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Tomato cybrids with mitochondrial DNA from *Lycopersicon pennelli*

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Summary. Cybrids have been regenerated following protoplast fusion of iodoacetamide-treated leaf mesophyll cells of *Lycopersion esculentum* cv UC82 and gamma-irradiated cell suspensions of *L. pennellii,* LA716. The cybrids were recovered in the regenerant population at a frequency of 19%, no selection pressure was applied for the persistence of the donor cytoplasm. The nuclear genotype of ten cybrids was characterized extensively using isozyme markers, cDNA-based restriction fragment length polymorphisms (RFLPs), and the morphology of the plants. No nuclear genetic information from *L. pennellii* was detected in the cybrids. The organellar genotype of the cybrids was determined using cloned probes and species-specific RFLPs. All the cybrids had inherited the tomato chloroplast genome and had varying amounts of *L. pennellii* mitochondrial DNA. The cybrids all had a diploid chromosome number of 24, produced pollen, and set seed.

Key words: Tomato – Mitochondrial DNA – Cybrid – Protoplast fusion – Cytoplasmic male sterility

Introduction

Tomato, *Lycopersicon esculentum,* is an important horticultural crop throughout the world. The genetic variation within this self-pollinated crop is very limited, as shown by the lack of isozyme variation among cultivars (Rick 1983). Wild *Lycopersicon* and *Solanum* species offer a wealth of genetic variability (Rick 1973), and some of these species have been used to introgress valuable characters into tomato by sexual hybridization (Rick 1982). In these crosses, tomato (*L. esculentum*) always functions as the female parent (Rick 1979; Hogenboom 1979). Because of the unilateral incongruity and maternal inheritance of the cytoplasmic genomes in *Lycopersicon* the interspecific hybrids always have cultivated tomato organellar genomes. Non sexual hybridization techniques, such as somatic hybridization, asymmetric somatic hybridization and cybridization, can create new combinations of cytoplasmic genomes (Galun and Aviv 1983, review) or new organizations of cytoplasmic genomes (Kumashiro et al. 1989; Morgan and Maliga 1987; O'Connell and Hanson 1987; Tanno-Suenaga et al. 1988; Thanh and Medgyesy 1989).

A number of labs have been successful in creating both interspecific (Kinsara et al. 1986; O'Connell and Hanson 1985; Wijbrandi 1989) and intergenetic (Guri et al. 1988; Handley et al. 1986; Melchers et al. 1978; O'Connell and Hanson 1987; Shepard et al. 1983; Schweizer et al. 1988) somatic hybrid plants with tomato. Wijbrandi (1989) has reported the construction of asymmetric somatic hybrid plants between tomato and *L. peruvianum,* and Melzer and O'Connell (1990) have characterized asymmetric somatic hybrids between tomato and *L. pennellii.*

Cybrid constructions have been made in tobacco (Aviv and Galun 1988; Fluhr et al. 1983; Glimelius et al. 1986; Menczel et al. 1982; Sidorov et al. 1981), in *Brassica* (Chetrit et al. 1985; Kemble et al. 1988; Morgan and Maliga 1987), and in rice (Kyozuka et al. 1989). These cybrids were all constructed by fusing a nucleus containing recipient protoplast with irradiated protoplasts, in which the radiation effectively inactivates the nuclear genetic information. In these cybrids, usually one of the parental chloroplast genomes is inherited, while the mitchondrial DNA is frequently a novel organization arising from recombination between the parental genomes (Galun and Aviv 1983). Plant mitochondrial genomes are quite large and variable in size and are

composed of circular molecules, which can recombine with one another through direct repeat sequences (Levings and Brown 1989). Morgan and Maliga (1987) constructed *Brassica* cybrids and revealed that the recombination involved a DNA region outside the repeats, which normally is not involved in recombination.

Mutschler and Cobb (1985) obtained an interspecific hybrid between tomato and *L. pennellii* with tomato as the pollen parent, using embryo rescue techniques. Further backcrosses were proposed to study the effect of *L. pennellii* cytoplasm with a tomato nuclear genome on several traits, including male sterility. Their early results indicated that *L. pennellii* organellar genomes do not reduce fertility in tomato. The reciprocal organization, *L. pennellii* nuclear genome and tomato cytoplasmic organelles, was generated sexually by Anderson (1964), and these plants displayed cytoplasmic male sterility.

In this paper, we describe the first successful construction of tomato cybrids, using *L. pennellii* as the cytoplasm donor. The nuclear genotype of the cybrids was extensively characterized using molecular markers, and the genotype and organization of the organellar genomes was determined using a battery of cloned organellar sequences. The phenotype, including male fertility, of these cybrids is described.

Materials and methods

Plant material

Lycopersicon pennellii, LA 716, collected in Atico, Peru, was generously supplied by C. Rick; Tomato Genetics Stock Center, University of California, Davis. *L. pennellii* is drought tolerant and is resistant to several insects (Rick 1973). Seeds of *L. esculentum* cv 'UC82,' a widely grown processing tomato, were provided by PetoSeed Co.

Protoplast isolation and treatments

Tomato plants were grown from seed in a growth chamber, 17 h light at 24 °C, 7 h dark at 19 °C, and watered with Nitsch salt solution daily (Nitsch 1965). Tomato protoplasts were isolated from leaves of 4-week-old plants as described by Tan et al. (1987a), and the protoplast fraction that floated on CPW salts (Tan et al. 1987a) and 15% sucrose solution was collected. Prior to use in fusions, UC82 protoplasts were treated with iodoacetamide to prevent growth of unfused parental protoplasts. The protoplasts were incubated with 1 m*M* iodoacetamide for 20 min at 4 °C. The iodoacetamide was removed by washing three times with W5 salts (18.2 g/l $CaCl_2 \cdot 2H_2O$; 8.69 g/l NaCl; 0.36 g/l KCl; 0.9 g/l glucose; 5 m*M* MES, pH 5.8).

Suspension cell cultures of *L. pennellii* were maintained in UM1a (Uchimaya and Murashige 1974) and subcultured at weekly intervals. The culture was 4.5 months old when used for the cybrid constructions. Four days after subculture, cells to be used for fusion were irradiated with 100 krad ^{60}Co gamma-radiation at a rate of 1.9 rad/s. The suspension cells were kept on ice during radiation and until enzyme digestion was started, 8 h later. The suspension cells (3 ml packed cell volume) were digested for 16 h with 6 vol. of enzyme solution (CPW salts, 9% mannitol, 1% cellulysin, 0.5% pectinol, 0.25% rhozyme, 0.05% pectolyase Y-23, 12.5 mg/ml fluoresceinisothiocyanate, 5 m*M* 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 5.8) in the dark at 25 °C at 40 rpm. Protoplasts were isolated as described by Tan et al. (1987b).

Protoplast fusion

Protoplasts of 'UC82' and *L. pennellii,* treated as described above, were mixed in a 1:1 ratio at 4×10^6 protoplasts per milliliter in W5 salts. Fusion was as described by Menczel and Wolfe (1984). After fusion, the protoplasts were pelleted, washed once, and resuspended in protoplast culture medium.

Cell culture and plant regeneration

Protoplasts were plated at a density of 1×10^5 cells per milliliter in Tmp medium, a modification of Tm-2, in which the sucrose was replaced by 59.43 g/l glucose (Shahin 1985). Dishes were diluted every 5 days with Tmd medium, a modification of Tm-3, in which 0.1 mg/l NAA and 0.5 mg/l zeatin were added, and 59.43 g/l glucose replaced sucrose (Shahin 1985). In some cases, the plates were solidified with 0.6% Seaplaque agarose. Microcalli were transferred to greening (JSC-12), shooting (TR-1), and rooting (MSO) medium as described by O'Connell and Hanson (1985). When roots formed, the plants were potted in soil and kept in plastic bags in a growth chamber for 1 week. Finally, when plants were established in soil, they were transferred to the greenhouse.

Starch gel analysis of isozymes

Shoots and calli on TR-1 medium were tested for the isozymes phosphoglucoisomerase (Pgi) and phosphoglucomutase (Pgm). A small amount of tissue was ground in 0.12 *M* reduced glutathione and 1 *M* TRIS-Cl, pH 7.4. The samples were absorbed into paper wicks, electrophoresed on TRIS-citrate starch gels, and stained as described by Vallejos (1983). The stained gels were dried between cellophane membranes and stored.

DNA isolation

Total DNA was extracted from fresh leaf tissue of dark treated plants. Routinely, 3 g of leaf material was ground in 12 ml proteinase K buffer (0.2 *M* TRIS-HCl, pH 8.0, 0.1 *M* EDTA, 1% sarcosyl, 100 µg/ml proteinase K, 1% mercaptoethanol) and incubated for 1 h at 45 °C, extracted twice with chloroform/isoamyl alcohol (24:1), and the aqueous layer precipitated with ethanol. The precipitate was dissolved in 3 ml TE (10 m*M* TRIS HCl, pH 8.0, 1 m*M* EDTA) and further purified on a CsCl gradient. The DNA was precipitated after the addition of 4 vol. of sterile distilled water. For some samples, the first precipitate was dissolved in 3 ml TE and treated with RNase for 1 h, after which a second ethanol precipitation followed.

DNA probes

A 27-kb SalI fragment of the tomato chloroplast (cp) genome was cloned into pUC8 and used to probe HindIII digests of total DNA. This SalI fragment contains the inverted repeat of the cpDNA (Phillips 1985) and allows the characterization of the chloroplast genotype of the regenerants.

Mitochondrial DNA was cloned into the cosmid cloning vector pHC79 (Hohn and Collins 1980). Seven nonoverlapping cosmid clones were selected from the cosmid library and used to analyze the mitochondrial (mt) DNA of the regenerants. These clones each cover about 30–35 kb of the mitochondrial genome, and useful species-specific RFLPs have been determined for each clone (Wachocki et al. 1991). Further, clone 2D4, a 2.1-kb *L. pennellii* mtDNA SalI fragment, which occurs in multiple

copies in the mitochondrial genome and contains no known coding sequence (McClean and Hanson 1986), and one plasmid clone carrying the 18S and 5S mitochondrial ribosomal genes from tomato, a repeated sequence in tomato (Estabrook and O'Connell 1986), were used as probes to analyze the mtDNA.

Mapped tomato cDNA clones (Bernatzky and Tanksley 1986) were used as probes to analyze the nuclear DNA of the cybrids.

DNA restriction, Southern transfer, and hybridization

DNA, 8 μg, was digested for at least 5 h at 37 °C with the indicated restriction endonucleases. The restricted DNA was electrophoresed in 0.8% agarose gels, and gels were denatured for 20 min (0.4 *N* NaOH, 0.6 *M* NaCl), after which the DNA was transferred to Zetabind by capillary transfer with denaturing solution. After transfer, the filters were neutralized (0.5 *M* Na_2PO_4, pH 7.5) and baked for 2 h. Hybridization with organellar probes as a described by O'Connell and Hanson (1986), and the blots were washed finally with 0.75 *M* NaCl, 0.0075 *M* Na citrate, pH 7.0 (0.5 × SSC). For hybridization with nuclear probes, the procedure of Melzer et al. (1989) was followed.

Chromosome counts and pollen staining

Root tips were collected and incubated for 4 h at 18 °C in 2 m*M* 8-hydroxyquinoline. The tissue was hydrolized for 20 min in 4 *N* HCl at room temperature, rinsed with water, and kept in water at 5 °C overnight. Tips were stained with acetocarmine and squashed. Percent viable pollen was determined after staining, as described by Alexander (1969).

Results

Culture and regeneration of fusion products

The construction of tomato cybrids was based on the double inactivation procedure described by Sidorov et al. (1981). Both parental protoplasts were inactivated prior to fusion: the cytoplasm donor, *L. pennellii*, was irradiated to inactivate the nuclear genome, and the recipient protoplast, 'UC82,' was poisoned with iodoacetamide to prevent growth of unfused protoplasts. Protoplast isolation from 100 krad irradiated suspension cell decreases the yield tenfold, compared to protoplast isolation from nonirradiated cells (data not shown). However, the protoplasts recovered from irradiated cells appear normal. Control cultures of protoplasts isolated from irradiated cells appear viable for over 10 days, form cell walls, but do not divide. Previously, it was determined that a 10-krad irradiation is sufficient to prevent cell division, but not inheritance of the irradiated genome in fusion products (O'Connell and Hanson 1985). The protoplast-derived microcalli were transfered to solid media, JSC-12, and green calli were transfered to shoot induction medium, TR-1. Shoot regeneration was observed 2.5–7 months after protoplast fusion, and rooted regenerants were transferred to the greenhouse 4–12 months after protoplast fusion.

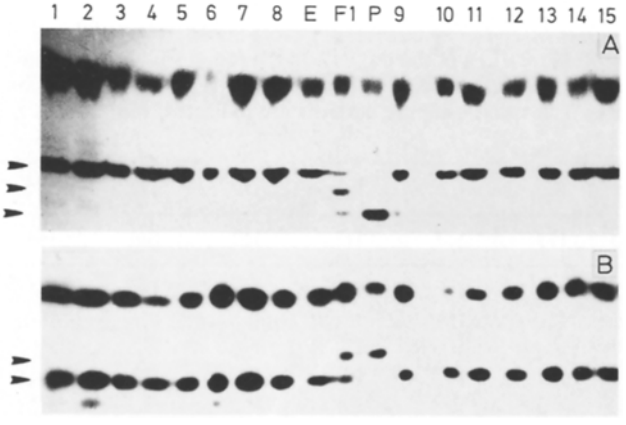

Fig. 1. Starch gel analysis of isozyme activities. The gel in panel *A* was stained for phosphoglucoisomerase activity, in panel *B* for phosphoglucomutase activity. Lanes E, F_1, and P contain extracts from *L. esculentum* cv UC82, the interspecific sexual hybrid, and *L. pennellii*, respectively. The numbered lanes contain extracts from leaf tissue of 15 regenerants. The position of the isozyme activities of interest, *Pgi-1* and *Pgm-2*, are indicated by *arrowheads*

Analysis of the nuclear genotype of the regenerants

As soon as the calli started to regenerate shoots, the genotype of the callus was determined using isozyme assays. Altogether, 250 regenerants and calli have been analyzed for the isozymes phosphoglucoisomerase (*Pgi-1*, located on chromosome 12) and phosphoglucomutase (*Pgm-2*, located on chromosome 4). In Fig. 1, *Pgm* and *Pgi* isozyme patterns of the regenerants are compared with the patterns of the fusion parents tomato, *L. pennellii* and the F_1. The majority, 246/250 (98%) of the samples tested, scored as tomato at these two loci. The four samples that displayed *L. pennellii* alleles at these loci were asymmetric somatic hybrids. Of the four calli showing *L. pennellii* nuclear genetic information, two calli were hybrid for *Pgi-1* and had the tomato *Pgm-2* allele. These calli have not regenerated. The two other calli (83 and 76) were hybrid for *Pgm-2* and had the tomato *Pgi-1* allele; these two calli have regenerated. Regenerants from 83 have lost the *L. pennellii Pgm-2* allele and now score as tomato at all tested loci. The genotype of one regenerant, 83B, has been determined at an additional 10 loci using cDNA RFLPs, with all loci scored as tomato. Regenerants from 76 have maintained the *L. pennellii Pgm* allele, but these plants have an aberrant phenotype, a weak root system, and are difficult to maintain.

Analysis of the chloroplast genome of the regenerants

The chloroplast genotype of the regenerants was determined using a species-specific HindIII polymorphism. Southern blots containing HindIII-restricted total DNA were probed with a cloned SalI fragment of the tomato chloroplast genome. This 27-kb SalI fragment hybridizes to several HindIII fragments, three of which display use-

ful polymorphisms, 10- and 3.8-kb HindIII fragments in tomato cpDNA and a 14-kb fragment in *L. pennellii* cpDNA (Fig. 2). The chloroplast genotype of ten regenerants is also shown in Fig. 2. All of these individuals displayed the 10- and 3.8-kb HindIII fragments and were scored as having the tomato chloroplast genome. Altogether, the chloroplast genotype of 65 regenerants was determined and all had inherited the tomato chloroplast genome.

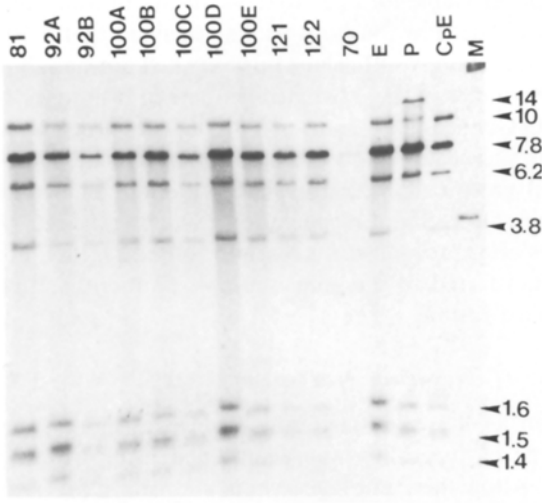

Fig. 2. Identification of the chloroplast genomes present in tomato cybrids. All lanes contain 8 μg of total DNA restricted with HindIII. The Southern blot was probed with a cloned fragment of tomato cpDNA, 27-kb Sal1 fragment. Lanes E, P, and CpE contain DNA from *L. esculentum* cv UC82, *L. pennellii*, or purified chloroplast DNA from tomato, respectively. The lanes containing DNA from the cybrids are labeled; the sizes of the hybridizing fragments are indicated in kb. Lane 70 contains DNA from a non-cybrid regenerant, lane M contains HindIII-digested lambda DNA

Analysis of the mitochondrial genome of the regenerants

The analysis of the mtDNA of the putative cybrids is complicated, since the tomato mitochondrial genome is large (350–450 kb, based on preliminary mapping data) and only partially mapped. Further, the mitochondrial genome of fusion products is often the result of rearrangements and recombination of both parental genomes; consequently, no single RFLP can accurately predict the genotype of the mitochondrial genome.

A collection of cloned tomato mtDNA fragments was used to determine the genotype of the mtDNA in the regenerants. Seven non overlapping cosmid clones containing 30–35 kb of tomato mtDNA were selected from a cosmid library constructed earlier (Wachocki et al. 1991). A plasmid clone carrying the tomato mitochondrial 18S+5S ribosomal RNA genes (Estabrook and O'Connell 1986), and 2D4, a plasmid clone carrying a 2.1-kb SalI fragment of *L. pennellii* mtDNA, were also used as probes. Altogether, the cloned mtDNA fragments used to characterize the mtDNA in the cybrid regenerants cover at least 60% of the mitochondrial genome (Wachocki et al. 1991). The results of the hybridizations with all nine mitochondrial probes is summarized in Table 1, and examples of the Southern hybridization patterns are shown in Figs. 3, 4, and 5. The summary only indicates the results of the analyses of regenerants that carried any detectable *L. pennellii* mtDNA. Only these ten regenerants were scored as cybrids; approximately 50 additional regenerants were test-

Table 1. Composition of cybrid mitochondrial genomes. The species-specific RFLPs of the mtDNA in the tomato cybrids are listed for the cosmid clones A1, A2, A3, B1, B3, C3, and D9, as well as the plasmid clones 2D4 and 18S+5S genes. The restriction endonucleases used to identify the RFLPs are indicated; H3=HindIII. The species-specific patterns are indicated; tomato=E, *L. pennellii*=P, and novel pattern=R

Cybrids	2D4	A1	A2	A3	B1	B3	B3	C3	C3	D9	D9	18S
	Sal	H3	Bam	H3	Sma	Bam	H3	Sal	H3	H3	Sal	H3
81	P+R	P	P	P	P	P	P	P	P	P	P	P
92A	E	E	E	E+R	E	E+R	R	E	E+R	E	E	P
92B	E	E	E	E+R	E	E+R	R	E+R	E+R	E	E	P
100A	P+R	P	E	P	E	P+E	P+E	P	P	–	P	P
100B	P+R	P	E	P	E	P+E	P+E	P	P	P	P	P
100C	P+R	P	P	P	P	P	P+E	P	P	P	P	P
100D	P+R	P	E	P	E	P+E	P+E	P	P	P	P	P
100E	P+R	P	E	P	E	P+E	P+E	P	P	–	P	P
121	P+R	P	P	P+R	P	P	P	P	P	P	P	P
122	P+R	P	P	P	P	P	P	P	P	–	P	P
83B[a]	E	E	E	E	E	E	E	E	E	E	E	E

[a] Regenerant 83B is not a cybrid

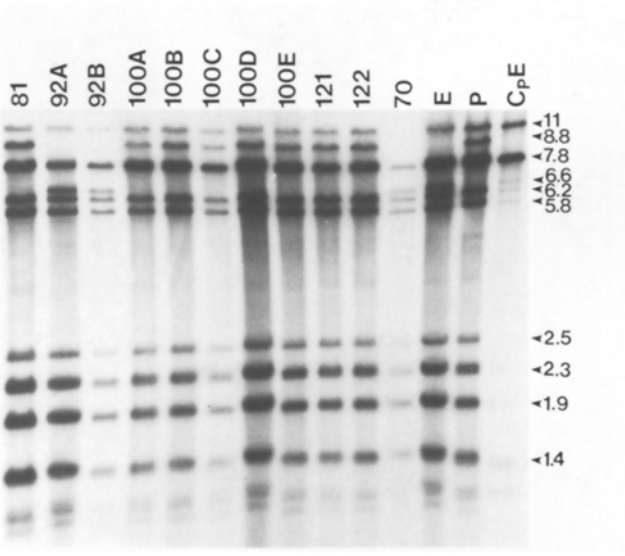

Fig. 3. Identification of the mitochondrial sequences present in tomato cybrids that hybridize to the cosmid clone A1. All lanes contain 8 µg of total DNA restricted with HindIII. Lanes E, P, and CpE contain DNA from *L. esculentum* cv UC82, *L. pennellii,* or purified chloroplast DNA from tomato, respectively. The lanes containing DNA from the cybrids are labeled; the sizes of the hybridizing fragments are indicated in kb. Lane 70 contains DNA from a non-cybrid regenerant

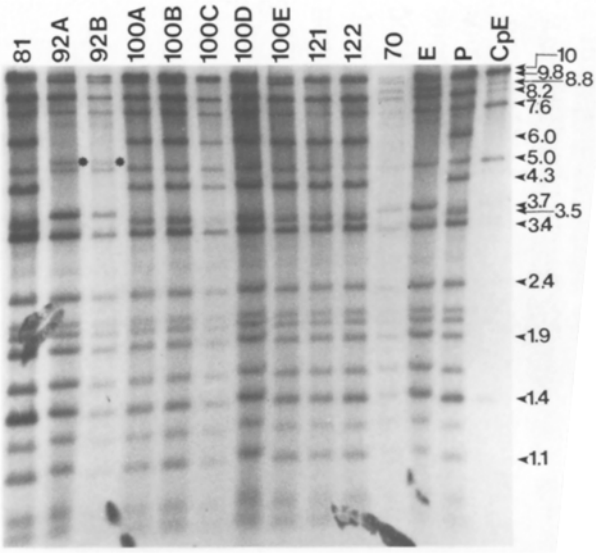

Fig. 4. Identification of the mitochondrial sequences present in tomato cybrids that hybridize to the cosmid clone B3. All lanes contain 8 µg of total DNA restricted with HindIII. Lanes E, P, and CpE contain DNA from *L. esculentum* cv UC82, *L. pennellii,* or purified chloroplast DNA from tomato, respectively. The lanes containing DNA from the cybrids are labeled; the sizes of the hybridizing fragments are indicated in kb. Novel fragments are indicated with an *asterisk*. Lane 70 contains DNA from a non-cybrid regenerant

ed with some of these mtDNA probes and displayed only the tomato hybridization pattern.

Figure 3 shows an autoradiogram of HindIII-digested total DNA of putative cybrids, tomato, and *L. pennellii*, probed with cosmid clone A1. The hybridization pattern of this probe is quite complex, hybridizing to about 50 kb of DNA. A1 hybridizes to two species-specific fragments, an 8.8-kb HindIII fragment in *L. pennellii* DNA and a 6.6-kb fragment in HindIII digests of tomato DNA. The hybridization pattern of eight of the cybrids is identical to the *L. pennellii* pattern; only 92A and 92B display the tomato-specific 6.6-kb fragment and lack the 8.8-kb *L. pennellii* fragment.

Figure 4 shows an autoradiogram of HindIII-digested total DNA of putative cybrids, tomato, and *L. pennellii* probed with cosmid clone B3. This probe hybridize to 60–70 kb with some cpDNA fragments identified by this probe as well. Cosmid clone B3 hybridized to several species-specific fragments, 9.8-, 8.8-, and 3.7-kb HindIII fragments in tomato DNA, and 6.0-, 4.3-, and 3.5-kb HindIII fragments in *L. pennellii* DNA. Cybrids 81, 121, and 122 shows a hybridization pattern identical to *L. pennellii*, while cybrids 100A–100E, all regenerated from the same callus, show a hybridization pattern similar, but not identical, to that of *L. pennellii*. These five regenerants, 100A-100E, also show the 9.8-kb tomato-specific fragment indicating the presence of mtDNA from 'UC82.' Cybrids 92A and 92B, also derived from one callus, display a novel fragment when probed with cosmid B3. A 5.4-kb HindIII fragment (indicated in Fig. 4 with an asterisk) is detected in DNA isolated from 92A and 92B; this fragment is not present in the parental DNAs. Further, 92A and 92B are missing all three *L. pennellii*-specific fragments, 6.0, 4.3, and 3.5 kb, and are missing only one tomato-specific fragment, 8.8 kb; the remaining two tomato-specific fragments, 9.8 and 3.7 kb, detected by cosmid B3 are present. The presence of the novel band and the absence of some parental bands suggests that a recombination event between the two parental mitochondrial genomes occurred in cybrids 92A and 92B involving sequences contained in cosmid B3.

Cosmid A3 hybridizes to 40–50 kb of HindIII-digested DNA. The hybridization pattern of this cosmid to HindIII-digested DNA of putative cybrids and the parents is shown in Fig. 5. This probe hybridizes to one tomato-specific fragment, a 7.3-kb HindIII fragment, and to four *L. pennellii*-specific HindIII fragments, 6.2, 3.8, 3.4, and 3.1 kb. The organization of the mtDNA contained in cosmid A3 in the cybrids 81, 100A–100E, and 122 is identical to *L. pennellii*. The mtDNA of cybrid 121 resembles the *L. pennellii* hybridization pattern when probed with cosmid A3, but lacks the 3.4-kb *L. pennellii*-specific band. The mtDNA of cybrids 92A and 92B is again aberrant; cosmid A3 hybridizes to the tomato-

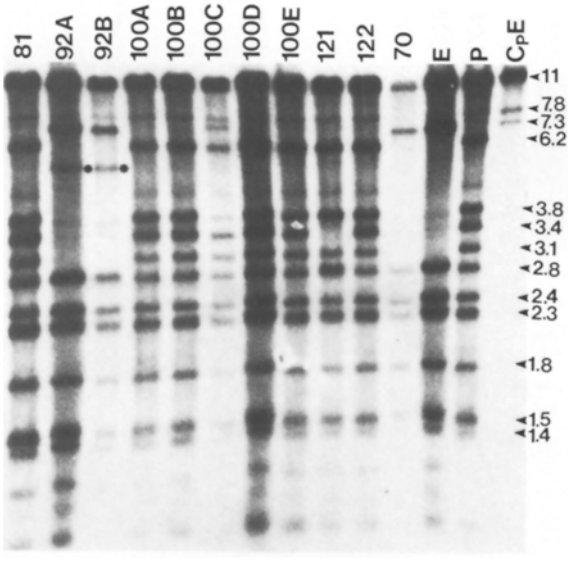

Fig. 5. Identification of the mitochondrial sequences present in tomato cybrids that hybridize to the cosmid clone A3. All lanes contain 8 μg of total DNA restricted with HindIII. Lanes E, P, and CpE contain DNA from *L. esculentum* cv UC82, *L. pennellii,* or purified chloroplast DNA from tomato, respectively. The lanes containing DNA from the cybrids are labeled; the sizes of the hybridizing fragments are indicated in kb. Novel fragments are indicated with an *asterisk*. Lane 70 contains DNA from a non-cybrid regenerant

specific 7.3-kb fragment and to none of the *L. pennellii*-specific fragments in DNA isolated from these two cybrids. However, a novel 8.8-kb fragment is detected in DNA from 92A and 92B (Fig. 5, asterisk).

A total of nine probes and 12 restriction digestions was used to characterize the mtDNA in the putative tomato cybrids (Table 1). Novel, nonparental fragments were detected with four probes, and novel fragments were observed in all ten putative cybrids.

Analysis of the nuclear genotype of the cybrids

The nuclear composition of selected putative cybrids was characterized extensively to determine if any nuclear information from *L. pennellii* was present. The cybrids 92A, 100A, and 122 were tested for nine cDNA loci, located on chromosomes 1, 2, 3, 6, 8 and 12. Cybrid 92B was analyzed with six cDNA probes, located on chromosomes 1, 2, 3, 6, and 12, and cybrid 121 was analyzed for three loci on chromosomes 2 and 8. In all cases, only the tomato alleles for these loci were present in the cybrids (data not shown). In addition to the cDNA and isozyme markers used to characterize the genotype of these cybrids, the morphology of the plants was used to indicate the presence of *L. pennellii* genetic information. Based on our experience characterizing protoplast regenerants of sexual and somatic hybrids between these two species (O'Connell and Hanson 1985, 1987; O'Connell et al. 1986; Melzer and O'Connell 1990), the cybrid regenerants did not display any *L. pennellii* morphological traits.

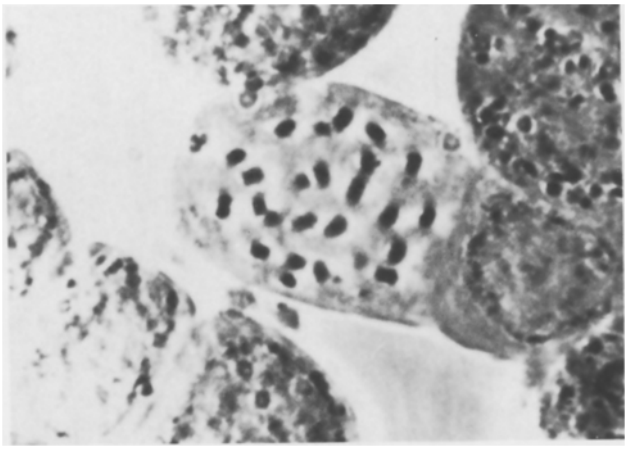

Fig. 6. Chromosome spread in a root-tip cell of tomato cybrid 122

Table 2. Summary of mitochondrial and chloroplast genotypes, chromosome numbers, and pollen viabilities in the tomato cybrids. The percent of mtDNA RFLPs identified as *L. pennellii*-specific is listed, %P, as well as the frequency of novel bands, %R

Cybrids	mtDNA		CPDNA	Chromosome #	% viable pollen
	%P	%R			
81	92	8	E	24	22
92A	7	27	E	24	50
92B	6	31	E	24	70
100A	64	7	E	24	69
100B	67	7	E	–	60
100C	86	7	E	–	30
100D	67	7	E	24	32
100E	64	7	E	24	–
121	86	14	E	–	57
122	92	8	E	24	40
83B[a]	0	0	E	42–46	10

[a] Regenerant 83B is not a cybrid

Cytological characterization of the cybrids

Chromosome counts were performed on root-tip squashes of several cybrids. All of the cybrids contained 24 chromosomes, the diploid chromosome number for tomato (Fig. 6 and Table 2).

Pollen viability of the cybrids ranged from 20 to 70% (Table 2). Tomato and *L. pennellii* have pollen viabilities of 90%. All but one cybrid produced selfed seeds (data not shown). None of the cybrids that carried at least some of the *L. pennellii* mitochondrial genome displayed male sterility.

Fig. 7. Comparison of growth habit between 'UC82' and tomato cybrid 100C

Phenotype of the cybrids

One of the objectives of this program was to determine if the presence of *L. pennellii* organellar DNA in a tomato nuclear background altered plant growth and development. While the cybrids had reduced pollen viability (Table 2), they were still able to produce selfed seed. Interestingly, the cybrids did have a unique morphology. The cybrids exhibited reduced plant height, shorter internodes, extensive branching, smaller leaves, and less vigor than non-cybrid regenerants (Fig. 7). This phenotype is associated with regenerants scored as cybrids. Two cybrids, 92A and 92B, are more elongated and show only extensive branching near the top of the plant. These two plant contain the least amount of detected *L. pennellii* mtDNA among the cybrids. Regenerants that do not contain any detectable *L. pennellii* cytoplasmic DNA did not display this phenotype. Reciprocal crosses with the tomato 'UC82' will be used to determine the inheritance of this altered morphology.

Discussion

Tomato cybrids were constructed based on the double inactivation procedure described by Sidorov et al. (1981). Unlike most cybridization constructions, no selection pressure was applied during culture for the persistence of the *L. pennellii* cytoplasm in the fusion products. In fusions within the genera *Nicotiana* and *Brassica*, where the organellar composition of cybrids and asymmetric somatic hybrids has been described, plants were regenerated under selection pressure (i.e., atrazine resistance, streptomycin resistance, and chlorophyll deficiency), which influences the organellar segregation (Chetrit et al. 1985; Kemble et al. 1988; Kumashiro et al. 1989; Sidorov et al. 1981). The tomato cybrids described here were recovered at a frequency of 19% and without influence on the organellar segregation.

A high dose of gamma-radiation was chosen for the inactivation of the *L. pennellii* nucleus, based on earlier reports from this lab (O'Connell and Hanson 1985, 1987; O'Connell et al. 1986) and based on results obtained in fusion systems with other species (Immamura et al. 1987). The 100-krad dose used in the construction of tomato cybrids is considerably higher than the doses used for the construction of cybrids in *Brassica* or *Nicotiana* (Sidorov et al. 1981; Menczel et al. 1982; Morgan and Maliga 1987). At lower doses, a percentage of the *Brassica* or *Nicotiana* fusion products were somatic hybrids, and a detailed characterization of the nuclear genome in the cybrids was frequently not performed. A 100-krad irradiation of *L. pennellii* suspension cells seems sufficient to completely eliminate its nuclear genome. When 250 calli and regenerants were scored for the two loci *Pgm-2* (chromosome 4) and *Pgi-1* (chromosome 12), 246 had the tomato alleles for both loci and only 4 were hybrid for one of the scored loci. The nuclear genomes of confirmed cybrids 92A, 100A, and 122 were tested for an additional nine loci, cybrid 92B for six loci, and cybrid 121 for three loci, and again all the cybrids contained only the tomato alleles (data not shown). Further, all the regenerants resemble tomato and do not shown any of the morphological traits of *L. pennellii*. All the cybrids have 24 chromosome; no aneuploidy or polyploidy was found in these plants. This is remarkable, since Koornneef et al. (1989) and O'Connell et al. (1986) showed that a high percentage of the regenerants from tomato and F_1 (tomato × *L. pennellii*) protoplasts have a tetraploid or higher ploidy level.

The cpDNA and a large part of the mtDNA was analyzed in 60 regenerants. Eleven plants, or 19%, of the 60 regenerants were scored as cybrids, i.e., they contained some *L. pennellii* mtDNA. The remaining plants were regenerants from fusions in which the *L. pennellii* cpDNA and mtDNA did not persist in the majority of the cells of the callus, or was not present in the cells that regenerated from the callus. A few of the plants could be regenerants from unfused tomato protoplasts that grew despite the iodoacetamide treatment.

The loss of genetic information during culture is demonstrated in the analysis of plant 83B. This plant initially contained the *Pgm-2* allele from *L. pennellii* and lost this allele during further development. The nuclear genome of this plant was analyzed at 10 loci (1 on chromosome 1; 3 on chromosome 3; 1 on chromosome 4; 1 on chromosome 6; 2 on chromosome 8; 1 on chromosome 9; and 1 on chromsome 12) and all loci contained only the tomato allele. This plant, originating from a fusion event, has tomato cpDNA and scores as tomato with all the mtDNA RFLPs (Tables 1 and 2). Therefore, the estimate of 19% recovery of cybrids in the population of regener-

ants is an underestimate of the fusion frequency, and if a more thorough analysis of the mtDNA were technically feasible, a higher frequency for the recovery of cybrids would be obtained.

There are several possible explanations why none of the regenerants have *L. pennellii* cpDNA. Even though there are many copies of the chloroplast and mitochondrial genomes in a cell, the 100-krad irradiation was sufficient to damage the *L. pennellii* organellar DNA. Morgan and Maliga (1987) created *Brassica* cybrids and also found that none of the cybrid clones contained chloroplasts of the irradiated parent, while the mtDNA was a combination of both parents. They used an X-ray source and administered 14 krads. Sidorov et al. (1981) fused *Nicotiana plumbaginifolia* protoplasts with 6-krad (^{60}Co gamma rays) irradiated *N. tabacum* protoplasts and found that the regenerants preferentially maintained the cpDNA of the irradiated parent. However, at this dose, 50% of the regenerants were somatic hybrids. They hypothesized that improved competitive ability of irradiated plastids may be the explanation for this phenemenon. And finally, Asahi et al. (1988) observed random inheritance of irradiated organellar genomes. They analyzed regenerants of a fusion between *N. tabacum* protoplasts and X-ray irradiated *N. debneyi* protoplast. Fifty percent of the regenerants contained the *N. tabacum* chloroplast genome and 50% contained the *N. debneyi* chloroplast genome, suggesting that radiation does not affect the random segregation of the chloroplast genomes. The results observed in the construction of tomato cybrids are consistent with those of Morgan and Maliga (1987): the cpDNA of the irradiated protoplast fusion partner is not inherited in the regenerants.

Another explanation for the limited transfer of *L. pennellii* organellar information after cybridization is nuclear-cytoplasmic incompatibility. The tomato nuclear background may favor tomato organelles and interact less efficiently with the *L. pennellii* chloroplasts and mitochondria. The reduced vigor of the cybrids is consistent with a nuclear-cytoplasmic incompatibility.

A third explanation for the absence of *L. pennellii* chloroplast in the regenerants is that the source of *L. pennellii* protoplasts was a suspension cell culture, and these cells contain proplastids. After protoplast fusion, the chloroplasts from the tomato mesophyll cell may have a growth advantage over the proplastids. However, in somatic hybrid constructions between these two species, either chloroplast genome can be inherited, and in these constructions the same protoplast sources were used (O'Connell and Hanson 1985, 1987). The three hypotheses to explain the skewed inheritance of cpDNA in the tomato cybrids are not mutually exclusive. The combination of the three influences listed above may have together resulted in the absence of *L. pennellii* cpDNA in the tomato cybrids.

The organization of the mitochondrial genome in the cybrids has been summarized in Table 1 and 2. The cybrids contain varying amounts of *L. pennellii* and tomato mtDNA and mtDNA rearrangements. Cybrids with almost exclusively *L. pennellii* mtDNA, with both *L. pennellii* and tomato mtDNA, and with mostly tomato mtDNA and many rearrangements are represented in the population. Although a map of the tomato mitochondrial genome is not available, a number of interesting observations about the structures of the cybrid mtDNA can be determined.

Brears et al. (1989) observed rearrangements in mtDNA in regenerants of sugar beet; they concluded the rearrangements were induced by tissue culture. Similarly, Shirzadegan et al. (1989) found major rearrangements of mtDNA molecules in cultured cells of *Brassica campestris*. In tissue-cultured cells of maize, the frequency of circular mtDNA molecules changed, but no rearrangements were detected (McNay et al. 1984). Are the novel bands observed in the analysis of mtDNA in these tomato cybrids the result of recombination events between *L. pennellii* and *L. esculentum* mtDNA? All analyzed regenerants containing no *L. pennellii* mtDNA had RFLP patterns identical to the tomato parent; no novel bands were observed (data not shown). If novel bands in the tomato cybrids were induced by tissue culture, then the frequency of rearrangements in the mtDNA should be the same in all regenerants. The mitochondrial genome was characterized in 60 regenerants; only regenerants with *L. pennellii*-specific mtDNA RFLPs showed novel bands. Therefore, the novel bands in the tomato cybrids are probably the result of intergenomic recombination.

The mtDNA organization of the individual cybrids was variable; each individual had a unique RFLP pattern. However, all of the cybrids had the *L. pennellii* 18 + 5S ribosomal RNA genes. The cybrids 92A and 92B contained the least amount of *L. pennellii*-specific mtDNA, yet had the highest percentage of mtDNA rearrangements (Table 2).

Probe 2D4, a repeat in *L. pennellii* mtDNA, reveals mtDNA rearrangements in all of the cybrids, except for 92A and 92B. These repeats in the *L. pennellii* mitochondrial genome could be involved in intragenomic recombination in *L. pennellii* mitochondria and in recombination between *L. pennellii* and *L. esculentum* mtDNA in the cybrids. The novel bands in the cybrid mtDNA could be the result of recombination at the 2D4 sequences in both parental genomes.

Cybrids 81 and 122 show only *L. pennellii* hybridization patterns for the seven cosmid probes. Also, cybrid 121 shows only *L. pennellii* hybridization patterns for these cosmid probes, but lacks a 3.4-kb *L. pennellii*-specific band for cosmid A3 (Fig. 5). Surprisingly, no tomato-specific or novel fragments were detected with

the cosmid probes, indicating the loss of some *L. pennellii* mtDNA.

The DNA hybridization patterns for the cybrids 100A–100E, all derived from one callus, are not identical. The hybridization pattern for total DNA, digested with SmaI and probed with cosmid B1 is identical to the tomato pattern for 100A, 100B, 100D, and 100E, but resembles the *L. pennellii* pattern for cybrid 100C. Similarly, probe B3 distinguishes 100C from 100A, 100B, 100D, and 100E in BamHI digests of total DNA of these cybrids. This implies that the mitochondrial genome of the callus was not fixed when the shoots regenerated. Every shoot originated from a different cell or small cluster of cells, and the mitochondrial genomes of these cells were not identical at the time of shoot regeneration.

Although the pollen fertility of nine of the ten described cybrids is reduced and ranges from 30% to 70% (Table 2), all these plants have set selfed fruits containing viable seeds (data not shown). The presence of some of the *L. pennellii* mitochondrial genome in a tomato nuclear background did not induce complete male sterility.

The cybrids have a different morphology from most of the regenerants containing no detectable *L. pennellii* mtDNA. The cybrids are shorter in height, have shorter internodes, have extensive branching and are less vigorous than non-cybrid regenerants (Fig. 7). Since alterations in the phenotype of the cybrids could be due to somaclonal variation instead of the presence of foreign mtDNA, progeny analysis of selfed and reciprocal crosses needs to be performed. These tests are currently underway. Maternal inheritance of the altered morphology will provide evidence for the induction of these traits by the *L. pennellii* mtDNA.

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