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Genome composition of asymmetric hybrids in relation to the phylogenetic distance between the parents. Nucleus-chloroplast interaction

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Summary. A series of fusion experiments were performed between protoplasts of a cytoplasmic albino mutant of tomato, *Lycopersicon esculentum* (ALRC), and gamma-irradiated protoplasts of *L. hirsutum* and the *Solanum* species *S. commersonii*, *S. etuberosum* and *S. nigrum*. These species were chosen for their different phylogenetic relationships to tomato. In all fusion combinations except from those between ALRC and *S. nigrum*, green calli were selected as putative fusion products and shoots regenerated from them. They were subsequently analyzed for their morphology, nuclear DNA composition and chloroplast DNA origin. The hybrids obtained between ALRC and *L. hirsutum* contained the chloroplasts of *L. hirsutum* and had the flower and leaf morphology of *L. esculentum*. After Southern blot analysis, using 13 restriction fragment length polymorphisms (RFLPs) randomly distributed over all chromosomes, all hybrids showed *L. esculentum* hybridization patterns. No chromosomes of *L. hirsutum* were found. These results indicate that these hybrids were true cybrids.

The putative asymmetric hybrids, obtained with *S. commersonii* and *S. etuberosum*, showed phenotypic traits of both parents. After hybridization with species-specific repetitive nuclear DNA probes it was found that nuclear material of both parents was present in all plants. In the case of *S. nigrum*, which combination has the greatest phylogenetic distance between the fusion parents, no hybrid plants could be obtained. The chloroplast DNA of all hybrid plants was of the donor type suggesting that chloroplast transfer by asymmetric protoplast fusion can overcome problems associated with large phylogenetic distances between parental plants.

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Key words: *Lycopersicon* – *Solanum* – Asymmetric somatic hybridization – Chloroplast DNA – nuclear DNA analysis

Introduction

Using asymmetric somatic hybridization or cybridization, new organelle-nuclear genome combinations can be obtained between sexually incongruent species. By this method useful agronomic traits of a wild species can be introduced into the gene pool of a crop species (Pelletier et al. 1988; Glimelius et al. 1991).

One method to produce asymmetric hybrids involves donor-recipient fusion (Zelcer et al. 1978). In this method, the donor protoplasts are inactivated by irradiation with X- or gamma-rays in order to eliminate the donor nuclear DNA. When fusion products are selected in favour of the donor organelles, hybrids can be obtained with the organelles of the donor and the nuclear DNA of the recipient. For this purpose selection systems, based on the chloroplast and mitochondrial composition of the hybrids, have to be used.

Using cytoplasmic traits, e.g., chlorophyll deficiency, antibiotic or herbicide resistance, fusion products with organelles of one species and the morphological phenotype of the other, species have to-date been selected from intergeneric and interspecific cybridizations (Medgyesy et al. 1980; Fluhr et al. 1985; Medgyesy et al. 1985; Menczel et al. 1986; Aviv and Galun 1988; Ratushnyak et al. 1991). Surprisingly, in none of these reports were extensive nuclear DNA analyses using nuclear DNA probes described.

In the genus *Lycopersicon*, cybrids were isolated after selection based on inactivation of both parents, or after

selection for chloroplast type. Bonnema et al. (1991) described tomato cybrids with mitochondrial DNA of *L. pennellii*. The nuclear genome of these fusion products was investigated using restriction fragment length polymorphism (RFLP) analysis. Ratushnuyak et al. (1991) obtained tomato hybrids with donor chloroplasts of *L. peruvianum*; however, the nuclear genome has not been extensively characterized.

In the present report, the relation between chloroplast transfer and the phylogenetic relationships between the fusion partners is examined. For this purpose, a series of asymmetric fusion experiments between protoplasts of a cytoplasmic albino mutant of tomato (ALRC) and protoplasts of *L. hirsutum* and several *Solanum* species is described. These cytoplasm donor species were chosen for their different phylogenetic relationships to tomato. Transfer of chloroplasts from *L. hirsutum* to *L. esculentum* is not possible by sexual crosses, since successful seed setting between these species occurs only when *L. hirsutum* is used as a pollinator (Martin 1961). Since chloroplasts are inherited maternally, chloroplasts of *L. hirsutum* can only be transferred to *L. esculentum* by protoplast fusion. Crosses between *L. esculentum* and the *Solanum* species employed are unsuccessful due to incongruity.

In these experiments, hybrids between *L. esculentum* and gamma-irradiated chloroplast donor species were analyzed for their morphology, nuclear DNA content, and chloroplast DNA. By using RFLP analysis or species-specific probes, the nuclear DNA of the fusion products was characterized for the presence of nuclear DNA of the donor. In this report the nucleus-chloroplast interaction in relation to the phylogenetic relationships between fusion parents is discussed.

Materials and methods

Plant material and growth

In vitro-grown plants of the cytoplasmic albino mutant *L. esculentum* cv. Large Red Cherry (ALRC) were obtained from Dr. M. R. Hansen (Section of Genetics and Development, Cornell University, Ithaca, USA). Axenic shoots of the ALRC lines were cultured as described by Derks et al. (1990). In vitro-grown plants of *S. commersonii* (PI 243503) and *S. etuberosum* (PI498311) were obtained from Prof. dr. E. Galun (Department of Plant Genetics, Weizmann Institute of Science, Rehovot, Israel). Shoots of these lines were cultured using the same culture conditions and the same medium as for ALRC with the addition of 2% (w/v) sucrose. Seeds of *L. hirsutum* LA1777 were supplied by Dr. W. H. Lindhout (CPRO-DLO, Wageningen, The Netherlands), and seeds of an atrazine-resistant mutant of *S. nigrum* (Wag. 3R) were obtained from Dr. ir. J.L.P. v. Oorschot (CABO-DLO, Wageningen, The Netherlands). The seeds were sterilized for 7 min in a 2% (v/v) sodium hypochlorite solution (commercial bleach), rinsed with sterile water and germinated at 25 °C in darkness on MS medium (Murashige and Skoog 1962) with 2% (w/v) sucrose, 0.7% (w/v) agar (Oxid) in the absence of hormones. After 1 week the seedlings were cut below the cotyledons and subcultured on the same medium under the culture conditions described by Derks et al. (1990).

Protoplast isolation, fusion and culture

In fusions the albino mutant ALRC was used as recipient and *L. hirsutum*, *S. commersonii*, *S. etuberosum* and *S. nigrum* were used as chloroplast donors. The procedures for protoplast isolation, culture and plant regeneration of all lines used were essentially as described previously (Derks et al. 1990); however, different enzyme concentrations were used for protoplast isolation of *S. commersonii*, *S. etuberosum* and *S. nigrum*, namely 0.25% (w/v) Cellulase 'Onozuka' R-10, 0.05% (w/v) Macerozyme R-10 (Yakult, Honsha Co. Ltd., Tokyo, Japan). The donor protoplasts were inactivated by gamma-irradiation (300 Gy, ^{60}Co source, 1750 Gy/h). The polyethylene glycol (PEG) fusion was performed according to the method of Menczel et al. (1981) with the following modifications: the PEG solution had a final concentration of 7.5% (w/v) PEG 4000 and was removed after 7 min. For the recognition of the fusion products the albino protoplasts were stained with fluorescein diacetate (FDA) (100 µg/ml) during the enzyme incubation.

Determination of ploidy level

The DNA content of nuclei isolated from mesophyll cells from in vitro-grown plants and from the regenerants was determined by flow cytometry. Leaf material was chopped with a sharp razor blade in a Petri dish in an ice-cold nuclear isolation buffer described by Saxena and King (1989) with some modifications: 0.2 *M* sucrose, 10 m*M* spermine-tetrahydrochloride and 0.25 µg/ml DAPI (4.6-Diamidino-2-phenylindole). After filtration through a 15 µm nylon filter, the samples were measured using a Partec PAS-II flow cytometer as described by Verhoeven et al. (1990). The fluorescent signals proportional to the quantity of DNA per nucleus were presented as histograms in which nuclear DNA content was expressed in arbitrary units. Nuclei of ALRC were added to some samples as an internal standard.

Chromosome counts

Chromosome numbers in metaphase cells of root-tips were determined in squash preparations as described by Sree Ramulu et al. (1983). The chromosomes were counted in at least ten metaphase cells of each hybrid.

DNA isolation

For the total DNA extraction a modified method was used obtained from Dr. S. D. Tanksley (Cornell University, Ithaca, New York, USA). Two gram of fresh young leaf tissue was frozen in liquid N_2 and 20 ml of cold extraction buffer (0.35 *M* sorbitol, 0.1 *M* TRIS-HCl pH 7.5, 0.005 *M* EDTA, 0.02 *M* $Na_2S_2O_5$) was added. The leaf material was fragmented in a Polytron for 20–30 s and the samples were centrifuged for 40 min at 4500 rpm (4500 g) in a labofuge. After centrifugation, 1.25 ml extraction buffer was added to the pellet and mixed for 5 s. Then 1.75 ml of lysis buffer [0.2 *M* TRIS-HCl pH 7.5, 0.05 *M* EDTA, 2 *M* NaCl, 2% (w/v) cetyl trimethylammonium bromide (CTAB)] and 0.6 ml of 5% (w/v) Na N-lauroylsarcosine was added. The samples were inverted 5–10 times and incubated at 65 °C for 20 min; then 7.5 ml chloroform/Isoamyl alcohol (24:1) was added. After inverting the samples 30–40 times and centrifugation at 4500 rpm (4500 g) for 10 min in a labofuge, the supernatant was carefully removed. The DNA was precipitated by adding 4 ml of isopropanol and, after inverting the tubes 5–10 times, the DNA was hooked out and resuspended in 100 µl 4 m*M* TRIS-HCl pH 8.0, 0.04 m*M* EDTA.

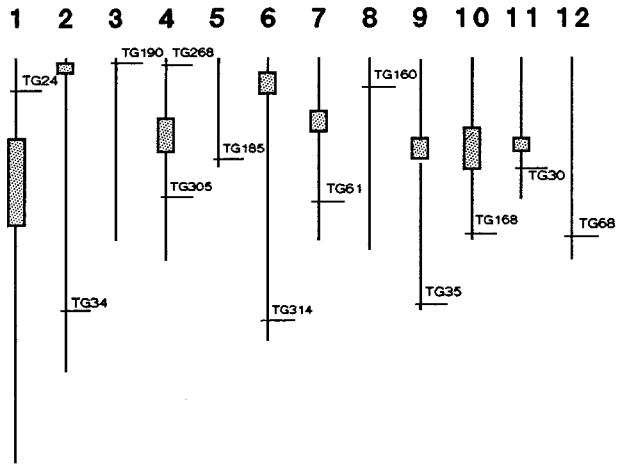

Fig. 1. Linkage map of the restriction fragment length polymorphism (RFLP) markers used in the analysis of the asymmetric hybrids. The *shaded areas* indicate putative locations of centromere regions. The positions are according the Young and Tanksley (1989) and Dr. S.D. Tanksley (Cornell University, Ithaca, New York, USA)

Hybridization probes

The following tomato genomic single-copy clones were used: TG24, TG30, TG34, TG35, TG61, TG68, TG160, TG168, TG185, TG190, TG268, TG305 and TG314. These probes were kindly supplied by Dr. S. D. Tanksley (Cornell University, Ithaca, New York, USA). The map positions of the clones used in the hybrid analysis are shown in Fig. 1. The inserts in each clone were excised from the vector with the appropriate restriction enzyme and separated from the vector on a 1% (w/v) agarose gel.

DNA inserts of the probes were labelled with α-^{32}P-dATP (Amersham, UK) at 37 °C for 10 min with a random primer kit (Stratagene, Heidelberg, Germany) as described by the supplier. Unincorporated nucleotides were removed by centrifugation on a Sephadex G50-M column (Pharmacia, Uppsala, Sweden). The probe was boiled for 10 min before being added to the hybridization mixture.

DNA restriction, Southern transfer and hybridization

Total DNA (15 µg) was digested with the restriction endonucleases *Eco*RI, *Eco*RV and *Hin*dIII (10 units/µg DNA) as described by the manufacturer (Amersham). DNA electrophoresis on an 0.8% (w/v) agarose gel, Southern blotting and DNA hybridization were performed as described previously by Derks et al. (1991).

Dot blot analysis of the nuclear DNA of the asymmetric somatic hybrids

Total DNA from the parents and hybrids was isolated according to Dellaporta et al. (1983). The DNA concentration was measured with Hoechst 33258 using a mini fluorometer (TKO 100, Hoefer Scientific Instruments, San Francisco, USA). Before DNA was applied to the Gene Screen Plus membrane (Dupont, Boston, USA), the membrane was soaked in 0.5 N NaOH for 5 min and subsequently placed in a dot-blotting apparatus (HYBRI-DOT™ Manifold, Gibco BRL, Breda, The Netherlands). The vacuum was then applied and the DNA solutions were spotted onto the membrane. DNA of the parents was applied in a concentration series ranging from 0.05 to 0.4 µg and ± 0.2 µg DNA of the fusion products (each hybrid three times) was spotted. The membranes were rinsed with 100 µl/well of 2 m*M* TRIS-HCl (pH 8.0), 0.04 m*M* EDTA. After 15 min the vacuum was removed and the membrane was soaked again in 0.5 N NaOH for 5 min. The membrane was air-dried for 2 min on Whatman 3 MM paper and incubated in 1 *M* TRIS-HCl (pH 7.5) for 2 min (two times). After drying for 2 min, the membrane was soaked in 0.5 *M* TRIS-HCl (pH 7.5)/1.5 *M* NaCl for 5 min and then air-dried for 1 h at 20 °C ready for hybridization. The following nuclear species-specific repetitive probes were used for the analysis of the fusion products: pTHG2, a tomato-specific repetitive DNA probe, obtained from Dr. P. Zabel, Department of Molecular Biology, Agricultural University, Wageningen, The Netherlands (Zabel et al. 1985), and two potato-specific reptitive DNA probes (P5L and P3), obtained from Dr. R. G. F. Visser, Department of Plant Breeding, Agricultural University, Wageningen, The Netherlands (Visser et al. 1988).

The labelling of the probe and hybridization were performed as described by Derks et al. (1991). The radioactivity per dot was measured by scintillation counting. Each dot was cut out of the filter and placed in a scintillation vial to which 5 ml of the liquid scintillation cocktail Ultima Gold (Packard, Downers Grove, Illinois, USA) was added. Each vial was counted for 15 min in a Tri-carb liquid scintillation analyzer 1600CA (Packard, Downers Grove, Illinois, USA). Data obtained from the scintillation counts were corrected using a membrane without DNA as a control for background.

Characterization of chloroplast DNA

The *Petunia* chloroplast DNA probes pPCY64, the *Pst*I fragments 1 and 4 (Haas de et al. 1986), pPCY20-1 (Overbeeke et al. 1984) and two *Sac*I fragments, S2a and S2b, of the chloroplast DNA of *Sedum album* (R. van Ham, Department of Population and Evolution Biology, Utrecht State University, Utrecht, The Netherlands) were used for the characterization of chloroplast DNA. Total DNA of all tested species was isolated according to Dellaporta et al. (1983) and 4 µg of DNA was digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII and *Hin*fI (10 units/µg DNA) as described by the manufacturer (Amersham). DNA electrophoresis, Southern blotting, labelling of the chloroplast DNA probes, and hybridization were all performed as described previously by Derks et al. (1991).

Chloroplast DNA restriction fragment data analysis

Differences between the restriction fragment patterns were used to reconstruct the phylogenetic relationship between the parental chloroplasts. Fragments were given the number 1 if present in the pattern and 0 if absent. Only informative homologous restriction fragments were compiled into a binary data matrix.

Phylogenetic analysis was performed on the data matrix using the Branch and Bound (BANDB) option of the computer program P.A.U.P. (version 2.4.1., D. Swofford, Illinois Natural History Survey). This option is guaranteed to find the shortest Wagner tree using the algorithm of Hendy and Penny (1982).

Results

Phylogenetic relationship

Hybridizations with six chloroplast DNA probes were carried out on Southern blots with total DNA of the two *Lycopersicon* species (ALRC and *L. hirsutum*), the three *Solanum* species (*S. commersonii*, *S. etuberosum* and *S.*

nigrum) and *Nicotiana tabacum* SR1, digested with four restriction enzymes. The presence or absence of the restriction fragments were compiled in a matrix (data not shown). Analyzing these data with the BANDB option of P.A.U.P. and by using *N. tabacum* SR1 as outgroup, a phylogenetic tree was obtained (Fig. 2). There are only two extra unique restriction fragments for the chloroplast DNA of *L. eculentum* compared to *L. hirsutum*, while there are 24 differences in the chloroplast DNA restriction patterns of *L. esculentum* and *S. nigrum*. These results indicate that the regard to chloroplast DNA *L. hirsutum* is closely related to *L. esculentum*, *S. nigrum* is the most distantly related species and the other two *Solanum* species are intermediate.

Asymmetric somatic hybridizations

Since the albino protoplasts were stained with FDA and the donor protoplasts were red, because of chlorophyll autofluorescence, it was possible to estimate the number of heterofusion products using double UV-fluorescence.

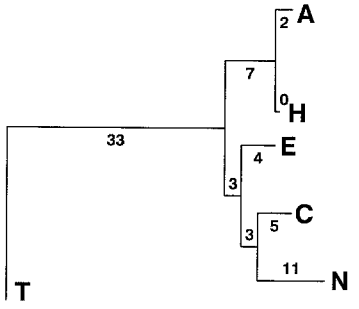

Fig. 2. The phylogenetic tree of *Lycopersicon* and *Solanum* chloroplast DNA restriction sites. The length of the tree is 68 steps with a consistency index of 0.926. The branch length is given by the numbers. *Nicotiana tabacum* SR1 was used as outgroup. A, *L. esculentum* (ALRC); C, *S. commersonii*; E, *S. etuberosum*; H, *L. hirsutum*; N, *S. nigrum*; T, *N. tabacum* SR1

A fusion frequency of about 15% was observed directly after fusion. The first green calli (putative cybrids) developed 8 weeks after fusion. Table 1 summarizes the percentage of green calli obtained 11 weeks after fusion. Green calli were obtained in all fusion combinations, although in that between ALRC and *S. nigrum* only seven were selected in one of the three experiments. These seven turned brown and stopped growing a few weeks after selection and no shoot primordia were obtained. In the control dishes, no sustained cell division or growth was observed after irradiation of the donor protoplasts, while albino protoplasts, plated in the presence and absence of irradiated donor protoplasts, resulted only in white calli. No green calli were found even in the presence of zeatin.

The percentage of green calli varied between 4.9 and 26.8% depending on the fusion combination (Table 1). They showed a more vigorous growth pattern than did white calli, except those from the fusion combination with *S. nigrum*. The first shoot primordia developed 7 to 25 weeks after plating on regeneration medium. The plant regeneration frequency, as determined 25 weeks after plating on regeneration medium, varied between 0 and 42% (Table 1). Many shoots could be obtained per regenerating callus.

Variegated hybrids with white and green sectors were not observed in the present study. Furthermore, no hybrids with a mixture of green and white plastids were found. These results indicate that sorting of the plastids had occurred at the callus level.

Morphology of the shoots

The morphology of leaves and flowers of the hybrid regenerants, obtained from the ALRC and *L. hirsutum* fusion, resembled those of tomato (Fig. 3A, B). No morphological features, i.e., hairiness of the *L. hirsutum* par-

Table 1. Asymmetric protoplast fusion between four Solanaceous species and the cytoplasmic albino mutant of *L. esculentum*

Asymmetric fusion[a]	Total number of calli[b]	Number of green calli (%)[c]	Regeneration percentage[d]	Number of shoots per callus	Total number of shoots
A. (+) H	407	19.4	10	1–12	46
A. (+) C	63	19.0	42	13–112	228
	892	5.9	13	1–47	73
A. (+) E	679	26.2	13	1–35	309
	652	26.8	6	1–22	112
A. (+) N	142	4.9	0		
	779	0			
	298	0			

[a] Gamma irradiation of 300 Gy. A., ALRC; H, *L. hirsutum*; C, *S. commersonii*; E, *S. etuberosum*; N, *S. nigrum*
[b] Total number of calli plated on regeneration medium, 4 weeks after fusion
[c] Percentage of green calli counted 11 weeks after fusion
[d] Regeneration percentage of the green calli determined after culturing for 25 weeks on regeneration medium

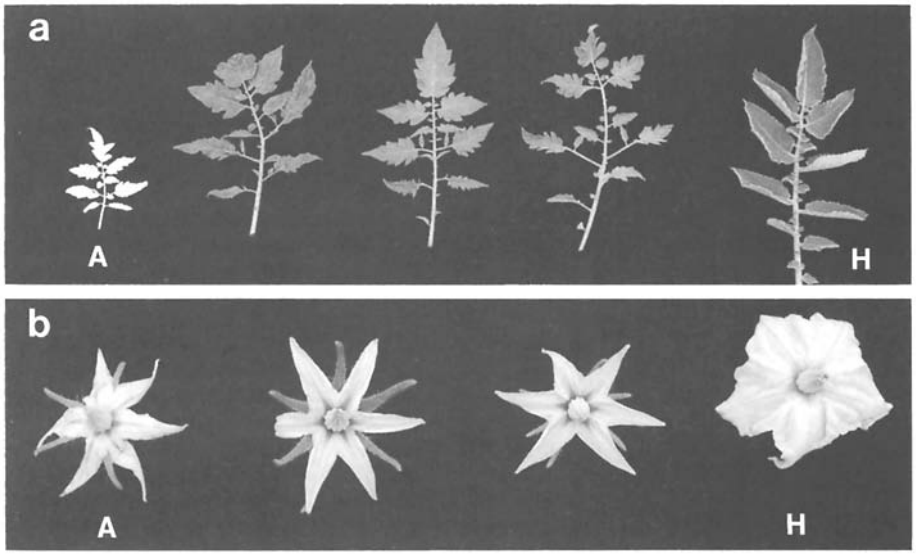

Fig. 3 a, b. Morphological analyses. **a** Leaves, ALRC (*A*), hybrids between the ALRC and *L. hirsutum* (AH38, AH39, AH47) and *L. hirsutum* (*H*). **b** Flowers, *L. esculentum* cv. Moneymaker (*A*), hybrids between the ALRC and *L. hirsutum* (AH39, AH47) and *L. hirsutum* (*H*)

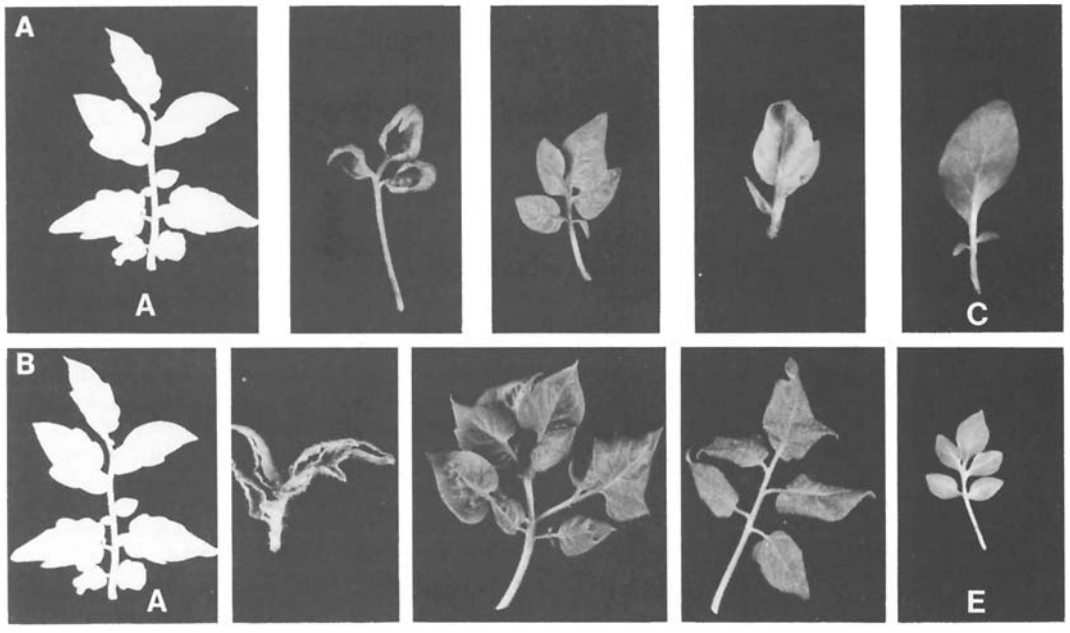

Fig. 4 A, B. Morphological analyses. **A** Leaves, ALRC (*A*), hybrids between the ALRC and *S. commersonii* (AC1A, AC1C, AC2B) and *S. commersonii* (*C*). **B** Leaves, ALRC (*A*), hybrids between the ALRC and *S. etuberosum* (AE20, AE48, AE6) and *S. etuberosum* (*E*)

ent were present. The flowers of all hybrids had viable pollen, and seeds were obtained (data not shown).

The leaves of the hybrid regenerants, obtained from the fusion between ALRC and *S. commersonii*, had an intermediate morphology (Fig. 4 A). Shoots of these hybrids rarely produced roots and, therefore, it was not possible to culture them in the greenhouse. The shoots started to flower in vitro, but the flowers had an aberrant morphology. One flower set an orange fruit in vitro, but unfortunately no seeds were obtained.

An intermediate leaf morphology was also found for in-vitro grown fusion products between ALRC and *S. etuberosum* (Fig. 4 B). Three hybrid plants from a total of 23 were successfully transferred to the greenhouse. Only one of these hybrids had flowers which had an aberrant morphology and aborted before any seeds could be produced.

Chloroplast DNA analysis

The chloroplast DNA of the parental species could be distinguished by restriction fragment length polymorphism (RFLP) when the chloroplast DNA probe pPCY64 was hybridized to *Hin*fI-digested total DNA (Fig. 5).

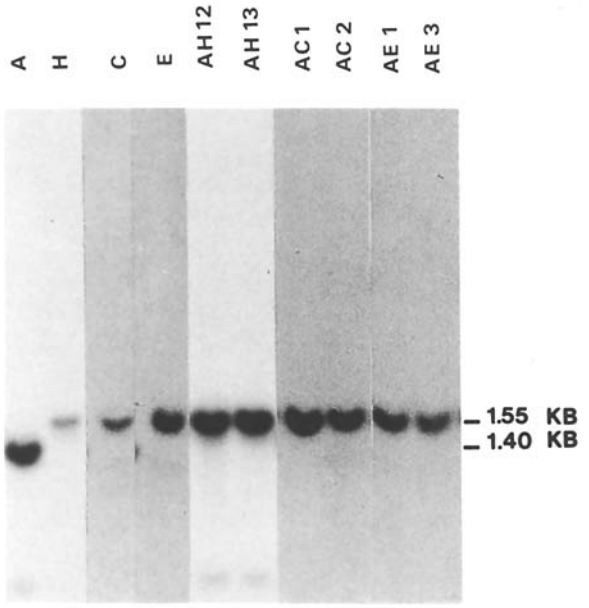

Fig. 5. Analysis of chloroplast DNA. Autoradiograph showing the hybridization pattern of the *Hin*fI digest probed with pPCY64 of *L. esculentum* (ALRC) (*A*), *L. hirsutum* (*H*), *S. commersonii* (*C*) and *S.* etuberosum (*E*), several asymmetric hybrids of ALRC with *L. hirsutum* (*AH12, AH13*), *S. commersonii* (*AC1A, AC2A*) and *S. etuberosum* (*AE1, AE2*)

Chloroplast DNA was analyzed from eight plants of independent hybrids, obtained from the fusion with *L. hirsutum*, ten green shoots, obtained from three independent calli from the fusion with *S. commersonii*, and 16 plants of independent hybrids, obtained from the fusion with *S. etuberosum*. All regenerants showed the chloroplast DNA restriction pattern of the donor (Fig. 5), indicating that in all three combinations the hybrids contained donor chloroplasts.

Determination of ploidy level and chromosome counts

To study the composition of the nuclear genome of the hybrids the nuclear DNA content was measured by flow cytometry. Figure 6A shows that the nuclear DNA content of *L. esculentum* (A) was lower than that of *L. hirsutum* (H). Five hybrids had a DNA content which was equivalent to the DNA content of the diploid *L. esculentum* (Fig. 6B), while the other three hybrids were tetraploid (Fig. 6C).

To determine the exact chromosome number, Feulgen-stained somatic metaphase cells were analyzed. Both *L. esculentum* and *L. hirsutum* have $2n = 2x = 24$ chromosomes. Of the eight hybrids analyzed, five had 24 chromosomes, while three contained 48 chromosomes. No aneuploid or higher polyploid cells were observed.

The nuclear DNA content of most of the hybrids between *L. esculentum* and *S. commersonii* or *S. etuberosum* varied between 3C and 8C (Tables 2 and 3, Fig. 6D,

Table 2. Analysis of nuclear DNA content in the hybrids of *S. commersonii* and ALRC

Plant[a]	Nuclear DNA content (in C-value)[b]	Nuclear DNA derived from *S. commersonii* (%)[c]
ALRC	2.0	0
S. comm.	2.0	100
AC1A	6.0	27
AC1B	4.0	30
AC1C	nd	29
AC1D	4.0	14
AC1E	5.6	nd
AC1H	5.6	37
AC2A	3.0	43
AC2B	3.0	51
AC2C	3.0	37
AC2D	nd	52
AC3	3.0	nd
AC7	8.6	28

[a] *S. comm., S. commersonii*; AC represents a hybrid between the ALRC and *S. commersonii*. Shoots AC1A–AC1H were all obtained from hybrid callus no. 1. Shoots AC2A–AC2D were obtained from callus no. 2
[b] C-value was determined, with the nuclear DNA content of ALRC as an internal standard, by flow cytometry
[c] Percentage nuclear DNA derived from the donor was determined by dot blot hybridization using the repetitive species-specific probes pTHG2 and P5.
nd, not determined

Table 3. Analysis of nuclear DNA content in the hybrids of *S. etuberosum* and ALRC

Plant[a]	Nuclear DNA content (in C-value)[b]	Nuclear DNA derived from *S. etuberosum* (%)[c]
ALRC	2.0	0
S. etub.	1.8	100
AE1	6.2	nd
AE2	8.0	27
AE3	5.4	31
AE4	7.8	43
AE5	7.0	41
AE6	5.6	38
AE7	5.6	19
AE20	5.8	28
AE48	5.2	26
AE82	5.6	25
AE102	6.8	nd
AE103	6.8	31

[a] *S. etub., S. etuberosum*; AE represents a hybrid between the ALRC and *S. etuberosum* (AE)
[b] C-value was determined, with the nuclear DNA of ALRC as an internal standard, by flow cytometry
[c] Percentage nuclear DNA derived from the donor was determined by dot blot hybridization using the repetitive species-specific probes pTHG2 and P3
nd, not determined

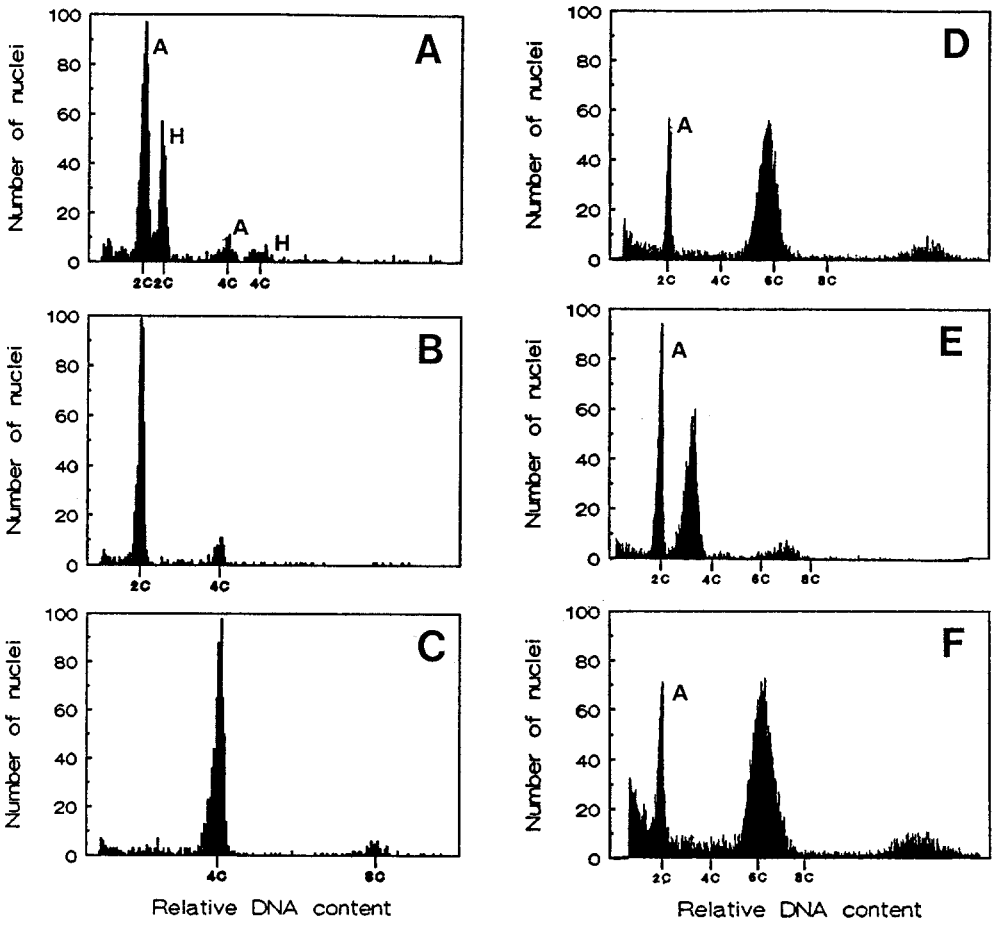

Fig. 6A–F. Flow cytometric determination of relative nuclear DNA content in parents and hybrids. **A** Mixture of ALRC (*A*) and *L. hirsutum* (*H*) cells; **B** Diploid hybrid (AH 12); **C** Tetraploid hybrid (AH 14); **D** Mixture of ALRC (*A*) and hybrid between ALRC and *S. commersonii* (AC2A); **E** Mixture of ALRC (*A*) and hybrid between ALRC and *S. commersonii* (AC2A); **F** Mixture of ALRC (*A*) and hybrid between ALRC and *S. etuberosum* (AE1). c = arbitrary units

Table 4. Restriction fragment length polymorphism (RFLP) between *L. hirsutum* LA1,777 and the cytoplasmic albino mutant of *L. esculentum* cv. Large Red Cherry (ALRC)

Chromosome number	Probe number	Restriction enzymes[a]		
		*Eco*RI	*Eco*RV	*Hin*dIII
1	TG24	+	+	+
2	TG34	−	+	−
3	TG190	+	nd	−
4	TG268	+	±	+
4	TG305	−	+	−
5	TG185	−	+	+
6	TG314	+	±	±
7	TG61	+	+	±
8	TG160	+	+	+
9	TG35	−	−	+
10	TG168	+	+	−
11	TG30	−	+	+
12	TG68	nd	+	+

[a] +, RFLP is present; −, no RFLP is present; ±, no useful RFLP (only one parent gives one or more specific bands); nd = not determined

E, F). Most hybrids were aneuploid. Furthermore, the hybrids AC 1A-1H, obtained from one callus, varied in their DNA content (Table 2).

Nuclear DNA analysis

Because there are no species-specific repetitive probes which discriminate between *L. esulentum* and *L. hirsutum*, the characterization of the hybrids between these species had to be performed with single-copy probes. First, single-copy probes were used to find RFLPs between the parental species. These probes were hybridized to total DNA of both parents that was digested with three restriction enzymes: *Eco*RI, *Eco*RV and *Hin*dIII. For each chromosome at least one single-copy probe was selected (the map positions of the probes used are given in Fig. 1). All tested probes showed that for the restriction enzymes used, at least one RFLP was useful for nuclear DNA analysis of the fusion products (Table 4). Those single-copy probes that gave useful RFLPs be-

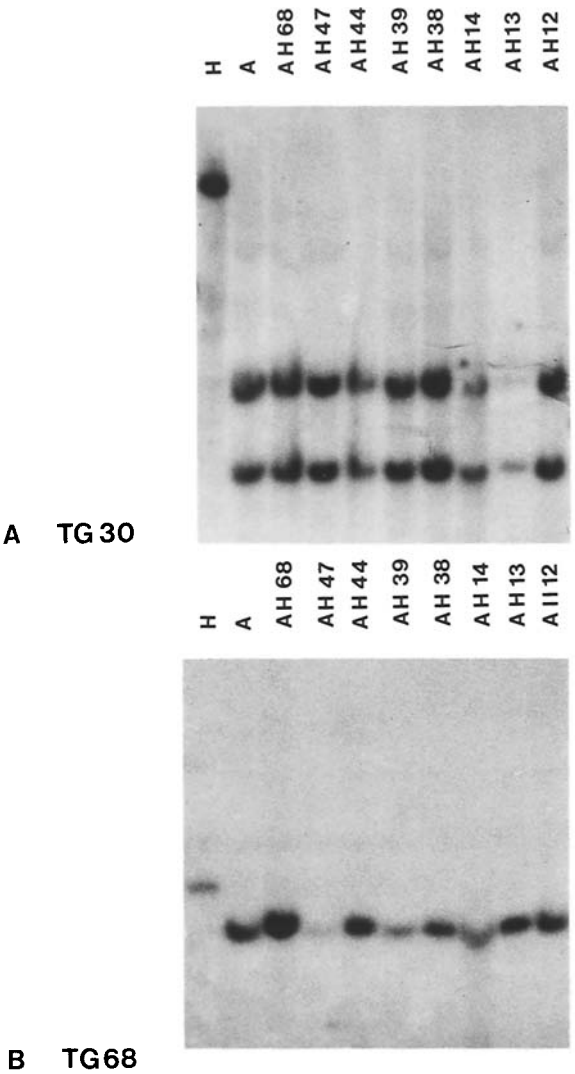

Fig.7 A, B. Restriction fragment length polymorphism (RFLP) pattern obtained after hybridization with the probes TG30 (**A**) and TG68 (**B**) to total DNA of the ALRC (*A*), *L. hirsutum* (*H*) and the eight fusion products (*AH12–AH68*), digested with the restriction enzyme *Eco*RV

tween the parents were used to analyze the fusion products for the presence of *L. hirsutum* chromosomes. Figure 7 shows hybridization patterns of eight fusion products, digested with *Eco*RV and probed with TG30 and TG68 (chromosomes 11 and 12 respectively). All eight hybrids showed the RFLP which resembled the pattern of the albino mutant. Hybrid AH 13 was not tested for the probes TG34, TG305, TG61 and TG168, because of the difficulty in DNA isolation. All hybrids, except AH 13, were tested for each chromosome by using the corresponding useful RFLP (listed in Table 4). In all hybrid-probe combinations only the RFLP pattern resembling that of tomato was found. These results indicate that hybrids contained no intact *L. hirsutum* chromosomes.

Species-specific repetitive probes, which discriminate between *Solanum* and *Lycopersicon* species, were used to estimate the amount of donor and recipient DNA in the hybrids from both combinations by dot blot analyses. The concentration range of parental DNA gave a linear correlation between the amount of target DNA content and the radioactivity measurements (Fig. 8 A, B). Using this correlation, the composition of the DNA in the fusion products was calculated (Fig. 8 C, D, Tables 2 and 3). The amount of nuclear DNA of *S. commersonii* in the hybrids varied between 14 and 52% (Table 2). Furthermore, the percentage of *S. commersonii* DNA varied between shoots even when they were regenerated from one fusion product (AC 1A-1H, Table 2). In the hybrids obtained between ALRC and *S. etuberosum*, nuclear DNA of both parents was also present and the amount of *S. etuberosum* nuclear DNA was very variable (between 19 and 43%; Table 3).

Discussion

This paper has described chloroplast transfer from *L. hirsutum*, *S. commersonii* and *S. etuberosum* to *L. esculentum* indicating that the problems associated with large phylogenetic distances between the parental plants can be surmounted. Unfortunately, no chloroplast transfer could be obtained in the more distantly related combination *S. nigrum* and *L. esculentum*.

RFLP analyses showed that no intact chromosomes of *L. hirsutum* were present in the cybrids. This suggests that gamma-radiation was effective in eliminating the chromosomes of the donor, *L. hirsutum*. This elimination of nuclear-donor DNA was probably due to the method of cybrid selection. However, in all hybrids of the fusion between *L. esculentum* and *S. commersonii* or *S. etuberosum* nuclear DNA of the donor species remained present, ranging from 14 to 52%. These results indicate that elimination of the donor chromosomes by gamma-irradiation was far from complete, although we selected for a cytoplasmic trait.

Probably, the phylogenetic relationship, based on plastome phylogeny, between species in the fusion combination influences the production of cybrids and asymmetric hybrids. RFLP analyses showed that the chloroplast DNA of *L. esculentum* is very similar to that of *L. hirsutum*. Possibly there is no incongruity of the plastome-nuclear genome between these two species. This indicates that gene products in our cybrids encoded by the nucleus of *L. esculentum* can function in association with those encoded by the chloroplast DNA of *L. hirsutum*. This means that there is a proper functional interplay of nucleus and chloroplast in the cybrids obtained between these two *Lycopersicon* species in which unilateral incongruity occurs.

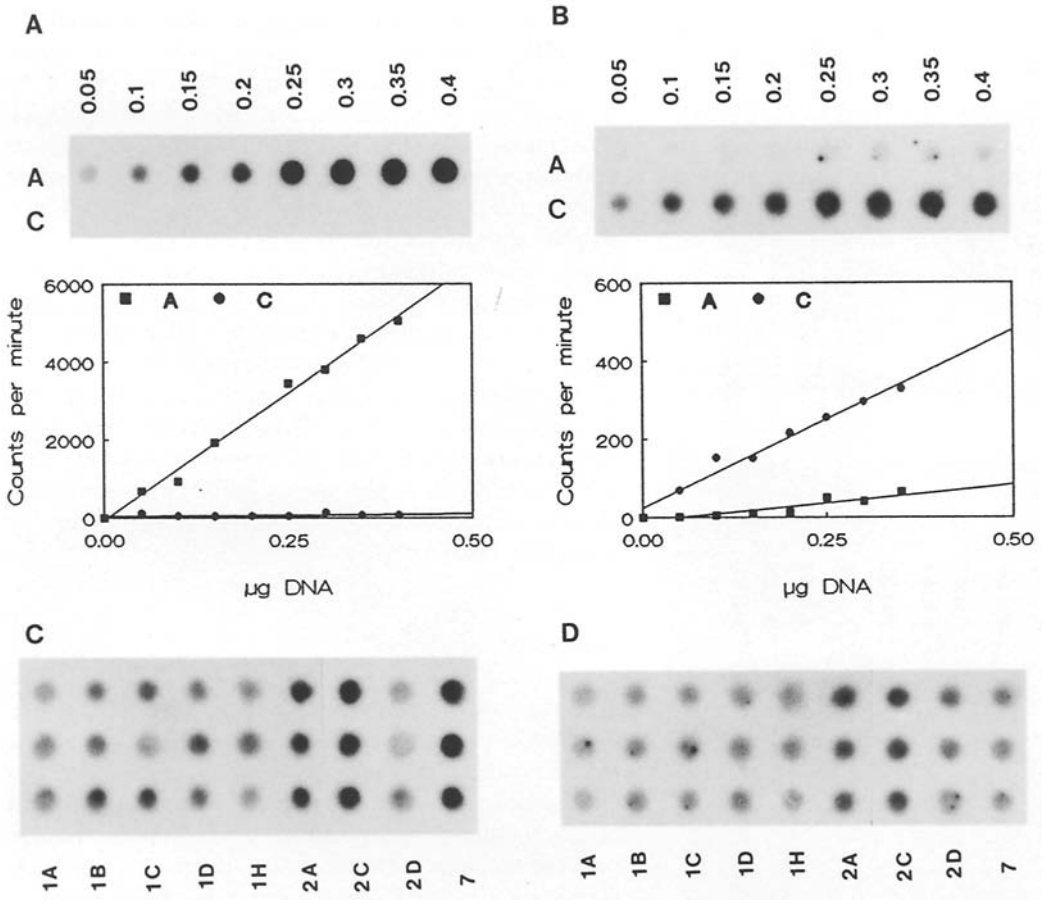

Fig. 8A–D. Dot blot analysis of *L. esculentum* (ALRC), *S. commersonii* and the hybrids. A concentration series of total DNA of ALRC (A) and *S. commersonii* (*C*) was spotted on the blots, which were radioactively labelled with a tomato-specific repetitive nuclear DNA probe pTHG2 (**A**), or with a potato-specific repetitive nuclear DNA probe P5L (**B**). Calibration plots were made by cutting the dots out of the filters and measuring the radioactivity. Total DNA of the hybrids was spotted on the blots, which were probed with pTHG2 (**C**) or with P5L (**D**)

A considerable phylogenetic distance exists between the chloroplast DNAs of *S. commersonii S. etuberosum*, and tomato. The fusions with ALRC and these *Solanum* species resulted in asymmetric hybrids with chloroplast DNA of the wild species and nuclear DNA of both parents. It seems that in these intergeneric combinations nuclear DNA of the donor is necessary for the donor chloroplasts to function. This suggests that, although selection for cytoplasmic traits was performed, no true cybrids can be selected between phylogenetically remote species. This incomplete elimination of the donor chromosomes was also described by Menczel et al. (1982), Cséplö et al. (1984), Glimelius and Bonnett (1986), and Ratushnuyak et al. (1991), all of who selected their cybrids for cytoplasmic traits.

S. nigrum was the most phylogenetically distant species to tomato used in our experiments and with this combination a few green calli were only obtained once and these stopped growing. Probably, chloroplast transfer between *S. nigrum* and *L. esculentum* is not possible, because of incongruity between the donor chloroplast and the recipient nuclear genome, or else of incongruity between both parental nuclear genomes. The results described by Thanh et al. (1988) also point to incongruity between the plastome and nuclear genome, since no cybrids or asymmetric hybrids could be obtained between *S. nigrum* as donor and *N. tabacum* as recipient. Both Kushnir et al. (1991) and Perl et al. (1990) found that their cybrids, obtained in fusions between two phylogenetically remote species, (between *Nicotiana* and *Atropa*, and between several *Solanum* species, respectively), showed chlorophyll-deficiency which could be due to incongruity between the chloroplast and the nucleus. Unfortunately, the presence of donor nuclear DNA in these hybrids was not analyzed using nuclear probes. Our results are in contrast to those of Jain et al. (1988), who reported atrazine-resistant asymmetric hybrids between tomato and a cytoplasm-encoded atrazine-resistant mutant of *S. nigrum*. However, chloroplast DNA analyses were not carried out. According to Dr. J. Hirschberg

(personal communication), it is possible that by selecting for atrazine resistance, hybrids were obtained with mixed chloroplast populations.

The variation in the composition of the nuclear DNA between the various hybrids, obtained with *S. commersonii* and *S. etuberosum* as donor species, could be the result of somaclonal variation (D'Amato 1985; Sree Ramulu 1987; Pijnacker and Sree Ramulu 1990), which is a common phenomenon in tissue culture. The nuclear DNA content could vary, even among the individual shoots from the same hybrid callus, as was observed in the fusion combination ALRC and *S. commersonii*. A possible explanation for this is that during callus growth the amount of nuclear DNA content per cell can change leading to a non-homogeneous DNA distribution in the callus. Different nuclear DNA rearrangements may also occur during differentiation into shoots. This report, for the first time, describes fusion experiments where the protoplast fusion method, the selection system, the culture method and the recipient plant were all kept constant. Therefore, the results can be compared to the phylogenetic relationships of the parental plants and conclusions can be drawn about chloroplast behaviour related to nuclear DNA composition. This study shows that the asymmetric somatic hybridization method, based on selection for a cytoplasmic trait, was successful in the production of true cybrids between *L. esculentum* (ALRC) and gamma-irradiated *L. hirsutum*. By developing these cybrids at the somatic cell level, it was demonstrated that crossing barriers were bypassed and new organellar-nuclear genome combinations obtained. Our results also indicate that transfer of organelles is possible between plants that are phylogenetically fairly distantly related, but this seems to be coupled with the maintenance of the nuclear DNA of the donor. This seems to be essential for proper functioning of the chloroplast. In more distantly related species the production of cybrids is prevented.

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