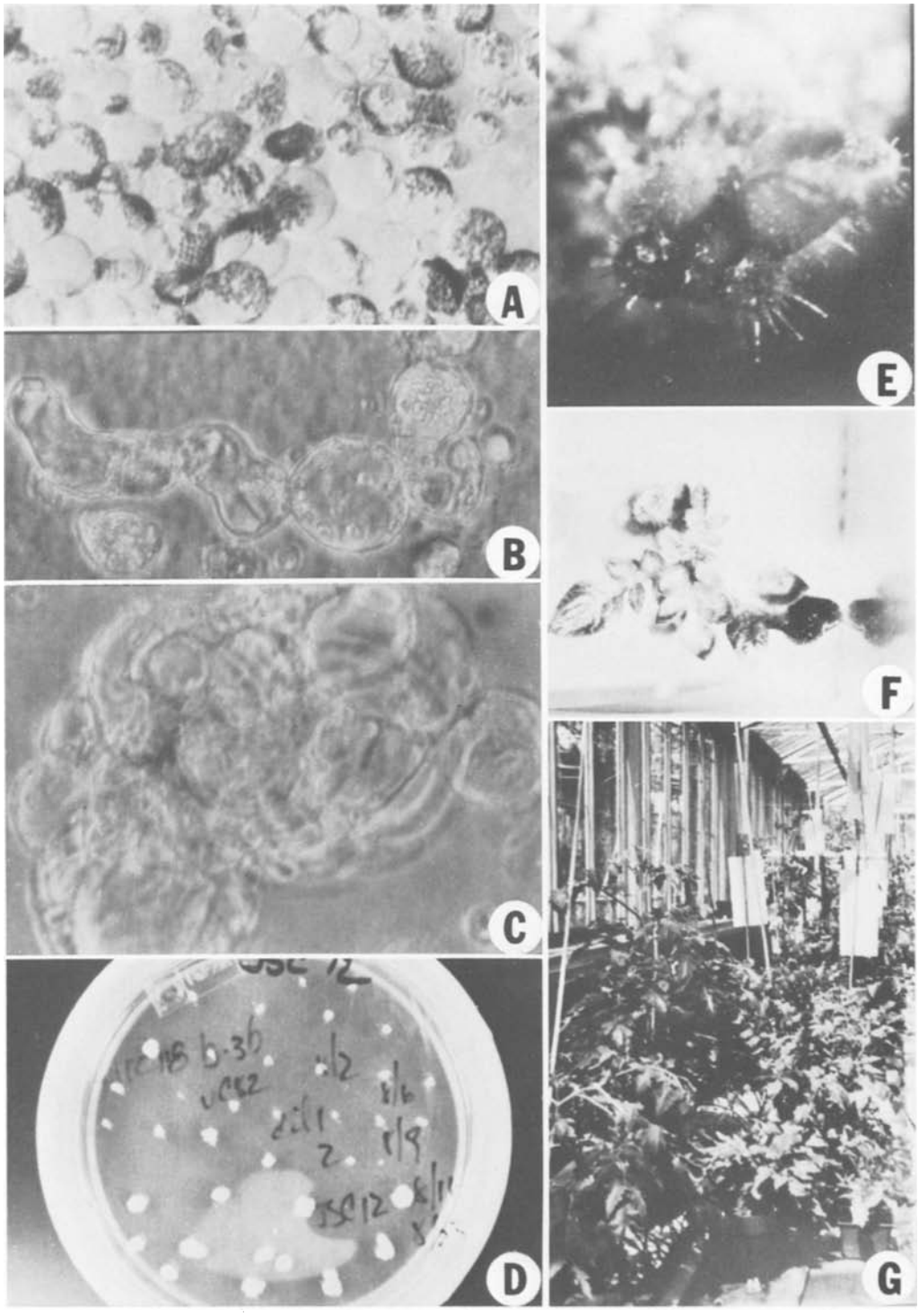

Fig. 1. **A** Protoplasts from mesophyllic cells of *L. esculentum.* **B** Dividing cells 5 days after planting. **C** Small colony 15 days after planting. **D** Small calli on greening media. **E** Shoot regenerating on callus. **F** Small plantlet on root inducing media. **G** Regenerated plants in greenhouse

hardt 1976) 6X SSC, total volume of 10 ml for 18 h at 65 °C. The blots were hybridized with α^{32}P-CTP labeled probe in 0.5% SDS, 0.2 mg/ml calf thymus DNA, 6X SSC, 5X Denhardt's, total volume 5 ml. Blots were hybridized at 65 °C for 18 h. The blots were then washed for 1 h with 10 ml 5X Denhardts, 6X SSC, 0.5% SDS, followed by three washes of 500 ml 2X SSC for 1 h each at 65 °C. The blots were air dried, wrapped in Saran Wrap® on cardboard mounts, exposed to X-ray film with a Cronex intensifying screen and stored at −70 °C.

Starch gel analysis of phosphoglucoisomerase isozymes

Approximately 0.5 gm (wet weight) of callus 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). Gels were stained for phosphoglucoisomerase activity as described by Tanksley (1980).

Results

Phenotypic differences in cultures of L. pennellii and L. esculentum

The suspension culture from which the *L. pennellii* protoplasts were made was two years old and grew rapidly as a finely divided suspension. While plants had been regenerated from this culture soon after it was established (E. Dowling and M. Hanson, unpublished), callus from this culture has not recently been induced to regenerate shoots, even on media which readily induced shoot regeneration for a number of related species. The microcalli which formed from protoplasts of unirradiated *L. pennellii* suspension cells were loose aggregates of cells, and broke up easily when the liquid culture was agitated gently. The microcalli which formed from protoplasts of *L. esculentum* mesophyll cells, on the other hand, were compact, discrete cell masses which did not break up to form single cells or smaller cell clusters. It was possible, therefore, to identify microcalli which were derived from protoplasts of *L. pennellii.* In fusion experiments, only compact microcalli were selected and transferred to solidified JSC-12 media. It was anticipated that microcalli formed by fused protoplasts would grow as discrete colonies. *L. pennellii* calli which grew from protoplasts which had been irradiated for 3 min (1.5 Krad) died when transferred to shoot inducing media (Fig. 2). *L. pennellii* protoplasts which had been irradiated for 20 min (10 Krad) did not divide more than once and did not form microcalli.

Fusion

Protoplasts prepared from mesophyll cells of the *L. esculentum* cultivars 'UC82B' or 'Petoseed No. 46' were fused with protoplasts prepared from suspension cells of *L. pennellii* that had been exposed to 1.5 or 10 Krad. Since the protoplasts prepared from suspension cells had been stained with FITC, it was possible to determine confidently an estimate of the number of fusion products by examining the cultures under fluorescing conditions. Cells fluorescing both red (chlorophyll autofluorescence) and green (FITC) were frequently seen, at least one for every ten cells observed (Fig. 3). Using the double fluorescent signal, fused protoplasts were observed that had undergone cells division after six days in culture. In many cases the *L. esculentum* chloroplasts were equally distributed between the two daughter cells; a few cell pairs were observed, though, in which one of the two daughter cells contained a greater number of the autofluorescing chloroplasts.

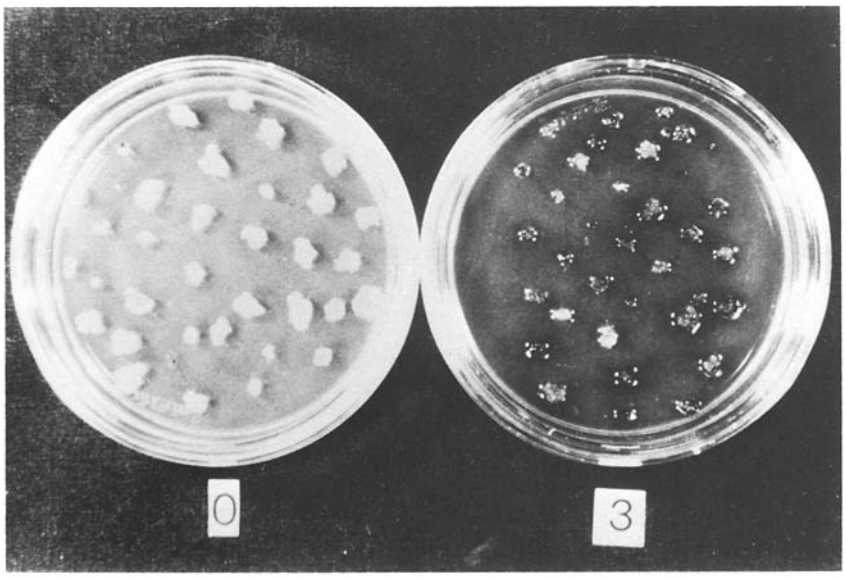

Fig. 2. Growth of *L. pennellii* calli derived from irradiated protoplasts on greening media. The dish on the *left* contains calli which grew from unirradiated protoplasts. The dish on the *right* contains calli which grew from protoplasts irradiated for 3 min. Both sets of calli had been growing on JSC–12, the greening media, for 1 month

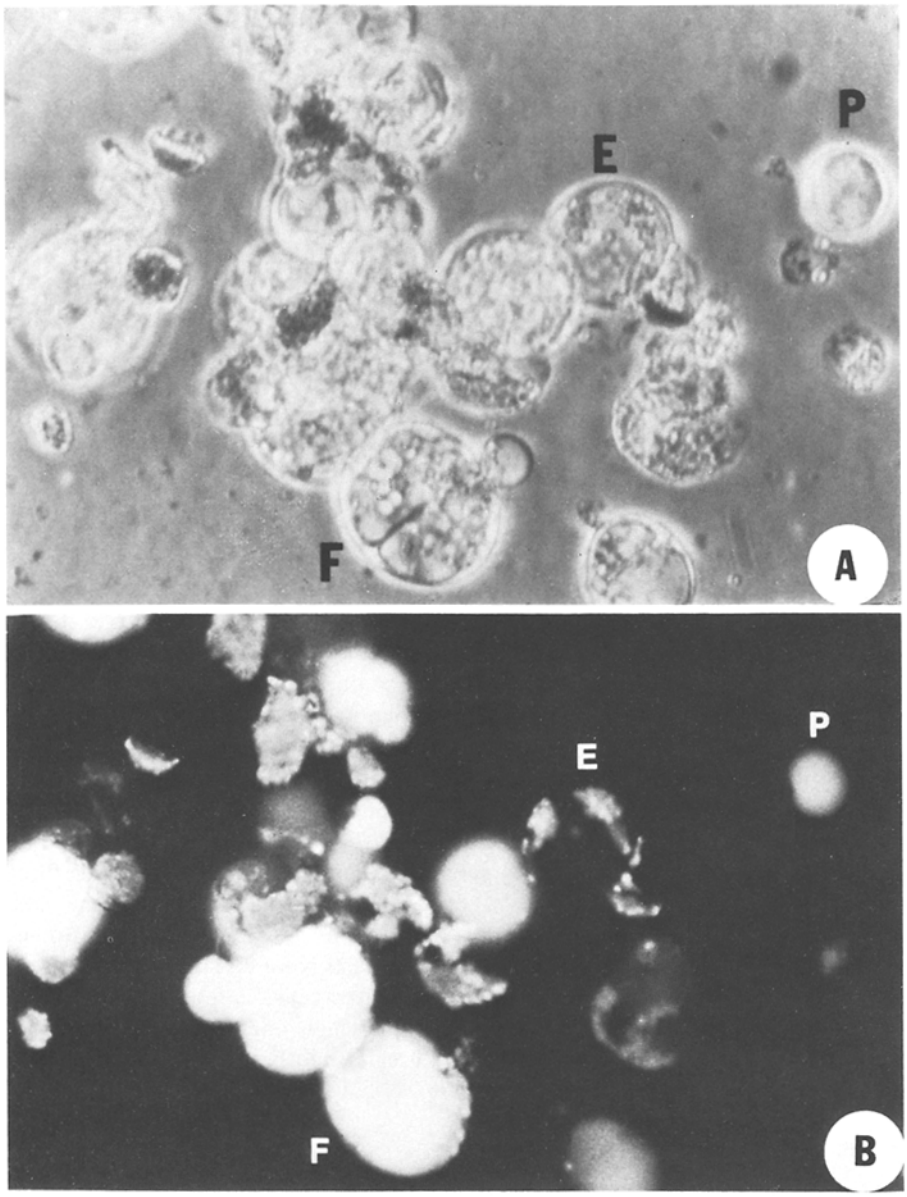

Fig. 3 A, B. Identification of fused protoplasts with fluorescence microscopy. **A** White light illumination of field with fused protoplasts 2 h after removal of polyethylene glycol, *E, L. esculentum, P, L. pennellii, F,* fused protoplast. **B** Same field illuminated with 390–490 nm light

Table 2. Number of calli transferred to JSC-12. Micro calli were selected as described in the text from cultures which had initially contained 1:1 mixtures of unfused protoplasts, 1:1 or 1:10 *L. esculentum* to *L. pennellii* protoplasts as fusion partners

Treatment		Fusion partners					
		L. pennellii-0		*L. pennellii-3*		*L. pennellii-20*	
		'UC82b'	'Peto-#46'	'UC82b'	'Peto-#46'	'UC82b'	'Peto-#46'
Unfused	1: 1	17	57	34	27	3	13
Fused	1: 1	148	215	107	241	229	163
Fused	1:10	180	48	95	171	131	11

After several weeks in culture, the microcalli were large enough to be transferred to solid media. In total, 1,800 microcalli were selected from the liquid cultures. All of the calli which grew as discrete colonies were transferred to greening media, JSC-12 (Table 2). Roughly equal numbers from each fusion were taken: for 'UC82B', 328, 302, 360 microcalli were selected from fusion dishes with 0, 3, and 20 min γ-irradiated protoplasts from *L. pennellii*, respectively. For the cultures of 'Petoseed No. 46', 263, 412, 174, microcalli were taken from fusion dishes with 0, 3, and 20 min γ-irradiated protoplasts from *L. pennellii*, respectively. The remainder of the microcalli in these dishes grew in a loose fashion and were considered to be *L. pennellii*. There were no cells growing as loose aggregates in the cultures which developed when 20 min γ-irradiated *L. pennellii* had been used as a fusion partner.

After transfer to TR-1, some calli immediately developed shoots within one week. Those calli which regenerated shoots did not visibly increase in the amount of callus cells after transfer; instead most of the new growth was in the form of shoots. A large number of calli did not regenerate shoots. These calli grew rapidly and formed light green friable masses. Small portions of both of these types of callus were analyzed using starch gel electrophoresis as described below.

Identification of somatic hybrid calli

L. pennellii and *L. esculentum* contain forms of the homodimeric enzyme phosphoglucoisomerase which can be distinguished by starch gel electrophoresis (Fig. 4, lanes 1, 2, 17 and 18). The sexual hybrid between these two species makes a third form of the enzyme, a heterodimer (Fig. 4, lanes 3 and 16). Mix-

Table 3. Phosphoglucoisomerase isoenzymes expressed in calli cultured from protoplasts after PEG fusion

Isozyme pattern	Fusion partners					
	L. pennellii-0		*L. pennellii-3*		*L. pennellii-20*	
	'UC82b'	'Peto-#46'	'UC82b'	'Peto-#46'	'UC82b'	'Peto-#46'
esculentum	14	3	3	–	–	21
pennellii	1	–	–	–	–	–
Somatic hybrid	14	37	29	23	1	–
Mixture	–	–	1	–	–	–

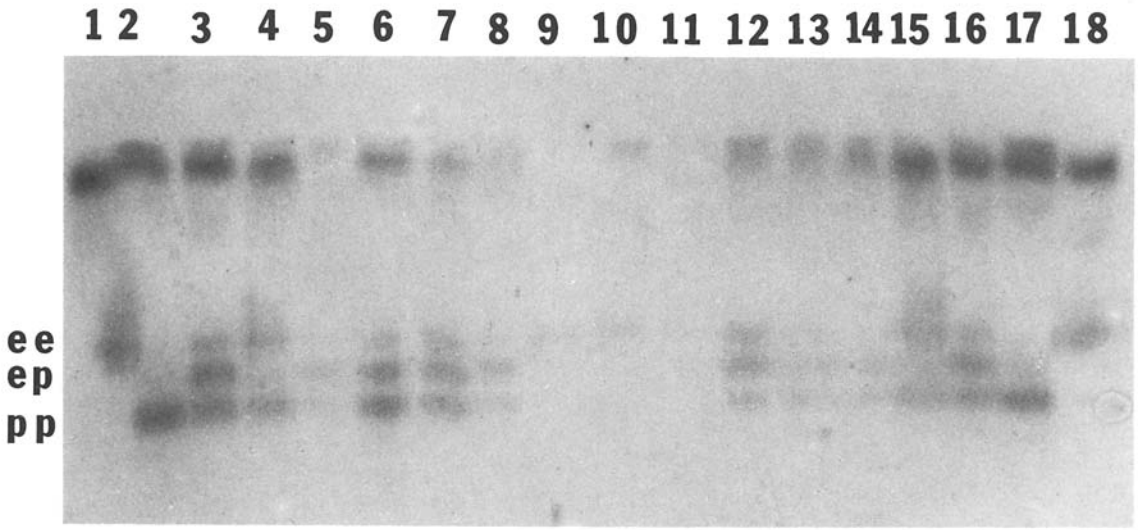

Fig. 4. Starch gel analysis of phosphoglucoisomerase activities. Extracts of callus were applied to a starch gel with wicks and electrophoresed; the gel was sliced and stained for phosphoglucoisomerase activities as described in the "Methods" section. *Lanes 1* and *18* contain *L. esculentum*; *lanes 2* and *17* contain *L. pennellii*. *Lanes 3* and *16* contain the sexual hybrid; *lanes 4* and *15* contain a mixture of *L. esculentum* and *L. pennellii*. *Lanes 5–14* contain extracts from calli which grew up in the fusion dishes. The homodimeric forms of phosphoglucoisomerase specified by gene *Pgi-1* (Tanksley 1980), are marked ee or pp and the heterodimeric form is marked ep. The additional band at the top of the gel is a different phosphoglucoisomerase isozyme specified by another locus

tures of extracts from these two species when electrophoresed and stained for phosphoglucoisomerase activity show only the parental forms, not the heterodimer (Fig. 4, lanes 4 and 15). *L. esculentum* callus was mixed with *L. pennellii* callus and the mixture was cultured for one month. When extracts of this mixed callus were analyzed for phosphoglucoisomerase isozymes, only the parental homodimers were seen; no heterodimer was detected (data not shown). Therefore, the presence of the heterodimer in an extract indicates that both alleles are transcribed and translated in the same cell and can be used to identify a somatic hybrid callus.

Calli which were selected from the fusion cultures were assayed for the presence of the phosphoglucoisomerase heterodimer. Lanes 5–14 in Fig. 4 are extracts from 10 different calli from this fusion experiment; lanes 5–8 and 12–14 were scored as somatic hybrids, lanes 9–11 were scored as *L. esculentum.* A summary of all these determinations is presented in Table 3. Of 147 calli tested, 60 calli were determined to be somatic hybrids of 'Petoseed No. 46' and *L. pennellii,* and 44 calli were determined to be somatic hybrids of 'UC82B' and *L. pennellii.* This represents a somatic hybrid frequency in those calli tested of 70%. Only 1 callus was determined to have arisen from an unfused *L. pennellii* protoplast. This indicates that distinguishing microcalli on the basis of their morphology is a reliable selection procedure. All of the calli which had regenerated shoots had the *L. esculentum* phosphoglucoisomerase pattern. The calli which grew rapidly and as friable masses had the phosphoglucoisomerase pattern of a somatic hybrid. None of the calli scored as somatic hybrid have regenerated shoots.

Analysis of chloroplast DNA in somatic hybrid calli

Those calli which were determined to have arisen from fused protoplasts were subcloned on MS564 (Murashige and Skoog salts, 0.15 M glucose, 12 μM thiamine, 4 μM nicotinic acid, 26 μM glycine, 550 μM myo-inositol, 4.5 μM zeatin, 1.1 μM naphthalene acetic acid, pH 5.8) a medium which caused the callus to proliferate but not regenerate (Hosticka 1982). The callus was then frozen in liquid nitrogen and lyophilized and DNA was isolated as described in the "Methods" section.

The chloroplast genomes of *L. esculentum* and *L. pennellii* have been well characterized (Palmer and Zamir 1982) and can be distinguished when cut with the restriction endonuclease Sst 1. Figure 5 is an autoradiograph of a blot containing DNA from *L. pennellii* (lane 6) and *L. esculentum* (lane 5) which have been cut with Sst 1 and probed with ^{32}P-labeled DNA purified from chloroplasts of *L. esculentum.* The fragments of chloroplast DNA (cpDNA) in total DNA preparations can be identified in this way. The upper arrow marks a fragment only observed when DNA from chloroplasts of *L. pennellii* is present, and the lower arrow marks a fragment only observed in preparations from *L. esculentum.* Lanes 1–4 contain DNA prepared from calli which were determined to be somatic hybrids. The callus analyzed in lane 1 contains both *L. esculentum* and *L. pennellii* chloroplast genomes whereas the calli analyzed in lanes 2–4 contain detectable levels of only *L. pennellii* chloroplast genomes.

Analysis of mitochondrial DNA in somatic hybrid calli

The tomato mitochondrial genome is larger than the chloroplast genome, making it difficult to interpret blots which had been probed with ^{32}P-labeled purified mitochondrial DNA (mtDNA). Instead, cloned fragments of *L. pennellii* mtDNA were used to identify the mitochondrial genomes present in the somatic hybrid calli. These cloned fragments were selected because

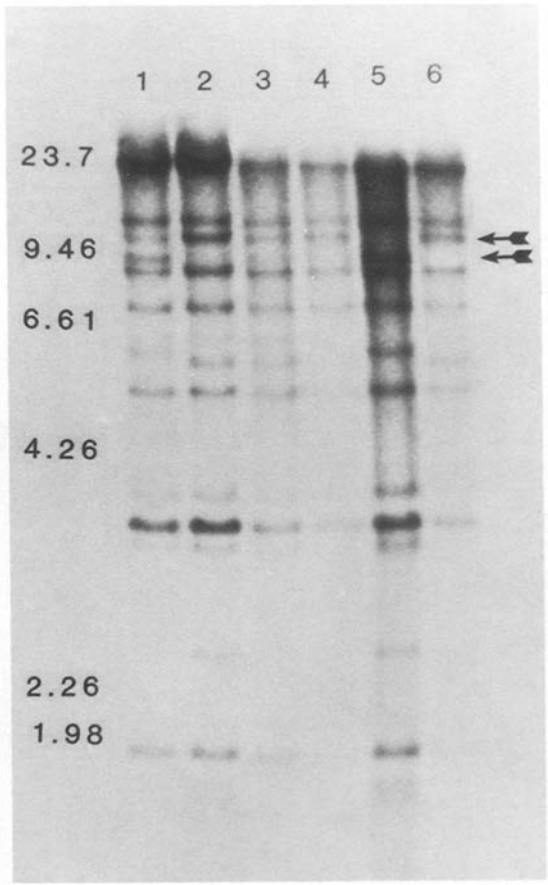

Fig. 5. Identification of the chloroplast genomes present in somatic hybrid calli. Samples were treated as described in the "Methods" section. *Lanes 1–4* contain DNA from somatic hybrid calli. *Lane 5* contains DNA from *L. esculentum; lane 6* contains DNA from *L. pennellii.* The *upper arrow* marks a fragment specific for chloroplast DNA from *L. pennellii,* the *lower arrow* marks a fragment specific for chloroplast DNA from *L. esculentum*

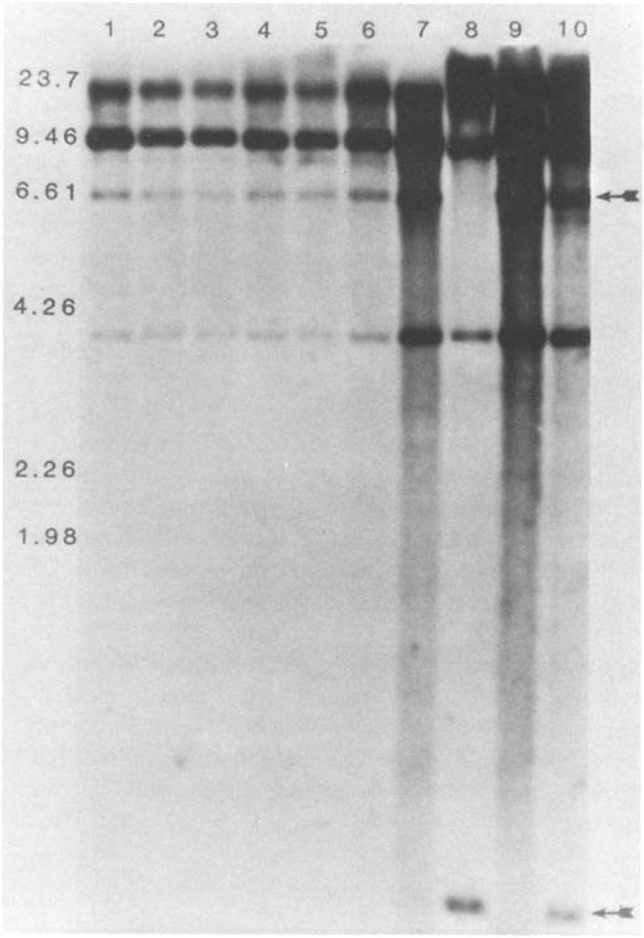

Fig. 6. Identification of the mitochondrial genomes present in somatic hybrid calli. Samples were treated as described in the "Method" section. *Lanes 1–7* contain DNA from somatic hybrid calli; *lane 8* contains DNA from *L. esculentum*. *Lane 9* contains DNA From *L. pennellii; lane 10* contains DNA from both *L. esculentum* and *L. pennellii*. The *upper arrow* marks a fragment specific for mitochondrial DNA from *L. pennellii;* the *lower arrow* marks a fragment specific for *L. esculentum* DNA

they hybridized to fragments of a different size in digests of *L. pennellii* than in digests of *L. esculentum* (McClean and Hanson, submitted). Figure 6 shows Sma I digests of DNA prepared from *L. pennellii* (lane 9) and *L. esculentum* (lane 8) which have been probed with one of these clones. The upper arrow marks a fragment which is only present in mtDNA prepared from *L. pennellii;* the lower arrow marks a fragment which is only present in mtDNA prepared from *L. esculentum*. Both fragments can be detected when DNA from both species is mixed and then electrophoresed (lane 10). Lanes 1–7 contain DNA prepared from somatic hybrid calli. All of the calli contain the *L. pennellii*-specific mtDNA fragment. Lanes 1–6 do not contain detectable quantities of *L. esculentum*-specific mtDNA fragment. Lane 7 has a very faint signal at a position characteristic of a *L. esculentum* mtDNA fragment. The results of organelle genome analyses of some somatic hybrid calli have been collected and are reported in Table 4.

Phenotypic stability of calli identified as somatic hybrid

Many of the calli identified as somatic hybrid were retested after months in culture. All of the somatic hybrids which were formed by fusion of a cultivar with either 0 or 3 min irradiated *L. pennellii* continued to express the heterodimer form of phosphoglucoisomerase. However, 6 calli were unstable somatic hybrids and expressed the heterodimer form of phosphoglucoisomerase in the initial stages of culture. After 4 months in culture they lost the heterodimer and only expressed the *L. esculentum* form of the enzyme and coordinately started to regenerate shoots. These 6 calli were formed by the fusion of a cultivar with 20 min irradiated *L. pennellii*. Either the progeny cells of the fusion product lost some or all of the *L. pennellii* chromo-

Table 4. Comparison of organelle genomes present in somatic hybrid calli

Sample	*L. esculentum* parent	*L. pennellii* min γ-ray	Pgi-2[a]	cp DNA	mt DNA
UC82b-Oc-4	UC82b	0	SH[b]	P[c]	P
UC82b-Oc-6	UC82b	0	SH	P	P+E
UC82b-Oc-34	UC82b	0	SH	P+E[d]	P
UC82b-Oc-105	UC82b	0	SH	P+E	P
UC82b-Oc-122	UC82b	0	SH	E	P
UC82b-3c-149	UC82b	3	SH	P+E	P
Peto-3b-47	Petoseed #46	3	SH	P+E	P
Peto-3b-48	Petoseed #46	3	SH	E	P
Peto-3b-157	Petoseed #46	3	SH	P+E	P

[a] PGI = phosphoglucoisomerase, [b] SH = somatic hybrids, [c] P = *L. pennellii*, [d] E = *L. esculentum*

somes, or these calli originated from two protoplasts, one fused and the other an unfused *L. esculentum.*

Regeneration of plants from L. esculentum protoplasts

Protoplasts prepared from tomato leaves can be induced to regenerate healthy fertile plants. Initial protoplast division frequencies range from 10 to 30%, and have been observed in a number of cultivars. Of 42 different *L. esculentum* cultivars tested, protoplasts prepared from 29 of them underwent cell division. Of those, 13 cultivars have been successfully cultured to form callus from protoplasts. In the experiments described here, several plants have been regenerated from control dishes of protoplasts obtained from 'UC82B' and 'Petoseed No. 46'. In comparison to cultures of fused protoplasts, control cultures grow slowly and tend to turn brown. While shoots do appear on calli grown from control cultures, the numbers of calli which develop are much less than in cultures of fused protoplasts (Table 2). Currently there are 79 plants in our greenhouse which regenerated from the fusion cultures described here. These plants all arose from calli which were determined to be *L. esculentum.* Of the 41 calli scored as *L. esculentum,* 28 have given rise to regenerated plants.

Discussion

Under the culture conditions we employed, visual selection for compact calli was sufficient to produce a population of callus clones highly enriched for somatic hybrids. Fifty-one out of 69 callus clones selected following fusion of unirradiated *L. pennellii* and *L. esculentum* protoplasts carried isozyme alleles from both parents (Table 3).

The enrichment for somatic hybrids is further increased by irradiating the *L. pennellii* protoplasts at a dose (1.5 Krad) which results in senescence and cessation of growth at the small callus colony stage (Fig. 2). Fifty-two of 56 callus colonies selected visually from fusions which had 3 min irradiated *L. pennellii* as a partner were identified as somatic hybrids. This experiment also revealed that a nuclear genome damaged by γ-irradiation could be rescued by fusion with and complementation by an unirradiated nuclear genome (Table 3).

A larger dose of γ-rays (10 Krad), however, effectively prevented transmission of genetic information from the irradiated nuclear genome. Most calli obtained when the *L. pennellii* fusion partner was irradiated for 20 min contained only the isozyme allele characteristic of the unirradiated *L. esculentum* nucleus, and the plants which regenerated from such callus exhibited cultivar phenotype. Menczel et al. (1982) used 5 to 30 Krad, to inactivate *N. tabacum* mesophyll protoplasts. They saw a decreased frequency of somatic hybrids and an increased frequency of cybrids with increased radiation doses.

The possibility remains that some undetected *L. pennellii* nuclear genetic information may be present in these callus clones and/or regenerated plants. Gupta et al. (1982) has successfully transferred a nuclear gene necessary for nitrate reductase activity from irradiated protoplasts of *Physalis minima* or *Datura innoxia* into an auxotrophic mutant of *N. tabacum.* No morphological traits indicative of *Physalis or Datura* were observed in the *N. tabacum* regenerates.

Six fusion products, containing a 20 min irradiated *L. pennellii* parent, gave rise to calli which contained somatic hybrid cells in the first months of culture. Later, subcultures of these calli were found to contain the isozyme allele only from the *L. esculentum* parent, and the plants regenerated from these subcultures did not appear to have any *L. pennellii* phenotypic traits. Either these subcultures arose from cells which lost *L. pennellii* nuclear genes during culture, or the initial callus was a mixture of somatic hybrid and *L. esculentum* cells. If the latter case is true, then the somatic hybrid cells must have ceased or had much reduced growth during subculture so that the progeny cells of the initial mesophyll cell became predominant. This scenario seems unlikely since the callus medium used favors growth of the comparable *L. pennellii-L. esculentum* sexual hybrid callus over *L. esculentum* callus.

The observed rate of 70% somatic hybrid calli among the selected calli is quite high, considering that there was no visual selection against the *L. esculentum* parent. The mesophyll-suspension culture fusion products may have a greater chance of survival than mesophyll protoplasts, which are visibly damaged by the fusion treatment. Furthermore, very few *L. esculentum* protoplasts in the co-cultivated controls grew to form microcalli (Table 2). The combination of these factors can explain why the population of microcalli was greatly enriched in somatic hybrids.

More *L. esculentum* plants regenerated from fusion experiments than from control cultures untreated with PEG and high calcium solutions. The stimulation of growth could have resulted from the presence of the *L. pennellii* cells or perhaps from the fusion treatment itself. Following fusion, the cells are aggregated in close contact at the bottom of the culture dish, unlike ordinary liquid cultures. The close proximity of other cells could be a factor improving growth and regeneration of *L. esculentum* in fusion treatments.

Three types of plastid genome compositions of callus clones were detected when nine clones were analyzed with probes to species-specific cpDNA restriction fragments. Either only *L. pennellii,* only *L. esculen-*

tum, or a mixture of cpDNA signals were observed, depending on the particular clone. The level of sensitivity of this probing method allows us to conclude that one parental genome had already become predominant in four of the nine clones analyzed. However, these clones may yet contain a small proportion of cpDNA from the other parent which was not detected.

In contrast to the cpDNA analyses, these same nine clones all exhibited a mtDNA signal from the *L. pennellii* parent, but only one of these also had a faint signal from the other parent. It cannot be concluded that these clones carry primarily *L. pennellii* mtDNA, however, because there is considerable evidence that parental mitochondrial genomes recombine in somatic hybrid plants (Belliard et al. 1979; Nagy et al. 1981; Galun et al. 1982; Boeshore et al. 1983). A cloned hybridization probe examines only a small part of the mitochondrial genome. While the *L. esculentum* restriction fragment identified by the probe used was in low proportion or absent from the calli analyzed, other undetected *L. esculentum* fragments might be present in high proportions. Whether or not these somatic hybrid clones contain novel mitochondrial genomes must await further subculture and detailed analysis of purified mtDNA.

The lack of shoot regeneration by somatic hybrid calli was unexpected because the comparable sexual hybrid regenerates at high frequency under the culture conditions used (O'Connell and Hanson 1984). The *L. pennellii* protoplasts utilized were derived from a non-regenerating culture selected for rapid growth and ease of protoplast production. The non-regenerating phenotype of the somatic hybrid calli indicates that lack of regeneration is dominant in this particular fusion combination. Previous reports within the related genus *Nicotiana* have indicated that lack of regeneration is recessive (Maliga et al. 1977; Glimelius and Bonnett 1981; Gleba and Evans 1983). In order to obtain shoots from *L. esculentum-L. pennellii* somatic hybrid calli, both protoplast sources may need be capable of regeneration.

Our analysis of organelle genomes in somatic hybrid clones indicates that novel organelle-nuclear combinations can be created by somatic hybridization in *Lycopersicon.* While a plant hybrid with respect to the nuclear genome can readily be created by a sexual cross with *L. esculentum* as the maternal parent, obtaining progeny from the reciprocal cross is inhibited by unilateral sexual incompatibility. Thus, *L. pennellii* organelle genomes are not present in the easily-regenerable hybrids we obtained by sexual crosses, but were present in the somatic hybrid clones. Therefore, an alternative explanation for the lack of regeneration of the somatic hybrid clones is disruption in the morphogenetic process resulting from the presence of *L. pennellii* mtDNA.

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