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Characterisation of a cytoplasmic male-sterile hybrid line between *Lycopersicon peruvianum* Mill. × *Lycopersicon pennellii* Corr. and its crosses with cultivated tomato

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Abstract The cytoplasmic male-sterile (CMS) line CMS-*pennellii* ($BC_{10}P_2$ *L. peruvianum* × *L. pennellii*) and its complex hybrids with *L. esculentum* were studied. The established sterility was classified as the sporogenous type. As a result of the interaction of the genome of *L. pennellii* and the cytoplasm of *L. peruvianum* clear changes were established in the profiles of malic enzyme and esterase. Restriction fragment length polymorphism (RFLP) was detected between the mitochondrial (mt) genomes of CMS-*pennellii* and the cytoplasm donor, *L. peruvianum*, for two mtDNA probes: *atpA* and *nad3*. The established differences in the isozyme pattern and mt genomes are considered as useful markers to distinguish fertile and sterile plants. A breakthrough in the unilateral incompatibility of CMS-*pennellii* and the incorporation of the genome of *L. esculentum* on a CMS background is reported. The analysis of the complex hybrids assumes the interaction of two dominant genes – a maintainer gene from *L. pennellii* and a restorer gene from cultivated tomato. The hybrids produced with *L. esculentum* provide the basis for the development of a CMS system in cultivated tomato.

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Introduction

CMS does not occur naturally in the genus *Lycopersicon*. The CMS phenotype is inherited through the female parent and results from an interaction between the nuclear and cytoplasmic genomes. It can arise by intraspecific, interspecific, or intergeneric crosses, either spontaneously or after mutagenic treatment (Hanson and Conde 1985; Kaul 1988).

In the genus *Lycopersicon* a number of previous experiments have been directed to reveal the interaction of the organellar genomes and the nucleus in backcrosses of interspecific hybrids between *L. esculentum*, *L. esculentum var. minor*, *L. hirsutum f. glabratum* as pistillate parents with *L. pennellii* as the staminate parent (Andersen 1963, 1964); *L. peruvianum* as the pistillate parent with *L. hirsutum f. glabratum* (Vulkova-Achkova and Stoeva 1978), *L. hirsutum typicum*, *L. pennellii* (Valkova-Achkova 1980), *L. chilense* (Valkova-Achkova 1982) and *L. esculentum* (Gradziel and Robinson 1991) as pollinating parents; *L. pennellii* as the pistillate parent with *L. esculentum* (Stoeva 1980, 1982; Mutschler 1990) or with *L. pimpinellifolium*, *L. pimpinellifolium var. Galapagos*, *L. cheesmanii typicum*, *L. cheesmanii var. minor* and *L. minutum* (Stoeva 1980, 1982) as the staminate parents.

In the genus *Lycopersicon* a description of the male-sterility phenotype occurring as a result of the incompatibility between the cytoplasm of one species and the nuclear genome of another was provided by the studies of Andersen (1963, 1964) and Valkova-Achkova (1980). Andersen (1963, 1964) reported a reduction of pollen fertility appearing in the first backcross of the crosses between *L. esculentum*, *L. esculentum var. minor*, *L. minutum* and *L. hirsutum f. glabratum* used as pistillate

parents and *L. pennellii* as the recurrent pollinating parent. Valkova-Achkova (1980) reported a progressive decrease in pollen viability from BC_1-P_2 to BC_3-P_2 in the *L. peruvianum* × *L. pennellii* cross and from BC_1-P_2 in the to BC_3-P_2 in the *L. peruvianum* × *L. hirsutum typicum* cross. Further introgression of the nuclear genome of the recurrent parents up to BC_5 confirmed the stability of the observed CMS phenotype.

Somatic hybridisation has been widely applied both to overcome the existing barriers of unilateral and bilateral incompatibility between cultivated tomato and species from the subgenus *Eriopersicon*, as well as from the genera *Solanum* and *Nicotiana*, in order to obtain novel recombinational cytoplasmic and nuclear genomes (reviewed in Lefrancois et al. 1993; Stoeva et al. 1997). In spite of the repored new recombinational patterns of the nuclear and mitochondrial genomes, in only one study (Melchers et al. 1992) was a CMS phenotype found to appear as a result of the fusion of cytoplasmically inactivated tomato protoplasts with nuclearly inactivated *Solanum* (*Solanum acuale* and *Solanum nigrum*) protoplasts. Among regenerated fusion products, plants with normal tomato characteristics were observed but with a complete lack, or else malformation, of anthers, with shrunken pollen and pollen that did not germinate. The female reproductive function, however, was normal. The restriction analysis of mitochondrial DNA revealed that the mitochondrial genome of CMS somatic hybrids did not combine all elements of the parental species and included new fragments with a recombinational nature.

In the present paper we have investigated the morphological, cytological, biochemical and molecular characteristics of CMS in the late backcross BC_{10}-P_2 of the hybrid *L. peruvianum* × *L. pennellii*. We present data for the introgression of the genome of *L. esculentum* both into the CMS background and in the first generations of the complex hybrids obtained.

Materials and methods

Plant material

In this study we used plants from BC_{10}-P_2 *L. peruvianum* (LA 446) × *L. pennellii* (LA 716) (denoted as CMS-*pennellii*) originating from the CMS line described in Valkova-Achkova (1980), as well as the following species and hybrids: *L. peruvianum* (LA 446), *L . pennellii* (LA 716), *L. esculentum* cv Merkurii, and the F_1 *L. esculentum* (cv Merkurii) × *L. pennellii* (LA 716).

All species and hybrid plants were grown in pots in the greenhouse.

Morphological characteristics

A comparative study of the phenotype of the CMS-*pennellii* and the parental species *L. pennellii* and *L. peruvianum* was carried out for leaf, inflorescence, and flower morphology. Were also in detail studied the morphological characteristics of the flower and fruit, and the fruit set.

Cytological studies and pollen staining

The meiotic division was investigated in pollen mother cells of CMS-*pennellii* by the acetocarmine smear technique as modified by Khush and Rick (1963). For pollen viability determinations, anthers from fully opened flowers were squashed in 1% acetocarmine in 45% acetic acid, and the number of grains absorbing the stain was recorded. For each plant, three independent acetocarmine squash analyses were carried out during the growing season.

Isozyme analysis

Isozymes were extracted from leaves. Fresh leaf material was ground with a mortar and pestle in 2 vol (v/w) of the extraction buffer: 0.05 M Tris-HCl, pH 6.8, 0.2% 2-mercaptoethanol and 12% glycerol. The homogenates were centrifuged at 15 000 g for 15 min. Discontinuous polyacrylamide-gel electrophoresis (PAGE) was used to separate the isoenzymes. The samples were loaded on a 10% polyacrylamide gel. The electrode buffer system employed was Tris-glycine (0.025 M Tris-0.19 M Glycine), pH 8.3. The enzyme-activity stains were prepared according to: Brown et al. (1978) for malate dehydrogenase (MDH), Wendel and Weenden (1989) for malic enzyme (ME) and esterase (EST), Hawkes et al. (1982) for peroxidase (PRX), Beauchamp and Fridovich (1971) for superoxide dismutase (SOD), and Decker and Rau (1963) for glutamate oxalacetate transaminase (GOT).

DNA isolation, mitochondrial DNA probes

Total DNA from leaves was isolated according to the protocol of Fulton et al. (1995). DNA was extracted from the bulked leaf material of 5–10 individual plants. Ten micrograms of DNA were digested with different restriction enzymes (Boehringer Mannheim and Stratagene) (Table 1) and fractionated by electrophoresis on 0.8% agarose gels. The fractionated DNA was blotted and hybridized to DIG-labeled heterologous mtDNA probes according to the manufacturer's protocols of the Gene Screen Plus membranes (DuPont) and of the Non-radioactive Labeling and Detection of Nucleic Acids system (Boehringer Mannheim).

The following heterologous mitochondrial gene probes were employed (Table 1): *atpA*, a 1.5-kb *Hin*dIII/*Eco*RI fragment, containing the coding region of subunit α of the ATPase gene of *Pisum sativum* (kindly provided by Dr. Toro Terachi, Kyoto Sangyo University, Japan); *atp6*, a 2.7-kb *Hin*dIII fragment containing subunit 6 of the ATPase gene and flanking sequences from *Zea mays* (Dewey et al. 1985); the *18S* + *5S* ribosomal gene region from the mtDNA of *Zea diploperennis*, a 6.0-kb *Bam*HI fragment (Gwynn et al. 1987); *nad3*, a 0.8-kb *Bam*HI/*Hin*dIII fragment containing the NADH dehydrogenase subunit 3 from *Petunia hybrida* (Yesodi et al. 1995); clone 2 from *P. hybrida* containing the *S-Pcf* locus (Yesodi et al. 1995) associated with CMS (a 2.9-kb *Sal*I fragment including part of the *coxII* gene – cytochrome oxidase subunit 2, urf-s – an unidentified open reading frame, the *nad3* gene and *rps12* gene – ribosomal protein); *cob*, a 0.68-kb *Eco*RI/*Hin*dIII intragenic fragment from apocytochrome *b* of maize (Dawson et al. 1984); *coxII*, a 1.9-kb *Eco*RI/*Hin*dIII fragment of cytochrome oxidase subunit II from maize (Fox and Leaver 1981), and *coxIII* a 1.1-kb *Eco*RI/*Pst*I fragment from the cytochrome oxidase subunit III of *Oenothera* (Heisel et al. 1987).

Table 1 Mt gene probes and restriction enzymes employed in the Southern-blot analysis of mt-DNA

Mitochondrial gene probes	Restriction endonucleases
18S+5S rRNA	*Dra*I, *Bam*HI, *Eco*RI, *Hin*dIII
atp6	*Bam*HI, *Eco*RI, *Hin*dIII
atpA	*Dra*I, *Hin*dIII, *Bam*HI/*Hin*dIII *Eco*RI/*Hin*dIII
nad3	*Bam*HI, *Hin*dIII, *Eco*RI, *Bam*HI/*Hin*dIII, *Eco*RI/*Hin*dIII
Clone no. 2 (*coxII*, *urf-s*, *nad3*, *rps12*)	*Hin*dIII
coxII	*Hin*dIII
coxIII	*Hin*dIII
cob	*Hin*dIII

Results

Morphological observations and cytological analysis of CMS-*pennellii*

There are no differences in the leaves (number, size and form of leaf segments and secondary leaf segments) and the inflorescence between CMS-*pennellii* and *L. pennellii*. The flowers of CMS-*pennellii* differ from those of the recurrent parent in the following features: the corolla size is smaller, pale yellow in color and the petals are not well opened; the anthers are reduced in size with a mean anther length of 2.3 mm, pale green in colour, and are not coalesced laterally to form a normal staminal cone (Figs. 1, 2). The anthers do not shed pollen. To study female fertility, CMS-*pennellii* plants have been pollinated with pollen from *L. pennellii*. The fruits formed do not differ phenotypically from those of the recurrent parent. The fruit set of the pollinated inflorescences is comparable to that of *L. pennellii*. The seed set and the size of the fruits formed indicate that the female fertility of CMS-*pennellii* is normal (Table 2). The fruit size and the seed set of the CMS line surpass those of *L. pennellii* because the CMS-*pennellii* flowers have been hand pollinated.

The cytological analysis showed that the meiotic and tetrad stages (Sawhney and Bhadula 1988) of pollen development were normal. Degeneration of the microspores was observed after tetrad disintegration. The anthers did not shed pollen. The studies on anther squash preparations showed that all CMS-*pennellii* pollen grains were shrunken and were not stained with 1% acetocarmine.

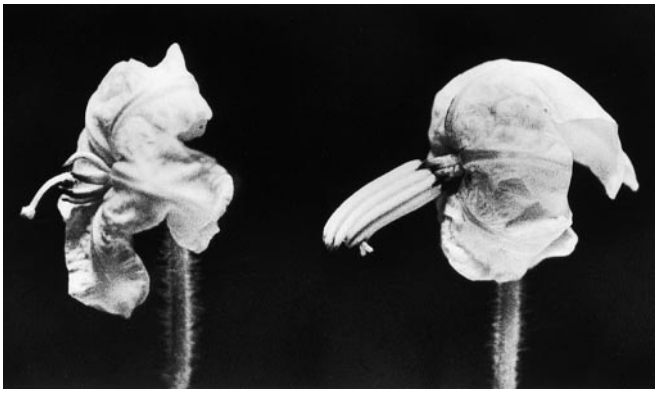

Fig. 1 Flowers from CMS-*pennellii* (left) and *L. pennellii* (right)

Fig. 2 Anthers from CMS-*pennellii* (up) and *L. pennellii* (down)

Isozyme analysis

This analysis was carried out in order to compare the activity of the enzymes in *L. pennellii* and its sterile analogue CMS-*pennellii*. The study of MDH shows that its activity decreased in CMS-*pennellii*. The two fractions with the highest intensity in *L. pennellii* were absent from the electrophoretic spectrum of the sterile form (Fig. 3). For ME a general decrease in the intensity and the activity of all fractions was detected in the sterile form. The two isoforms with the highest mobility in the profile of the CMS line were absent in *L. pennellii*. One fraction from the profile of *L. pennellii* was missing in the CMS line (Fig. 3).The peroxidases preserved the same qualitative composition in both forms. The activity of the two fractions with lowest mobility was increased in the CMS line (data not shown). The isozyme profile of GOT was the same for

Table 2 Characteristics of female fertility in CMS-*pennellii*

Species	Fruit size	Seed set
L. peruvianum	13.44 ± 0.11	88.8 ± 0.23
L. pennellii	9.54 ± 0.05	87.97 ± 0.46
CMS-*pennellii*	13.33 ± 0.24	108.63 ± 0.57

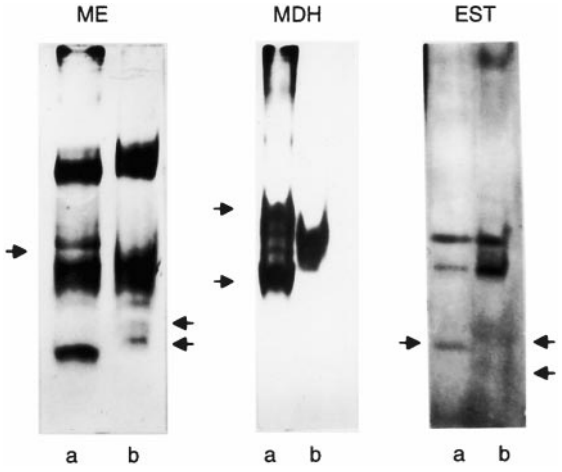

Fig. 3 Isozyme analysis of MDH, ME, EST in leaves of *L. pennellii* (**a**) and CMS-*pennellii* (**b**)

the fertile and sterile analogues. In the latter, the intensity of the high-mobility fractions decreased (data not shown). No differences were detected in the qualitative and quantitative composition of SOD in leaves (data not shown). An increased activity and the appearance of two new fractions fraction characterized the profile of EST in the CMS line in comparison with *L. pennellii* (Fig. 3).

The studies of the isozyme profile of *L. peruvianum* were included as data validating that the nuclear genome of *L. peruvianum* had been fully replaced in the CMS-*pennellii* line. The isozyme study of *L. peruvianum* showed clear differences between *L. peruvianum* and *L. pennellii* for the profiles of MDH, ME, EST, PRX and SOD. None of the specific *L. peruvianum* isoforms were detected in the CMS-*pennellii* profile (data not shown).

Studies of the mitochondrial genome

The molecular studies of the organization of the mitochondrial genome of the CMS-*pennellii* line with *L. peruvianum* as a donor of the cytoplasm were carried out with *atpA*, *atp6*, *nad3*, *clone 2*, *18S + 5S* rRNA, *coxII*, *coxIII* and *cob* as heterologous gene probes (Table 1).

Differences were established in the hybridization pattern of the *atpA* gene with *Hin*dIII-, *Dra*I-, and *Eco*RI/*Hin*dIII-digested total DNA. In CMS-*pennellii* *atpA* hybridized to a 5.44-kb *Hin*dIII fragment, while in *L. peruvianum* the probe hybridized to a 5.34-kb fragment (Fig. 4). After double-enzyme digestion with *Hin*dIII and *Eco*RI a single fragment of 3.6 kb appeared in the profile of CMS-*pennellii*. This provided evidence that the *Eco*RI site(s) is (are) internal to the *Hin*dIII fragment. In *L. peruvianum* the same 3.6-kb fragment was present but an additional fragment of 5.0 kb was also detected. The *atpA* hybridization profile of the *Dra*I-digested CMS -*pennellii* DNA revealed four fragments (10.0 kb, 5.2 kb, 3.4 kb and 2.5 kb), while the hybridization pattern of the donor of the *L. peruvianum* cytoplasm had two additional fragments (6.34 kb and 4.4 kb) (data not shown).

With the *nad3* gene a polymorphism between CMS-*pennellii* and *L. peruvianum* was detected on the blots with *Hin*dIII- and with *Eco*RI/*Hin*dIII-digested DNA (Fig 4). The *nad3* gene hybridised to the same *Hin*dIII fragments detected in the *atpA* *Hin*dIII profiles of CMS-*pennellii* and L. *peruvianum*. The hybridization pattern of *nad3* for the double-digested CMS-*pennellii* DNA duplicated the results obtained with *atpA*. The *Eco*RI/*Hin*dIII profile of *L. peruvianum* had three fragments: 3.6 kb and 5.0 kb (duplicating the *atpA* hybridization) as well as an additional one of 3.0 kb.

As expected, the results obtained with the clone 2 probe, comprising the *S-Pcf* locus from petunia, demonstrated multiple bands. The hybridization pattern showed that the most intensive band had the same length as the band detected with the *nad3* probe, 5.34 kb for *L. peruvianum* and 5.44 kb for the sterile analogue (data not shown).

For all other heterologous probes studied no polymorphism was established between the sterile line and *L. peruvianum*.

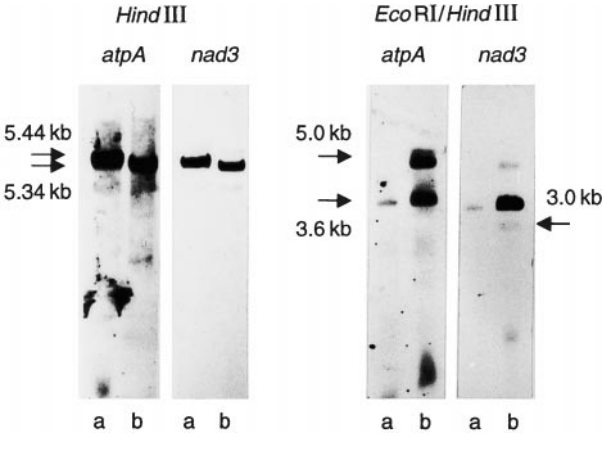

Fig. 4 Southern-blot analysis of total DNA from CMS-*pennellii* (**a**) and *L. pennellii* (**b**) with *atpA* gene probe and *nad3* gene probe

Table 3 Segregation for fertility in the F_1, F_2 and F_3 progenies of the cross CMS-*pennellii* × (F_1 *L. esculentum* × *L. pennellii*)

Progeny	Number of plants			Ratio S:SF:F	χ^2	$P >$
	Sterile	Semi-fertile	Fertile			
F_1	14	10	10	1.4:1:1	1.05	0.5
F_2	18	8	8	2.2:1:1	0.12	0.80
F_3	19	13	10	1.9:1:1	0.81	0.50
Theoretically expected ratio				2:1:1		

Introgression of *L. esculentum*

We overcame the unilateral incompatibility of CMS-pennellii with *L. esculentum* by the use of pollen from the F_1 *L. esculentum* × *L. pennellii* (Martin 1964; Stoeva 1980, 1982; Lemke and Mutschler 1983). The pollination of 578 flowers of CMS-*pennellii* with the *L. esculentum* × *L. pennellii* F_1 yielded 15 fruits with 1–5 seeds per fruit. Thirty four hybrid F_1 CMS-*pennellii* × (F_1 *L. esculentum* × *L. pennellii*) plants were grown and studied. The morphological observations showed a predomination of the *L. pennellii* phenotype. The characteristics of *L. esculentum* were observed in the range of leaf, flower and fruit morphology variation. According to pollen stainability the plants in the F_1 segregating progeny fell into three groups: sterile (S) – plants with 0–20% pollen stainability, semi-fertile (SF) – 20–70%, and fertile (F) – 70–100%. The grounds for accepting 70% pollen stainability as the lower range value for the fertile plants is the 70–75% pollen stainability of the interspecific F_1 *L. esculentum* × *L. pennellii* hybrid. A study of the pollen stainability in the progeny of the complex F_1 CMS-*pennellii* × (F_1 *L. esculentum* × *L. pennellii*) hybrid revealed that the proportion of plants in the defined groups S:SF:F was 1.4:1:1 (Table 3). F_2 and F_3 generations of this complex hybrid were produced by the sib-mating of sterile plants (with a pollen stainability of 0–2 %) with fertile plants (with a pollen stainability of 80–90%). In the F_2 and F_3, 34 and 43 plants, respectively, were studied. The segregation for sterile, semi-fertile and fertile plants in these progenies reproduced the segregation observed in the F_1 (Table 3). The analysis of the distribution of plants within the groups from each of the progenies studied demonstrated that most of the sterile plants had a pollen stainability of 0–10%. In the semi-fertile group plants with a pollen stainability ranging from 50 to 70% predominated. The fertile plants grouped mainly in the range from 80 to 100% pollen stainability (Fig. 5).

The plants from the F_2 and F_3 segregating progenies were used as pistillate parents for the further introgression of *L. esculentum* by backcrossing to the *L. esculentum* × *L. pennellii* F_1. As female parents both sterile (with pollen stainability 0–10%) and fertile plants (80–100% pollen stainability) were chosen. The percent of successful backcrosses to the F_1 *L. esculentum* × *L. pennellii* was variable ranging from 0.05% for the sterile female partners to 1% for the fertile female partners. Sterile and fertile segregants from these backcross generations were successfully pollinated with the cultivated tomato *L. esculentum.*

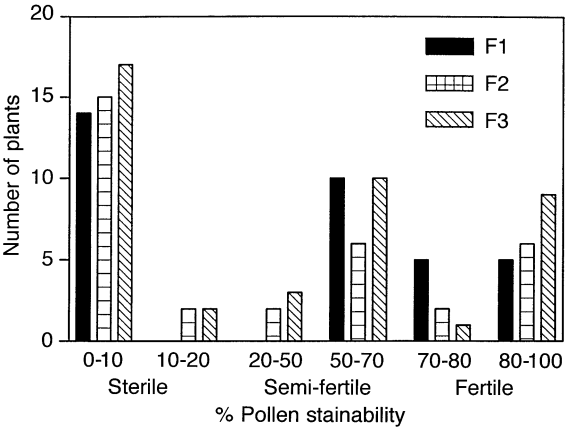

Fig. 5 Frequency distribution within the groups of sterile, semi-fertile and fertile plants in F_1, F_2 and F_3 progenies

Discussion

Analysis of the CMS-*pennellii* line

The basis for our studies was the *L. peruvianum* × *L. pennellii* CMS hybrid (Valkova 1980). The maternal inheritance of the male sterility over the subsequent backcross generations with *L. pennellii* proves that the CMS is stable. The phenotype of the anthers and the cytological analysis classifies the male sterility as the sporogenous type (Kaul 1988). The normal fruit and seed set of the plant pollinated with the *L. pennellii* CMS line (Table 2) proves that it is fully female-fertile. In the backcross of CMS-*pennellii* to the female parent (*L. peruvianum*) male fertility is restored (unpublished data). The data provide evidence that the cause of male sterility is a result of the interaction between the cytoplasm of *L. peruvianum* and a nuclear gene(s) from *L. pennellii.*

The morphological studies and the comparative analysis of different isozyme systems (EST, PRX, MDH, ME, SOD, GOT) in *L. pennellii*, *L. peruvianum* and the CMS-*pennellii* line indicate that the nuclear genome of the female parent has been fully replaced in

the CMS line. Part of the enzyme systems studied in total leaf extracts show quantitative changes (GOT, PRX, SOD); while for MDH, EST and ME both quantitative and qualitative changes have been observed. The new fractions in the electrophoretic spectrum of EST and ME, produced as a result of the altered expression of *L. pennellii* nuclear genes in the alien cytoplasm (Fig. 3), can be used as isozyme markers for the CMS line.

An investigation of the mitochondrial genomes of the CMS line and the donor of the cytoplasm, *L. peruvianum*, has been carried out in order to distinguish the changes which have taken place as a result of the interaction between the cytoplasm of *L. peruvianum* and the genome of *L. pennellii*. On genomic DNA blots, differences in the hybridization profiles between *L. peruvianum* and CMS-*pennellii* are detected with the 1.5-kb *atpA* gene probe from *P. sativum* and the 0.8-kb *nad3* gene probe from *P. hybrida* (Yesodi et al. 1995) (Fig. 4). The results reveal that the *nad3* and *atpA* genes are proximal and lie on the same *Hin*dIII fragment in *L. peruvianum*, 5.34 kb, and in CMS-*pennellii*, 5.44 kb. The presence of polymorphism between *L. peruvianum* and CMS-*pennellii* for these two probes is confirmed by the results from the double-digestion with the *Hin*dIII and *Eco*RI restriction enzymes. With both probes additional fragments appear in *L. peruvianum* (Fig. 4). The absence of these fragments from the RFLP pattern of the CMS-*pennellii* can be considered as evidence for the generation of diversity in the mitochondrial genome due the interaction of the cytoplasm of *L. peruvianum* with the nuclear genome of *L. pennellii*.

Sterile or fertile segregants from the backcross populations BC_2-P_2 [CMS-*pennellii* × (F_1 *L. esculentum* × *L. pennellii*] have been used as female bridge hybrid plants in crosses with the cultivated tomato. Recurrent backcrosses are in progress for the introduction of the genome of *L. esculentum* into a CMS-*pennellii* background. The complex hybrids obtained will provide a basis for the development of the CMS system in cultivated tomato.

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