Analysis of nuclear and organellar DNA in somatic hybrids between solanaceous species

Analyse van kern- en organel-DNA in somatische hybriden tussen soorten van de familie *Solanaceae*

Promotoren: dr. ir. M. Koornneef
persoonlijk hoogleraar bij de
vakgroep Erfelijkheidsleer

dr. C. Heyting
hoogleraar in de generatieve en
somatische celgenetica

Anne-marie Wolters

Analysis of nuclear and organellar DNA in somatic hybrids between solanaceous species

Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
dr. C.M. Karssen,
in het openbaar te verdedigen
op woensdag 21 september 1994
des namiddags te half twee in de Aula
van de Landbouwuniversiteit te Wageningen

Aan mijn ouders
Aan Johan

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Wolters, Anne-marie

Analysis of nuclear and organellar DNA in somatic hybrids between solanaceous species / Anne-marie Wolters. - [S.l.: s.n.]. - Ill.
Thesis Wageningen. - With ref. - With summary in Dutch.
ISBN 90-5485-278-X
Subject headings: somatic hybridization / somatic incongruity / *Lycopersicon esculentum*.

Omslagontwerp: Ernst van Cleef

Stellingen

1. De mogelijkheden om via somatische hybridisatie een voor de plantenveredeling bruikbaar genotype te produceren, waarin genetische informatie van twee weinig verwante soorten is gecombineerd, zijn beperkt.
   Dit proefschrift

2. Veranderde kern-organel interacties leiden vaak tot verminderde levensvatbaarheid.
   Dit proefschrift

3. Van de tomaat-achtige planten met kern-DNA en mitochondriaal DNA geheel afkomstig van tomaat, die door Melchers et al. (1992) verkregen zijn na fusie van met joodaceetamide behandelde tomaat en gamma-bestraalde tabak, is niet overtuigend aangetoond dat ze werkelijk het resultaat zijn van een fusiegebeurtenis tussen tomaat- en tabaksprotoplasten.
   Melchers et al. (1992) Proc Natl Acad Sci USA 89:6832-6836

4. De notatie "*Lycopersicon esculentum* x *L. peruvianum* hybrid" gebruikt door Smith et al. (1986) voor genotype IVT 741505 suggereert ten onrechte dat het hier gaat om een nakomeling van een kruising tussen *L. esculentum* als vrouwelijke ouder en *L. peruvianum* als mannelijke ouder.
   Smith et al. (1986) Plant Cell Rep 5:298-301
   Koornneef et al. (1986) Plant Sci 45:201-208

5. De omschrijving van het begrip "mitochondriën" in de "Dikke Van Dale" doet geen recht aan de onmisbaarheid van deze organellen voor de meeste eukaryote organismen.
   Van Dale Groot Woordenboek der Nederlandse Taal, 11^{e} herziene druk, 1989

6. Vóór het schrijven van het eerste wetenschappelijke artikel dient men niet alleen "Writing a Scientific Paper" van Booth (1975) te lezen, maar ook "The Mystery of Marie Rogêt" van Edgar Allan Poe (1840-45).
   Booth V (1975) Biochem Soc Transact 3:1-26
   Poe AE (1840-45) Tales of Mystery and Imagination

7. Gedeelde kennis is dubbele wijsheid.

8. De belangstelling voor reizen naar exotische bestemmingen met de bedoeling kennis te maken met vreemde volkeren en culturen lijkt omgekeerd evenredig te zijn met de tolerantie voor vreemdelingen en hun culturen in ons eigen land.

9. Links/rechtshandigheid is een continu variërende eigenschap.

10. Beter één artikel in de hand dan tien in de maak.

11. The proof of the thesis is in the reading.

12. Door de traditie om "vermakelijke" stellingen als laatste te plaatsen worden veel stellingen niet gelezen.

Stellingen behorende bij het proefschrift "Analysis of nuclear and organellar DNA in somatic hybrids between solanaceous species" door Anne-marie Wolters, in het openbaar te verdedigen op woensdag 21 september 1994, te Wageningen.

Abstract

This thesis describes an analysis of the possibilities and limitations of somatic hybridization of solanaceous species. Emphasis was laid on the elucidation of the interactions between nuclei, chloroplasts and mitochondria in the obtained somatic hybrids. Hybridization experiments between tomato (*Lycopersicon esculentum*) and potato (*Solanum tuberosum*) revealed that cybrids combining a tomato nucleus with potato chloroplasts were not viable. Symmetric and asymmetric somatic hybrids between these two species contained chloroplasts from either parent, and mitochondrial DNA fragments from either parent or from both parents, without any relation to the nuclear DNA constitution. Chloroplasts and mitochondria sorted out independently. During mitosis and meiosis of tomato (+) potato somatic hybrids many irregularities were observed: chromosome fragments, bridges, laggards and a high percentage of First Division Restitution during male meiosis. One hybrid proved to contain a tomato/potato translocation chromosome. Many hybrids were aneuploid and all were sterile. Somatic hybrids between distantly related species (*Lycopersicon* (+) *Nicotiana* and *Solanum* (+) *Nicotiana* hybrids) showed uni- or biparental chromosome elimination, often in combination with multiplication of the genome of one of the parents. A strong correlation was apparent between organelle DNA and nuclear DNA; the hybrids contained chloroplasts and mitochondrial DNA only or mainly derived from the parental species that predominated in the nucleus. Regenerants were very difficult to obtain. The two hybrid plants that were produced grew weakly and were sterile. These results indicate that phylogenetic divergence and somatic incongruity impose strong restrictions on the potential of somatic hybridization.

These investigations were supported by the Dutch Programme Committee on Agricultural Biotechnology (PcLB) and performed at the Department of Genetics, Wageningen Agricultural University, the Netherlands.

Contents

CHAPTER 1

General introduction

Somatic hybridization

Transfer of desirable characteristics from a non-cultivated species into a crop species can be achieved by several methods. When the two species are closely related and sexually compatible, crosses are made and the hybrid is backcrossed with the crop species in order to obtain a new genotype of the cultivated species with the desired trait. When the gene coding for the trait of interest (e.g. disease resistance) has been cloned, it can be transferred to the crop species by means of transformation. When, however, the trait of interest is present in a species that is sexually incompatible with the crop species, and the responsible gene has not been cloned, or the characteristic is polygenic, transfer of this trait can in theory be achieved by somatic hybridization, i.e. fusion of protoplasts of both species.

By symmetric somatic hybridization the complete genomes of two species are combined in one hybrid cell, which subsequently must be able to divide. The resulting callus must be able to proliferate and differentiate, in order to obtain somatic hybrid plants. In general, species hybrids possess not only the desired trait, but also many undesired characteristics. Therefore, a symmetric somatic hybrid usually is not the ultimate goal, but backcrosses with the crop species are required to achieve elimination of the unfavourable traits. This implies that somatic hybrids must be, at least partially, fertile.

A way to shorten this procedure is the production of asymmetric somatic hybrids. In this case the genetic material of the species possessing the desired trait (the donor species) is fragmented, e.g. by gamma- or X-irradiation, or reduced, e.g. by using haploid pollen-derived protoplasts (Pirrie and Power 1986) or microprotoplasts (Ramulu et al. 1992, 1993). After fusion with protoplasts from the crop species (the recipient species) asymmetric somatic hybrids are obtained. When these are highly asymmetric, the improved genotype of the cultivated species might be obtained immediately, or after a few backcrosses.

When the desired trait is encoded by chloroplast or mitochondrial DNA, only the organelles have to be transferred from the donor species to the recipient species. In this case production of cybrids is aimed at, i.e. fusion products that combine nuclear DNA exclusively derived from the recipient with chloroplasts and/or mitochondria from the donor. To obtain cybrids, the nucleus of the donor species has to be excluded or removed from the fusion product. When in a heterokaryon no nuclear fusion takes place, sorting out of the nuclei can result in cybrids. However, since nuclear fusion is the rule (Constabel et al. 1975; Medgyesy 1990), the removal of the donor nucleus has to be enforced. This can be achieved by irradiation or enucleation of the donor species, often in combination with inactivation of protoplasts of the recipient by iodoacetate or iodoacetamide. Alternatively, isolated chloroplasts or mitochondria can be injected into the recipient protoplast (Verhoeven and Blaas 1992). The transferred organelles subsequently have to be able to function in coordination with the nucleus from the recipient species.

In this thesis the results are presented of experiments aimed at the combination of genetic information of different solanaceous species by somatic hybridization. Emphasis is laid on the elucidation of the interaction between nuclei, chloroplasts and mitochondria in the obtained somatic hybrids.

Somatic incongruity and phylogenetic distance between fusion parents

Technically, there are no or almost no limitations for fusion of protoplasts of different plant species (Harms 1983; Gleba and Sytnik 1984), or even of plant protoplasts and animal cells (Dudits et al. 1976; Ward 1984). However, the further development of a fusion product depends on the potential of the genomes that are brought together to collaborate in such a way that metabolic and developmental functions needed for cell division and cell differentiation are possible. When these functions are impaired, somatic incongruity is involved, i.e. a condition whereby well-regulated physiological and biochemical interactions fail to function in a coordinated way because of non-matching information between the genomes of two species brought together by hybridization (Hogenboom 1984). Incongruity may exist between the nuclei of two different species, but also between the nucleus of one species and chloroplasts and/or mitochondria of the other species (Harms 1983).

Genetic divergence is a major factor determining somatic incongruity. Therefore, viability and performance of somatic hybrids is largely influenced by the phylogenetic relatedness of the fusion parents. On the basis of this relatedness five classes of somatic hybrids can be distinguished.

(1) Intraspecific hybrids are obtained after fusion of two different genotypes of one species. The resulting hybrid contains, in principle, the sum of the number of chromosomes of both parents. This type of fusion can be useful in tetraploid species like potato (e.g. Möllers and Wenzel 1992) or tobacco, to combine favourable traits present in two diploid genotypes.

(2) Interspecific hybrids are obtained after fusion of two different species of the same genus. Interspecific hybridization has been performed on a large

scale for transfer of nuclear or organellar traits from a wild relative to a crop species, for the resynthesis of amphidiploid species (e.g. *Brassica napus* (Jourdan et al. 1989), *B. carinata* (Narasimhulu et al. 1992)), and, in the case of potato, for combination of characteristics of a diploid wild *Solanum* species with traits of a diploid *S. tuberosum* genotype (e.g. Helgeson et al. 1986; Mattheij et al. 1992; Cardi et al. 1993).

(3) Intergeneric hybridization involves two species from different genera of the same tribe. Success with this type of hybridization is highly variable. Generally, complete plants can be regenerated, but they may show an aberrant morphology (Dudits 1988).

(4) Intertribal hybrids result from fusion between species from different tribes. In this case regeneration of complete plants often is difficult and flowering of the resulting plants is limited (Gleba and Sytnik 1984). When symmetric somatic hybrid plants cannot be obtained for a given species combination, sometimes spontaneous or enforced elimination of part of the nuclear genome of one of the parents results in viable asymmetric hybrid plants (e.g. Krumbiegel and Schieder 1981; Gupta et al. 1984; Potrykus et al. 1984).

(5) Interfamiliar hybrid cell lines have been obtained by symmetric hybridization and in some combinations these could be cultured for several months or even years. In none of these cell lines morphogenetic activity was observed (Gleba and Sytnik 1984). However, in some cases viable and fertile recipient-like hybrid plants can be produced by asymmetric hybridization (Dudits et al. 1987).

The phylogenetic relations between some well-known solanaceous species are shown in Fig. 1. Interspecific, intergeneric and intertribal somatic hybrids or cybrids with tomato that are described in literature, are listed in Tables 1,2 and 3 respectively. The species are ordered on the basis of increasing phylogenetic distance from tomato, according to McClean and Hanson (1986), Derks et al. (1992) and Spooner et al. (1993). Most interspecific hybrids with tomato were viable and fertile (Table 1). Intergeneric somatic hybrids with tomato mostly showed partial or no fertility, and only in a few cases progeny plants were obtained. Cybrids combining a tomato nucleus with chloroplasts from a *Solanum* species could not be obtained (Table 2). Only a limited number of intertribal hybrids with tomato (*L. esculentum*) or with *L. peruvianum* have been reported. None of these were symmetric (Table 3).

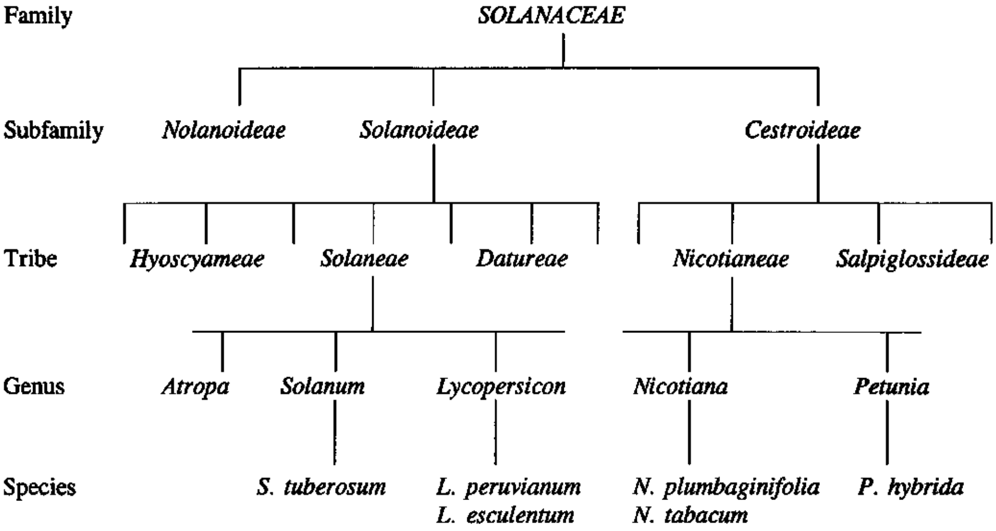

Fig. 1. Phylogenetic tree of some solanaceous species, adapted from D'Arcy (1991)

Fate of chromosomes/nuclear DNA after somatic hybridization

Symmetric somatic hybrids

When a protoplast fusion experiment is performed with two closely related parental species, the resulting somatic hybrids are expected to be genetically balanced and therefore to contain the sum of the number of chromosomes of both parents. However, often some type of chromosome instability occurs, possibly induced during in vitro culture, resulting in chromosome fragmentation, elimination and/or translocation. The extent of this type of chromosome instability, which is an aspect of somaclonal variation, may depend on the duration of the callus phase, the genotype and the protoplast source.

In nuclei of somatic hybrids of distantly related species the chromosomes of each parent are often assembled in blocks or clusters instead of being well mixed in a common metaphase plate (Gleba and Sytnik 1984; Gleba et al. 1987). This can result in maldistribution of chromosomes in the following mitoses, leading to an extensive uniparental or biparental chromosome elimination (Gleba and Sytnik 1984; Gilissen et al. 1992). Since the callus phase of such hybrids usually is relatively long, the possibility of karyotypic aberrations is larger than in the case of somatic hybrids between closely related species.

Table 1. Interspecific somatic hybridizations with tomato

L. esculentum (+)	Reference	Selection and identification[a]	Regeneration	Fertility[b]	Progeny[b]	Nuclear DNA	cpDNA (no. of hybrids)	mtDNA[c] (no. of hybrids)
L. hirsutum	Derks et al. 1992	green calli	yes	yes	–	cp cybrids[c]	8 *L hirs*	ND
	Jourdan et al. 1993	growth & reg.	yes	yes	yes	symmetric	18 *L hirs*	*L esc+L hirs*
L. pennellii	O'Connell & Hanson 1985	isozymes	no	–	–	symmetric	1 *L esc* 2 *L penn* 2 *L esc+L penn*	4 *L penn* 1 *L esc+L penn*
						asymmetric	1 *L esc* 3 *L esc+L penn*	4 *L penn*
	O'Connell & Hanson 1987	growth & isozymes	yes	yes	no	symmetric	2 *L esc* 1 *L penn* 1 *L esc+L penn*	1 *L esc* 4 *L penn*
						asymmetric	ND	ND
	Melzer & O'Connell 1990	growth & isozymes	yes	yes	yes	asymmetric	2 *L esc*	2 *L penn*
	Melzer & O'Connell 1992	growth	yes	yes	–	asymmetric	ND	ND
	Wachocki et al. 1991	growth & isozymes	yes	–	–	asymmetric	8 *L esc* 1 *L penn* 1 *L esc+L penn*	4 *L penn* + R 6 *L esc+L penn*
						mt cybrids[d]	10 *L esc*	3 *L penn* + R 7 *L esc+L penn*
	Bonnema et al. 1991	growth & isozymes	yes	yes	–	mt cybrids[d]	65 *L esc*	3 *L penn* + R 7 *L esc+L penn*
	Bonnema et al. 1992	growth & isozymes	yes	yes	–	asymmetric	56 *L esc* 26 *L penn*	7 *L esc* (+ R) 58 *L penn* (+ R) 17 *L esc+L penn*
L. peruvianum	Kinsara et al. 1986	morphology	yes	yes	–	symmetric	ND	ND
	San et al. 1990	morphology	yes	yes	–	symmetric	24 *L esc* 19 *L per*	43 *L esc+L per*
	Wijbrandi 1989; Wijbrandi et al. 1990a	growth & reg.	yes	yes	yes	symmetric	ND	ND
	Wijbrandi et al. 1990b,c,d	growth & reg.	yes	no	no	asymmetric	ND	ND

Table 1. (continued)

L. esculentum (+)	Reference	Selection and identification[a]	Regene- ration	Fertility[b]	Progeny[b]	Nuclear DNA	cpDNA (no. of hybrids)	mtDNA[e] (no. of hybrids)
L. peruvianum	Derks et al. 1991	growth & reg.	yes	–	–	symmetric	12 *L esc*	3 *L esc*
							21 *L per*	8 *L per*
							1 *L esc*+*L per*	1 R
						asymmetric	19 *L esc*	6 *L esc*
							20 *L per*	2 *L per*
								4 R
	Ratushnyak et al. 1991	growth & greening	yes	no	no	asymmetric	6 *L per*	6 *L esc*
						cp cybrids[c]	1 *L per*	ND
	Ratushnyak et al. 1993	growth & reg.	yes	yes	yes	asymmetric	1 *L esc*	*L esc*+*L per*
							9 *L per*	
	Giddings & Rees 1992a,b	morphology	yes	–	–	symmetric	ND	ND
	Sakata et al. 1991; Sakata & Monma 1993	morphology	yes	yes	yes	symmetric	ND	ND
L. chilense	Bonnema & O'Connell 1992	growth & RFLPs	yes	no	no	symmetric	2 *L esc*	2 *L esc*+*L chil*

[a] reg., regeneration

[b] –, no information available

[c] cp cybrids, fusion products containing *L. esculentum* nuclear DNA and chloroplasts of donor species

[d] mt cybrids, fusion products containing *L. esculentum* nuclear DNA and at least some mtDNA of donor species

[e] R, novel, non-parental fragment(s)

ND, not determined

Table 2. Intergeneric somatic hybridizations with tomato

L. esculentum (+)	Reference	Selection and identification^{a}	Regeneration	Fertility^{b}	Progeny^{b}	Nuclear DNA	cpDNA (no. of hybrids)	mtDNA^{f} (no. of hybrids)
S. lycopersicoides	Handley et al. 1986; Levi et al. 1988; Moore & Sink 1988a,b	vigorous growth	yes	yes	–	symmetric	68 *L esc* 1 *S lyc* 1 *L esc+S lyc*	45 *S lyc* 1 *S lyc* + R
	Tan 1987	manual selection	yes	–	–	symmetric	3 *S lyc*	ND
	Li & Sink 1992	vigorous growth	yes	–	–	symmetric S+M^{c}	40 *S lyc* 2 *L esc+S lyc*	ND
						M+M	18 *L esc* 16 *S lyc* 2 *L esc+S lyc*	ND
						S+S	22 *L esc*	ND
S. rickii	O'Connell & Hanson 1986	growth & morph.	yes	–	–	symmetric	4 *S rick*	4 *S rick* + R
S. acaule	Schweizer et al. 1988	cold tolerance & isozymes	yes	–	–	symmetric	ND	ND
	Melchers et al. 1992	growth	yes	♂ sterile/ fertile ♀ fertile	yes	mt cybrids^{d}	ND	*L esc+S ac*
S. tuberosum	Melchers et al. 1978; Schiller et al. 1982	growth & reg.	yes	no	no	symmetric	4 *L esc* 8 *S tub*	ND
	Melchers et al. 1992	growth	yes	♂ sterile ♀ fertile	–	mt cybrids^{d}	ND	*L esc+S tub*
	Shepard et al. 1983	growth & reg.	yes	no	no	symmetric	2 *S tub*	2 *S tub*

Table 2. (continued)

L. esculentum (+)	Reference	Selection and identification[a]	Regeneration	Fertility	Progeny	Nuclear DNA	cpDNA (no. of hybrids)	mtDNA[f] (no.of hybrids)
S. tuberosum	Schoenmakers et al. 1992,93	growth	yes	no	no	symmetric	ND	ND
	Schoenmakers et al. 1994a	Kan^R & Hyg^R	some	no	no	asymmetric:		
						150 Gy	45 *L esc* 2 *S tub*	ND
						500 Gy	32 *L esc* 30 *S tub*	ND
	Jacobsen et al. 1992	growth & greening; growth & Kan^R	yes	no	no	symmetric	ND	ND
	Jacobsen et al. 1994a,b	growth	yes	yes	yes	symmetric	ND	ND
S. etuberosum	Derks et al. 1992	green calli	yes	no	no	asymmetric[e]	16 *S etub*	ND
	Gavrilenko et al. 1992	vigorous growth & morphology	yes	yes	yes	symmetric	ND	ND
S. commersonii	Derks et al. 1992	green calli	yes	no	no	asymmetric[e]	10 *S comm*	ND
S. muricatum	Sakomoto & Taguchi 1991	growth & reg.	yes	no	no	symmetric	ND	ND
S. nigrum	Guri et al. 1988	growth & reg.	yes	no	no	symmetric	3 *L esc*	*L esc*+*S nigr*
	Jain et al. 1988	morphology	yes	no	no	symmetric	ND	ND
						asymmetric[e]	ND	ND
	Derks et al. 1992	green calli	no	no	no	asymmetric[e]	–	–

[a] reg., regeneration; morph., morphology; Kan^R, kanamycin resistance; Hyg^R, hygromycin resistance

[b] –, no information available

[c] S, suspension culture protoplasts; M, mesophyll protoplasts

[d] mt cybrids, fusion products containing *L. esculentum* nuclear DNA and at least some mtDNA of donor species

[e] no cp cybrids (fusion products containing *L. esculentum* nuclear DNA and chloroplasts of donor species) obtained

[f] R, novel, non-parental fragments(s)

ND, not determined

Table 3. Intertribal somatic hybridizations with tomato

L. esculentum (+)	Reference	Selection and identification[b]	Regeneration	Fertility[c]	Progeny[c]	Nuclear DNA	cpDNA (no. of hybrids)	mtDNA (no. of hybrids)
N. plumbaginifolia	Negrutiu et al. 1989a, 1992[a]	growth & Kan^R	yes	yes	yes	asymmetric	ND	ND
N. tabacum	Hassanpour-Estahbanati et al. 1986; Turpin 1986	morphology	yes	–	–	asymmetric	4 *N tab*	ND
	Melchers et al. 1992	growth	yes	yes	–	mt cybrids?	ND	4 *L esc*
L. peruvianum (+)								
Petunia hybrida	Tabaeizadeh et al. 1985, 1989	chromosome number & size	yes	no	no	asymmetric	2 *L per* 1 R[d]	ND
	Tan 1987	no selection	yes	–	–	no cybrids obtained	*L per*	*L per*

[a] *L. esc.* irradiated, *N. plum.*-like plants obtained

[b] Kan^R, kanamycin resistance

[c] –, no information available

[d] hybrid showing a non-parental form of the large subunit of Ribulose-1,5-biphosphate carboxylase/oxygenase

ND, not determined

Asymmetric somatic hybrids

When one of the fusion parents is irradiated before fusion, the resulting somatic hybrid is expected to be more or less asymmetric, depending on the irradiation dose. However, the effect of irradiation may be influenced by several other factors, e.g. cell cycle stage and phylogenetic relatedness of the parents. Although a high dose of gamma- or X-ray irradiation results in a strong fragmentation of the nuclear DNA, repair can take place during the long period (several days for plant cells as compared with several hours for animal cells) before the first nuclear and cell divisions of the fusion product (Schoenmakers et al. 1994b). When the irradiated cells or protoplasts possess an efficient repair system, chromosome (fragment) elimination may be limited, but instead translocation chromosomes may result. Chromosome rearrangements in asymmetric somatic hybrids, involving chromosomes of both the irradiated and the unirradiated parent, have been demonstrated by RFLP analysis (Wijbrandi et al. 1990d; Fehér et al. 1992) and by *in situ* hybridization (Piastuch and Bates 1990; Parokonny et al. 1992). When fusion products are selected on the basis of a chloroplast-encoded trait of the donor, elimination of nuclear DNA of this parent may be easier to accomplish than when a nuclear-encoded trait of the donor is selected for.

Cybrids

Cybrids are most commonly produced by fusing heavily irradiated protoplasts of the donor species with untreated (Zelcer et al. 1978) or iodoacetate/iodoacetamide-inactivated (Sidorov et al. 1981) protoplasts of the recipient species. Regenerants with a recipient-like morphology, containing chloroplasts and/or mitochondria of the donor, are considered to be cybrids. However, even if these regenerants contain the number of chromosomes of the recipient species, it cannot always be excluded that some nuclear DNA of the donor is present. Chromosome rearrangements may have occurred in these fusion products, in a comparable way as in asymmetric somatic hybrids.

Characteristics of chloroplasts/chloroplast DNA and their fate after somatic hybridization

Chloroplasts are the sites of photosynthetic activity, i.e. the conversion of solar energy into chemical energy. They play a vital physiological and metabolic role during plant growth and development. Chloroplasts of most plants contain 60-100 copies (Parthier 1982) of a single circular DNA molecule of 120-160 kb, which consists of a large single-copy region (70-90 kb) and a small single-copy region (12-25 kb), separated from each other by a large inverted repeat of 10-25 kb (Palmer 1983, 1985). Chloroplast DNA encodes 3-5 chloroplast ribosomal RNA genes, ca 30 transfer RNA genes and approximately 100 structural genes (Shinozaki et al. 1986b; Sugiura 1992). However, the majority (80-90%) of the chloroplast proteins is encoded by nuclear DNA, synthesized in the cytoplasm and transported into the chloroplast (Parthier 1982). Per mesophyll cell 50-200 chloroplasts may be present, depending on the plant species and on age, developmental stage and physiological state of the cell (Possingham 1980).

When protoplasts of two different species are fused, the two types of chloroplasts are combined and become intermixed. During the following cell divisions, when there is no selection for one type of chloroplasts, usually random segregation of the chloroplasts occurs (Akada and Hirai 1986). This ultimately results in calli or regenerated plants containing chloroplasts of only one or the other parental species. Sometimes sorting out of chloroplasts is not completed in regenerants and chimaeric plants are obtained, as can be visualized in variegated somatic hybrids between a cytoplasmic albino parent and a normal green parent (Gleba and Sytnik 1984).

Recombination between cpDNA molecules of different chloroplasts has only rarely been observed. After selection for characteristics of both types of chloroplasts, Medgyesy et al. (1985) obtained one somatic hybrid plant of *N. tabacum* and *N. plumbaginifolia* with chloroplasts containing recombined cpDNA. A detailed analysis of this cpDNA revealed that it consisted of a fine mosaic of the parental chloroplast genomes with a recombination site approximately every 2 kb (Fejes et al. 1990). In a similar way Thanh and Medgyesy (1989) produced one *N. tabacum* (+) *S. tuberosum* cybrid with a recombined chloroplast genome. Cybrids combining a *N. tabacum* nucleus with non-recombinant *S. tuberosum* chloroplasts could not be obtained, indicating the presence of nucleo-cytoplasmic incongruity between these two species. Kirti et al. (1993) produced a *Brassica juncea* cybrid with chloroplasts containing some *B. juncea* cpDNA fragments in addition to some *B. oxyrrhina* cpDNA fragments. Probably, cpDNA recombination is rare because plastid fusion is a rare event (Fejes et al. 1990).

Characteristics of mitochondria/mitochondrial DNA and their fate after somatic hybridization

Mitochondria are the centers of respiratory activity in the cell. In these organelles ATP is synthesized from stored energy in the form of fats, carbohydrates and proteins. The number and morphology of mitochondria vary between different cells of the same plant. While most differentiated plant tissues that have been examined contain hundreds of small oval mitochondria, large mitochondrial networks may be a general feature of meristematic cells. Fusion and fragmentation of plant mitochondria have been observed microscopically (Hanson 1984).

Plant mitochondrial genomes range in size between 200-2500 kb (Ward et al. 1981; Lonsdale 1984). Therefore, they are significantly larger than chloroplast genomes. Nevertheless, plant mitochondrial DNAs encode only ca 20 proteins: three ribosomal proteins and some subunits of complexes associated with the inner mitochondrial membrane, in addition to three ribosomal RNAs and ca 20 transfer RNAs (Levings and Brown 1989). The remainder of the 300 to 400 mitochondrial proteins, including the enzymes involved in mtDNA replication, transcription, processing and translation, are encoded by nuclear genes (Leaver 1991).

The organization of mtDNA is much more complex than that of cpDNA. Although circular physical maps of mitochondrial genomes have been constructed for several plant species (e.g. Lonsdale et al. 1984; Palmer and Shields 1984; Palmer and Herbon 1987; Palmer 1988; Folkerts and Hanson 1989, 1991), evidence that plant mitochondrial genomes mainly consist of one or a few circular molecules has not been obtained. Bendich (1993) argues that linear molecules actually represent the major form of DNA within plant mitochondria, because of the following observations. When mtDNA was isolated from intact plant tissues, no circles the size of the genome or predicted subgenomic circles were reported. The circles that were obtained were usually small, a few kb in size, and they never constituted more than a few percent of total mtDNA of the plant species in question. Of three forms of a genome that yield circular physical maps, two are linear: a head-to-tail concatemeric array, a collection of circularly permuted molecules (sequences a....z, b....za, c....zab, etc.) and a circle. Thus, restriction fragment mapping cannot be used as the only criterion on which to establish circularity as the form of a genome. The circles that are found could be products of recombination events between directly repeated sequences within a mtDNA molecule.

Between plant species there are large differences in mitochondrial gene order, even when closely related species are compared. Highly conserved coding sequences are often flanked by completely different sequences in mtDNAs of different plant species (Palmer 1990). This may be the result of the frequent intra- and intermolecular recombination occurring in plant mitochondrial genomes (Levings and Brown 1989). There may be two different mechanisms occurring simultaneously: a reversible recombination that occurs with high frequency across large repeated sequences, and recombination that occurs less often and is not readily reversible because it involves small repeats (André et al. 1992). Probably, these irreversible rearrangements are responsible for the rapid evolutionary changes of plant mitochondrial DNA.

When mitochondria of two species are combined in a somatic hybrid, fusion of mitochondria of different parents and subsequent recombination between the two mitochondrial genomes may occur. When this happens frequently, and the resulting rearranged mtDNA molecules have no selective disadvantage in the somatic hybrid, eventually the hybrid may have a non-parental mtDNA composition, which is often observed in somatic hybrids. A predominant mtDNA configuration can be achieved in a callus clone prior to plant regeneration, but segregation is not always complete in the initial regenerant, and further segregation can continue to occur for several generations (Hanson 1984).

Information on amount or size of nuclear, chloroplast and mitochondrial genomes of some solanaceous species is given in Table 4. Tomato and potato are very similar with respect to chromosome morphology, chromosome number and gene order (Tanksley et al. 1992).

Fertility and progeny of somatic hybrids

Fertility of a somatic hybrid is influenced by several factors: the phylogenetic relatedness of the parental species, the mitochondrial DNA composition and the nuclear DNA composition. Interspecific somatic hybrids are more likely to be fertile than intergeneric and intertribal somatic hybrids (see Tables 1-3). In cybrids, cytoplasmic male sterility may occur as a result of incongruity between the nucleus of one species and mitochondria of an other species (Spangenberg et al. 1992), or because of the presence of a rearranged mitochondrial genome (Melchers et al. 1992). Chromosomal imbalance, as occurs in aneuploid hybrids, often results in reduced vigour and sterility. In the case of somatic hybrids between distantly related species, e.g. intertribal hybrids, a symmetric or slightly asymmetric somatic hybrid may be male and female sterile because of incompatibility between the two nuclear genomes. In a highly asymmetric somatic hybrid this problem can sometimes be overcome (Negrutiu et al. 1992).

Table 4. Amount and size of nuclear, chloroplast and mitochondrial DNA of tomato, potato, tobacco and *N. plumbaginifolia*

Species	Haploid chromosome number (n)	Nuclear DNA content per haploid genome (pg)[a]	Size cpDNA	Size mtDNA
L. esculentum	12	1.0	157-159 kb[b]	at least 270 kb[f] at least 300 kb[g]
S. tuberosum	24	1.8	155-156 kb[c]	ca 400 kb[h]
N. tabacum	24	4.6	156 kb[d]	ca 250 kb[i] at least 270 kb[j]
N. plumbaginifolia	10	2.4	156 kb[e]	unknown

[a] Arumuganathan and Earle 1991
[b] Palmer and Zamir 1982; Phillips 1985; Piechulla et al. 1985
[c] Schiller et al. 1982,; Hosaka et al. 1984; Heinhorst et al. 1988
[d] Shinozaki et al. 1986a,b
[e] Yang et al. 1992
[f] Hause et al. 1986
[g] McClean and Hanson 1986
[h] Kemble and Shepard 1984
[i] Dale et al. 1983
[j] Satoh et al. 1993

Exchange of nuclear DNA between the two parental genomes facilitates introgression of traits from a donor species into the recipient species. To achieve this, pairing of chromosomes of different parents and subsequent recombination is required. Multivalents present during meiotic metaphase I of a somatic hybrid of two diploid species suggest that recombination occurs not only between homologous, but also between homoeologous or heterologous chromosomes. When the two parental species are sufficiently related, multivalent formation can sometimes be observed (Ehlenfeldt and Helgeson 1987; Giddings and Rees 1992a,b,c; Narasimhulu et al. 1992; de Jong et al. 1993). Indications that intergenomic pairing occurs during meiosis of *S. tuberosum* (+) *S. brevidens* somatic hybrids have been obtained by RFLP analysis of a number of progeny plants (Williams et al. 1990).

Seeds formed after selfing or backcrossing of a somatic hybrid often degenerate upon maturation, which may be caused by an endosperm imbalance. When in this case embryo rescue is performed, progeny may be obtained (Gavrilenko et al. 1992; Jacobsen et al. 1994a,b). Often all or part of the progeny plants are more fertile than the original somatic hybrid (Negrutiu et al. 1989b; Giddings and Rees 1992c).

Outline of the thesis

Many somatic hybrids have been produced between different plant species, mostly from the *Solanaceae* and *Brassicaceae* families (Puite 1992).

Problems that are often encountered are the sterility of the resulting hybrids, the impossibility to obtain cybrids between species of different genera and the difficulty to obtain somatic hybrid plants between species belonging to different tribes. We wanted to get more insight into the causes of these problems and thereby into the limitations of somatic hybridization as a tool for plant breeding. Therefore, analyses of nuclear, chloroplast and mitochondrial genomes were performed of intergeneric somatic hybrids between tomato (*L. esculentum*) and potato (*S. tuberosum*), and of intertribal somatic hybrids between tomato and *N. tabacum*/*N. plumbaginifolia* and between potato and *N. plumbaginifolia*.

Chapter 2 describes a fusion experiment between a cytoplasmic albino tomato genotype and a potato genotype that had been irradiated or left untreated. Green calli were selected, i.e. hybrids containing potato chloroplasts. The effect of irradiation on nuclear DNA composition of the resulting hybrids was analyzed.

In chapter 3 the organellar DNA compositions of triploid ($2n=2x+x$) and tetraploid ($2n=2x+2x$) somatic hybrids of tomato and potato are described and related to their nuclear DNA composition.

Chapter 4 describes a cytogenetic analysis of tomato (+) potato somatic hybrids: the number of euploid versus aneuploid hybrids and irregularities observed during mitosis and meiosis of some of these hybrids. Genomic *in situ* hybridization was performed to distinguish tomato and potato chromosomes in three hybrids. The occurrence of multivalents was studied at diakinesis and metaphase I stage of anthers of these three hybrids. The presence of multivalents would indicate that exchange of nuclear DNA between tomato and potato is possible during meiosis.

In chapter 5 fusion experiments between *L. esculentum* or *L. peruvianum* and *N. tabacum* or *N. plumbaginifolia* are described. A detailed analysis of nuclear DNA composition and organellar DNA constitution of the obtained hybrid calli is presented.

In chapter 6 the chloroplast and mitochondrial DNA compositions of *S. tuberosum* (+) *N. plumbaginifolia* somatic hybrid calli are related to their nuclear DNA composition.

Chapter 7 contains a general discussion on the possibilities and limitations of somatic hybridizations between distantly related solanaceous species and the importance of nucleo-cytoplasmic interactions determining somatic incongruity.

Account

Chapters 2-6 of this thesis have been published before or will be published. Parts of chapters 1 and 7 have been used in a review article that will appear in Euphytica. These and related publications, in which I was involved, are given below.

de Jong JH, Wolters AMA, Kok JM, Verhaar H, van Eden J (1993) Chromosome pairing and potential for intergeneric recombination in some hypotetraploid somatic hybrids of *Lycopersicon esculentum* (+) *Solanum tuberosum*. Genome 36:1032-1041

Schoenmakers HCH, Wolters AMA, Koornneef M (1990) Transfer of genetic information between unrelated plant species by means of asymmetric somatic hybridization. In: Dekkers JJ, van der Plas HC, Vuijk DH (eds) Agricultural Biotechnology in focus in the Netherlands. Pudoc, Wageningen, pp 30-32

Schoenmakers HCH, Wolters AMA, Nobel EM, de Klein CMJ, Koornneef M (1993) Allotriploid somatic hybrids of diploid tomato (*Lycopersicon esculentum* Mill.) and monoploid potato (*Solanum tuberosum* L.). Theor Appl Genet 87:328-336

Schoenmakers HCH, Wolters AMA, de Haan A, Saiedi AK, Koornneef M (1994) Asymmetric somatic hybridization between tomato (*Lycopersicon esculentum* Mill.) and gamma irradiated potato (*Solanum tuberosum* L.): a quantitative analysis. Theor Appl Genet 87:713-720

Wijbrandi J, Wolters AMA, Koornneef M (1990) Asymmetric hybrids between *Lycopersicon esculentum* and irradiated *Lycopersicon peruvianum*. 2. Analysis with marker genes. Theor Appl Genet 80:665-672

Wolters AMA, Schoenmakers HCH, van der Meulen-Muisers JJM, van der Knaap E, Derks FHM, Koornneef M, Zelcer A (1991) Limited DNA elimination from the irradiated potato parent in fusion products of albino *Lycopersicon esculentum* and *Solanum tuberosum*. Theor Appl Genet 83:225-232 (Chapter 2)

Wolters AMA, Koornneef M, Gilissen LJW (1993) The chloroplast and mitochondrial DNA type are correlated with the nuclear composition of somatic hybrid calli of *Solanum tuberosum* and *Nicotiana plumbaginifolia*. Curr Genet 24:260-267 (Chapter 6)

Wolters AMA, Vergunst AC, van der Werff F, Koornneef M (1993) Analysis of nuclear and organellar DNA of somatic hybrid calli and plants between *Lycopersicon* spp. and *Nicotiana* spp. Mol Gen Genet 241:707-718 (Chapter 5)

Wolters AMA, Schoenmakers HCH, Kamstra S, van Eden J, de Jong JH, Koornneef M (1994). Mitotic and meiotic irregularities in somatic hybrids of *Lycopersicon esculentum* and *Solanum tuberosum*. Genome (in press) (Chapter 4)

Wolters AMA, Schoenmakers HCH, Koornneef M. Chloroplast and mitochondrial DNA composition of triploid and tetraploid somatic hybrids between *Lycopersicon esculentum* and *Solanum tuberosum*. Theor Appl Genet (in press)

(Chapter 3)

Wolters A, Jacobsen E, O'Connell M, Bonnema G, Ramulu KS, de Jong H, Schoenmakers H, Wijbrandi J, Koornneef M (1994) Somatic hybridization as a tool for tomato breeding. Euphytica (in press)

References

Akada S, Hirai A (1986) Studies on the mode of separation of chloroplast genomes in parasexual hybrid calli III. Random separation of two types of chloroplast genomes in a hybrid callus. Jpn J Genet 61:437-445

André C, Levy A, Walbot V (1992) Small repeated sequences and the structure of plant mitochondrial genomes. Trends Genet 8:128-132

Arumuganathan K, Earle ED (1991) Nuclear DNA content of some important plant species. Plant Mol Biol Rep 9:208-218

Bendich AJ (1993) Reaching for the ring: the study of mitochondrial genome structure. Curr Genet 24:279-290

Bonnema AB, O'Connell MA (1992) Molecular analysis of the nuclear organellar genotype of somatic hybrid plants between tomato (*Lycopersicon esculentum*) and *Lycopersicon chilense*. Plant Cell Reports 10:629-632

Bonnema AB, Melzer JM, O'Connell MA (1991) Tomato cybrids with mitochondrial DNA from *Lycopersicon pennellii*. Theor Appl Genet 81:339-348

Bonnema AB, Melzer JM, Murray LW, O'Connell MA (1992) Non-random inheritance of organellar genomes in symmetric and asymmetric somatic hybrids between *Lycopersicon esculentum* and *L. pennellii*. Theor Appl Genet 84:435-442

Cardi T, D'Ambrosio F, Consoli D, Puite KJ, Ramulu KS (1993) Production of somatic hybrids between frost-tolerant *Solanum commersonii* and *S. tuberosum*: characterization of hybrid plants. Theor Appl Genet 87:193-200

Constabel F, Dudits D, Gamborg OL, Kao KN (1975) Nuclear fusion in intergeneric heterokaryons. A note. Can J Bot 53:2092-2095

Dale RMK, Wu M, Kiernan MCC (1983) Analysis of four tobacco mitochondrial DNA size classes. Nucl Acids Res 11:1673-1685

D'Arcy WG (1991) The Solanaceae since 1976, with a review of its biogeography. In: Hawkes JG, Lester RN, Nee M, Estrada N (eds) Solanaceae III: taxonomy, chemistry, evolution. Royal Botanic Gardens, Kew, pp 75-137

Derks FHM, Wijbrandi J, Koornneef M, Colijn-Hooymans CM (1991) Organelle analysis of symmetric and asymmetric hybrids between *Lycopersicon peruvianum* and *Lycopersicon esculentum*. Theor Appl Genet 81:199-204

Derks FHM, Hakkert JC, Verbeek WHJ, Colijn-Hooymans CM (1992) Genome composition of asymmetric hybrids in relation to the phylogenetic distance between the parents. Nucleus-chloroplast interaction. Theor Appl Genet 84:930-940

Dudits D (1988) Increase of genetic variability by asymmetric cell hybridization and isolated chromosome transfer. Plant Cell Tissue Organ Cult 12:205-211

Dudits D, Rasko I, Hadlaczky Gy, Lima-de Faria A (1976) Fusion of human cells with carrot protoplasts induced by polyethylene glycol. Hereditas 82:121-124

Dudits D, Maroy E, Praznovszky T, Olah Z, Gyorgyey J, Cella R (1987) Transfer of resistance traits from carrot into tobacco by asymmetric somatic hybridization: Regeneration of fertile plants. Proc Natl Acad Sci USA 84:8434-8438

Ehlenfeldt MK, Helgeson JP (1987) Fertility of somatic hybrids from protoplast fusions of

Solanum brevidens and *S. tuberosum*. Theor Appl Genet 73:395-402

Fehér A, Preiszner J, Litkey Z, Csanádi Gy, Dudits D (1992) Characterization of chromosome instability in interspecific somatic hybrids obtained by X-ray fusion between potato (*Solanum tuberosum* L.) and *S. brevidens* Phil. Theor Appl Genet 84:880-890

Fejes E, Engler D, Maliga P (1990) Extensive homologous chloroplast DNA recombination in the pt14 *Nicotiana* somatic hybrid. Theor Appl Genet 79:28-32

Folkerts O, Hanson MR (1989) Three copies of a single recombination repeat occur on the 443 kb mastercircle of the *Petunia hybrida* 3704 mitochondrial genome. Nucl Acids Res 17:7345-7357

Folkerts O, Hanson MR (1991) The male sterility-associated *pcf* gene and the normal *atp9-1* gene in *Petunia* are located on different mitochondrial DNA molecules. Genetics 129:885-895

Gavrilenko TA, Barbakar NI, Pavlov AV (1992) Somatic hybridization between *Lycopersicon esculentum* and non-tuberous *Solanum* species of the *Etuberosa* series. Plant Science 86:203-214

Giddings GD, Rees H (1992a) The cytology of *Lycopersicon* somatic hybrids. Proc R Soc Lond B 248:63-67

Giddings GD, Rees H (1992b) The cytology of *Lycopersicon* somatic hybrids II. A detailed analysis of chromosome pairing at meiosis in pollen mother cells of somatic hybrids of *Lycopersicon esculentum* and *Lycopersicon peruvianum*. Proc R Soc Lond B 248:255-259

Giddings GD, Rees H (1992c) A *Nicotiana* gametosomatic hybrid and its progenies. J Exp Bot 43:419-425

Gilissen LJW, van Staveren MJ, Verhoeven HA, Sree Ramulu K (1992) Somatic hybridization between potato and *Nicotiana plumbaginifolia*. 1. Spontaneous biparental chromosome elimination and production of asymmetric hybrids. Theor Appl Genet 84:73-80

Gleba YY, Sytnik KM (1984) Protoplast fusion. Genetic engineering in higher plants. Springer Verlag, Berlin Heidelberg New York Tokyo

Gleba YY, Parokonny A, Kotov V, Negrutiu I, Momot V (1987) Spatial separation of parental genomes in hybrids of somatic plant cells. Proc Natl Acad Sci USA 84:3709-3713

Gupta PP, Schieder O, Gupta M (1984) Intergeneric nuclear gene transfer between somatically and sexually incompatible plants through asymmetric protoplast fusion. Mol Gen Genet 197:30-35

Guri A, Levi A, Sink KC (1988) Morphological and molecular characterization of somatic hybrid plants between *Lycopersicon esculentum* and *Solanum nigrum*. Mol Gen Genet 212:191-198

Handley LW, Nickels RL, Cameron MW, Moore PP, Sink KC (1986) Somatic hybrid plants between *Lycopersicon esculentum* and *Solanum lycopersicoides*. Theor Appl Genet 71:691-697

Hanson MR (1984) Stability, variation, and recombination in plant mitochondrial genomes via tissue culture and somatic hybridisation. Oxford Surveys of Plant Molecular and Cell Biology 1:33-52

Harms CT (1983) Somatic incompatibility in the development of higher plant somatic hybrids. Q Rev Biol 58:325-353

Hassanpour-Estahbanati A, Turpin C, Demarly Y (1986) Hybridizations by protoplast fusions in *Solanaceae*. Acta Hortic 191:369-376

Hause B, Baldauf F, Stock K, Wasternack C, Metzlaff M (1986) Molecular analysis of mitochondrial DNA from tomato cell suspension cultures. Curr Genet 10:785-790

Heinhorst S, Gannon GC, Galun E, Kenschaft L, Weissbach A (1988) Clone bank and physical and genetic map of potato chloroplast DNA. Theor Appl Genet 75:244-251

Helgeson JP, Hunt GJ, Haberlach GT, Austin S (1986) Somatic hybrids between *Solanum brevidens* and *Solanum tuberosum*: Expression of a late blight resistance gene and potato leaf roll resistance. Plant Cell Rep 3:212-214

Hogenboom NG (1984) Incongruity: non-functioning of intercellular and intracellular partner relationships through non-matching information. In: Linskens HF, Heslop-Harrison J (eds) Cellular interactions. Springer, Berlin Heidelberg New York, pp 640-654

Hosaka K, Ogihara Y, Matsubayashi M, Tsunewaki K (1984) Phylogenetic relationship between the tuberous *Solanum* species as revealed by restriction endonuclease analysis of chloroplast DNA. Jpn J Genet 59:349-369

Jacobsen E, Reinhout P, Bergervoet JEM, de Looff J, Abidin PE, Huigen DJ, Ramanna MS (1992) Isolation and characterization of potato-tomato somatic hybrids using an amylose-free potato mutant as parental genotype. Theor Appl Genet 85:159-164

Jacobsen E, Daniel MK, Bergervoet-van Deelen JEM, Huigen DJ, Ramanna MS (1994a) The first and second backcross progeny of the intergeneric fusion hybrids of potato and tomato after crossing with potato. Theor Appl Genet 88:181-186

Jacobsen E, de Jong JH, Kamstra SA, van den Berg PMMM, Ramanna MS (1994b) Genomic *in situ* hybridization (GISH) and RFLP analysis for the identification of alien chromosomes in the backcross progeny of potato (+) tomato fusion hybrids. Heredity (in press)

Jain SM, Shahin EA, Sun S (1988) Interspecific protoplast fusion for the transfer of atrazine resistance from *Solanum nigrum* to tomato (*Lycopersicon esculentum* L.). Plant Cell Tissue Organ Cult 12:189-192

de Jong JH, Wolters AMA, Kok JM, Verhaar H, van Eden J (1993) Chromosome pairing and potential for intergeneric recombination in some hypotetraploid somatic hybrids of *Lycopersicon esculentum* (+) *Solanum tuberosum*. Genome 36:1032-1041

Jourdan PS, Earle ED, Mutschler MA (1989) Synthesis of male sterile, triazine-resistant *Brassica napus* by somatic hybridization between cytoplasmic male sterile *B. oleracea* and atrazine-resistant *B. campestris*. Theor Appl Genet 78:445-455

Jourdan P, Montagno T, Berry S (1993) Somatic hybrids produced between *Lycopersicon esculentum* and *L. hirsutum*. Plant Science 91:55-65

Kemble RJ, Shepard JF (1984) Cytoplasmic DNA variation in a potato protoclonal population. Theor Appl Genet 69:211-216

Kinsara A, Patnaik SN, Cocking EC, Power JB (1986) Somatic hybrid plants of *Lycopersicon esculentum* Mill. and *Lycopersicon peruvianum* Mill. J Plant Physiol 125:225-234

Kirti PB, Narasimhulu SB, Mohapatra T, Prakash S, Chopra VL (1993) Correction of chlorophyll deficiency in alloplasmic male sterile *Brassice juncea* through recombination between chloroplast genomes. Genet Res 62:11-14

Krumbiegel G, Schieder O (1981) Comparison of somatic and sexual incompatibility between *Datura innoxia* and *Atropa belladonna*. Planta 153:466-470

Leaver CJ (1991) Mitochondrial genetic information. In: Huang AHC, Taiz L (eds) Molecular approaches to compartmentation and metabolic regulation. The American Society of Plant Physiologists, Rockville, pp. 48-58

Levi A, Ridley BL, Sink KC (1988) Biased organelle transmission in somatic hybrids of *Lycopersicon esculentum* and *Solanum lycopersicoides*. Curr Genet 14:177-182

Levings III CS, Brown GG (1989) Molecular biology of plant mitochondria. Cell 56:171-179

Li Y, Sink KC (1992) Cell type determines plastid transmission in tomato intergeneric somatic hybrids. Curr Genet 22:167-171

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

Lonsdale DM, Hodge TP, Fauron CM-R (1984) The physical map and organisation of the mitochondrial genome from the fertile cytoplasm of maize. Nucl Acids Res 12:9249-9261

Mattheij WM, Eijlander R, de Koning JRA, Louwes KM (1992) Interspecific hybridization between the cultivated potato *Solanum tuberosum* subspecies *tuberosum* L. and the wild species *S. circaeifolium* subsp. *circaeifolium* Bitter exhibiting resistance to *Phytophthora infestans* (Mont.) de Bary and *Globodera pallida* (Stone) Behrens. 1. Somatic hybrids. Theor Appl Genet 83:459-466

McClean PE, Hanson MR (1986) Mitochondrial DNA sequence divergence among *Lycopersicon* and related *Solanum* species. Genetics 112:649-667

Medgyesy P (1990) Selection and analysis of cytoplasmic hybrids. In: Dix PJ (ed) Plant Cell Line Selection. Procedures and Applications. VCH, Weinheim New York Basel Cambridge, pp 287-316

Medgyesy P, Fejes E, Maliga P (1985) Interspecific chloroplast recombination in a *Nicotiana* somatic hybrid. Proc Natl Acad Sci USA 82:6960-6964

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

Melchers G, Mohri Y, Watanabe K, Wakabayashi S, Harada K (1992) One-step generation of cytoplasmic male sterility by fusion of mitochondrial-inactivated tomato protoplasts with nuclear-inactivated *Solanum* protoplasts. Proc Natl Acad Sci USA 89:6832-6836

Melzer JM, O'Connell MA (1990) Molecular analysis of the extent of asymmetry in two asymmetric somatic hybrids of tomato. Theor Appl Genet 79:193-200

Melzer JM, O'Connell MA (1992) Effect of radiation dose on the production of and the extent of asymmetry in tomato asymmetric somatic hybrids. Theor Appl Genet 83:337-344

Möllers C, Wenzel G (1992) Somatic hybridization of dihaploid potato protoplasts as a tool for potato breeding. Bot Acta 105:133-139

Moore PP, Sink KC (1988a) Characterization of a *Lycopersicon esculentum* x *Solanum lycopersicoides* somatic hybrid lacking a glutamate oxaloacetate transaminase isozyme. Plant Cell Tissue Organ Cult 13:39-47

Moore PP, Sink KC (1988b) Molecular analysis of single copy and repetitive genes on chromosome 2 in intergeneric tomato somatic hybrid plants. Plant Mol Biol 11:139-145

Narasimhulu SB, Kirti PB, Prakash S, Chopra VL (1992) Resynthesis of *Brassica carinata* by protoplast fusion and recovery of a novel cytoplasmic hybrid. Plant Cell Rep 11:428-432

Negrutiu I, Hinnisdaels S, Mouras A, Gill BS, Gharti-Chhetri GB, Davey MR, Gleba YY, Sidorov V, Jacobs M (1989a) Somatic versus sexual hybridization: features, facts and future. Acta Bot Neerl 38:253-272

Negrutiu I, Mouras A, Gleba YY, Sidorov V, Hinnisdaels S, Famelaer Y, Jacobs M (1989b) Symmetric versus asymmetric fusion combinations in higher plants. In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry. Vol 8, Plant Protoplasts and Genetic Engineering I. Springer-Verlag, Berlin Heidelberg, pp 304-319

Negrutiu I, Hinnisdaels S, Cammaerts D, Cherdshewasart W, Gharti-Chhetri G, Jacobs M (1992) Plant protoplasts as genetic tool: selectable markers for developmental studies. Int J Dev Biol 36:73-84

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

O'Connell MA, Hanson MR (1986) Regeneration of somatic hybrid plants formed between *Lycopersicon esculentum* and *Solanum rickii*. Theor Appl Genet 72:59-65

O'Connell MA, Hanson MR (1987) Rgeneration of somatic hybrid plants formed between *Lycopersicon esculentum* and *L. pennellii*. Theor Appl Genet 75:83-89

Palmer JD (1983) Chloroplast DNA exists in two orientations. Nature 301:92-93

Palmer JD (1985) Comparative organization of chloroplast genomes. Ann Rev Genet 19:325-354

Palmer JD (1988) Intraspecific variation and multicircularity in *Brassica* mitochondrial DNAs.

Genetics 118:341-351

Palmer JD (1990) Contrasting modes and tempos of genome evolution in land plant organelles. Trends Genet 6:115-120

Palmer JD, Herbon LA (1987) Unicircular structure of the *Brassica hirta* mitochondrial genome. Curr Genet 11:565-570

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

Palmer JD, Zamir D (1982) Chloroplast DNA evolution and phylogenetic relationships in *Lycopersicon*. Proc Natl Acad Sci USA 79:5006-5010

Parokonny AS, Kenton AY, Gleba YY, Bennett MD (1992) Genome reorganization in *Nicotiana* asymmetric somatic hybrids analysed by *in situ* hybridization. Plant J 2:863-874

Parthier B (1982) The cooperation of nuclear and plastid genomes in plastid biogenesis and differentiation. Biochem Physiol Pflanzen 177:283-317

Phillips AL (1985) Restriction map and clone bank of tomato plastid DNA. Curr Genet 10:147-152

Piastuch WC, Bates GW (1990) Chromosomal analysis of *Nicotiana* asymmetric somatic hybrids by dot blotting and in situ hybridization. Mol Gen Genet 222:97-103

Piechulla B, Chonoles Imlay KR, Gruissem W (1985) Plastid gene expression during fruit ripening in tomato. Plant Mol Biol 5:373-384

Pirrie A, Power JB (1986) The production of fertile, triploid somatic hybrid plants (*Nicotiana glutinosa* (n) + *N. tabacum* (2n)) via gametic, somatic protoplast fusion. Theor Appl Genet 72:48-52

Possingham JV (1980) Plastid replication and development in the life cycle of higher plants. Ann Rev Plant Physiol 31:113-129

Potrykus I, Jia J, Lazar GB, Saul M (1984) *Hyoscyamus muticus* + *Nicotiana tabacum* fusion hybrids selected via auxotroph complementation. Plant Cell Rep 3:68-71

Puite KJ (1992) Progress in plant protoplast research. Physiol Plant 85:403-410

Ramulu KS, Dijkhuis P, Verhoeven HA, Famelaer I, Blaas J (1992) Microprotoplast isolation, enrichment and fusion for partial genome transfer in plants. Physiol Plant 85:315-318

Ramulu KS, Dijkhuis P, Famelaer I, Cardi T, Verhoeven HA (1993) Isolation of sub-diploid microprotoplasts for partial genome transfer in plants: Enhancement of micronucleation and enrichment of microprotoplasts with one or a few chromosomes. Planta 190:190-198

Ratushnyak YI, Latypov SA, Samoylov AM, Piven NM, Gleba YY (1991) Introgressive hybridization of tomatoes by 'gamma-fusion' of *Lycopersicon esculentum* Mill. and *Lycopersicon peruvianum* var. *dentatum* Dun. protoplasts. Plant Science 73:65-78

Ratushnyak YI, Cherep NN, Zavgorodnyaya AV, Latypov SA, Borozenko IV, Rachkovskaya RI, Gleba YY (1993) Fertile asymmetric somatic hybrids between *Lycopersicon esculentum* Mill. and *Lycopersicon peruvianum* var. *dentatum* Dun. Mol Gen Genet 236:427-432

Sakata Y, Monma S (1993) Genetic instability of a progeny from a somatic hybrid between tomato (*Lycopersicon esculentum*) and *L. peruvianum*. Plant Science 90:225-232

Sakata Y, Nishio T, Narikawa T, Monma S (1991) Cold and disease resistance of somatic hybrids between tomato (*Lycopersicon esculentum*) and *L. peruvianum*. J Jpn Soc Hort Sci 60:329-335

Sakomoto K, Taguchi T (1991) Regeneration of intergeneric somatic hybrid plants between *Lycopersicon esculentum* and *Solanum muricatum*. Theor Appl Genet 81:509-513

San LH, Vedel F, Sihachakr D, Rémy R (1990) Morphological and molecular characterization of fertile tetraploid somatic hybrids produced by protoplast electrofusion and PEG-induced fusion between *Lycopersicon esculentum* Mill. and *Lycopersicon peruvianum* Mill. Mol Gen Genet 221:17-26

Satoh M, Nemoto Y, Kawano S, Nagata T, Hirokawa H, Kuroiwa T (1993) Organization of heterogeneous mitochondrial DNA molecules in mitochondrial nuclei of cultured tobacco cells. Protoplasma 175:112-120

Schiller B, Herrmann RG, Melchers G (1982) Restriction endonuclease analysis of plastid DNA from tomato, potato and some of their somatic hybrids. Mol Gen Genet 186:453-459

Schoenmakers HCH, Nobel EM, Koornneef M (1992) Use of leaky nitrate reductase-deficient mutants of tomato (*Lycopersicon esculentum* Mill.) for selection of somatic hybrid cell lines with wild type potato (*Solanum tuberosum* L.). Plant Cell Tissue Organ Cult 31:151-154

Schoenmakers HCH, Wolters AMA, Nobel EM, de Klein CMJ, Koornneef M (1993) Allotriploid somatic hybrids of diploid tomato (*Lycopersicon esculentum* Mill.) and monoploid potato (*Solanum tuberosum* L.). Theor Appl Genet 87:328-336

Schoenmakers HCH, Wolters AMA, de Haan A, Saiedi AK, Koornneef M (1994a) Asymmetric somatic hybridization between tomato (*Lycopersicon esculentum* Mill.) and gamma-irradiated potato (*Solanum tuberosum* L.): a quantitative analysis. Theor Appl Genet 87:713-720

Schoenmakers HCH, van der Meulen-Muisers JJM, Koornneef M (1994b) Asymmetric fusion between protoplasts of tomato (*Lycopersicon esculentum* Mill.) and gamma-irradiated protoplasts of potato (*Solanum tuberosum* L.): the effects of gamma irradiation. Mol Gen Genet 242:313-320

Schweizer G, Ganal M, Ninnemann H, Hemleben V (1988) Species-specific DNA sequences for identification of somatic hybrids between *Lycopersicon esculentum* and *Solanum acaule*. Theor Appl Genet 75:679-684

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

Shinozaki K, Ohme M, Tanaka M, Wakasugi T, Hayshida N, Matsubayasha T, Zaita N, Chunwongse J, Obokata J, Yamaguchi-Shinozaki K, Ohto C, Torazawa K, Meng BY, Sugita M, Deno H, Kamogashira T, Yamada K, Kusuda J, Takaiwa F, Kata A, Tohdoh N, Shimada H, Sugiura M (1986a) The complete nucleotide sequence of the tobacco chloroplast genome. Plant Mol Biol Rep 4:111-147

Shinozaki K, Ohme M, Tanaka M, Wakasugi T, Hayshida N, Matsubayasha T, Zaita N, Chunwongse J, Obokata J, Yamaguchi-Shinozaki K, Ohto C, Torazawa K, Meng BY, Sugita M, Deno H, Kamogashira T, Yamada K, Kusuda J, Takaiwa F, Kata A, Tohdoh N, Shimada H, Sugiura M (1986b) The complete nucleotide sequence of the tobacco chloroplast genome: its gene organization and expression. EMBO J 5:2043-2049

Sidorov VA, Menczel L, Nagy F, Maliga P (1981) Chloroplast transfer in *Nicotiana* based on metabolic complementation between irradiated and iodoacetate treated protoplasts. Planta 152:341-345

Spangenberg G, Pérez Vicente R, Oliveira MM, Osusky M, Nagel J, Pais MS, Potrykus I (1992) CMS system in *Nicotiana*: flower development, patterns of mitochondrial DNA and mitochondrial gene expression. I. Analysis of parental *Nicotiana* species and alloplasmic cms analogs of tobacco. Sex Plant Reprod 5:13-26

Spooner DM, Anderson GJ, Jansen RK (1993) Chloroplast DNA evidence for the interrelationships of tomatoes, potatoes, and pepinos (Solanaceae). Am J Bot 80:676-688

Sugiura M (1992) The chloroplast genome. Plant Mol Biol 19:149-168

Tabaeizadeh Z, Perennes C, Bergounioux C (1985) Increasing the variability of *Lycopersicon peruvianum* Mill. by protoplast fusion with *Petunia hybrida* L. Plant Cell Reports 4:7-11

Tabaeizadeh Z, Bergounioux C, Demarly Y (1989) *Lycopersicon* + *Petunia* somatic hybrids. In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry 8. Plant Protoplasts and Genetic Engineering I. Springer Verlag, Berlin Heidelberg, pp. 336-355

Tan MMC (1987) Somatic hybridization and cybridization in some Solanaceae. PhD Thesis, Free University Amsterdam, the Netherlands

Tanksley SD, Ganal MW, Prince JP, de Vicente MC, Bonierbale MW, Broun P, Fulton TM, Giovannoni JJ, Grandillo S, Martin GB, Messeguer R, Miller JC, Miller L, Paterson AH, Pineda O, Röder MS, Wing RA, Wu W, Young ND (1992) High density molecular linkage maps of the tomato and potato genomes. Genetics 132:1141-1160

Thanh ND, Medgyesy P (1989) Limited chloroplast gene transfer via recombination overcomes plastome-genome incompatibility between *Nicotiana tabacum* and *Solanum tuberosum*. Plant Mol Biol 12:87-93

Turpin C (1986) Attempt of male cytoplasmic sterility introduction by intergeneric fusion in cultivated tomato. Acta Hortic 191:377-379

Verhoeven HA, Blaas J (1992) Direct cell to cell transfer of organelles by microinjection. Plant Cell Rep 10:613-616

Wachocki SE, Bonnema AB, O'Connell (1991) Comparison of the organization of the mitochondrial genome in tomato somatic hybrids and cybrids. Theor Appl Genet 81:420-427

Ward M (1984) Fusion of plant protoplasts with animal cells. In: Beers RF, Bassett EG (eds) Cell fusion: gene transfer and transformation. Raven, New York, pp 189-197

Ward BL, Anderson RS, Bendich AJ (1981) The mitochondrial genome is large and variable in a family of plants (*Cucurbitaceae*). Cell 25:793-803

Wijbrandi J (1989) Isolation and characterisation of somatic hybrids between *Lycopersicon esculentum* and *Lycopersicon peruvianum*. PhD Thesis, Wageningen Agricultural University, The Netherlands, pp 29-40

Wijbrandi J, van Capelle W, Hanhart CJ, van Loenen Martinet-Schuringa EP, Koornneef M (1990a) Selection and characterization of somatic hybrids between *Lycopersicon esculentum* and *Lycopersicon peruvianum*. Plant Science 70:197-208

Wijbrandi J, Posthuma A, Kok JM, Rijken R, Vos JGM, Koornneef M (1990b) Asymmetric somatic hybrids between *Lycopersicon esculentum* and irradiated *Lycopersicon peruvianum* 1. Cytogenetics and morphology. Theor Appl Genet 80:305-312

Wijbrandi J, Wolters AMA, Koornneef M (1990c) Asymmetric somatic hybrids between *Lycopersicon esculentum* and irradiated *Lycopersicon peruvianum* 2. Analysis with marker genes. Theor Appl Genet 80:665-672

Wijbrandi J, Zabel P, Koornneef M (1990d) Restriction fragment length polymorphism analysis of somatic hybrids between *Lycopersicon esculentum* and irradiated *L. peruvianum*: Evidence for limited donor genome elimination and extensive chromosome rearrangements. Mol Gen Genet 222:270-277

Williams CE, Hunt GJ, Helgeson JP (1990) Fertile somatic hybrids of *Solanum* species: RFLP analysis of a hybrid and its sexual progeny from crosses with potato. Theor Appl Genet 80:545-551

Yang DI, Feng TY, Chen CC, Lai YK (1992) Physical maps of *Nicotiana* chloroplast DNA constructed by an efficient procedure. Theor Appl Genet 83:515-527

Zelcer A, Aviv D, Galun E (1978) Interspecific transfer of cytoplasmic male sterility by fusion between protoplasts of normal *Nicotiana sylvestris* and X-ray irradiated protoplasts of male-sterile *N. tabacum*. Z Pflanzenphysiol 90:397-408

CHAPTER 2

Limited DNA elimination from the irradiated potato parent in fusion products of albino *Lycopersicon esculentum* and *Solanum tuberosum*

A.M.A. Wolters, H.C.H. Schoenmakers, J.J.M. van der Meulen-Muisers, E. van der Knaap, M. Koornneef
Department of Genetics, Wageningen Agricultural University, Dreijenlaan 2,
NL-6703 HA Wageningen, The Netherlands

F.H.M. Derks
Centre for Plant Breeding and Reproduction Research CPRO, P.O. Box 16,
NL-6700 AA Wageningen, The Netherlands

A. Zelcer
Division of Genetics and Plant Breeding, Institute of Field and Garden Crops,
Volcani Centre, Agricultural Research Organization, Bet-Dagan 50250, Israel

Published in: Theor Appl Genet (1991) 83:225-232

Summary. This paper describes the analysis of the elimination of potato DNA from potato (+) tomato somatic cell hybrids. The hybrids were obtained by fusion of protoplasts of a cytoplasmic albino tomato genotype with leaf mesophyll protoplasts of a potato genotype carrying the kanamycin-resistance gene NPTII and the ß-glucuronidase (GUS) gene. The potato protoplasts were either isolated from unirradiated plants or from plants irradiated with 50 or 500 Gy of gamma-rays. Green calli were selected as putative fusion products. The hybridity of these calli was confirmed by isoenzyme analysis. All tested green calli contained a potato-specific chloroplast DNA restriction fragment and most of the analyzed calli showed ß-glucuronidase activity. In 72 hybrid calli we determined the percentage of potato nuclear DNA using species-specific probes. All of the tested green calli contained a considerable amount of potato genomic DNA, irrespective of the dose of irradiation of the potato parent. The limited degree of potato DNA elimination and the absence of true cybrids are discussed.

Introduction

For the improvement of cultivated plant species it is often desirable to add one or a few favourable traits from another more or less related species. If the trait concerned is present in a species which cannot be hybridized sexually with the crop plant, the desired gene(s) can be combined with the genome of the recipient by means of somatic hybridization. Since the donor will contain not only the trait of interest but also many unfavourable genes, in most cases the transfer of only a small part of the donor genome to the recipient species is intended.

One way to achieve partial genome transfer is to irradiate the donor species with a high dose of X- or gamma-rays. Several investigators have used this strategy. In some cases, only one or a few donor chromosomes were found to be retained within the hybrid (Bates et al. 1987; Dudits et al. 1980; Gupta et al. 1984), whereas in others, elimination of the irradiated genome appeared to be limited (Famelaer et al. 1989; Imamura et al. 1987; Müller-Gensert and Schieder 1987; Wijbrandi et al. 1990; Yamashita et al. 1989). In all these studies, the fusion products were selected on the basis of nuclear-encoded traits of both parents; therefore the limited elimination of the donor genome may be explained in part by "dragging" of nuclear DNA by the genes for which selection was carried out.

To investigate quantitatively the elimination of genetic material from the irradiated donor species without any bias for a selected or dragged nuclear-encoded trait, experiments were performed in which the isolation of hybrid calli was based on a cytoplasmically controlled trait of the donor species. For this purpose protoplasts derived from suspension cultures of *Lycopersicon esculentum* genotype ALRC, which has a cytoplasmically inherited albino mutation, were fused with leaf protoplasts of *Solanum tuberosum* plants that had been irradiated with a low (50 Gy) or high (500 Gy) dose of gamma-rays, or left unirradiated. Selection was based on the transfer of functional chloroplasts from potato to the albino tomato, i.e. green calli were selected. The amount of potato nuclear DNA in the obtained fusion calli was determined in dot blot analyses (Saul and Potrykus 1984), by means of probes recognizing species-specific repetitive DNA sequences.

Tomato and potato cannot be hybridized sexually, but somatic hybrids between both species have been obtained (Melchers et al. 1978; Shepard et al. 1983). This indicates that somatic incongruity does not prevent the formation of viable hybrid plants of these species.

Materials and methods

Plant materials. As recipient we used the maternally inherited albino mutant of *Lycopersicon esculentum* cv 'Large Red Cherry' (ALRC) (originating from Dr. M.R. Hanson, Cornell University, Ithaca, USA (Hosticka and Hanson 1984)). Callus cultures of this genotype were initiated on solid UM2D medium (the "callus and cell culture medium" of Uchimiya and Murashige 1974). Subsequently, cell suspensions were established by culturing callus tissue in liquid UM2D medium at 100 rpm on a gyratory shaker at a light intensity of 0.25 W/m^2 (16 h) at 25°C. These cell suspensions were diluted 1:5 with fresh UM2D medium every 7 days. Flow cytometric analysis showed that the ALRC suspension consisted of a mixture of cells with a 4C (16%), 8C (58%) or 16C (26%) DNA content (results not shown).

The donor species used was *Solanum tuberosum* 7322-K1. This genotype was obtained by transforming monoploid H7322 (originating from Dr. G. Wenzel, Grünbach, Germany (de Vries et al. 1987)) with *Agrobacterium tumefaciens* strain C58 containing the plasmid pZ707C, with a kanamycin-resistance (NPTII) gene and a ß-glucuronidase (GUS) gene on one T-DNA. Apparently polyploidization had occurred during transformation or tissue culture, since the regenerated transformed plant was tetraploid. Shoots of this genotype were subcultured aseptically every four weeks in plastic containers on medium containing MS salts (Murashige and Skoog 1962), vitamins according to Tewes et al. (1984) and 20 g/l sucrose, supplemented with 150 mg/l kanamycin and solidified with 8 g/l agar, and grown at a light intensity of 10 W/m^2 (Philips TLD 36W; 16 h) at 25°C.

Isolation, fusion and culture of protoplasts. For protoplast isolation of the ALRC genotype, 50 ml of a fast growing, 3- to 4-day old cell suspension culture was centrifuged for 5 min at 600 rpm (45 *g*). The pellet was resuspended in 30 ml AM medium (CPW salts (Frearson et al. 1973), 0.4 M mannitol, 10 mM $CaCl_2.2H_2O$ and 100 mg/l 2[*N*-morpholino]-ethane-sulfonate (MES)), supplemented with 10 g/l Cellulase R10 and 2 g/l Macerozym R10 (both enzymes from Yakult, Japan), pH 5.6, and incubated for 16 h in the dark at 25°C. The suspension was filtered through a 50-ml syringe containing glass wool into 10-ml plastic tubes and centrifuged for 5 min at 600 rpm (45 *g*). The pelleted protoplasts were resuspended in BM medium (0.4 M mannitol, 0.5 mM $CaCl_2.2H_2O$, pH 5.8), centrifuged for 5 min at 600 rpm (45 *g*) and resuspended again in BM medium. A sample was counted in a haemocytometer to determine the concentration. The suspension was centrifuged once more and resuspended in BM medium to a final concentration of 0.5 x 10^6 protoplasts/ml.

Prior to protoplast isolation 7322-K1 plants were irradiated with 50 or 500 Gy of gamma-rays from a ^{60}Co source at a dose rate of approximately 2000 Gy/h at the Pilot-Plant for Food Irradiation, Wageningen (the Netherlands). These plants were placed at 4°C in the dark for 8 h, together with unirradiated 7322-K1 plants. Subsequently, the leaves (± 1 g/petri dish) were cut in small pieces and incubated for 16 h in the dark at 25°C in 12 ml AS medium (= AM medium with 0.4 M sucrose instead of mannitol) plus enzymes. The protoplasts were separated from cell debris by filtration through a nylon filter with a pore size of 50 μm and centrifuged for 10 min at 1000 rpm (125 *g*). The floating protoplasts were rinsed once in AS medium without enzymes and twice in BS medium (0.4 M sucrose, 0.5 mM $CaCl_2.2H_2O$, pH 5.8), after which the concentration was determined with a haemocytometer. After another centrifugation for 10 min at 1000 rpm (125 *g*) BS medium was added to the protoplast layer to a final concentration of 0.5 x 10^6 protoplasts/ml.

Prior to electrofusion protoplasts from ALRC and 7322-K1 were mixed 1:1; 0.4 ml of this protoplast mixture was then transferred to a fusion chamber with five 2-mm-wide gaps between parallel stainless steel electrodes glued in a glass petri dish. The fusion apparatus was a combination of a function generator, a generator of direct current (DC) pulses and the fusion chamber. The electrofusion was performed as follows: the protoplasts were aligned in an alternating current (AC) field (1 MHz) of 100-150 V/cm; one or two DC pulses of 1500 V/cm (20-30 μs) were applied, after which the AC field was slowly reduced to zero. The protoplast suspension was then transferred to a petri dish with an equal volume of culture medium in which all of the medium components, except glucose, were at double concentrations.

The protoplasts were cultured at a density of 2.5 x 10^5 protoplasts/ml in TM2G medium, which is modified TM-2 medium (Shahin 1985) containing 0.3 M glucose instead of 0.2 M sucrose, in the dark at 25°C. When the first cell divisions were observed, mostly after 3-4 days, the cultures were diluted 1:1 with TM2G medium and exposed to dim light. After another 3-4 days the cultures were again diluted 1:1 in TM2G medium and solidified with 0.6% (final concentration) agarose (SeaPlaque, FMC BioProducts, USA). When microcalli were visible (after 3-4 days) the agarose was cut in pieces, transferred to a 9-cm diameter petri dish and diluted 1:1 with TMD medium (modified TM-2 medium, containing 100 mg/l myo-inositol, 0.3 M sucrose and 0.1 mg/l NAA). Three to four days later, one-half volume TMD medium was added. Subsequently the liquid medium was replaced once a week with TMD medium until calli with a diameter of approximately 3 mm were obtained. Calli of this size were placed on solid medium: TMcμZ (modified TM-3 medium (Shahin 1985) containing 2.5 g/l sucrose, 36.4 g/l mannitol, 0.1 mg/l NAA instead of 2,4-D, 0.5 mg/l zeatin riboside instead of BAP) or TMcGZ (like TMcμZ, but containing 41 g/l glucose instead of sucrose and mannitol). Every 4 weeks the green calli were subcultured on new medium. Part of the calli was transferred to solid UM2D medium (the "callus and cell culture medium" of Uchimiya and Murashige 1974).

Chloroplast DNA analysis. Total DNA from leaves of ALRC and 7322 and from 13 putative fusion calli was isolated according to Dellaporta et al. (1983). After the last step NaCl was added to the DNA solution to a final concentration of 1 M, the solution was kept at -20°C for 15 minutes and subsequently centrifuged at maximum speed in an Eppendorf centrifuge at 4°C for 5 min. The DNA in the supernatant was ethanol precipitated, and the pellet was resuspended in TE buffer. Four micrograms of total DNA were digested with *Hin*fI. DNA fragments were separated on a 1.2% agarose gel and transferred to Gene Screen Plus filter (DuPont) as described by Derks et al. (1991). Hybridization was carried out with pPCY64, a chloroplast DNA probe originating from *Petunia hybrida* (de Haas et al. 1986), which was radioactively labeled by nick translation.

Screening for GUS activity. Callus of 39 putative fusion products (approximately 20 mg) was ground in an Eppendorf tube in 20 μl GUS-extraction buffer (Jefferson 1987) to which 1 μl of a 25 mM MUG (= 4-methyl umbelliferyl glucuronide) solution was added. After incubation at 37°C for 30 min the reaction was stopped by adding 20 μl 1 M Na_2CO_3. The tubes were centrifuged at maximum speed in an Eppendorf centrifuge for 5 min and subsequently placed in UV light, together with a negative and a positive control tube. The presence or absence of fluorescence was determined.

Isoenzyme analysis. To determine whether the green calli, obtained after fusion of albino ALRC protoplasts and leaf protoplasts of 7322-K1, expressed genes from both parents, isoenzyme analyses were performed for two enzymes: glutamate oxaloacetate transaminase (GOT) and malate dehydrogenase (MDH). Callus tissue (0.5 mg) was ground in 0.5 ml of ice cold buffer consisting of 0.1 M TRIS-HCl, 5% w/v Ficoll 400, 1% v/v ß-mercaptoethanol and 0.05% Bromophenol Blue. The pH of the extraction buffer was 8.9 for GOT analysis and 7.0 for MDH analysis. After centrifugation in an Eppendorf centrifuge for 5 min at maximum speed, 20 μl of the supernatants was applied to a non-denaturing polyacrylamide gel, 6% for GOT, 8% for MDH.

Electrophoresis was carried out in a Desaphor VA (Desaga) separation system at 4°C. The electrophoresis buffer consisted of 0.02 M TRIS-Glycine, pH 8.75 for GOT or TRIS-Citric acid buffer, pH 7.0 for MDH. To test for GOT a voltage of 150 V was applied until the tracking dye band had migrated through the stacking gel (± 30 min), then the voltage was increased to 180 V (62 mA) for 16 h. When MDH analysis was performed the starting voltage was 30 V, which after 30 min was increased to 190 V (350 mA) for 6 h. The gels were incubated in staining solutions prepared after Vallejos (1983) for 30 min (GOT) or several hours (MDH) at 37°C in darkness.

Dot blot analysis using species-specific repetitive DNA probes. In order to determine the percentage of potato DNA in the fusion products dot blot analyses were performed. Total DNA from the fusion parents was isolated according to Rogers and Bendich (1988) after which the DNA was purified on a cesium chloride gradient. The DNA concentration was measured spectrophotometrically. Total DNA from 72 fusion calli was obtained using the method of Mettler (1987).

Twelve concentrations, ranging from 0 ng up to 275 ng, of ALRC or 7322 DNA, in addition to ± 200 ng DNA from fusion calli, were applied to Gene Screen Plus (GS^+) filter (DuPont), using the Hybridot 96-well filtration manifold from Gibco/BRL, as follows. Whatman 3MM paper and GS$^+$ filter, soaked in 0.5 N NaOH, were placed in the manifold and clamped tightly. After the DNA solutions (50 μl) were pipetted in the wells, vacuum was applied. The wells were rinsed with 100 μl TE, pH 7.8. After another 5-10 min under vacuum the manifold was disassembled and the filter was placed on 3 MM paper that had been soaked in 0.5 N NaOH, for 5 min. Then the filter was air dried for 2 min, after which it was placed on 3 MM paper prewetted in 1 M TRIS-HCl (pH 7.5), for 2 min (2 times). The filter was again air dried for 2 min and subsequently placed on 3 MM paper soaked in 0.5 M TRIS-HCl (pH 7.5), 1.5 M NaCl, for 5 min. Finally the filter was air dried for 1 h at room temperature, wrapped in Saran-Wrap and kept at 4°C until use.

Two identical filters were prepared for every analysis. One was probed with pTHG2, a tomato-specific repetitive DNA probe, kindly provided by Dr. P. Zabel, Dept. of Molecular Biology, Wageningen Agricultural University (Zabel et al. 1985). The insert of pTHG2 represents a moderately repetitive DNA fragment that is evenly dispersed on all tomato chromosomes. The other filter was probed with P5L, a potato-specific repetitive DNA probe, kindly provided by Dr. R.G.F. Visser, Dept. of Plant Breeding, Wageningen Agricultural University (Visser et al. 1988). On potato chromosomes hybridization with this fragment occurs predominantly in the telomeric and centromeric regions. All chromosomes of the *Solanum phureja* genome (a tuber-bearing *Solanum* species) can be labeled.

Probes were radioactively labeled using the Boehringer Mannheim Random Primed DNA Labeling kit. Hybridization was performed in glass bottles using a Hybaid hybridization oven at 65°C for 16 h. The blots were rinsed in 2x SSC, 1% SDS, 0.1% tetra-sodium pyrophosphate (Na-PPi) at 65°C for 10 min (2 times), in 0.2x SSC, 1% SDS, 0.1% Na-PPi at 65°C for 30 min (2 times), in 0.1x SSC, 1% SDS, 0.1% Na-PPi at 65°C for 30 min (1 time) and finally in 0.1x SSC at 65°C for 30 min. Autoradiography was performed on Konica X-ray film. Dots were cut out of the filters and put in scintillation vials, 5 ml of the liquid scintillation cocktail Ultima Gold (Packard) was added and radioactivity per dot was measured in a Tri-Carb Liquid Scintillation Analyzer (Packard), 10 min per dot.

The analysis was performed in duplo for 36 of the 72 calli tested and in triplo for 23 calli.

Results and discussion

Protoplast fusion and callus culture

By using albino cell suspension protoplasts and green leaf mesophyll protoplasts as fusion parents it was possible to detect heterofusion products under a UV microscope. The cytoplasm of the albino cell suspension protoplasts showed a yellow autofluorescence, while the chloroplasts of the leaf mesophyll protoplasts showed a red autofluorescence. Twenty-four hours after fusion the cytoplasms of both protoplasts involved in the fusion were completely mixed, and in part of the fusion products the two nuclei had already fused. Two months after protoplast fusion the first green calli were visible. Irradiation does not seem to reduce the frequency of green calli (putative hybrids) that could be obtained in the different fusion experiments (Table 1).

Only white calli were obtained from ALRC protoplast cultures. In previous experiments neither irradiated 7322-K1 protoplasts nor unirradiated protoplasts of this genotype ever yielded microcalli. Only white calli developed in mixtures of unfused ALRC and 7322-K1 protoplasts. Because of these results it was assumed that all green calli obtained after fusion were products of a fusion event between ALRC and 7322-K1 protoplasts.

Table 1. Number of protoplasts used for fusion of ALRC and 7322-K1, number of green calli obtained and number of GUS positive and GUS negative calli in symmetric (0 Gy) or asymmetric (50, 500 Gy) fusion experiments

Irradiation dose of 7322-K1 plants	Number of protoplasts used for fusion	Number of green calli	Number of calli	
			GUS$^+$	GUS$^-$
0 Gy	6.1×10^6	28	13	0
50 Gy	5.6×10^6	32	17	3
500 Gy	8.0×10^6	120	6	0

Chloroplast DNA analysis

In order to confirm that the green calli obtained after fusion contained 7322-K1 chloroplasts, total DNA was isolated from 13 putative fusion calli (three 0 Gy calli; six 50 Gy calli; four 500 Gy calli) and from leaves of the fusion parents. Chloroplast DNA probe pPCY64 showed a polymorphism between *Hin*fI-digested tomato and potato DNA (see Derks et al. 1990). When *Hin*fI-digested DNA from the green calli was probed with pPCY64 all 13 calli contained the potato chloroplast DNA band, and none of them contained the tomato chloroplast DNA band (Fig. 1), indicating the presence of only potato chloroplasts in all of the calli tested.

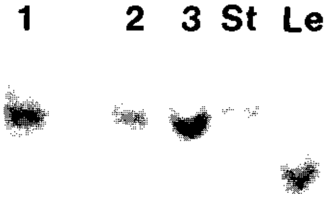

Fig. 1. Chloroplast DNA analysis of the putative fusion calli. Total DNA was isolated from *Lycopersicon esculentum* ALRC (**Le**), *Solanum tuberosum* 7322 (**St**) and from three putative fusion calli: **1** 50 Gy, nr 4; **2** 50 Gy, nr 13; **3** 500 Gy, nr 13. Approximately 4 μg DNA was digested with *Hin*fI, the fragments were separated on a 1.2% agarose gel and subsequently Southern blotted. Hybridization was carried out with chloroplast DNA probe pPCY64

GUS activity screening and isoenzyme analysis

To assay whether nuclear-encoded traits from 7322-K1 were still present in the green calli, a GUS activity test and isoenzyme analyses were performed. All thirteen 0 Gy calli, seventeen out of twenty of the 50 Gy calli and all six 500 Gy calli tested were positive for GUS activity (Table 1). This indicated that in most of the green calli a nuclear-encoded trait derived from 7322-K1 was expressed.

Isoenzyme analyses of the green calli are shown in Fig. 2. All 18 calli analysed for MDH isoenzymes showed a sum of the bands from both fusion parents (Fig. 2A). In all 36 calli tested for the isoenzymes of GOT (a dimeric enzyme) not only parental bands were visible, but also a new hybrid band could be seen (Fig. 2B). This new band confirms the hybrid nature of the green calli.

Quantitative analysis of the nuclear composition by dot blotting

All calli tested for MDH and GOT isoenzymes not only contained tomato nuclear DNA, but also potato genomic DNA, indicating that elimination of genetic material caused by the irradiation treatment was limited. To estimate the percentage of potato DNA in the nuclei of the fusion products species-specific repetitive DNA probes were used as a measure of the total amount of DNA

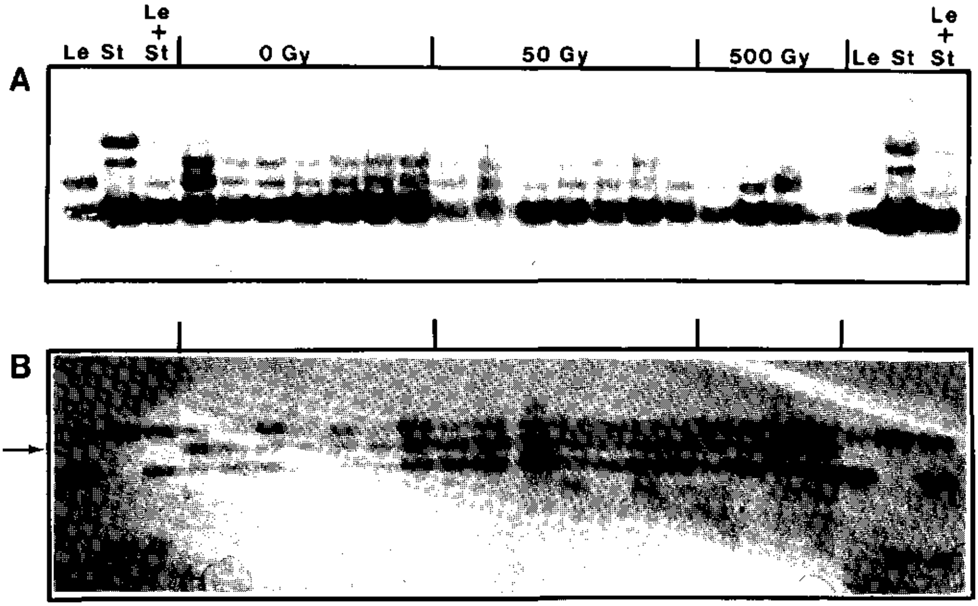

Fig. 2A,B. Isoenzyme analysis of the putative fusion calli. **A** Stained for MDH activity; **B** stained for GOT activity. **Le**: enzymes of *Lycopersicon esculentum* ALRC; **St**: enzymes of *Solanum tuberosum* 7322-K1; **Le + St**: mixture of enzymes of ALRC and 7322-K1; **0 Gy, 50 Gy, 500 Gy**: enzymes of some green calli obtained after fusion of ALRC protoplasts with 0 Gy, 50 Gy or 500 Gy gamma-irradiated 7322-K1 protoplasts, respectively. *Arrow* in **B** indicates a new intermediate sized band present in all fusion calli

from one species. The species-specificity of probes pTHG2 and P5L was tested by Southern blot analysis (data not shown). Cross-hybridization of pTHG2 with potato DNA and of P5L with tomato DNA was negligible compared to the hybridization signal with homologous DNA.

Total DNA was isolated from the fusion parents and from fifteen 0 Gy calli, twenty-four 50 Gy calli and thirty-three 500 Gy calli. Several concentrations of parental DNA were applied to two identical dot blots (Fig. 3) to make a calibration plot of the radioactivity per dot in relation to the amount of DNA from one species (Fig. 4). Total DNA from the fusion products was applied to the same dot blots. With the calibration plots for both species-specific probes the amount of tomato and potato DNA per dot could be estimated, and the percentage of nuclear DNA of the fusion products that originated from potato

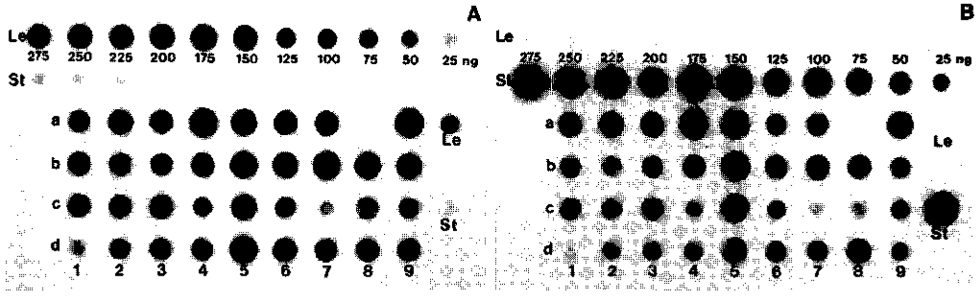

Fig. 3A,B. Dot blot analysis of fusion calli. Total DNA was isolated from the fusion parents *Lycopersicon esculentum* ALRC (**Le**) and *Solanum tuberosum* 7322 (**St**) and from green calli obtained after fusion of ALRC protoplasts with 0 Gy, 50 Gy or 500 Gy gamma-irradiated 7322-K1 protoplasts. Concentration series of ALRC DNA or 7322 DNA were applied to two identical dot blots in addition to ± 200 ng DNA of 0 Gy (a1-7), 50 Gy (a9, b1-9, c1-2) and 500 Gy (c3-9, d1-9) fusion products. Blot **A** was probed with pTHG2, a tomato-specific repetitive DNA probe; blot **B** was hybridized with P5L, a potato-specific repetitive DNA probe

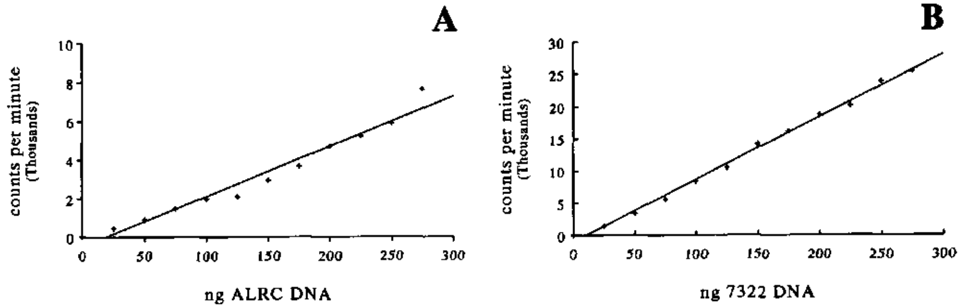

Fig. 4A,B. Calibration plots of the radioactivity per dot in relation to the amount of ALRC DNA (**A**) or 7322 DNA (**B**). Dots were cut out of the filters in Fig. 3, and radioactivity per dot was measured in a Liquid Scintillation Analyzer. Background radioactivity was subtracted from the obtained values

could be determined. Averages of the obtained data per fusion callus are shown in Fig. 5. The average difference between duplicate or triplicate determinations of the percentage potato DNA in the nuclei of individual fusion calli was 2.0%.

All fusion calli contained potato nuclear DNA. The large variation in percentage potato DNA in the group of symmetric fusion calli (Fig. 5A) can be explained by the fact that the ALRC protoplasts were isolated from a suspension

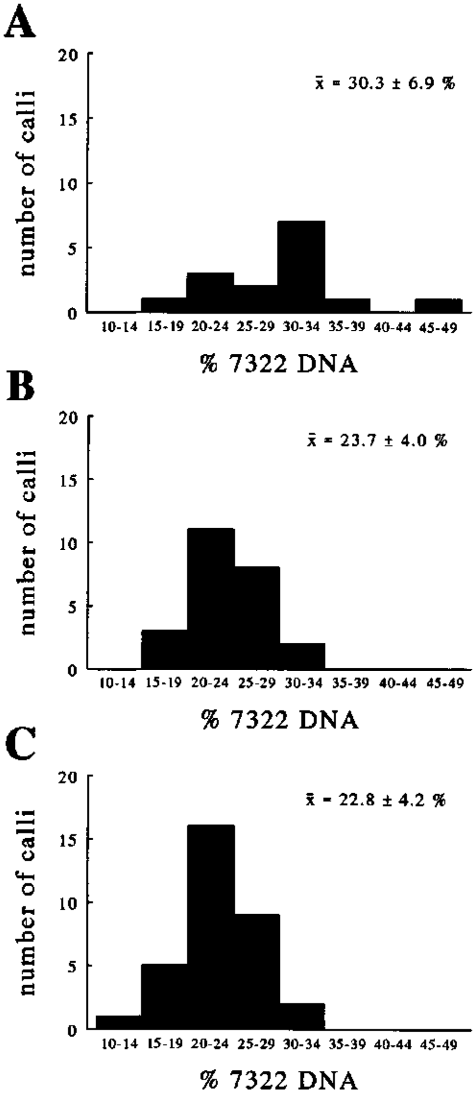

Fig. 5A-C. Mean percentage potato DNA in calli obtained after fusion of protoplasts of tomato genotype ALRC with protoplasts of 0 Gy (**A**), 50 Gy (**B**) or 500 Gy (**C**) gamma-irradiated potato 7322-K1

culture in which different ploidy levels were present and/or due to multiple fusion events. A moderate decrease in mean percentage potato DNA was detected in asymmetric hybrids (50 Gy and 500 Gy calli) as compared to symmetric hybrids (0 Gy calli). Interestingly, there seems to be no difference in mean percentage potato DNA between 50 Gy and 500 Gy calli.

These results show a remarkable feature: no true cybrids lacking potato nuclear DNA have been obtained. Since selection of fusion products was based exclusively on the correction of the cytoplasmically encoded albino phenotype of the tomato parent, transfer of functional potato chloroplasts to the tomato cells should presumably be sufficient to obtain green calli. However, even when the potato protoplasts had been heavily irradiated before fusion, a relatively large amount of potato nuclear DNA was retained in the hybrid calli.

A possible explanation for the results presented here is that a dose of 500 Gy of gamma rays is not sufficient to cause total elimination of potato chromosomes from the fusion products. Menczel et al. (1982) fused gamma-irradiated *N. tabacum* SR1 protoplasts ($2n=4x=48$) carrying a chloroplast DNA-encoded streptomycin resistance with *N. plumbaginifolia* protoplasts. Four doses of irradiation were used: 50, 120, 210 and 300 Gy. Similarly to the work described herein, selection of hybrids was based exclusively on a cytoplasmic trait, i.e. streptomycin resistance. However, following all four irradiation regimes, cybrids containing *N. plumbaginifolia* nuclear DNA and *N. tabacum* chloroplasts could be obtained. Although only a low dose of irradiation was needed to achieve complete elimination of *N. tabacum* chromosomes, we cannot discard the possibility that higher doses are needed to obtain the same result with *Solanum tuberosum.*

Alternatively, it is conceivable that for potato chloroplasts to remain functional in a tomato cell, a certain amount of potato nuclear traits is required. The majority of chloroplast proteins is of nucleo-cytoplasmic origin (Parthier 1982). Thylakoid membranes of higher plants are composed of approximately 45 unique proteins, most of which exist as four multiprotein complexes. Approximately half of the proteins are encoded by chloroplast DNA, the other half by nuclear DNA (Herrmann et al. 1985). Therefore, *S. tuberosum* chloroplasts presumably require nuclear-encoded chloroplast proteins from the same or a very closely related species in order to be functional (green).

However, it is somewhat surprising that the relatively closely related *Lycopersicon* species cannot replace these (in potato chloroplasts). The genera *Lycopersicon* and *Solanum* are members of the same tribe (*Solaneae*) in the family of *Solanaceae*, have nearly identical karyotypes and contain comparable monoploid amounts of nuclear DNA (0.9-1.0 pg). In addition to the homology of DNA sequences throughout the tomato and potato genomes, the linkage order of those sequences on the chromosomes is remarkably well conserved (Bonierbale et al. 1988). Analysis of restriction digests demonstrated also that, while the plastomes of potato and tomato are distinct, they appear to be closely related (Schiller et al. 1982).

Symmetric tomato (+) potato hybrids carrying chloroplasts from either species could be obtained (Schiller et al. 1982), presumably because the corresponding nuclear genome was always present. However, Derks et al. (1992) could not obtain true cybrids when the albino tomato ALRC was fused with *Solanum commersonii* or *S. etuberosum*. Similarly, Jain et al. (1988) could not isolate cybrids containing the tomato nucleus with atrazine-resistant chloroplasts derived from *S. nigrum*.

Genome-plastome incongruity has been reported in several other cases after protoplast fusion of different solanaceous species. Examples are *Nicotiana/Solanum* (Thanh and Medgyesy 1989), *Atropa/Nicotiana* (Kushnir et al. 1991) and even some combinations within the genus *Solanum*, studied by Perl et al. (1990). An indication that within the genus *Lycopersicon* some chloroplast-nucleus combinations may not result in viable cybrids comes from the study of Bonnema et al. (1991), who fused 1000 Gy gamma-irradiated *Lycopersicon pennellii* protoplasts with iodoacetamide-treated *L. esculentum* protoplasts. The chloroplast genotype of 65 regenerants was determined, and all contained the tomato plastome.

This genome-plastome incongruity, which has also been reported for some sexual hybrids (reviewed by Kirk and Tilney-Bassett 1978) could be overcome in the *Nicotiana/Solanum* somatic hybrids by means of recombination between parental chloroplast DNA types (Thanh and Medgyesy 1989). However, chloroplast DNA recombination is presumably a very rare event in higher plants (Medgyesy 1990), and the present study provides further evidence on strong limitations in combining plastomes from one species with the nucleus from another, not very closely related species.

It can not be excluded that the limited elimination of nuclear DNA in asymmetric hybrids is also due to the fact that many "collaborating" nuclear genes of the donor species have to stay together in the hybrid to yield viable asymmetric somatic hybrid plants.

Acknowledgements. The authors thank C. Heyting for critically reading the manuscript. This research was supported by the Programme Committee on Agricultural Biotechnology (PcLB). Support from the Agricultural University, Wageningen, and the Israel-USA Binational Agricultural Research and Development Fund (BARD) to A.Z. is gratefully acknowledged.

References

Bates GW, Hasenkampf CA, Contolini CL, Piastuch WC (1987) Asymmetric hybridization in *Nicotiana* by fusion of irradiated protoplasts. Theor Appl Genet 74: 718-726

Bonierbale MW, Plaisted RL, Tanksley SD (1988) RFLP maps based on a common set of clones reveal modes of chromosomal evolution in potato and tomato. Genetics 120: 1095-1103

Bonnema AB, Melzer JM, O'Connell MA (1991) Tomato cybrids with mitochondrial DNA from *Lycopersicon pennellii*. Theor Appl Genet 81: 339-348

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1: 19-21

Derks FHM, Hakkert JC, Colijn-Hooymans CM (1990) Organelle transfer from Solanaceous and *Lycopersicon* species to tomato via protoplast fusion. In: Cuartero J, Gomez-Guillamon ML, Fernandez-Munoz R (eds) Eucarpia Tomato '90. Proceedings of the XIth meeting on tomato genetics and breeding. Torremolinos, Spain, March 6-8 1990, pp 179-184

Derks FHM, Wijbrandi J, Koornneef M, Colijn-Hooymans CM (1991) Organelle analysis of symmetric and asymmetric hybrids between *Lycopersicon peruvianum* and *Lycopersicon esculentum*. Theor Appl Genet 81: 199-204

Derks FHM, Hakkert JC, Verbeek WHJ, Colijn-Hooymans CM (1992) Genome composition of asymmetric hybrids in relation to the phylogenetic distance between the parents. Nucleus-chloroplast interaction. Theor Appl Genet 84:930-940

Dudits D, Fejér O, Hadlaczky G, Koncz C, Lázár GB, Horváth G (1980) Intergeneric gene transfer mediated by plant protoplast fusion. Mol Gen Genet 179: 283-288

Famelaer I, Gleba YY, Sidorov VA, Kaleda VA, Parokonny AS, Boryshuk NV, Cherep NN, Negrutiu I, Jacobs M (1989) Intrageneric asymmetric hybrids between *Nicotiana plumbaginifolia* and *Nicotiana sylvestris* obtained by 'gamma-fusion'. Plant Sci 61: 105-117

Frearson EM, Power JB, Cocking EC (1973) The isolation, culture and regeneration of *Petunia* leaf protoplasts. Dev Biol 33: 130-137

Gupta PP, Schieder O, Gupta M (1984) Intergeneric nuclear gene transfer between somatically and sexually incompatible plants through asymmetric protoplast fusion. Mol Gen Genet 197: 30-35

de Haas JM, Boot KJM, Haring MA, Kool AJ, Nijkamp HJJ (1986) A *Petunia hybrida* chloroplast DNA region, close to one of the inverted repeats, shows sequence homology with the *Euglena gracilis* chloroplast DNA region that carries the putative replication origin. Mol Gen Genet 202: 48-54

Herrmann RG, Westhoff P, Alt J, Tittgen J, Nelson N (1985) Thylakoid membrane proteins and their genes. In: van Vloten-Doting L, Groot GSP, Hall TC (eds) Molecular form and function of the plant genome. Plenum Publ Corp, New York, pp 233-256

Hosticka LP, Hanson MR (1984) Induction of plastid mutations in tomatoes by nitrosomethylurea. J Hered 75: 242-246

Imamura J, Saul MW, Potrykus I (1987) X-ray irradiation promoted asymmetric somatic hybridisation and molecular analysis of the products. Theor Appl Genet 74: 445-450

Jain SM, Shahin EA, Sun S (1988) Interspecific protoplast fusion for the transfer of atrazine resistance from *Solanum nigrum* to tomato (*Lycopersicon esculentum* L.) Plant Cell, Tissue and Organ Culture 12: 189-192

Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep 5: 387-405

Kirk JTO, Tilney-Bassett RAE (1978) The plastids. Their chemistry, structure, growth and inheritance. Elsevier/North-Holland Biomedical Press, Amsterdam New York Oxford, pp 460-486

Kushnir S, Babiychuk E, Bannikova M, Momot V, Komarnitsky I, Cherep N, Gleba Y (1991) Nucleo-cytoplasmic incompatibility in cybrid plants possessing an *Atropa* genome and a *Nicotiana* plastome. Mol Gen Genet 225: 225-230

Medgyesy P (1990) Selection and analysis of cytoplasmic hybrids. In: Dix PJ (ed) Plant cell line selection. Procedures and applications. VCH, Weinheim New York Basel Cambridge, pp 287-316

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

Menczel L, Galiba G, Nagy F, Maliga P (1982) Effect of radiation dosage on efficiency of chloroplast transfer by protoplast fusion in *Nicotiana*. Genetics 100: 487-495

Mettler IJ (1987) A simple and rapid method for minipreparation of DNA from tissue cultured plant cells. Plant Mol Biol Rep 5: 346-349

Müller-Gensert E, Schieder O (1987) Interspecific T-DNA transfer through plant protoplast fusion. Mol Gen Genet 208: 235-241

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15: 473-497

Parthier B (1982) The cooperation of nuclear and plastid genomes in plastid biogenesis and differentiation. Biochem Physiol Pflanzen 177: 283-317

Perl A, Aviv D, Galun E (1990) Protoplast-fusion-derived *Solanum* cybrids: application and phylogenetic limitations. Theor Appl Genet 79: 632-640

Rogers SO, Bendich AJ (1988) Extraction of DNA from plant tissues. Plant Molecular Biology Manual A6. Kluwer Academic Publ, Dordrecht, pp 1-10

Saul MW, Potrykus I (1984) Species-specific repetitive DNA used to identify interspecific somatic hybrids. Plant Cell Rep 3: 65-67

Schiller B, Herrmann RG, Melchers G (1982) Restriction endonuclease analysis of plastid DNA from tomato, potato and some of their somatic hybrids. Mol Gen Genet 186: 453-459

Shahin EA (1985) Totipotency of tomato protoplasts. Theor Appl Genet 69: 235-240

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

Tewes A, Glund K, Walther R, Reinbothe H (1984) High yield isolation and rapid recovery of protoplasts from suspension cultures of tomato (*Lycopersicon esculentum*). Z Pflanzenphysiol 113: 141-150

Thanh ND, Medgyesy P (1989) Limited chloroplast gene transfer via recombination overcomes plastome-genome incompatibility between *Nicotiana tabacum* and *Solanum tuberosum*. Plant Mol Biol 12: 87-93

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

Vallejos CE (1983) Enzyme activity staining. In: Tanksley SD, Orton TJ (Eds) Isozymes in plant genetics and breeding, part A. Elsevier Science Publ, Amsterdam, pp 469-516

Visser RGF, Hoekstra R, Van der Leij FR, Pijnacker LP, Witholt B, Feenstra WJ (1988) In situ hybridization to somatic metaphase chromosomes of potato. Theor Appl Genet 76: 420-424

de Vries SE, Ferwerda MA, Loonen AEHM, Pijnacker LP, Feenstra WJ (1987) Chromosomes in somatic hybrids between *Nicotiana plumbaginifolia* and a monoploid potato. Theor Appl Genet 75: 170-176

Wijbrandi J, Zabel P, Koornneef M (1990) Restriction fragment length polymorphism analysis of somatic hybrids between *Lycopersicon esculentum* and irradiated *L. peruvianum*: Evidence for limited donor genome elimination and extensive chromosome rearrangements. Mol Gen Genet 222: 270-277

Yamashita Y, Terada R, Nishibayashi S, Shimamoto K (1989) Asymmetric somatic hybrids of *Brassica*: partial transfer of *B. campestris* genome into *B. oleracea* by cell fusion. Theor Appl Genet 77: 189-194

Zabel P, Meyer D, Van de Stolpe O, Van der Zaal B, Ramanna MS, Koornneef M, Krens F, Hille J (1985) Towards the construction of artificial chromosomes for tomato. In: van Vloten-Doting L, Groot GSP, Hall TC (eds) Molecular form and function of the plant genome. Plenum Publ Corp, New York, pp 609-624

CHAPTER 3

Chloroplast and mitochondrial DNA composition of triploid and tetraploid somatic hybrids between *Lycopersicon esculentum* and *Solanum tuberosum*

A.M.A. Wolters, H.C.H. Schoenmakers, M. Koornneef
Department of Genetics, Wageningen Agricultural University, Dreijenlaan 2,
NL-6703 HA Wageningen, The Netherlands

Theor Appl Genet (in press)

Summary. The chloroplast (cp) DNA type and mitochondrial (mt) DNA composition of 17 somatic hybrids between a cytoplasmic albino tomato and monoploid potato (A7-hybrids) and 18 somatic hybrids between a nitrate reductase-deficient tomato and monoploid potato (C7-hybrids) were analyzed. Thirteen A7-hybrids and nine C7-hybrids were triploids (with one potato genome); the other hybrids were tetraploid. As expected, all A7-hybrids contained potato cpDNA. Of the C7-hybrids seven had tomato cpDNA, ten had potato cpDNA and one hybrid contained both tomato and potato cpDNA. The mtDNA composition of the hybrids was analyzed by hybridization of Southern blots with four mtDNA-specific probes. The mtDNAs in the hybrids had segregated independently from the cpDNAs. Nuclear DNA composition (i.e. one or two potato genomes) did not influence the chloroplast type in the C7-hybrids, nor the mtDNA composition of A7- or C7-hybrids. From the cosegregation of specific mtDNA fragments we inferred that both tomato and potato mtDNAs probably have a *cox*II gene closely linked to 18S+5S rRNA genes. In tomato *atpA*, and in potato *atp6* seems to be linked to these mtDNA genes.

Introduction

By somatic hybridization it is possible to bring the nuclei, chloroplasts and mitochondria from two different species together in one cell. When the two species involved in the protoplast fusion are closely related, the resulting somatic hybrids generally contain the nuclear DNA complements of both parents (e.g. Wijbrandi et al. 1990; Daunay et al. 1993; Taguchi et al. 1993). However, when the fusion parents are distantly related, uni- or biparental chromosome elimination may occur (Gilissen et al. 1992; Wolters et al. 1993b, Chapter 5). The heteroplasmic state of two types of chloroplasts is unstable, and usually sorting out of chloroplasts occurs rapidly (Akada and Hirai 1986), resulting in somatic hybrid plants that contain either one or the other parental chloroplast type (e.g. Belliard et al. 1978; Pehu et al. 1989; San et al. 1990). Similarly, segregation of mtDNA molecules takes place. The mtDNA composition of somatic hybrids can range from purely parental to a combination of some, but not all, mtDNA fragments from both parents (e.g. Kemble et al. 1986; Wachocki et al. 1991). Frequently new, non-parental mtDNA fragments are present, most likely resulting from recombination (Rothenberg et al. 1985; Rothenberg and Hanson 1987). To obtain viable somatic hybrid plants, organelle segregation must result in cells in which the remaining chloroplasts and mitochondrial genes can function well with genes encoded by the hybrid nucleus.

We have been investigating the possibilities of somatic hybridization between tomato and other more or less related solanaceous species, such as potato, tobacco and *Nicotiana plumbaginifolia* and have studied nucleo-cytoplasmic interactions in the resulting hybrids. Symmetric somatic hybrid plants between tomato and potato were easily obtained (Schoenmakers et al. 1993), whereas it was much more difficult to produce shoot-regenerating hybrids between tomato and *N. tabacum* or *N. plumbaginifolia* (Wolters et al. 1993b, Chapter 5). In the *Lycopersicon* (+) *Nicotiana* hybrids, but also in *S. tuberosum* (+) *N. plumbaginifolia* somatic hybrids (Wolters et al. 1993a, Chapter 6), which showed an extensive elimination of chromosomes of one of the parents, we found a strong correlation between the origins of nuclear, chloroplast and mitochondrial DNA. The hybrids contained cpDNA and most mtDNA fragments of the parent predominating in the nucleus.

Apparently, a viable somatic hybrid between distantly related species requires chloroplasts, mtDNA fragments and nuclear DNA to be mainly or solely derived from one parental species. This raised the question whether there is also a preference for mtDNA and cpDNA of the same parental species in somatic hybrids between relatively related species, e.g. between *L. esculentum* and *S. tuberosum*. The presence of nucleo-cytoplasmic incongruity between these two species was suggested by the impossibility to obtain true cybrids combining a *L. esculentum* nucleus with *S. tuberosum* chloroplasts (Wolters et al. 1991, Chapter 2).

Here we report the analysis of organellar DNA composition of (near) triploid and (near) tetraploid somatic hybrid plants between diploid tomato and monoploid potato. Hybrids were selected in two ways. After fusion of potato with a cytoplasmic albino tomato genotype, green calli were selected, i.e. calli containing potato chloroplasts (A7-hybrids). The same potato genotype was also fused with a nitrate reductase-deficient mutant of tomato. In this case calli which proliferated on medium containing nitrate as the sole nitrogen source were selected, and thus selection was based on a nuclear encoded trait from potato (C7-hybrids, Schoenmakers et al. 1992).

We investigated (*i*) if tomato (+) potato hybrids selected on nuclear-encoded traits show a random distribution of chloroplast types, (*ii*) if selection for the presence of potato chloroplasts influences the mtDNA composition of the resulting tomato (+) potato hybrids, and (*iii*) if there is a correlation between nuclear and organellar DNA composition in these hybrids. (*iv*) Furthermore, recombination between tomato and potato mtDNAs was analyzed by (co)segregation analysis of mtDNA restriction fragments.

Materials and methods

Plant materials. The triploid somatic hybrids between *L. esculentum* ALRCxM8-7 (a cytoplasmic albino mutant) and monoploid *S. tuberosum* 7322 (designated as A7-hybrids) and between *L. esculentum* C31-244 (a nitrate reductase-deficient mutant) and monoploid *S. tuberosum* 7322 (designated as C7-hybrids) that were used in this study, have been described by Schoenmakers et al. (1993). The tetraploid hybrids were obtained from the same fusion combinations. Individual A7- or C7-hybrid calli were numbered consecutively. Independent shoots regenerated from a single hybrid callus were distinguished by different letters: A, B, C or D. The hybrid nature of the somatic hybrid calli was confirmed by isozyme analyses (Schoenmakers et al. 1993). For organelle DNA analyses we used *L. esculentum* ALRC, the female parent of ALRCxM8-7, as parental species for the A7-hybrids. We used *L. esculentum* cultivar GT, the wild type of C31-244 (Schoenmakers et al. 1993), as parental species for the C7-hybrids. No differences in cpDNA or mtDNA RFLP pattern were detected between these two tomato genotypes. Chromosome numbers of the triploid and tetraploid somatic hybrids used in this study were analyzed as described in Schoenmakers et al. (1993).

DNA isolation, restriction and Southern transfer. Leaves of the hybrids and of parental species *L. esculentum* ALRC grown in vitro, and of *L. esculentum* GT and *S. tuberosum* 7322 grown in the greenhouse, were used for isolation of total DNA according to Rogers and Bendich (1988). DNA concentrations were measured with a mini-fluorometer (Hoefer) according to the supplier's instructions. Total DNA (4 μg) was digested with *Eco*RI or *Hin*dIII. The DNA fragments were separated on 0.8% agarose gels and subsequently alkaline-blotted onto Gene Screen Plus (DuPont) membranes, according to Chomczynski and Qasba (1984).

Organelle DNA probes and hybridization. For the characterization of cpDNA the 2.4-kb *Bam*HI fragment of *Petunia* cpDNA probe pPCY64 (de Haas et al. 1986) and the 1.8-kb *Eco*RI/*Hin*dIII fragment of *Petunia* cpDNA probe pPCY20-1 (Overbeeke et al. 1984) were used. We characterized the mtDNA composition using the 0.45-kb *Eco*RI-*Sal*I fragment of the *Pcf* gene from *Petunia* (Young and Hanson 1987), the 6.0-kb *Bam*HI fragment of *Zea* *diploperennis* mtDNA containing the 18S+5S rRNA genes (Gwynn et al. 1987), the 1.2-kb *Bam*HI/*Hin*dIII fragment of the ATPase α-subunit (*atpA*) gene from *Oenothera* mtDNA (Schuster and Brennicke 1986) and the 2.7-kb *Hin*dIII fragment of *Zea mays* mtDNA containing the gene encoding ATPase subunit 6 (*atp6*, Dewey et al. 1985). In order to isolate inserts from the plasmids the correct restriction fragment was cut out of an agarose gel and the DNA was recovered with the Prep-A-Gene DNA Purification kit from Bio-Rad. Probes were radioactively labeled with the Boehringer Mannheim Random Primed DNA Labeling kit. We performed hybridizations in glass bottles using a Hybaid hybridization oven, at 65°C for 16 h. The blots were rinsed to a stringency of 0.2 x SSC, 1% SDS at 65°C. After autoradiography the probe was removed from the blots according to the supplier's instructions and the membranes were re-used.

Results

Table 1 shows the chromosome numbers of the 17 A7-hybrids and 18 C7-hybrids used in this study. Thirteen A7-hybrids and nine C7-hybrids were triploid ($2n=2x+x=36$) or near-triploid, with 1 or 2 chromosomes missing or with 1 chromosome extra. One A7-hybrid (A7-74B) formed roots with cells containing 36 chromosomes in addition to roots with cells containing 37 chromosomes. Four A7-hybrids were tetraploid ($2n=2x+2x=48$) and nine C7-hybrids were tetraploid or hypotetraploid, with 1 or 2 chromosomes missing.

Nuclear hybridity of all 35 plants was confirmed by the presence of tomato and potato satellite chromosomes. The satellite of tomato chromosome 2 of the genotypes used in this study is significantly larger than the satellite of potato chromosome 2 (cf. Schoenmakers et al. 1993). All triploid A7- and C7-hybrids, except C7-149A and C7-167A, possessed two tomato and one potato satellite chromosomes. Hybrids C7-149A and C7-167A, which both had 35 chromosomes, contained one tomato and one potato chromosome 2. All tetraploid A7- and C7-hybrids, except C7-49A, possessed two tomato and two potato satellite chromosomes, indicating that they resulted from fusion of one diploid tomato protoplast with one dihaploid or two monoploid potato protoplasts. Hybrid C7-49A contained two tomato and one potato chromosome 2.

Table 1. Chromosome complement, chloroplast DNA (cpDNA) type and mitochondrial DNA (mtDNA) composition of parental genotypes and somatic hybrids between *L. esculentum* ALRCxM8-7 and *S. tuberosum* 7322 (A7-hybrids) and between *L. esculentum* C31-244 and *S. tuberosum* 7322 (C7-hybrids)

Genotype	No. of chromosomes	cpDNA	No. of mtDNA fragments[a]					mtDNA group[b]
			C	L	S	R	total	
ALRCxM8-7/C31-244	24	L	8	10	-	-	18	L
7322	12	S	8	-	9	-	17	S
A7-hybrids								
58A	36	S	8	7	6	-	21	LS
73C	36	S	8	6	3	-	17	L
74B	36/37	S	8	9	5	1	23	L
82A	36	S	8	10	-	-	18	L
105B	37	S	8	9	2	-	19	L
105C	37	S	8	9	4	1	22	L
120D	35	S	8	7	2	2	19	L
146D	34	S	8	2	9	-	19	S
169A	35	S	8	10	-	-	18	L
170B	34	S	8	9	1	-	18	L
194A	36	S	8	10	2	-	20	L
196B	35	S	8	10	-	-	18	L
208A	35	S	8	9	2	1	20	L
3D	48	S	8	8	1	-	17	L
76B	48	S	8	8	1	-	17	L
97B	48	S	8	9	3	1	21	L
98C	48	S	8	7	2	-	17	L
C7-hybrids								
22A	35	L	8	4	9	-	21	S
72B	34	L+S	8	8	3	-	19	L
76A	36	S	8	9	1	-	18	L
112B	36	S	8	3	9	-	20	S
113A	36	S	8	5	7	-	20	S
116A	36	S	7	9	3	-	19	L
133A	36	S	8	3	8	-	19	S
149A	35	L	7	8	3	-	18	L
167A	35	L	7	7	3	-	17	L
15C	48	L	8	7	2	-	17	L
23A	48	S	8	2	9	-	19	S
42B	48	L	7	9	2	-	18	L
49A	47	S	8	3	8	-	19	S
74A	47	L	8	4	7	-	19	S
107B	47	S	7	4	8	-	19	S
109C	46	S	8	2	9	-	19	S
142A	48	L	8	2	9	2	21	S
147A	48	S	8	1	8	-	17	S

[a] C, common; L, *L. esculentum*-specific; S, *S. tuberosum*-specific; R, new fragment

[b] L group: number of L mtDNA fragments/number of S mtDNA fragments (L/S) > 1.4; LS group: $0.8 <$ L/S < 1.4; S group: L/S < 0.8

Total DNA from the A7-hybrids, the C7-hybrids and their respective parents was digested with *Eco*RI, electrophoresed and subsequently blotted. The blots were hybridized with the inserts of plasmids pPCY64 and pPCY20-1 (Fig. 1). The results obtained with the first probe were confirmed by the second probe. As expected, all A7-hybrids possessed the cpDNA fragments specific for *S. tuberosum*. The C7-hybrids showed a random distribution of chloroplast types (Fig. 1, Table 1): 10 hybrids contained chloroplasts from potato, 7 had tomato chloroplasts and 1 hybrid possessed chloroplasts from both parents (C7-72B).

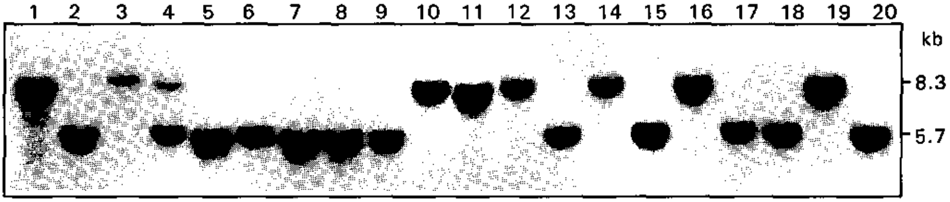

Fig. 1. Autoradiogram of a blot containing *Eco*RI-digested total DNA of *L. esculentum* GT (lane 1), *S. tuberosum* 7322 (lane 2) and C7-hybrids 22A, 72B, 76A, 112B, 113A, 116A, 133A, 149A, 167A, 15C, 23A, 42B, 49A, 74A, 107B, 109C, 142A and 147A (lanes 3-20), hybridized with chloroplast probe pPCY20-1. Sizes of tomato- and potato-specific fragments are indicated

Blots containing *Eco*RI- or *Hin*dIII-digested total DNA of the hybrids and their parents were hybridized with four mtDNA-specific probes: the *Pcf* gene of *Petunia* mtDNA (consisting of part of the *cox*II gene and part of the *atp9-1* gene), *atp6*, *atpA* and the 18S+5S rRNA genes (Fig. 2). Fig. 2A shows an autoradiogram of total DNA from *L. esculentum* (lane 1), *S. tuberosum* (lane 2) and A7-hybrids (lanes 3-19), digested with *Eco*RI and hybridized with mtDNA probe *atpA*. The tomato parent shows a single band of 5.4 kb (L8, see Table 2) and the potato parent one band of 4.9 kb (S7). The hybrids contain only the tomato-specific band (lanes 4,6-9,11,12,14,16-18), only the potato-specific band (lanes 10,19), or both mtDNA bands (lanes 3,5,13,15). In this enzyme/probe combination we found no new, non-parental fragments.

Fig. 2B shows an autoradiogram of the same blot as in Fig. 2A, but hybridized with the 18S+5S rRNA genes. The strongly hybridizing band of 3.1 kb is the cpDNA fragment carrying the chloroplast 16S rRNA gene (this fragment has the same size as the tobacco cpDNA fragment containing this gene, data not shown). Two tomato-specific mtDNA fragments of 5.0 kb (L5) and 2.0 kb (L6) and two potato-specific mtDNA fragments of 4.3 kb (S5) and 2.9 kb (S6) can be seen. Thirteen hybrids contain both L5 and L6 bands and one hybrid (A7-146D, lane 10) contains both S5 and S6 bands. Hybrid A7-74B (lane 5) contains the S5 band besides the L5 and L6 bands, and hybrid A7-105B (lane 7) contains the S6 band besides the L5 and L6 bands. Finally, one hybrid (A7-208A, lane 15) contains one tomato-specific band (L5) and one potato-specific band (S6). Hybrid A7-120D (lane 9) shows a new, non-parental band of about 5.7 kb.

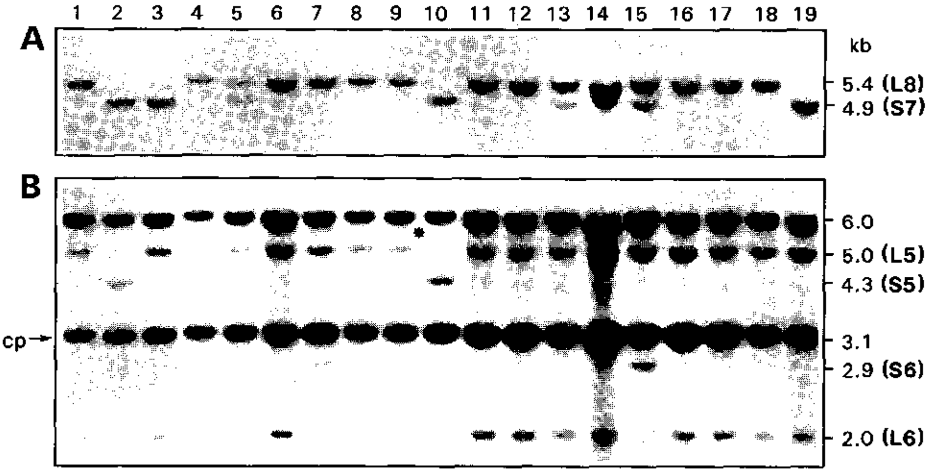

Fig. 2A,B. Autoradiograms of a blot containing *Eco*RI-digested total DNA of *L. esculentum* ALRC (lane 1), *S. tuberosum* 7322 (lane 2) and A7-hybrids 58A, 73C, 74B, 82A, 105B, 105C, 120D, 146D, 169A, 170B, 194A, 196B, 208A, 3D, 76B, 97B and 98C (lanes 3-19), hybridized with mtDNA probes *atpA* (**A**) or 18S+5S rRNA (**B**). Sizes of fragments are indicated. In **B** the cpDNA fragment carrying the 16S rRNA gene is indicated with an *arrow*. A novel, non-parental fragment in hybrid A7-120D (lane 9) is indicated with an *asterisk*

For each enzyme/probe combination the number of common bands (bands of a particular size displayed by both parents), *L. esculentum*-specific bands, *S. tuberosum*-specific bands and novel (non-parental) bands were counted per hybrid. The sum of these numbers per hybrid are shown in Table 1. Details of the tomato-specific and the potato-specific mtDNA and cpDNA fragments are given in Table 2.

Table 2. Details of species-specific cpDNA and mtDNA fragments

Fragment	Size (kb)	Digest	Probe	Remarks
cpDNA				
tomato-specific				
cpL1	0.86	*Eco*RI	pPCY64	
cpL2	8.3	*Eco*RI	pPCY20-1	
potato-specific				
cpS1	0.73	*Eco*RI	pPCY64	
cpS2	5.7	*Eco*RI	pPCY20-1	
mtDNA				
tomato-specific				
L1	3.4	*Eco*RI	*Pcf*[a]	*cox*II containing fragment, cf. Guri et al. 1988[b]
L2	1.6	*Eco*RI	*Pcf*	*cox*II containing fragment, cf. Guri et al. 1988
L3	7.7	*Hin*dIII	*Pcf*	
L4	3.0	*Hin*dIII	*Pcf*	*cox*II containing fragment, cf. Guri et al. 1988
L5	5.0	*Eco*RI	18S+5S rRNA	
L6	2.0	*Eco*RI	18S+5S rRNA	
L7	10.5	*Hin*dIII	18S+5S rRNA	cf. Bonnema and O'Connell 1992
L8	5.4	*Eco*RI	*atpA*	
L9	3.2	*Eco*RI	*atp6*	
L10	1.4	*Hin*dIII	*atp6*	
potato-specific				
S1	0.76	*Eco*RI	*Pcf*	*atp9* containing fragment, cf. Dell'Orto et al. 1993
S2	8.8	*Hin*dIII	*Pcf*	
S3	2.3	*Hin*dIII	*Pcf*	*cox*II containing fragment, cf. Guri et al. 1988
S4	1.6	*Hin*dIII	*Pcf*	*cox*II containing fragment, cf. Guri et al. 1988
S5	4.3	*Eco*RI	18S+5S rRNA	
S6	2.9	*Eco*RI	18S+5S rRNA	
S7	4.9	*Eco*RI	*atpA*	
S8	1.5	*Eco*RI	*atp6*	
S9	6.3	*Hin*dIII	*atp6*	

[a] The 0.45-kb *Eco*RI-*Sal*I fragment of the *Pcf* gene used for hybridization consists of part of the *cox*II gene and part of the *atp9-1* gene of *Petunia* mtDNA (Young and Hanson 1987)

[b] Guri et al. (1988) mention the size of fragments obtained after hybridization of *Eco*RI- and *Hin*dIII-digested mitochondrial DNA of *L. esculentum* and *S. nigrum* with mtDNA probe *cox*II

For each hybrid the number of *L. esculentum*-specific mtDNA fragments was plotted against the number of *S. tuberosum*-specific mtDNA fragments, and the cpDNA type indicated (Fig. 3A). No correlation between chloroplast type and mtDNA composition in the hybrids was observed. Some hybrids possessing tomato chloroplasts contained predominantly tomato mtDNA fragments and others contained predominantly potato mtDNA fragments. When only the A7-hybrids, which all possessed potato chloroplasts, are considered it is remarkable that almost all of them contained predominantly *L. esculentum*-specific mtDNA fragments (see Table 1). A similar plot as in Fig. 3A was made in which, instead of the chloroplast type, the ploidy level of the hybrids was indicated (Fig. 3B). Again, no correlation was apparent between nuclear DNA content, i.e. the presence of one or two potato genomes, and mtDNA composition.

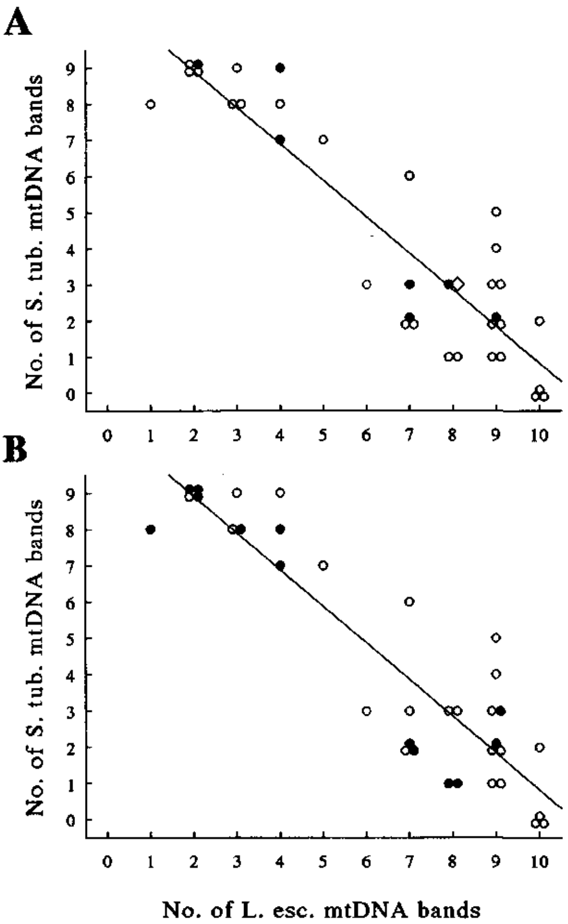

Fig. 3A,B. Scatter diagrams showing the number of tomato-specific and potato-specific mtDNA fragments per hybrid, and related to chloroplast type (**A**) or ploidy level of the hybrids (**B**). **A**: ○, hybrid containing potato chloroplasts; ●, hybrid containing tomato chloroplasts; ◇, hybrid containing both tomato and potato chloroplasts. **B**: ○, triploid hybrid; ●, tetraploid hybrid

The total number of mtDNA fragments per hybrid observed in our hybridizations varied between 17 and 23 (Table 1), which is equal to or slightly higher than the total numbers of the parents. This is a clear indication that in these somatic hybrids the heteroplasmic state of two complete mtDNA genomes was unstable and segregation took place. Hybrids containing many tomato mtDNA fragments had few potato mtDNA fragments, and vice versa. The regression lines in Fig. 3A and B have a correlation coefficient (r) of −0.91.

Random segregation per mtDNA fragment was tested in relation to the mean segregation ratio of all *Lycopersicon*-specific (L) or all *S. tuberosum*-specific (S) mtDNA fragments analyzed (Table 3), to find out if any of the mtDNA fragments showed a biased transmission.

Table 3. Segregation of mtDNA fragments and χ^2 assay to test for a biased transmission of specific mtDNA fragments from *L. esculentum* (L1-L10) or from *S. tuberosum* (S1-S9), described in Table 2

Fragment	Number of hybrids		χ^2_1
	Present	Absent	
L1	14	21	7.13[a]
L2	23	12	0.21
L3	22	13	0.01
L4	24	11	0.66
L5	24	11	0.66
L6	24	11	0.66
L7	5	30	33.67[a]
L8	25	10	1.34
L9+10	34	1	18.42[a]
Mean	21.7	13.3	
S1	20	15	1.03
S2	18	17	0.11
S3	13	22	1.83
S4	30	5	19.33[a]
S5	11	24	4.12[a]
S6	13	22	1.83
S7	16	19	0.11
S8+9	15	20	0.46
Mean	17	18	

[a] Significant deviation ($P < 0.05$) from the mean segregation ratio

In Table 3 the number of hybrids containing a specific mtDNA fragment (L1-L10 or S1-S9, see Table 2) and the number of hybrids in which this fragment was absent, are indicated. A χ^2 test was performed in which these numbers were compared with the mean ratio for L or for S mtDNA fragments. Fragments L1, L7, L9+10, S4 and S5 showed a significant deviation of the mean ratio. These deviations were also found when A7- and C7-hybrids were tested separately (data not shown).

To evaluate a possible cosegregation of specific mtDNA-fragments, the presence or absence of each mtDNA fragment in the hybrids was compared with the presence or absence of every other mtDNA fragment and of the cpDNA fragments. Independent assortment was tested with a χ^2 assay. Because of the large number of pairwise comparisons an error rate $P=0.001$ was used for each combination in order to obtain an approximate overall error rate $P=0.05$. The results are shown in Table 4. A strong cosegregation ($P<10^{-4}$) is apparent between L2 and L4 (both *cox*II-containing tomato mtDNA fragments), L2 and L5, L2 and L6, L4 and L5 (tomato *cox*II and tomato 18S+5S rRNA), L5 and L6 (both 18S+5S rRNA-containing tomato mtDNA fragments) and S3 and S5 (potato *cox*II and potato 18S+5S rRNA). Strong repulsion exists between L2 and S3, L4 and S3 (tomato *cox*II vs. potato *cox*II), L5 and S3, L7 and S4 (tomato 18S+5S rRNA vs. potato *cox*II), L2 and S5, L4 and S5 (tomato *cox*II vs. potato 18S+5S rRNA), L5 and S5, L6 and S5, L6 and S6 (tomato 18S+5S rRNA vs. potato 18S+5S rRNA). L8, the tomato mtDNA fragment containing *atpA*, cosegregates, although less frequently, with L2, L5 and L6, and is in repulsion with S3, S5 and S6, whereas S8 and S9 (potato *atp6*-carrying fragments) are in repulsion with L2, L4 (tomato *cox*II) and L5 (tomato 18S+5S rRNA) and cosegregate with S2 and S5 (potato 18S+5S rRNA). Furthermore, a strong cosegregation is apparent between L9 and L10 (the *Eco*RI respectively *Hin*dIII tomato mtDNA fragment carrying the *atp6* gene), between S8 and S9 (the *Eco*RI respectively *Hin*dIII potato mtDNA fragment containing the *atp6* gene) and between S1 (potato *atp9*) and S3 (potato *cox*II). A strong repulsion exists between L3 and S2 (probably tomato *atp9* vs. potato *atp9*), L8 and S7 (tomato *atpA* vs. potato *atpA*) and between cpL and cpS.

Table 4. Pairwise comparison of presence or absence of specific mtDNA fragments from *L. esculentum* (L1-L10) or from *S. tuberosum* (S1-S9) and of cpDNA fragments (cpL, cpS), described in Table 2. Results of a χ^2 test for independent assortment are indicated. 0, independent assortment; +, − dependent assortment ($P < 10^{-3}$); ++, −− dependent assortment ($P < 10^{-4}$); +, ++ cosegregation; −, −− repulsion

Fragment	L1	L3	L2	L4	L5	L6	L7	L8	L9	L10
L3	+									
L2	0	0								
L4	0	0	++							
L5	0	0	++	++						
L6	0	0	++	+	++					
L7	0	0	0	0	0	0				
L8	0	0	+	0	+	+	0			
L9	0	0	0	0	0	0	0	0		
L10	0	0	0	0	0	0	0	0	++	
cpL	0	0	0	0	0	0	0	0	0	0

Fragment	S1	S4	S3	S2	S5	S6	S7	S8	S9
S4	0								
S3	++	0							
S2	0	0	0						
S5	+	0	++	+					
S6	0	0	0	0	+				
S7	0	0	0	0	0	+			
S8	0	0	0	+	+	0	0		
S9	0	0	0	+	+	0	0	++	
cpS	0	0	0	0	0	0	0	0	0

Fragment	L1	L3	L2	L4	L5	L6	L7	L8	L9	L10	cpL
S1	0	0	−	−	−	0	0	0	0	0	0
S4	0	0	0	0	0	0	−−	0	0	0	0
S3	0	0	−−	−−	−−	−	0	−	0	0	0
S2	−	−−	−	0	−	0	0	0	0	0	0
S5	0	0	−−	−−	−−	−−	0	−	0	0	0
S6	0	0	−	0	−	−−	0	−	0	0	0
S7	0	0	0	0	0	−	0	−−	0	0	0
S8	0	0	−	−	−	0	0	0	0	0	0
S9	0	0	−	−	−	0	0	0	0	0	0
cpS	0	0	0	0	0	0	0	0	0	0	−−

Discussion

The analysis of chloroplast DNA type of 18 tomato (+) potato somatic hybrids selected for nuclear traits from both parents (the C7-hybrids), revealed that the chloroplast segregation ratio in these hybrids is approximately 1:1. This is in accordance with the results of Schiller et al. (1982) who reported that the frequency distribution of cpDNAs of either parent among 12 tomato (+) potato somatic hybrids was compatible with random selection. Both the triploid and the tetraploid hybrids we analyzed showed this 1:1 segregation. The tetraploid hybrids resulted from fusion of one diploid tomato protoplast with two monoploid potato protoplasts or with one somatically doubled and thus dihaploid potato protoplast. Since the number of chloroplasts in a leaf mesophyll cell is related to the number of genomes (Butterfass 1973), it seems likely that the original heterokaryons which yielded triploid hybrids contained on average fewer potato chloroplasts than the heterokaryons which gave rise to tetraploid hybrids. Therefore it is remarkable that the triploid C7-hybrids showed the same segregation ratio with respect to cpDNA as the tetraploid C7-hybrids. Hung et al. (1993) observed the same phenomenon. They produced diploid, triploid and tetraploid somatic hybrids between haploid *Nicotiana plumbaginifolia* and haploid *N. sylvestris*, and observed a 1:1 segregation of chloroplast types, irrespective of the ratio of parental nuclear genomes.

One somatic hybrid contained chloroplasts from both parents, even though the total DNA for organelle analysis was isolated from hybrids which had been maintained as shoot cultures in vitro for 2½ years. Although chloroplast segregation is usually completed before the onset of shoot regeneration, occasionally hybrid plants containing chloroplasts from both parents are found, up to three years after fusion (Glimelius et al. 1981; Derks et al. 1991; Wachocki et al. 1991).

The mtDNA composition of the hybrids seemed not to be influenced by nuclear DNA composition, i.e. one or two potato genomes. Similarly, Landgren and Glimelius (1990) could find no correlation between nuclear constitution and mtDNA type in *Brassica napus* (+) *Eruca sativa* somatic hybrids that showed preferential elimination of *E. sativa* chromosomes (Fahleson et al. 1988). However, Bonnema et al. (1992) reported that highly asymmetric somatic hybrids between *L. esculentum* and *L. pennellii* had more tomato-specific mitochondrial sequences than symmetric somatic hybrids. Probably, specific nuclear genes of one parental species are required for mitochondrial genes of that species to be functional.

The mtDNA composition of our hybrids seemed not to be affected by the chloroplast type. Independent segregation of mitochondria and chloroplasts was also reported by Walters and Earle (1993) for somatic hybrids obtained after fusion of *Brassica oleracea* genotypes containing either a *B. oleracea* cytoplasm or a *Raphanus sativus* cytoplasm. The A7-hybrids, which were selected for the presence of potato chloroplasts, did not contain more potato-specific mtDNA fragments than the C7-hybrids, but on the contrary showed mostly tomato-specific mtDNA fragments. Both the A7- and C7-hybrids were obtained from fusions between leaf mesophyll protoplasts of both parents, thus eliminating the influence of different sources of protoplasts on mitochondrial segregation, as proposed by Landgren and Glimelius (1990). Possibly, leaf cells of the albino tomato parent of the A7-hybrids, which contain only proplastids and grow heterotrophically, possess more mitochondria or mitochondria with a higher replication rate as compared with leaf cells of normal green tomato or potato genotypes.

Some specific mtDNA fragments show a biased segregation (Table 3). L9 and L10, the tomato mtDNA fragments carrying the *atp6* gene, are present in 34 of the 35 hybrids analyzed. In contrast, the corresponding potato *atp6* gene (fragments S8 and S9) is present in 15 of the 35 hybrids. The only hybrid that lacks the tomato *atp6* gene possesses the potato *atp6* gene. Although tomato-specific fragments are on average retained in 60% of the hybrids, the L7 fragment is only present in five A7-hybrids that lack S4. The S4 fragment is significantly more frequently retained in the hybrids than the other potato-specific mtDNA fragments. Temple et al. (1992) reported that some regions in the mtDNA genomes of *Brassica campestris* (+) *B. oleracea* (Ogura cms) somatic hybrids are always derived from one fusion partner. Possibly, some specific mtDNA regions of one or the other parent are needed for hybrid callus growth, for regeneration of for some other essential process. This suggests that hybrids with particular mtDNA compositions are more viable than others with a different mtDNA.

Between individual *Lycopersicon* (+) *Solanum* hybrids large differences in greenhouse performance, leaf and flower morphology were observed (Schoenmakers et al. 1993). Possibly, the mtDNA composition determines some of these traits. For example, in tobacco it was shown that mutations in the mtDNA can cause changes in floral morphology (Bonnett et al. 1991; Kofer et al. 1991,1992) or leaf variegation and abnormal vegetative growth (Bonnett et al. 1993). However, it cannot be excluded that differences in chromosome complements also affect these traits. Hybrid A7-82A, a euploid triploid hybrid containing potato chloroplasts and only tomato-specific mtDNA fragments (Table 1), showed a good viability in the greenhouse and regularly shaped leaves and flowers, whereas A7-146D, an aneuploid triploid hybrid with 34 chromosomes, potato chloroplasts and mtDNA fragments mainly from potato, was less viable and showed abnormal leaves and flowers (Schoenmakers et al. 1993).

The present analysis does not indicate that in tomato (+) potato somatic hybrids there is a requirement for organelles to be predominantly derived from one or the other parental species. Many possible nucleus-chloroplast-mitochondrion combinations seem to be functional in these fusion products. This could be a factor explaining the higher frequency of *Lycopersicon* (+) *Solanum* somatic hybrids as compared with *Lycopersicon* (+) *Nicotiana* somatic hybrids (Wolters et al. 1993b, Chapter 5). The latter hybrids apparently require a more specific fine-tuning of nucleus and organelles, and thus the fraction of viable fusion products in the total population of fusion products is lower.

When, by asymmetric somatic hybridization or by backcrossing, many nuclear genes from one of the parents are lost, the situation may be different for tomato (+) potato somatic hybrids. In that case nucleo-cytoplasmic interactions may be more critical for chloroplasts than for mtDNA genomes, because mtDNAs recombine easily, whereas cpDNAs usually do not recombine. In previous experiments we could not obtain true cybrids combining a tomato nucleus with potato chloroplasts, not even when the potato protoplasts had been heavily irradiated (Wolters et al. 1991, Chapter 2). Probably, potato chloroplasts can only be functional when a certain amount of potato nuclear DNA is present. In contrast, cybrids combining a tomato nucleus with mtDNA fragments from both tomato and potato were reported by Melchers et al. (1992). Thus, the proteins encoded by genes located on the potato mtDNA fragments in these cybrids can be functional when only tomato nuclear DNA is present, or at least do not interfere with the interaction between tomato mtDNA-encoded and nuclear DNA-encoded proteins.

It is remarkable that only six of the 35 analyzed tomato (+) potato hybrids showed one or two new, non-parental mtDNA fragments. The majority of the intertribal hybrids between *Lycopersicon* spp. and *Nicotiana* spp. (Wolters et al. 1993b, Chapter 5), which were analyzed with three mtDNA probes, but also of intrageneric hybrids between tomato and *L. pennellii*, analyzed with seven cosmid clones containing tomato mtDNA (Bonnema et al. 1992), showed several novel mtDNA fragments. Possibly, more A7- and C7-hybrids could be shown to contain new mtDNA fragments by using additional mtDNA probes, since novel fragments mainly seem to occur in distinct regions of the mitochondrial genome (Temple et al. 1992).

Not only novel fragments are an indication of recombination between the parental mtDNAs, but also new combinations of specific mtDNA fragments from both parents. Thus, recombination can also be observed when analyzing cosegregation of mtDNA restriction fragments. Closely linked genes will often be retained together in a somatic hybrid, whereas genes between which recombination occurs frequently, e.g. because they are located on different subgenomic molecules, are more often separated. The results of the cosegregation analysis suggest that on both tomato and potato mitochondrial DNA genes encoding *cox*II and 18S+5S rRNA are closely linked (on the same subgenomic molecule). Segregation of the original mixture of parental mtDNAs therefore resulted in most cases in hybrid cells containing either tomato *cox*II and 18S+5S rRNA genes, or potato *cox*II and 18S+5S rRNA genes. Probably, two tomato 18S+5S rRNA genes and two potato *cox*II genes are involved, since L7 (tomato 18S+5S) is not cosegregating with L5 and L6, and S4 (potato *cox*II) segregates independently from S3. The L8 fragment seems to be linked to the tomato *cox*II and 18S+5S rRNA genes, but at a larger genetic distance than the distance between *cox*II and 18S+5S rRNA. Similarly, S8 and S9, the *Eco*RI and respectively *Hin*dIII potato mtDNA fragments carrying the *atp6* gene, seem to be linked to the potato 18S+5S rRNA gene.

Cosegregation of mtDNA fragments may not only result from physical linkage, but may also occur when for the proper functioning of the somatic hybrid cell mitochondrial genes encoding different proteins have to originate from the same parental species. To distinguish between linkage and somatic incongruity as an explanation for cosegregation, a comparison of cosegregation data with physical maps of *Lycopersicon* and *Solanum* mitochondrial genomes, which are not yet available, will be necessary.

Acknowledgements. The authors thank J.W. van Ooijen for statistical advice and C. Heyting for critically reading the manuscript. This work was supported by the Dutch Programme Committee on Agricultural Biotechnology (PcLB).

References

Akada S, Hirai A (1986) Studies on the mode of separation of chloroplast genomes in parasexual hybrid calli III. Random separation of two types of chloroplast genomes in a hybrid callus. Jpn J Genet 61:437-445

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

Bonnema AB, O'Connell MA (1992) Molecular analysis of the nuclear organellar genotype of somatic hybrid plants between tomato (*Lycopersicon esculentum*) and *Lycopersicon chilense*. Plant Cell Reports 10:629-632

Bonnema AB, Melzer JM, Murray LW, O'Connell MA (1992) Non-random inheritance of organellar genomes in symmetric and asymmetric somatic hybrids between *Lycopersicon esculentum* and *L. pennellii*. Theor Appl Genet 84:435-442

Bonnett HT, Kofer W, Håkansson G, Glimelius K (1991) Mitochondrial involvement in petal and stamen development studied by sexual and somatic hybridization of *Nicotiana* species. Plant Science 80:119-130

Bonnett HT, Djurberg I, Fajardo M, Glimelius K (1993) A mutation causing variegation and abnormal development in tobacco is associated with an altered mitochondrial DNA. Plant J 3:519-525

Butterfass T (1973) Control of plastid division by means of nuclear DNA amount. Protoplasma 76:167-195

Chomczynski P, Qasba PK (1984) Alkaline transfer of DNA to plastic membrane. Biochem Biophys Res Commun 122:340-344

Daunay MC, Chaput MH, Sihachakr D, Allot M, Vedel F, Ducreux G (1993) Production and characterization of fertile somatic hybrids of eggplant (*Solanum melongena* L.) with *Solanum aethiopicum* L. Theor Appl Genet 85:841-850

Dell'Orto P, Moenne A, Graves PV, Jordana X (1993) The potato mitochondrial ATP synthase subunit 9: gene structure, RNA editing and partial protein sequence. Plant Sci 88:45-53

Derks FHM, Wijbrandi J, Koornneef M, Colijn-Hooymans CM (1991) Organelle analysis of symmetric and asymmetric hybrids between *Lycopersicon peruvianum* and *Lycopersicon esculentum*. Theor Appl Genet 81:199-204

Dewey RE, Levings III CS, Timothy DH (1985) Nucleotide sequence of ATPase subunit 6 gene of maize mitochondria. Plant Physiol 79:914-919

Fahleson J, Råhlén L, Glimelius K (1988) Analysis of plants regenerated from protoplast fusions between *Brassica napus* and *Eruca sativa*. Theor Appl Genet 76:507-512

Gilissen LJW, van Staveren MJ, Verhoeven HA, Sree Ramulu K (1992) Somatic hybridization between potato and *Nicotiana plumbaginifolia* 1. Spontaneous biparental chromosome elimination and production of asymmetric hybrids. Theor Appl Genet 84:73-80

Glimelius K, Chen K, Bonnett HT (1981) Somatic hybridization in *Nicotiana*: segregation of organellar traits among hybrid and cybrid plants. Planta 153:504-510

Guri A, Levi A, Sink KC (1988) Morphological and molecular characterization of somatic hybrid plants between *Lycopersicon esculentum* and *Solanum nigrum*. Mol Gen Genet 212:191-198

Gwynn B, Dewey RE, Sederoff RR, Timothy DH, Levings III CS (1987) Sequence of the 18S-5S ribosomal gene region and the cytochrome oxidase II gene from mtDNA of *Zea diploperennis*. Theor Appl Genet 74:781-788

de Haas JM, Boot KJM, Haring MA, Kool AJ, Nijkamp HJJ (1986) A *Petunia hybrida* chloroplast DNA region, close to one of the inverted repeats, shows sequence homology with the *Euglena gracilis* chloroplast DNA region that carries the putative replication origin. Mol Gen Genet 202:48-54

Hung CY, Lai YK, Feng TY, Chen CC (1993) Chloroplast segregation in somatic hybrids of *Nicotiana plumbaginifolia* and *N. sylvestris* having different ratios of parental nuclear genomes. Plant Cell Rep 13:83-86

Kemble RJ, Barsby TL, Wong RSC, Shepard JF (1986) Mitochondrial DNA rearrangements in somatic hybrids of *Solanum tuberosum* and *Solanum brevidens*. Theor Appl Genet 72:787-793

Kofer W, Glimelius K, Bonnett HT (1991) Modifications of mitochondrial DNA cause changes in floral development in homeotic-like mutants of tobacco. Plant Cell 3:759-769

Kofer W, Glimelius K, Bonnett HT (1992) Fusion of male-sterile tobacco causes modifications of mtDNA leading to changes in floral morphology and restoration of fertility in cybrid plants. Physiol Plant 85:334-338

Landgren M, Glimelius K (1990) Analysis of chloroplast and mitochondrial segregation in three different combinations of somatic hybrids produced within *Brassicaceae*. Theor Appl Genet 80:776-784

Melchers G, Mohri Y, Watanabe K, Wakabayashi S, Harada K (1992) One-step generation of cytoplasmic male sterility by fusion of mitochondrial-inactivated tomato protoplasts with nuclear-inactivated *Solanum* protoplasts. Proc Natl Acad Sci USA 89:6832-6836

Overbeeke N, Haring MA, Nijkamp HJJ, Kool AJ (1984) Cloning of *Petunia hybrida* chloroplast DNA sequences capable of autonomous replication in yeast. Plant Mol Biol 3:235-241

Pehu E, Karp A, Moore K, Steele S, Dunckley R, Jones MGK (1989) Molecular, cytogenetic and morphological characterization of somatic hybrids of dihaploid *Solanum tuberosum* and diploid *S. brevidens*. Theor Appl Genet 78:696-704

Rogers SO, Bendich AJ (1988) Extraction of DNA from plant tissues. Plant molecular biology manual A6. Kluwer Academic Publ, Dordrecht, pp 1-10

Rothenberg M, Hanson MR (1987) Recombination between parental mitochondrial DNA following protoplast fusion can occur in a region which normally does not undergo intragenomic recombination in parental plants. Curr Genet 12:235-240

Rothenberg M, Boeshore ML, Hanson MR, Izhar S (1985) Intergenomic recombination of mitochondrial genomes in a somatic hybrid plant. Curr Genet 9:615-618

San LH, Vedel F, Sihachakr D, Rémy R (1990) Morphological and molecular characterization of fertile tetraploid somatic hybrids produced by protoplast electrofusion and PEG-induced fusion between *Lycopersicon esculentum* Mill. and *Lycopersicon peruvianum* Mill. Mol Gen Genet 221:17-26

Schiller B, Herrmann RG, Melchers G (1982) Restriction endonuclease analysis of plastid DNA from tomato, potato and some of their somatic hybrids. Mol Gen Genet 186:453-459

Schoenmakers HCH, Nobel EM, Koornneef M (1992) Use of leaky nitrate reductase-deficient mutants of tomato (*Lycopersicon esculentum* Mill.) for selection of somatic hybrid cell lines with wild type potato (*Solanum tuberosum* L.). Plant Cell Tissue Org Cult 31:151-154

Schoenmakers HCH, Wolters AMA, Nobel EM, de Klein CMJ, Koornneef M (1993) Allotriploid somatic hybrids of diploid tomato (*Lycopersicon esculentum* Mill.) and monoploid potato (*Solanum tuberosum* L.). Theor Appl Genet 87:328-336

Schuster W, Brennicke A (1986) Pseudocopies of the ATPase α-subunit gene in *Oenothera* mitochondria are present on different circular molecules. Mol Gen Genet 204:29-35

Taguchi T, Sakamoto K, Terada M (1993) Fertile somatic hybrids between *Petunia hybrida* and a wild species, *Petunia variabilis*. Theor Appl Genet 87:75-80

Temple M, Makaroff CA, Mutschler MA, Earle ED (1992) Novel mitochondrial genomes in *Brassica napus* somatic hybrids. Curr Genet 22:243-249

Wachocki SE, Bonnema AB, O'Connell MA (1991) Comparison of the organization of the mitochondrial genome in tomato somatic hybrids and cybrids. Theor Appl Genet 81:420-427

Walters TW, Earle ED (1993) Organellar segregation, rearrangement and recombination in protoplast fusion-derived *Brassica oleracea* calli. Theor Appl Genet 85:761-769

Wijbrandi J, van Capelle W, Hanhart CJ, van Loenen Martinet-Schuringa EP, Koornneef M (1990) Selection and characterization of somatic hybrids between *Lycopersicon esculentum* and *Lycopersicon peruvianum*. Plant Science 70:197-208

Wolters AMA, Schoenmakers HCH, van der Meulen-Muisers JJM, van der Knaap E, Derks FHM, Koornneef M, Zelcer A (1991) Limited DNA elimination from the irradiated potato parent in fusion products of albino *Lycopersicon esculentum* and *Solanum tuberosum*. Theor Appl Genet 83:225-232

Wolters AMA, Koornneef M, Gilissen LJW (1993a) The chloroplast and mitochondrial DNA type are correlated with the nuclear composition of somatic hybrid calli of *Solanum tuberosum* and *Nicotiana plumbaginifolia*. Curr Genet 24:260-267

Wolters AMA, Vergunst AC, van der Werff F, Koornneef M (1993b) Analysis of nuclear and organellar DNA of somatic hybrid calli and plants between *Lycopersicon* spp. and *Nicotiana* spp. Mol Gen Genet 241:707-718

Young EG, Hanson MR (1987) A fused mitochondrial gene associated with cytoplasmic male sterility is developmentally regulated. Cell 50:41-49

CHAPTER 4

Mitotic and meiotic irregularities in somatic hybrids of *Lycopersicon esculentum* and *Solanum tuberosum*

A.M.A. Wolters, H.C.H. Schoenmakers, S. Kamstra, J. van Eden, M. Koornneef, J.H. de Jong
Department of Genetics, Wageningen Agricultural University, Dreijenlaan 2,
NL-6703 HA Wageningen, The Netherlands

Genome (in press)

Summary. Chromosome numbers were determined in metaphase complements of root tip meristems of 107 tomato (+) potato somatic hybrids, obtained from five different combinations of parental genotypes. Of these hybrids 79% were aneuploid, lacking one or two chromosomes in most cases. All four hybrids that were studied at mitotic anaphase of root tips showed laggards and bridges; the three aneuploids in a higher frequency than the single euploid. Hybrid K2H2-1C, which showed the highest percentage of aberrant anaphases, possessed 46 chromosomes. Fluorescence *in situ* hybridization with total genomic DNA showed that this hybrid contained 23 tomato, 22 potato, and one recombinant chromosome consisting of a tomato chromosome arm and a potato chromosome arm. The potato parent of K2H2-1C was aneusomatic in its root tips with a high frequency of monosomic and trisomic cells and a relatively high frequency of cells with one fragment or telosome. Meiotic analyses of three tomato (+) potato somatic hybrids revealed laggards, which occurred most frequently in the triploid hybrids, and bridges, which were frequently present in pollen mother cells (PMCs) at anaphase I of hypotetraploid K2H2-1C. We observed putative trivalents in PMCs at diakinesis and metaphase I of eutriploid A7-82A and quadrivalents in part of the PMCs of hypotetraploid K2H2-1C, suggesting that homoeologous recombination between tomato and potato chromosomes occurred in these hybrids. All three hybrids showed a high percentage of First Division Restitution (FDR), giving rise to unreduced gametes. However, shortly after tetrad stage all microspores completely degenerated, resulting in exclusively sterile pollen.

Introduction

The complete genomes of two different species can be combined in a single cell by symmetric somatic hybridization. If the two species concerned are sufficiently related, the resulting hybrids are expected to be genetically balanced and, consequently, to contain the sum of their parental chromosome complements. Accordingly, some papers report on the production of exclusively euploid somatic hybrids (e.g. Jacobsen et al. 1992; Jadari et al. 1992; Daunay et al. 1993; Taguchi et al. 1993). However, there are also numerous reports of a high percentage of aneuploid somatic hybrids obtained after symmetric hybridization of two species from the same genus (e.g. Puite et al. 1986; Sundberg et al. 1987; Pijnacker et al. 1989; Lee and Chen 1990; Sproule et al. 1991; Mattheij et al. 1992; Pupilli et al. 1992; Waara et al. 1992; Cardi et al. 1993).

For breeding purposes, introgression of genes from one species into an other species usually is the ultimate goal, rather than combining the complete genomes of two species. For this, euploidy can be important, but fertility even more, i.e. the possibility to obtain progeny, preferably via backcrossing with one parental species. Cardi et al. (1993) reported that hypotetraploid *Solanum tuberosum* (+) *S. commersonii* somatic hybrids showed a reduced degree of flowering compared with eutetraploid hybrids. All hybrids except one were male sterile; the one male fertile hybrid was a euploid tetraploid. However, euploid hybrids are not necessarily male fertile. The eutetraploid somatic hybrids of tomato and potato produced by Jacobsen et al. (1992) were all male sterile. On the other hand, Mattheij et al. (1992) reported that both euploid and aneuploid tetraploid somatic hybrids of *S. tuberosum* and *S. circaeifolium* were male sterile, but female fertile. This suggests that aneuploidy is not necessarily related to (female) sterility.

When male fertile or female fertile somatic hybrids are obtained, the occurrence of homoeologous recombination between chromosomes of both parents is advantageous, since it enables introgression. Homoeologous recombination can cytogenetically be established in electron micrographs of late pachytene nuclei showing multiple synaptonemal complex associations with late recombination nodules at both ends of a pairing partner exchange, and in light microscope preparations of pollen mother cells at diakinesis or metaphase I showing multivalents (cf. de Jong et al. 1993).

We have obtained a large number of tomato (+) potato somatic hybrids from five separate fusion experiments. In this study we address the following questions: (*i*) Are the tomato (+) potato somatic hybrids euploid or aneuploid, and how many tomato and potato chromosomes are involved? (*ii*) In the case of aneuploid hybrids, do they result from chromosomal irregularities in one or both parental genotypes, or were they produced during tissue culture of the hybrids? (*iii*) What happens to the chromosomes during mitosis and meiosis? (*iv*) Does recombination occur between homoeologous chromosomes of tomato and potato? (*v*) Is viable pollen produced?

Materials and methods

Plant materials. Protoplast fusions were performed according to Wolters et al. (1991, Chapter 2) with leaf protoplasts from five tomato genotypes and three potato genotypes. Characteristics of the parental genotypes are given in Table 1.

Table 1. Characteristics of the tomato and potato parental genotypes used for fusion

Genotype	Ploidy	Selectable markers[a]
L. esculentum		
MsK9-K1	diploid	Kan^R, Reg
MsK9-K2	diploid	Kan^R, Reg
Xa11-H1	diploid	Hyg^R, Reg
ALRCxM8-7	diploid	cytoplasmic albino, Reg
C31-244	diploid	nitrate reductase deficient (*nia*), Reg
S. tuberosum		
7322	monohaploid	—
7322-H2	dihaploid	Hyg^R
7322-K3	tetraploid	Kan^R

[a] Kan^R = kanamycin resistance, Hyg^R = hygromycin resistance, Reg = good regeneration capacity

The tomato genotypes MsK9-K1 and MsK9-K2 are kanamycin-resistant transformants of MsK9 (Koornneef et al. 1987). Xa11-H1 is a hygromycin-resistant transformant of Xa11 (Schoenmakers et al. 1994). ALRCxM8-7 was obtained after crossing the cytoplasmic albino tomato genotype ALRC (Hosticka and Hanson 1984) with MsK8 (Koornneef et al. 1987). C31-244 is described by Schoenmakers et al. (1993). The potato genotypes 7322-H2 and 7322-K3 are transformants of the monoploid potato 7322 (described by de Vries et al. 1987). Five fusion combinations of a tomato and a potato genotype were made. These are listed in Table 2. Some of the K1H2- and K2H2-hybrids have been decribed by de Jong et al. (1993), and the A7- and C7-hybrids by Schoenmakers et al. (1993).

Table 2. Details of somatic hybrids

Somatic hybrids	*L. esculentum* parent	*S. tuberosum* parent	Selection	Expected ploidy of somatic hybrids
K1H2	MsK9-K1	7322-H2	Kan^R, Hyg^R	tetraploid
K2H2	MsK9-K2	7322-H2	Kan^R, Hyg^R	tetraploid
H1K3	Xa11-H1	7322-K3	Kan^R, Hyg^R	hexaploid
A7	ALRCxM8-7	7322	green calli	triploid
C7	C31-244	7322	well growing calli (*nia*$^+$)	triploid

Cytological analyses. Chromosome counts and karyotype analyses of somatic hybrids and parental genotypes were performed on chromosome preparations made of root tip meristems according to a procedure of Pijnacker and Ferwerda (1984), with some slight modifications, as described in Wolters et al. (1993, Chapter 5). For studying mitotic anaphases, we used root tip meristems directly fixed in ethanol/acetic acid (3:1). For studying male meiosis, anthers were treated as described by Ramanna and Prakken (1967). Anthers containing pollen mother cells (PMCs) at prophase I - tetrad stage were fixed in a 3:1 96% ethanol/Fe^{3+} acetate-saturated propionic acid mixture for at least 1 week at room temperature. Chromosome preparations were made by squeezing out the content of one anther in 1% acetocarmine. The cells were spread by tapping on the cover glass and carefully squashed after gently heating the slide. Pollen morphology and viability were studied in lactophenol-acid fuchsin stained preparations of young microspores (Sass 1964).

Genomic in situ hybridization. DNA denaturation, *in situ* hybridization and detection/amplification steps were performed according to Leitch and Heslop-Harrison (1993), Schwarzacher and Heslop-Harrison (1993) and Schwarzacher and Leitch (1993). We used total genomic DNA from tomato as probe, and total DNA from potato as blocking DNA. The tomato DNA was sonicated until fragments of 1-10 kb were obtained (10 sec). The potato DNA was autoclaved for 5 min, which yielded fragments of 100-500 bp. The hybridization mix contained 50% deionized formamide, 10% (w/v) sodium dextran sulphate (Sigma), 2xSSC, 0.25% (w/v) sodium dodecyl sulphate (SDS), 2.5 ng/μl probe DNA and 0.1-0.25 μg/μl blocking DNA. This mix was denatured for 10 min at 70°C and then placed on ice for 5 min. Hybridization was performed overnight at 37°C. Subsequently, the slides were washed as follows: 30 min in 2xSSC at room temperature, 30 min in 0.1xSSC at 42°C, followed by 15 min in 2xSSC at room temperature. We used labeling with digoxigenin-11-dUTP (Boehringer-Mannheim), detected with anti-digoxigenin-FITC (fluorescein isothiocyanate) raised in sheep (Boehringer-Mannheim), and amplified with anti-sheep-FITC raised in rabbit (Nordic Immunological Laboratories), or labeling with FITC-11-dUTP (Amersham). The latter method with direct fluorochrome-labeled DNA probes gave less background fluorescence and was preferred in most experiments. Chromosomes were counterstained with 2 μg/ml DAPI (4',6-diamidino-2-phenylindole) and 1-5 μg/ml propidium iodide.

Results

Somatic chromosome numbers

Chromosome counts were made of 9 K1H2-, 15 K2H2-, 9 H1K3-, 37 A7- and 37 C7-hybrids (in total 107 hybrids). The K1H2-, K2H2- and H1K3-hybrids were chosen at random from the total population of these hybrids. Of the A7- and C7-hybrids we first established the exact number of chromosomes of hybrids that were classified as triploids by flow cytometric analysis (see Schoenmakers et al. 1993). Subsequently, chromosomes were counted of A7- and C7-hybrids that showed a more or less "normal" leaf morphology in vitro. The results are presented in Fig. 1. Most hybrids (79%) proved to be aneuploid; some were hyperploid, but most of these were hypoploid. Ten of the 26 triploids (38%), 9 of the 64 tetraploids (14%) and 3 of the 17 hexaploids (18%) were euploid. All K1H2- and K2H2-hybrids were hypoploid. The four hyperploids were A7-hybrids.

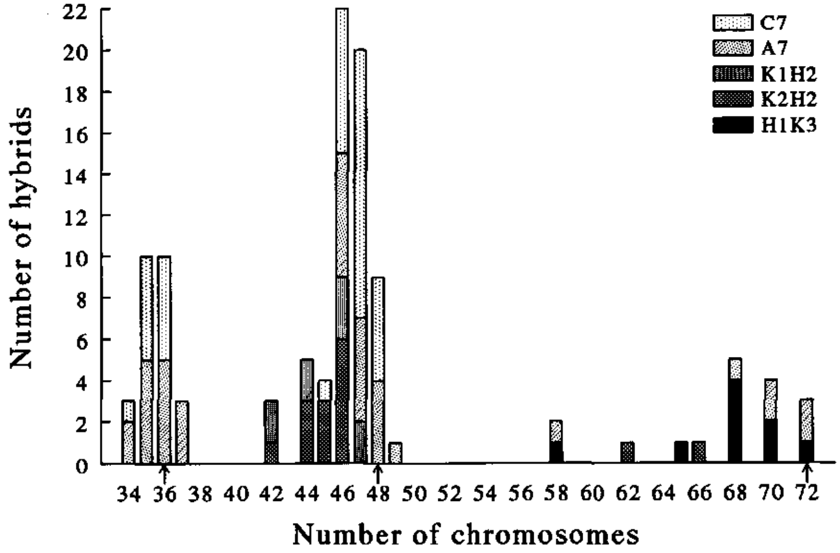

Fig. 1. Distribution of tomato (+) potato hybrids A7, C7, K1H2, K2H2 and H1K3 on the basis of chromosome numbers. Euploid chromosome numbers are indicated with an *arrow*

Homoeologous chromosomes of tomato and potato are morphologically very similar, except for the satellite chromosomes (the chromosomes 2 of both species). The tomato chromosome 2 contains a significantly larger satellite than the potato equivalent (cf. Schoenmakers et al. 1993). The tomato satellite chromosome is the most conspicuous chromosome in a metaphase plate of a tomato (+) potato somatic hybrid, and the numbers of these chromosomes could be established in the hypoploid triploid and tetraploid hybrids. The results are shown in Table 3. When the hypotriploid hybrids (A7 and C7) are considered, all three hybrids with 34 chromosomes possessed both chromosomes 2 of tomato; three of the ten hybrids containing 35 chromosomes possessed only one tomato chromosome 2 (Schoenmakers et al. 1993). In three of the 44 hypotetraploid hybrids one tomato nucleolar chromosome had been eliminated. From a total of 57 hypoploid hybrids analyzed six hybrids contained only one tomato chromosome 2. This suggests that loss of a tomato nucleolar chromosome occurs randomly.

Table 3. Number of hypoploid somatic hybrids in which one nucleolar chromosome of tomato has been eliminated

Type of hybrids	Total no. of hybrids	No. of hybrids with one tomato chromosome 2
A7:		
hypotriploids	7	1
hypotetraploids	11	0
C7:		
hypotriploids	6	2
hypotetraploids	21	3
K1H2 + K2H2:		
hypotetraploids	12	0
Total	57	6

To find out whether the high frequency of aneuploid somatic hybrids was (partly) caused by chromosome instability in the parental species, we studied the chromosome complements of some tomato and potato genotypes in more detail. First the number of chromosomes in a large number of metaphases was counted. The results are given in Table 4. In the tomato genotypes a low frequency of cells containing 23, 25 or 48 chromosomes was found besides cells with the expected 2n=24 chromosomes.

Table 4. Frequency of euploid and aneuploid metaphase cells in root-tip meristems of tomato and potato genotypes

Genotype	Percentage of cells with following number of chromosomes									No. of cells
	12	22	23	24	24+fr[a]	25	26	46	48	
L. esculentum										
"Tiny Tim"	–	–	4.0	92.0	–	1.0	–	1.0	2.0	100
MsK93	–	–	2.0	95.0	–	–	–	–	3.0	200
MsK8	–	–	1.7	96.5	–	0.4	–	–	1.3	230
S. tuberosum										
7322, doubled	5.8	–	–	94.2	–	–	–	–	–	52
7322-H2:										
Preparation I	–	1.0	5.0	80.0	1.0	7.0	3.5	–	2.5	200
Preparation II	–	–	7.3	86.0	1.3	5.3	–	–	–	150
Preparation III	–	–	7.0	83.0	3.0	6.0	–	–	1.0	200
Mean	–	0.4	6.4	82.7	1.8	6.2	1.3	–	1.3	550

[a] fr = fragment or telosome

A spontaneously doubled root tip of the monohaploid potato 7322 showed only euploid cells with 24 chromosomes and a few cells still containing 12 chromosomes. A dihaploid hygromycin-resistant transformant of this genotype, 7322-H2, proved to be aneusomatic. On the average 6.4% of the cells had 23 chromosomes, and 6.2% had 25 chromosomes (see Fig. 2A). In one preparation a small number of cells containing 22 or 26 chromosomes was found. Another interesting observation is that 1.8% of the metaphase cells of 7322-H2 showed one fragment or whole chromosome arm besides 24 normal chromosomes (Fig. 2B). In 1.2% of the anaphases of this genotype one or two bridges were present, and 8.6% of the anaphases showed one laggard (Table 5, Fig. 2C). We observed no anaphases with more than one laggard.

Anaphase

Mitotic anaphases were studied in two triploid hybrids (A7-82A with 36 chromosomes and A7-146A with 34 chromosomes) and two tetraploid hybrids (C7-23C with 49 chromosomes and K2H2-1C with 46 chromosomes). The results are shown in Table 5. The eutriploid A7-82A showed 95.1% normal anaphases, 3.3% anaphases with bridges, and 1.6%

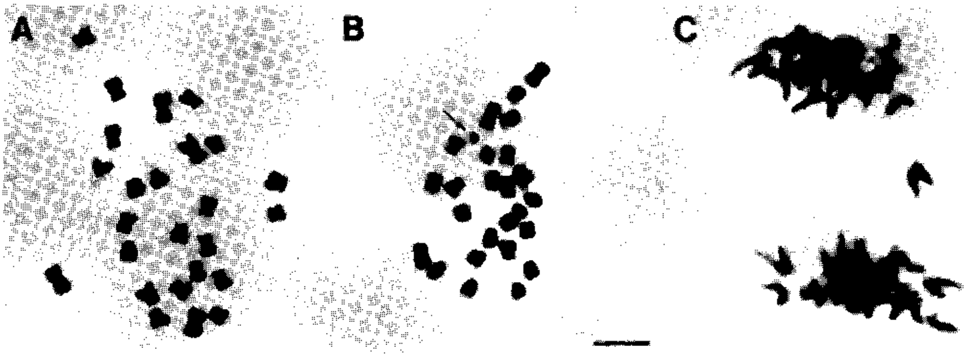

Fig. 2. Aberrant metaphases (A,B) and anaphase (C) in potato genotype 7322-H2. **A**, metaphase showing 25 chromosomes; **B**, metaphase showing 24 chromosomes plus one fragment or telosome, indicated with an *arrow*; **C**, anaphase with one laggard chromosome. Bar = 5 μm

Table 5. Percentages of normal and aberrant mitotic anaphases in potato genotype 7322-H2 and in tomato (+) potato somatic hybrids A7-82A, A7-146A, C7-23C and K2H2-1C

Genotype	Percentage of mitotic anaphases			No. of cells
	Normal	With 1-3 bridges	With 1-2 laggards	
7322-H2 (2n=24)	90.2	1.2	8.6	256
A7-82A (2n=36)	95.1	3.3	1.6	751
A7-146A (2n=34)	88.8	6.2	5.0	615
C7-23C (2n=49)	91.4	7.3	1.3	385
K2H2-1C (2n=46)	82.3	13.0	4.7	598

anaphases with 1-2 laggards. In hybrid A7-146A 88.8% of the anaphases were normal, in 6.2% one or two bridges and in 5.0% one or two laggards were observed. In prometaphases of this hybrid frequently one and sometimes two ring chromosomes were observed. Hybrid C7-23C showed a considerable number of anaphases with bridges: 7.3%. On average 1.3% of the anaphases contained 1-2 laggards. Hybrid K2H2-1C showed an even higher number of anaphases with 1-3 bridges, on average 13.0%, and in 4.7% of the anaphases 1-2 laggards. In eight of the 78 anaphases showing bridges a small fragment was clearly visible. Some of the aberrant anaphases of K2H2-1C are shown in Fig. 3.

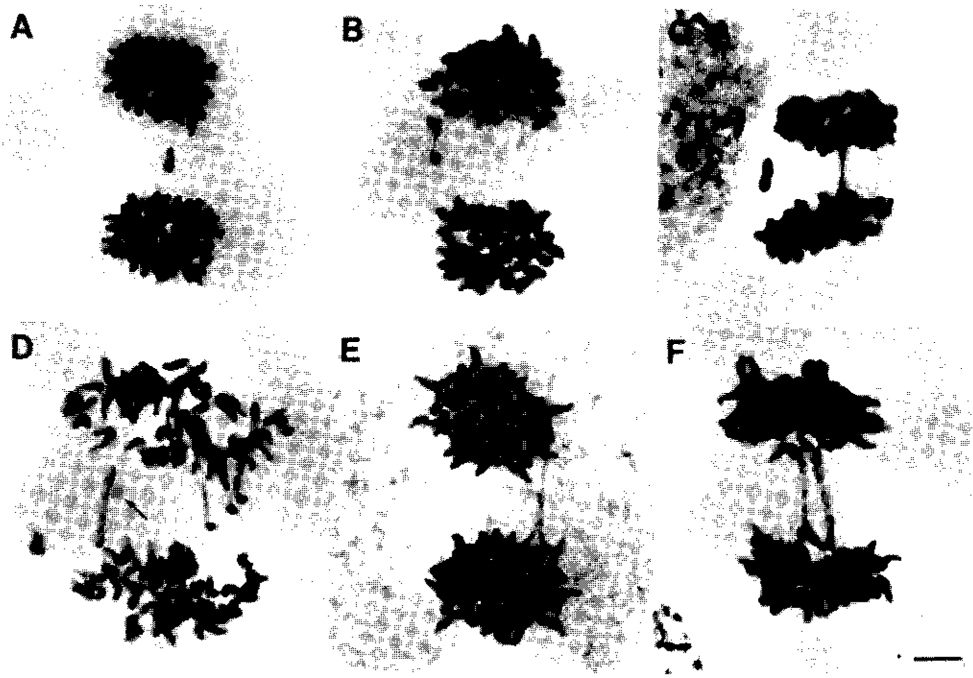

Fig. 3. Aberrant mitotic anaphases in tomato (+) potato hybrid K2H2-1C. **A,B**, anaphases with one laggard chromosome; **C**, anaphase showing one bridge and one laggard chromosome; **D**, anaphase with one bridge plus fragment (indicated with an *arrow*), one laggard and three "hockey stick-like" chromosomes; **E**, anaphase with one double bridge; **F**, anaphase with two double bridges. Bar = 5 μm

Genomic in situ hybridization

To find out whether tomato or potato chromosomes were lost, genomic *in situ* hybridization was performed on metaphase chromosomes of hybrids A7-82A, A7-146A and K2H2-1C with total DNA of tomato as a probe. The allotriploid A7-82A proved to contain the expected 24 chromosomes of tomato and 12 chromosomes of potato. Hybrid A7-146A possessed 23 tomato chromosomes and 11 potato chromosomes (Fig. 4A). By this technique it was shown that K2H2-1C had not simply lost two complete chromosomes, but possessed, in addition to 23 tomato chromosomes and 22 potato chromosomes, one recombinant chromosome with the short arm derived from a tomato chromosome and the long arm derived from a potato chromosome (Fig. 4B). This intergenomic translocation chromosome was observed in every analyzed complete metaphase of K2H2-1C. The satellites of chromosome 2 of tomato fluoresced less intensely than other tomato chromosome parts (Fig. 4B). Genomic separation of the tomato and potato chromosomes was very conspicuous in a few metaphase complements and interphase nuclei (Fig. 4C), but could not be demonstrated unambiguously in most other cells.

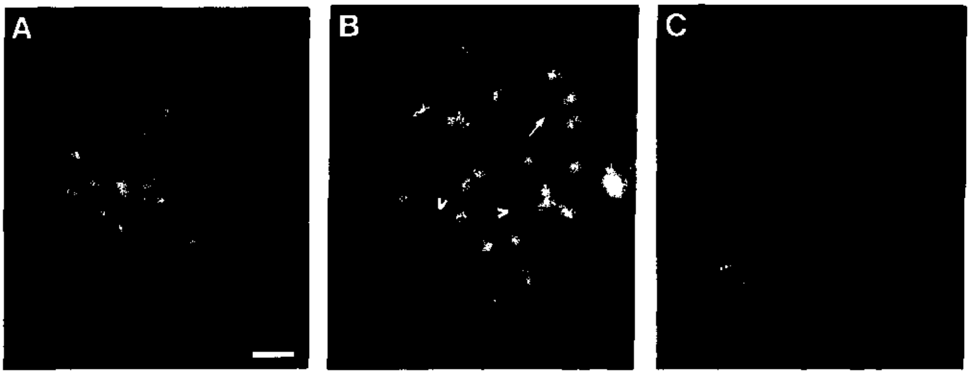

Fig. 4. Detection of tomato chromosomes and chromosome fragments in metaphase cells by genomic *in situ* hybridization. The tomato chromosomes show a yellow fluorescence, the potato chromosomes a red fluorescence. **A**, 23 tomato and 11 potato chromosomes in hypotriploid hybrid A7-146A; **B**, 23 tomato chromosomes, 22 potato chromosomes and one translocation chromosome (*arrow*) in hypotetraploid hybrid K2H2-1C. Tomato satellites are indicated with *arrowheads*; **C**, spatial separation of tomato and potato genomes in hybrid K2H2-1C. Bar = 5 μm

Meiosis

Meiosis was studied in three somatic hybrids that showed abundant flowering: A7-82A, a eutriploid with regularly shaped leaves and flowers; A7-146A, a hypotriploid with irregular leaf and flower morphology (comparable with A7-146D, described by Schoenmakers et al. 1993) and K2H2-1C, a hypotetraploid which showed vigorous growth, slightly deformed leaves and regular flowers in addition to irregular ones.

We analyzed meiosis from diakinesis to tetrad stage. However, PMCs of the hybrids possessed a rigid and narrow spindle, making it difficult or impossible to spread chromosomes at metaphase I. Thus, we were unable to quantify uni-, bi- and multivalent formation in these hybrids. In hybrid A7-82A putative tri- or quadrivalents were observed at diakinesis and metaphase I (Fig. 5A,B), whereas in hybrid A7-146A only uni- and bivalents were found. In hybrid K2H2-1C thirteen PMCs with 1-3 quadrivalents were observed among 60 PMCs at late diakinesis that could be analyzed unequivocally. No trivalents or multivalents involving five or more chromosomes were present in this hypotetraploid hybrid (de Jong et al. 1993). Bridges were observed at anaphase I of A7-82A and A7-146A, but at a lower frequency than in K2H2-1C. In K2H2-1C 42% of the anaphase I PMCs showed 1-3 bridges (de Jong et al. 1993), whereas in A7-82A one of the 29 (3%, see Fig. 5C) and in A7-146A four of the 30 (13%) anaphase I cells analyzed contained one or two bridges.

Table 6. Distribution of chromosomes to the poles at telophase I of somatic hybrid A7-146A (2n=34)

Combination with no laggards	No. of cells	Combination with laggards at one position	No. of cells	Combination with laggards at two positions	No. of cells
17/17	14	17/16/1	15	16/16/1/1	2
18/16	27	18/15/1	6	20/12/1/1	1
19/15	17	19/14/1	7	16/14/2/2	1
20/14	6	20/13/1	1		
21/13	0	21/12/1	0		
22/12	1	22/11/1	1		
19/16	1	18/14/1	1		
		20/14/1	1		
		17/15/2	2		
		19/13/2	1		
		16/15/3	1		
		18/13/3	1		
		20/11/3	1		
		16/14/4	1		
Total	66	Total	39	Total	4

Distribution of chromosomes to the poles at telophase I was determined in 109 PMCs of hybrid A7-146A (Table 6). The distribution with the highest frequency was a 18/16 division, followed by 19/15, 17/16/1 and 17/17 divisions. In 60% of these PMCs all chromosomes reached the two poles, in 36% 1-4 chromosomes lagged at one position in the PMC and the remaining 4% showed lagging chromosomes at two separate positions in the PMC.

Spindle orientation at metaphase II and anaphase II, production of reduced and unreduced gametes at telophase II, and the number of dyads, triads and tetrads at the tetrad stage are presented in Table 7.

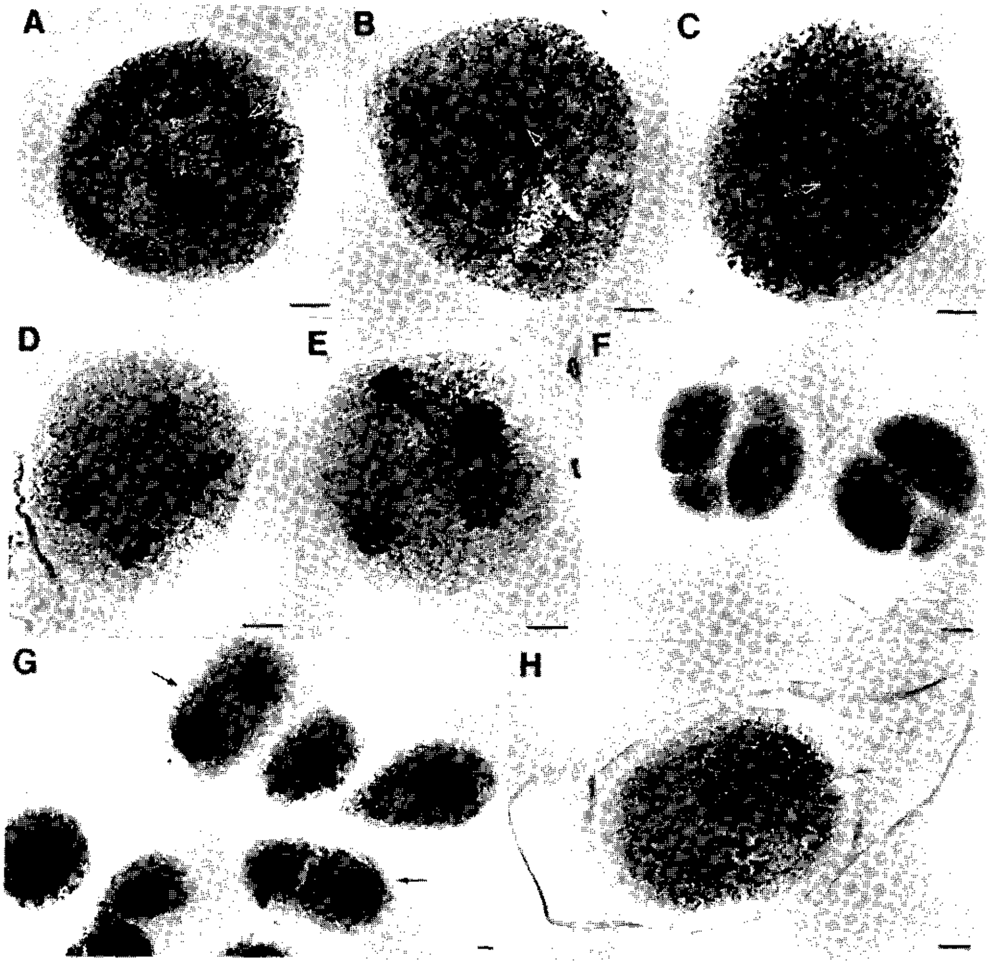

Fig. 5. **A-F**, Microsporogenesis in hybrid A7-82A. **A**, early diakinesis with one multivalent (*arrow*); **B**, metaphase I with one putative trivalent (*arrow*); **C**, anaphase I with bridge plus small fragment (*arrow*) and laggards; **D**, anaphase II with fused spindles; **E**, telophase II with one bridge; **F**, dyads with micronuclei. **G-H**, Syncytes in hybrid A7-146A. **G**, two syncytes in addition to normal PMCs; **H**, "octad", i.e. PMC at telophase II showing eight nuclei. Bars = 5 μm

Table 7. Frequency of pollen mother cells (PMCs) at metaphase II/anaphase II with fused, parallel and normal spindles, of unreduced and reduced PMCs at telophase II, and of dyads, triads and tetrads in anthers of somatic hybrids A7-82A, A7-146A and K2H2-1C

Genotype	Metaphase II/Anaphase II			No. of PMCs	Telophase II		No. of PMCs	Tetrad stage			Total number
	fused spindles	parallel spindles	normal		unreduced	reduced		dyads	triads	tetrads	
A7-82A:											
5/4-1[a]	36(47%)	16(21%)	25(32%)	77	34(68%)	16(32%)	50	-	-	-	-
5/4-2	15(43%)	7(20%)	13(37%)	35	113(64%)	63(36%)	176	-	-	-	-
5/7-1	139(80%)	6 (3%)	29(17%)	174	100(76%)	32(24%)	132	-	-	-	-
5/7-2	-	-	-	-	167(84%)	31(16%)	198	-	-	-	-
5/14-1	306(79%)	24 (6%)	58(15%)	388	41(79%)	11(21%)	52	-	-	-	-
9/6-1	-	-	-	-	-	-	-	147(74%)	27(13%)	26(13%)	200
9/6-2	-	-	-	-	-	-	-	160(80%)	23(12%)	17 (8%)	200
Total	496(74%)	53 (8%)	125(19%)	674	455(75%)	153(25%)	608	307(77%)	50(12%)	43(11%)	400
A7-146A:											
5/4-1	12(40%)	10(33%)	8(27%)	30	-	-	-	-	-	-	-
5/4-2	191(57%)	42(13%)	101(30%)	334	39(30%)	90(70%)	129	-	-	-	-
5/4-3	-	-	-	-	248(64%)	137(36%)	385	-	-	-	-
5/4-4	-	-	-	-	132(61%)	85(39%)	217	-	-	-	-
5/4-5	-	-	-	-	-	-	-	69(57%)	5(4%)	48(39%)	122
5/18-1	-	-	-	-	-	-	-	177(55%)	6(2%)	137(43%)	320
5/18-2	-	-	-	-	-	-	-	148(74%)	2(1%)	50(25%)	200
5/25-1	150(46%)	53(16%)	120(37%)	323	-	-	-	-	-	-	-
Total	353(51%)	105(15%)	229(33%)	687	419(57%)	312(43%)	731	394(61%)	13(2%)	235(37%)	642
K2H2-1C	49%	42%	9%	100				30%	6%	64%	200

[a] date of collecting - anther number

All three hybrids showed a high frequency of First Division Restitution (FDR), with a high percentage of metaphase II and anaphase II cells having fused spindles (see Fig. 5D), a high percentage of unreduced gametes at telophase II, and a high percentage of dyads instead of tetrads. Of 55 anaphase II cells of one anther of A7-82A 22 showed 1-4 laggards (40%). No bridges were observed in these PMCs. At tetrad stage of A7-82A micronuclei were observed in 33% of the 400 PMCs analyzed: 28% showed one micronucleus and 5% showed two micronuclei (Fig. 5F).

Occasionally syncytes were found in anthers of triploid hybrid A7-146A, i.e. one PMC containing two separate nuclei at the same stage of microsporogenesis. One PMC with two nuclei at pachytene, two PMCs each with two nuclei at diakinesis, six PMCs each with two nuclei at metaphase I (Fig. 5G) and one "octad" (at tetrad stage, Fig. 5H) were observed.

Pollen of the hybrids was stained with lactophenol-acid fuchsin to assess their viability. Pollen size of the hybrids was highly variable. Among very young pollen grains of A7-82A a few regularly shaped and viable ones were present, but at later stages only shrunken pollen was observed. No viable pollen was detected in hybrids A7-146A and K2H2-1C; these hybrids showed only shrunken pollen. Anthers of A7-146A produced much less pollen than anthers of A7-82A and K2H2-1C.

Discussion

A high percentage of aneuploidy was observed in the tomato (+) potato somatic hybrids. In the case of A7- and C7-hybrids, which were obtained from fusions between untransformed parental genotypes, this high frequency reflects a significant level of somaclonal variation induced during cell culture. In protoplast cultures of potato genotypes karyotypic aberrations occur frequently (reviewed by Pijnacker and Sree Ramulu 1990). In the case of K1H2-, K2H2- and H1K3-hybrids chromosomal instability in one or both of the transformed parental genotypes may also play an important role. The potato transgenic genotype 7322-H2 is aneusomatic, with 14.3% of the root-tip cells analyzed containing an aneuploid number of chromosomes and 1.8% of the cells showing one fragment or telosome. In addition, bridges and laggards were observed in 1.2% and respectively 8.6% of the mitotic anaphases. These irregularities may have arisen during the callus phase of the transformation process.

Aneuploidy may be caused by non-disjunction, lagging of chromosomes at anaphase, multipolar spindle formation, or anaphase bridges resulting from rearranged, dicentric chromosomes (Sunderland 1977). Alternatively, some form of stickiness may prevent the chromatids to separate at anaphase, possibly as a result of delayed replication due to hybridity or in vitro conditions (Lee and Phillips 1988; Peschke and Phillips 1992).

Among the K1H2- and K2H2-hybrids, which have 7322-H2 as the potato parent, there was not one euploid hybrid present. This may indicate that in these combinations of parental genotypes the loss of one or a few specific chromosomes promotes viability of the hybrid. If this is the case, it would be interesting to know which chromosomes are involved. Pijnacker et al. (1987, 1989) reported that in somatic hybrids of *Solanum tuberosum* and *S. phureja* nucleolar chromosomes of *S. phureja* were preferentially eliminated. In our tomato (+) potato somatic hybrids tomato nucleolar chromosomes do not seem to be preferentially, but randomly eliminated. Loss of a nucleolar chromosome does not seem to promote viability, since hypotetraploid hybrid K2H2-1C, which contains both tomato and both potato satellite chromosomes and 42 other chromosomes, is the most vigorous hybrid obtained from all fusion combinations. By genomic *in situ* hybridization it was shown that this hybrid lacks two complete tomato and one potato chromosome, and that a recombinant chromosome is present, consisting of a tomato and a potato chromosome arm. In hybrid A7-146A ($2n=34$) one tomato and one potato chromosome are lost. This suggests that there is no preferential elimination of exclusively tomato or potato chromosomes.

Intergenomic translocation chromosomes have been observed in asymmetric somatic hybrids, obtained after fusion of X- or gamma-irradiated donor protoplasts with untreated recipient protoplasts (Piastuch and Bates 1990; Parokonny et al. 1992), but evidence for this type of translocation in symmetric somatic hybrids is sparse, e.g. White and Rees (1985). We have shown that one of our symmetric tomato (+) potato somatic hybrids contained an intergenomic translocation chromosome, by performing genomic *in situ* hybridization (GISH). Without the use of GISH no rearranged chromosome was apparent in metaphase plates of this hybrid. This clearly shows the supplementary value of GISH.

Our genomic *in situ* hybridization experiments show a separation of parental genomes in at least a part of the analyzed somatic hybrid nuclei. Possibly, in more nuclei the potato and tomato chromosomes are spatially separated, but in the case of a top-bottom orientation of the two genomes, the spatial information will be lost in spread cell preparations, resulting in completely intermixed genomes. In addition, genomic separation may be restricted to specific cell types and therefore occur only in parts of a root-tip meristem. Future experiments will yield additional information on this phenomenon in these somatic hybrids.

The recombinant chromosome of K2H2-1C probably is a Robertsonian translocation chromosome, resulting from concomitant breakage and fusion involving a tomato and a potato chromosome at their centromeres. A protoplast of 7322-H2 containing a fragment or telosome may have been the source of the potato fragment, which in the hybrid cell possibly induced centromere instability of other chromosomes. Shepard et al. (1983) already anticipated translocations between potato and tomato chromosomes in somatic hybrids of these two species, because Shepard (1982) had observed octavalents in PMCs of some potato plants regenerated from protoplasts, which was suggestive of a translocation of genetic information between nonhomologous chromosomes. Indeed, translocated chromosomes were observed in some protoplast-derived potato plants by Creissen and Karp (1985). In K2H2-1C the resulting abnormal chromosome might be one of the causes of irregularities during mitotic anaphase, since this hybrid shows the highest percentage of aberrant anaphases of the four hybrids analyzed. Interestingly, hybrid A7-82A, which is a stable eutriploid with 24 tomato and 12 potato chromosomes and no distinct karyotypic aberrations, shows laggards and bridges at mitotic anaphase, although at a lower frequency than the other, aneuploid, hybrids.

Despite the high percentage of aberrant mitotic anaphases in root tips of the somatic hybrids, chromosome numbers of K2H2-1C, A7-82A and A7-146A have been stable for 1-2 years, while they were propagated by shoot culture in vitro or by cuttings in the greenhouse. An explanation for this can be that shoot meristems show less irregularities during mitosis, and therefore are karyotypically more stable, than root meristems, as was shown by Wilkinson (1992) for potato cultivar "Torridon".

During microsporogenesis of hybrids A7-82A, A7-146A and K2H2-1C many irregularities were observed. Most obvious is the high percentage of FDR in all three hybrids. In solanaceous species cell wall formation only starts after the second meiotic division, thus facilitating formation of dyads. Considerable variation in percentages of fused, parallel and normal spindles at metaphase II/anaphase II was sometimes observed between anthers from buds collected on different days (Table 7). This variability in percentage FDR may depend on the localization of the flower from which the anthers were taken, and may represent epigenetic variation (de Jong, unpublished observations). Veilleux et al. (1982) observed that in two *S. phureja* genotypes the frequency of 2n gametes was highly variable among buds and even among anthers from the same bud.

In anthers of A7-82A and A7-146A more laggards were observed at metaphase I and anaphase I than in anthers of hybrid K2H2-1C. Most probably, this is caused by the different chromosome compositions of the hybrids. In K2H2-1C most potato chromosomes have a homologous partner, whereas in the triploid hybrids A7-82A and A7-146A potato chromosomes can only pair, and possibly recombine, with homoeologous or heterologous chromosomes, or do not recombine at all. The latter possibility seems to be most frequent.

The number of bridges at anaphase I appears to be related to the percentage of aberrant anaphases in mitosis. Hybrid K2H2-1C with the highest percentage of mitotic anaphases containing bridges shows the highest percentage of anaphase I PMCs with bridges, whereas hybrid A7-82A with the lowest percentage of aberrant mitotic anaphases also shows the lowest percentage of anaphase I cells with bridges.

Recombination between chromosomes of potato and tomato seems to occur in part of the PMCs, as is indicated by the putative trivalents at metaphase I of A7-82A and by the quadrivalents at metaphase I in part of the PMCs of K2H2-1C (de Jong et al. 1993). This is in contrast with the observations of Jacobsen et al. (1992), who reported the presence of exclusively bivalents and some univalents at pachytene and metaphase I stages of 14 of the 15 analyzed eutetraploid somatic hybrids of tomato and potato. However, our hybrids do not produce viable pollen and are also female sterile, since no progeny was obtained after crossing. Only when progeny can be obtained, can this recombination be valuable, by facilitating introgression of potato genes into a tomato background, or vice versa.

Acknowledgements. The authors thank C.J. Hanhart for grafting hybrid shoots onto tomato rootstocks, H.M. Verhaar for collecting buds and fixing anthers, and C. Heijting and J. Sybenga for critically reading the manuscript. This work was supported by the Dutch Programme Committee on Agricultural Biotechnology (PcLB).

References

Cardi T, D'Ambrosio F, Consoli D, Puite KJ, Sree Ramulu K (1993) Production of somatic hybrids between frost-tolerant *Solanum commersonii* and *S. tuberosum*: characterization of hybrid plants. Theor Appl Genet 87:193-200

Creissen GP, Karp A (1985) Karyotypic changes in potato plants regenerated from protoplasts. Plant Cell Tissue Organ Cult 4:171-182

Daunay MC, Chaput MH, Sihachakr D, Allot M, Vedel F, Ducreux G (1993) Production and characterization of fertile somatic hybrids of eggplant (*Solanum melongena* L.) with *Solanum* *aethiopicum* L. Theor Appl Genet 85:841-850

Hosticka LP, Hanson MR (1984) Induction of plastid mutations in tomatoes by nitrosomethylurea. J Hered 75:242-246

Jacobsen E, Reinhout P, Bergervoet JEM, de Looff J, Abidin PE, Huigen DJ, Ramanna MS (1992) Isolation and characterization of potato-tomato somatic hybrids using an amylose-free potato mutant as parental genotype. Theor Appl Genet 85:159-164

Jadari R, Sihachakr D, Rossignol L, Ducreux G (1992) Transfer of resistance to *Verticillium dahliae* Kleb. from *Solanum torvum* S.W. into potato (*Solanum tuberosum* L.) by protoplast electrofusion. Euphytica 64:39-47

de Jong JH, Wolters AMA, Kok JM, Verhaar H, van Eden J (1993) Chromosome pairing and potential for intergeneric recombination in some hypotetraploid somatic hybrids of *Lycopersicon esculentum* (+) *Solanum tuberosum*. Genome 36:1032-1041

Koornneef M, Hanhart CJ, Martinelli L (1987) A genetic analysis of cell culture traits in tomato. Theor Appl Genet 74:633-641

Lee FM, Chen CC (1990) Somatic hybridization between *Nicotiana sylvestris* and *N. otophora* without the application of selection. J Hered 81:313-317

Lee M, Phillips RL (1988) The chromosomal basis of somaclonal variation. Ann Rev Plant Physiol Plant Mol Biol 39:413-437

Leitch IJ, Heslop-Harrison JS (1993) Detection of digoxigenin-labeled DNA probes hybridized to plant chromosomes *in situ*. In: Isaac PG (ed) Methods in Molecular Biology, vol. XX: Protocols for Nucleic Acid Analysis by Nonradioactive Probes. Humana Press Inc, Totowa NJ, Chapter 27

Mattheij WM, Eijlander R, de Koning JRA, Louwes KM (1992) Interspecific hybridization between the cultivated potato *Solanum tuberosum* subspecies *tuberosum* L. and the wild species *S. circaeifolium* subsp. *circaeifolium* Bitter exhibiting resistance to *Phytophthora infestans* (Mont.) de Bary and *Globodera pallida* (Stone) Behrens. 1. Somatic hybrids. Theor Appl Genet 83:459-466

Parokonny AS, Kenton AY, Gleba YY, Bennett MD (1992) Genome reorganization in *Nicotiana* asymmetric somatic hybrids analysed by *in situ* hybridization. Plant J 2:863-874

Peschke VM, Phillips RL (1992) Genetic implications of somaclonal variation in plants. Adv Genet 30:41-75

Piastuch WC, Bates GW (1990) Chromosomal analysis of *Nicotiana* asymmetric somatic hybrids by dot blotting and in situ hybridization. Mol Gen Genet 222:97-103

Pijnacker LP, Ferwerda MA (1984) Giemsa C-banding of potato chromosomes. Can J Genet Cytol 26:415-419

Pijnacker LP, Sree Ramulu K (1990) Somaclonal variation in potato: a karyotypic evaluation. Acta Bot Neerl 39:163-169

Pijnacker LP, Ferwerda MA, Puite KJ, Roest S (1987) Elimination of *Solanum phureja* nucleolar chromosomes in *S. tuberosum* + *S. phureja* somatic hybrids. Theor Appl Genet 73:878-882

Pijnacker LP, Ferwerda MA, Puite KJ, Schaart JG (1989) Chromosome elimination and mutation in tetraploid somatic hybrids of *Solanum tuberosum* and *Solanum phureja*. Plant Cell Rep 8:82-85

Puite KJ, Roest S, Pijnacker LP (1986) Somatic hybrid potato plants after electrofusion of diploid *Solanum tuberosum* and *Solanum phureja*. Plant Cell Rep 5:262-265

Pupilli F, Scarpa GM, Damiani F, Arcioni S (1992) Production of interspecific somatic hybrid plants in the genus *Medicago* through protoplast fusion. Theor Appl Genet 84:792-797

Ramanna MS, Prakken R (1967) Structure and homology between pachytene and somatic metaphase chromosomes of the tomato. Genetica 38:115-133

Sass JE (1964) Botanical microtechnique. Iowa State University Press, Ames Iowa, p 228

Schoenmakers HCH, Wolters AMA, Nobel EM, de Klein CMJ, Koornneef M (1993) Allotriploid somatic hybrids of diploid tomato (*Lycopersicon esculentum* Mill.) and monoploid potato (*Solanum tuberosum* L.). Theor Appl Genet 87:328-336

Schoenmakers HCH, Wolters AMA, de Haan A, Saiedi AK, Koornneef M (1994) Asymmetric somtic hybridization between tomato (*Lycopersicon esculentum* Mill.) and gamma-irradiated potato (*Solanum tuberosum* L.): a quantitative analysis. Theor Appl Genet 87:713-720

Schwarzacher T, Heslop-Harrison JS (1993) Direct fluorochrome-labeled DNA probes for direct fluorescent *in situ* hybridization to chromosomes. In: Isaac PG (ed) Methods in Molecular Biology, vol. XX: Protocols for Nucleic Acid Analysis by Nonradioactive Probes. Humana Press Inc, Totowa NJ, Chapter 26

Schwarzacher T, Leitch AR (1993) Enzymatic treatment of plant material to spread chromosomes for *in situ* hybridization. In: Isaac PG (ed) Methods in Molecular Biology, vol. XX: Protocols for Nucleic Acid Analysis by Nonradioactive Probes. Humana Press Inc, Totowa NJ, Chapter 24

Shepard JF (1982) The regeneration of potato plants from leaf-cell protoplasts. Sci Am 246:112-121

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

Sproule A, Donaldson P, Dijak M, Bevis E, Pandeya R, Keller WA, Gleddie S (1991) Fertile somatic hybrids between transgenic *Nicotiana tabacum* and transgenic *N. debneyi* selected by dual-antibiotic resistance. Theor Appl Genet 82:450-456

Sundberg E, Landgren M, Glimelius K (1987) Fertility and chromosome stability in *Brassica napus* resynthesised by protoplast fusion. Theor Appl Genet 75:96-104

Sunderland N (1977) Nuclear cytology. In: Street HE (ed) Plant Tissue and Cell Culture. Blackwell, Oxford, pp 177-205

Taguchi T, Sakamoto K, Terada M (1993) Fertile somatic hybrids between *Petunia hybrida* and a wild species, *Petunia variabilis*. Theor Appl Genet 87:75-80

Veilleux RE, McHale NA, Lauer FI (1982) 2n gametes in diploid *Solanum*: frequency and types of spindle abnormalities. Can J Genet Cytol 24:301-314

de Vries SE, Ferwerda MA, Loonen AEHM, Pijnacker LP, Feenstra WJ (1987) Chromosomes in somatic hybrids between *Nicotiana plumbaginifolia* and a monoploid potato. Theor Appl Genet 75:170-176

Waara S, Pijnacker L. Ferwerda MA, Wallin A, Eriksson T (1992) A cytogenetic and phenotypic characterization of somatic hybrid plants obtained after fusion of two different dihaploid clones of potato (*Solanum tuberosum* L.). Theor Appl Genet 85:470-479

White J, Rees H (1985) The chromosome cytology of a somatic hybrid petunia. Heredity 55:53-59

Wilkinson MJ (1992) The partial stability of additional chromosomes in *Solanum tuberosum* cv. Torridon. Euphytica 60:115-122

Wolters AMA, Schoenmakers HCH, van der Meulen-Muisers JJM, van der Knaap E, Derks FHM, Koornneef M, Zelcer A (1991) Limited DNA elimination from the irradiated potato parent in fusion products of albino *Lycopersicon esculentum* and *Solanum tuberosum*. Theor Appl Genet 83:225-232

Wolters AMA, Vergunst AC, van der Werff F, Koornneef M (1993) Analysis of nuclear and organellar DNA of somatic hybrid calli and plants between *Lycopersicon* spp. and *Nicotiana* spp. Mol Gen Genet 241:707-718

CHAPTER 5

Analysis of nuclear and organellar DNA of somatic hybrid calli and plants between *Lycopersicon* spp. and *Nicotiana* spp.

A.M.A. Wolters, A.C. Vergunst, F. van der Werff, M. Koornneef
Department of Genetics, Wageningen Agricultural University, Dreijenlaan 2,
NL-6703 HA Wageningen, The Netherlands

Published in: Mol Gen Genet (1993) 241:707-718

Summary. Protoplast fusion experiments between *Lycopersicon esculentum* or *L. peruvianum* and *Nicotiana tabacum* or *N. plumbaginifolia* were performed to investigate the possibility of producing symmetric and asymmetric somatic hybrids between these genera. These fusions, which involved 1.7 x 10^8 protoplasts, yielded 35 viable hybrid calli. Plant regeneration was successful with two calli. One of these regenerants flowered, but developed no fruits. Analysis of the nuclear DNA by means of dot blot hybridization with species-specific repetitive DNA probes combined with flow cytometry, revealed that the nuclei of most hybrid calli contained the same absolute amount of *Nicotiana* DNA as the *Nicotiana* parent or (much) less, whereas the amount of *Lycopersicon* DNA per nucleus was 2-5 times that of the parental genotype. Eighteen of the 34 hybrids analyzed possessed *Lycopersicon* chloroplast DNA (cpDNA), whereas the other 16 had DNA from *Nicotiana* chloroplasts. The cpDNA type was correlated with the nuclear DNA composition; hybrids with more than 2C *Nicotiana* nuclear DNA possessed *Nicotiana* chloroplasts, whereas hybrids with 2C or less *Nicotiana* nuclear DNA contained *Lycopersicon* chloroplasts. Mitochondrial DNA (mtDNA) composition was correlated with both nuclear DNA constitution and chloroplast type. Hybrids possessed only or mainly species-specific mtDNA fragments from the parent predominating in the nucleus and often providing the chloroplasts. The data are discussed in relation to somatic incongruity which could explain the low frequency at which hybrids between *Lycopersicon* and *Nicotiana* species are obtained and the limited morphogenetic potential of such hybrids.

Introduction

Somatic hybridization is a technique by which genetic information of sexually incompatible species can be combined. It has often been applied in attempts to achieve (limited) gene or organelle transfer from a donor species to a recipient species. The probability of obtaining viable and fertile somatic hybrid plants is influenced by the phylogenetic distance between the parental species. Although hardly any data are available on the success rate of somatic hybridization between species belonging to different tribes of the same family, intertribal hybrids appear to be difficult to obtain and they are almost exclusively sterile. These problems may be caused by somatic incongruity (reviewed by Harms 1983), resulting from defective nucleo-cytoplasmic interactions or from a defective nucleus-nucleus interaction. In the latter case, spontaneous and/or induced elimination of chromosomes of one of the parents is likely to improve the functioning of the hybrid. However, even when hybrid plants are obtained, it remains questionable whether these can be used in plant breeding programs, since they need to be fertile to achieve further elimination of unfavourable traits and stable introgression of favourable traits by homoeologous recombination or translocation.

We are investigating the possibilities of transferring genetic information from increasingly unrelated solanaceous species to tomato, and the somatic incongruity problems encountered in the fusion experiments. Tomato is a useful model species for the study of these problems because the biosystematics and (cyto)genetics of this species are well known, and tomato and many related solanaceous species are amenable to cell culture. Fertile symmetric hybrids, asymmetric hybrids and/or cybrids have been produced between *Lycopersicon esculentum* and *L. hirsutum* (Derks et al. 1992), *L. pennellii* (Bonnema et al. 1991) and *L. peruvianum* (Wijbrandi et al. 1990a; Ratushnyak et al. 1993), but symmetric somatic hybrids between *L. esculentum* and the least related *Lycopersicon* species *L. chilense* were sterile (Bonnema and O'Connell 1992).

It is much more difficult to obtain fertile somatic hybrids between *L. esculentum* and a *Solanum* species. Fruit set was reported for somatic hybrid plants of *L. esculentum* and *S. lycopersicoides* (Handley et al. 1986), *S. muricatum* (Sakomoto and Taguchi 1991), *S. etuberosum* (Gavrilenko et al. 1992) and *S. tuberosum* (Jacobsen et al. 1992), but only in a few cases were progeny obtained via embryo rescue (Gavrilenko et al. 1992). When cytoplasmic albino *L. esculentum* protoplasts were fused with irradiated protoplasts of *Solanum* spp., true cybrids combining *Solanum* chloroplasts with a *Lycopersicon* nucleus could not be obtained (Wolters et al. 1991, Chapter 2; Derks et al. 1992), which indicates the presence of nucleo-cytoplasmic incongruity between these genera.

Few intertribal fusions have been reported with *Lycopersicon*. Tabaeizadeh et al. (1985; 1989) obtained sterile asymmetric hybrid plants of *L. peruvianum* and *Petunia hybrida*. Negrutiu et al. (1989; 1992) mention the production of 14 highly asymmetric hybrid plants between *Nicotiana plumbaginifolia* and irradiated, kanamycin-resistant *L. esculentum*, which resembled *N. plumbaginifolia*. These hybrids were fertile and kanamycin-resistant progeny were obtained. Melchers et al. (1992) obtained fertile regenerants from ten calli after fusion of iodoacetamide-treated *L. esculentum* protoplasts and gamma-irradiated *N. tabacum* protoplasts, but no genetic information of *N. tabacum* was demonstrated in these plants.

Here we report the production and analysis of intertribal somatic hybrids between *L. esculentum* or *L. peruvianum* and *N. tabacum* or *N. plumbaginifolia*. Symmetric fusions were performed, as well as asymmetric fusions (using gamma-irradiation of the *Nicotiana* protoplasts), to investigate whether elimination of *Nicotiana* nuclear DNA would circumvent a possible nucleus-nucleus incongruity, resulting in a higher frequency of viable (*Lycopersicon*-like) hybrids. We used cell-selectable markers such as complementation of nitrate reductase deficiency and antibiotic resistances for an efficient selection of somatic hybrids. The nuclear, chloroplast and mitochondrial DNA constitution of the hybrids was analyzed to investigate which combination of genetic parameters allowed growth and development of these hybrids between species belonging to different tribes of the *Solanaceae* family.

Materials and methods

Plant materials. Eleven diploid *L. esculentum* genotypes were used as fusion parents: MsK93, which shows a very efficient protoplast regeneration capacity (Koornneef et al. 1986); ATWH1,2,3,4,5,6, which are hygromycin-resistant transformants of MsK93; and LA291, LA1164, LA1166, LA1189, which are homozygous recessive for several morphological marker genes (Rick 1982; Wijbrandi et al. 1990b). The *L. peruvianum* parents were diploid PI128650, and two diploid hygromycin-resistant transformants of this genotype: ATWH101 and ATWH102. The *N. tabacum* parent was SR1HK, a haploid kanamycin-resistant genotype obtained by anther culture of a kanamycin-resistant transformant of SR1 (streptomycin-resistant mutant of cultivar 'Petit Havana'; Maliga et al. 1975). Two *N. plumbaginifolia* genotypes were used: R20-K6 and R20-K10, which are tetraploid kanamycin-resistant transformants of R20 (*cnx*20; Dirks et al. 1985; Negrutiu et al. 1983), a nitrate reductase-deficient mutant. These genotypes can be used as "universal hybridizers". Transformations were carried out according to Koornneef et al. (1987) with the *Agrobacterium tumefaciens* strain LBA 4404 (pAL4404, pAGS112) (van den Elzen et al. 1985) to confer kanamycin resistance, or strain Ach5, containing the plasmid pJW6 (pAGS112, in which the NPTII gene has been replaced by the HPT gene) for hygromycin resistance.

The *L. esculentum*, *L. peruvianum* and *N. tabacum* genotypes were subcultured every 4 weeks on medium containing MS salts (Murashige and Skoog 1962), vitamins according to Tewes et al. (1984), 10 g/l sucrose and

8 g/l agar, supplemented with 100 mg/l kanamycin or 25 mg/l hygromycin for resistant plants. The *N. plumbaginifolia* genotypes were cultured on MSN medium, which is modified MS medium without hormones, containing 20 g/l sucrose and supplemented with 10 mM ammonium succinate, 0.2 mM $Na_2MoO_4.2H_2O$ and 100 mg/l kanamycin. The plants were grown at a light intensity of 10 W/m^2 (Philips TLD 36 W; 16 h) at 25°C.

Isolation, fusion and culture of protoplasts. Leaf mesophyll protoplasts were isolated as described in Wolters et al. (1991, Chapter 2). In some of the experiments *Nicotiana* protoplasts were irradiated with 25, 50, 100, 300 or 1000 Gy of gamma-rays from a ^{60}Co source at a dose rate of approximately 2000 Gy/h. Prior to fusion, *Lycopersicon* and *Nicotiana* protoplasts were mixed to a final density of 1.3-1.8x10^6/ml with a ratio of *Lycopersicon*: *Nicotiana* protoplasts of 2:1, 1:1, 4:3, 3:2, 2:1, 3:1, 4:1 or 8:1. Electrofusion was carried out as described in Wolters et al. (1991, Chapter 2) with the following modifications. The protoplasts were aligned in an alternating current (AC) field of 75-100 V/cm and one direct current (DC) pulse of 1500 V/cm (30-50 μs) was applied.

The protoplasts were cultured at densities of 0.6-0.9x10^6/ml in TMP medium, which is a modified TM-2 medium (Shahin 1985) containing 103 g/l sucrose, in the dark at 25°C. When the first cell divisions were observed (ca 7 days after fusion) the cultures were exposed to dim light and diluted 1:1 with TMP medium. When microcalli had developed the cultures were again diluted 1:1 in TMP medium and solidified with 0.6% (final concentration) agarose (SeaPlaque, FMC BioProducts, USA). Two to three weeks after fusion, the agarose was cut into four pieces and transferred to a 9-cm diameter petri dish containing 2 ml TMD medium [modified TM-2 medium containing 100 mg/l myo-inositol, 0.3 M sucrose and 0.1 mg/l naphthaleneacetic acid (NAA)], with 50 mg/l kanamycin and 12.5 mg/l hygromycin. During the next week, 4 ml TMD medium plus antibiotics was added twice. Subsequently the liquid medium was replaced once a week with TMD medium plus antibiotics until calli with a diameter of approximately 3 mm were obtained (ca 7 weeks after fusion). Calli of this size were transferred to solid TMcμ medium (modified TM-3 medium (Shahin 1985) containing 2.5 g/l sucrose, 36.4 g/l mannitol, 0.1 mg/l NAA instead of 2,4-dichlorophenoxyacetic acid, 0.5 mg/l 6-benzylaminopurine and 8 g/l purified agar) supplemented with 100 mg/l kanamycin and 25 mg/l hygromycin. Every 4 weeks the calli were subcultured on new medium. Eleven to 12 months after fusion, green calli were transferred to 1Z medium (modified 2Z medium (Thomas and Pratt 1981) with 1 instead of 2 mg/l zeatin) with the aforementioned antibiotics, for shoot induction.

In some fusion experiments in which R20-K6 or R20-K10 were used as universal hybridizers, selection was carried out in TMP and TMD medium in which L-glutamine, adenine sulfate and casein hydrolysate were omitted (to select for nitrate reductase-expressing calli), supplemented with 50 mg/l kanamycin.

Shoot regeneration and transfer of plants to the greenhouse. The shoot-like structures that developed on several hybrid calli were transferred to solid MS medium (Murashige and Skoog 1962) without the described hormones, but with 0.1 mg/l NAA for 3 days for root induction, after which they were placed on MS20 medium (MS medium without hormones, with 20 g/l sucrose and 8 g/l agar). On this medium some shoots developed roots. These plants were transferred to the greenhouse where some shoots were grafted onto tomato rootstocks.

Chromosome analysis. Chromosome preparations were made according to Pijnacker and Ferwerda (1984) with some minor modifications. Root tips were collected from the regenerants and their parents, incubated in 2 mM 8-hydroxyquinoline for 4 h at 17°C and afterwards fixed in cold Carnoy's solution (ethanol:glacial acetic acid, 3:1) for 18 h or more at 4°C. The fixed root tips were rinsed in water and subsequently incubated in 10 mM citric acid-sodium citrate buffer pH 4.2-4.5 containing 0.1% each of cellulase RS, pectolyase Y23 and cytohelicase enzymes for 1 h at 37°C. Then the enzyme solution was carefully removed and water was added. Root meristems were transferred to a slide cleaned in 70% ethanol, excess water was removed and the cells were separated with the aid of fine needles. A small drop of 60% acetic acid was added in which the cells were suspended. Then the suspension was surrounded with ice-cold Carnoy's solution, after which one drop of Carnoy's solution was put on top of the suspension. The slide was air dried for approximately 1 h. For staining, the slides were first incubated for 15 min in 2 x SSC at 60°C and subsequently rinsed for 1-2 min in water. The chromosomes were stained in 2% Giemsa in 10 mM Sörensen's phosphate buffer pH 6.8 for 30 min at room temperature, rinsed in Sörensen's buffer and water, air-dried and mounted in Entellan with a 24 x 50 mm cover slip.

Dot blot analysis and flowcytometry. In order to determine the percentage of

Nicotiana nuclear DNA in the fusion products, dot blot analyses were performed. Total DNA from the fusion parents was isolated according to Rogers and Bendich (1988) after which the DNA was purified on a cesium chloride gradient. Total DNA from the hybrid calli was isolated using the method of Mettler (1987). Eight concentrations, ranging from 0 ng up to 300 or 600 ng, of the *Lycopersicon* fusion parent and of the *Nicotiana* parent, together with 300-500 ng DNA from fusion calli, were applied to a Gene Screen Plus (GS^+) membrane (DuPont) using the Hybridot 96-well filtration manifold from Gibco/BRL, as described in Wolters et al. (1991, Chapter 2). Two identical filters were prepared for every analysis. One was probed with pTHG2, a tomato-specific repetitive DNA probe (Zabel et al. 1985). The insert of pTHG2 represents a moderately repetitive DNA fragment that is evenly dispersed on all tomato chromosomes. The other filter was probed with H10 when hybrids with *N. tabacum* were analyzed, or with pCF1 when hybrids with *N. plumbaginifolia* were involved. H10 is a plasmid containing a moderately repetitive DNA fragment from tobacco (Saul and Potrykus 1984). *In situ* hybridization on tobacco SR1 chromosomes showed that many chromosomes contain this repetitive element (Mouras et al. 1987). pCF1 is a plasmid containing a highly repetitive DNA fragment from *N. plumbaginifolia* (Marchesi et al. 1989).

Probes were radioactively labeled using the Boehringer-Mannheim Random Primed DNA Labeling kit. Hybridization was performed in glass bottles using a Hybaid hybridization oven at 65°C for 16 h. The blots were rinsed as described in Wolters et al. (1991, Chapter 2). Autoradiography was performed on Konica X-ray films. Dots were cut out of the filters and radioactivity per dot was measured in a Tri-Carb liquid scintillation analyzer (Packard). With the data obtained, calibration plots were constructed showing the relationship between the amount of *Lycopersicon* or *Nicotiana* nuclear DNA and the amount of radioactivity. By means of these calibration plots the amount of *Lycopersicon* nuclear DNA and the amount of *Nicotiana* nuclear DNA per hybrid dot could be calculated. From these results the percentage of nuclear DNA originating from *Nicotiana* was determined for each hybrid. The analysis was repeated three or four times for each hybrid. The average difference between the three or four calculations of the percentage *Nicotiana* DNA in the nuclei of individual hybrid calli was 4.7%.

In order to determine the total amount of DNA per nucleus for each somatic hybrid, flow cytometry was performed on the calli, using 4,6-diamidino-2-phenylindole (DAPI) staining of the DNA, by Plant Cytometry Services (Schijndel, The Netherlands). Calf thymocytes were included as a standard, in order to make all measurements comparable.

Analysis of chloroplast and mitochondrial DNA. Total DNA from the somatic hybrids was isolated from 0.5 g callus by a modified procedure of Dellaporta et al. (1983) as described in Wolters et al. (1991, Chapter 2). For the characterization of chloroplast DNA (cpDNA) the *Petunia* probes pPCY64 and P4 (*Pst*I fragment 4; de Haas et al. 1986) were used. The following probes were used for mitochondrial DNA (mtDNA) analysis: the 14.7-kb *Bam*HI fragment of the maize 26S rDNA gene (Dale et al. 1984), the 0.45-kb *Eco*RI-*Sal*I fragment of the *Pcf* gene (Young and Hanson 1987) and the 2.2-kb *Xba*I fragment of the maize ATPase subunit 9 gene (Dewey et al. 1985). In order to isolate inserts from the plasmids the correct restriction fragment was cut out of an agarose gel and the DNA retrieved using the Prep-A-Gene DNA purification kit from Bio-Rad. Five micrograms of total DNA from parents and fusion products were digested with *Bam*HI or *Hin*fI and run in 0.6% or 0.8% agarose gels, respectively. The DNA fragments were alkali-blotted onto GS^+ membranes. Hybridization was performed as described for the dot blots. The blots were rinsed to a stringency of 1 x SSC, 1% SDS. After autoradiography the probe was removed from the blots according to the supplier's instructions and the membranes were re-used.

Results

Selection of hybrids

Fifty-six fusion experiments between *L. esculentum* or *L. peruvianum* protoplasts and *N. tabacum* or *N. plumbaginifolia* protoplasts were performed. In these experiments we used five different doses of gamma-irradiation of the *Nicotiana* parent (with the intention of obtaining asymmetric hybrids) and eight different ratios of *Lycopersicon:Nicotiana* protoplasts, mostly with *Lycopersicon* protoplasts in excess. Many experiments failed to yield hybrids (Table 1), although an efficient selection system was used and parental protoplasts grew vigorously in all combinations, except the LA genotypes of tomato and the irradiated parents. Six months after fusion, 126 calli were obtained out of 10 of the 56 fusion experiments. Details of these successful experiments are presented in Table 2. Eighteen months after fusion, 35 calli were still growing well. Most of these (32) originated from symmetric fusions (four combinations: A, C, D, E). The other three were derived from a single asymmetric fusion experiment in which the *N. plumbaginifolia* protoplasts had been irradiated with 300 Gy of gamma-rays (B).

Table 1. Number of fusion experiments between *Lycopersicon peruvianum* (*L. per.*) or *L. esculentum* (*L. esc.*) protoplasts and untreated (- irr.) or gamma-irradiated (+ irr.) protoplasts of *Nicotiana plumbaginifolia* (*N. plum.*) or *N. tabacum* (*N. tab.*)

Fusion combination	Total no. of protoplasts	No. of experiments		No. of successful experiments[a]	
		- irr.	+ irr.	- irr.	+ irr.
L. per. (+) *N. plum.*	1.4 x 10^7	2	2	2	1
L. per. (+) *N. tab.*	1.9 x 10^7	2	4	0	0
L. esc. (+) *N. plum.*	3.4 x 10^7	8	6	4	0
L. esc. (+) *N. tab.*	10.4 x 10^7	8	24	3	0

[a] Experiments from which hybrid calli were obtained which survived for at least 6 months

Table 2. Details of the fusion experiments between *L. peruvianum* or *L. esculentum* genotypes and *N. plumbaginifolia* or *N. tabacum* genotypes from which hybrid calli were obtained

Fusion combination			Dose of gamma irr. (Gy)	No. of pp[a] (x 10^6)	Ratio[b]	Selection[c]	No. of calli 6 months after fusion	No. of calli 18 months after fusion[d]	No. of regenerating calli
L. per.	(+)	*N. plum.*							
ATWH101	(+)	R20-K10	0	6.0	2:1	Hm^R, Km^R	45	23 (A)	–
ATWH102	(+)	R20-K10	300	3.0	2:1	Hm^R, Km^R	5	3 (B)	1
PI128650	(+)	R20-K10	0	2.4	2:1	cnx$^+$, Km^R	2	1 (C)	–
L. esc.	(+)	*N. plum.*							
LA1189	(+)	R20-K6	0	3.6	2:1	cnx$^+$, Km^R	9	–	–
LA1166	(+)	R20-K6	0	2.4	2:1	cnx$^+$, Km^R	12	–	–
MsK93	(+)	R20-K6	0	1.5	4:1	cnx$^+$, Km^R	1	–	–
ATWH4	(+)	R20-K6	0	1.6	3:1	Hm^R, Km^R	10	2 (D)	–
L. esc.	(+)	*N. tab.*							
ATWH3	(+)	SR1HK	0	1.6	1:1	Hm^R, Km^R	22	–	–
ATWH4	(+)	SR1HK	0	7.0	4:3	Hm^R, Km^R	12	6 (E)	1
ATWH5	(+)	SR1HK	0	4.8	1:1	Hm^R, Km^R	8	–	–

[a] pp = protoplasts

[b] ratio of *Lycopersicon:Nicotiana* protoplasts during fusion

[c] selection for hygromycin resistance (Hm^R), kanamycin resistance (Km^R) or complementation of nitrate reductase deficiency (cnx$^+$)

[d] A, B, C, D, E, designation of fusion experiments

Thirteen green and firm calli developed shoot primordia (Table 3). On four hybrid calli these turned into shoots (with a stem and leaves) and two regenerants could be induced to form roots. One was a *L. peruvianum* (+) *N. plumbaginifolia* hybrid from an asymmetric fusion experiment (B3) and the other a *L. esculentum* (+) *N. tabacum* hybrid from a symmetric fusion experiment (E3). The B3 regenerant was successfully transferred to the greenhouse, but grew slowly. When grafted onto tomato rootstocks the plants grew more vigorously. The leaves showed a morphology similar to those of *L. peruvianum* (Fig. 1A), but were thicker, smaller and more blueish green. Trichomes on the leaves were similar to those from *L. peruvianum*. Flower buds were frequently formed, but abscission occurred at an early stage. Only during a short period of 2 weeks did the flowers develop fully (Fig. 1B). These were yellow and resembled *L. peruvianum* flowers. No functional pollen was formed and pollination of the flowers with *L. peruvianum* pollen did not result in fruit set. The E3 regenerant could only be grown in vitro. This slowly growing hybrid plant had a *L. esculentum*-like leaf morphology (Fig. 1C), with irregularly serrated leaves that were much smaller than *L. esculentum* leaves and with relatively short internodes.

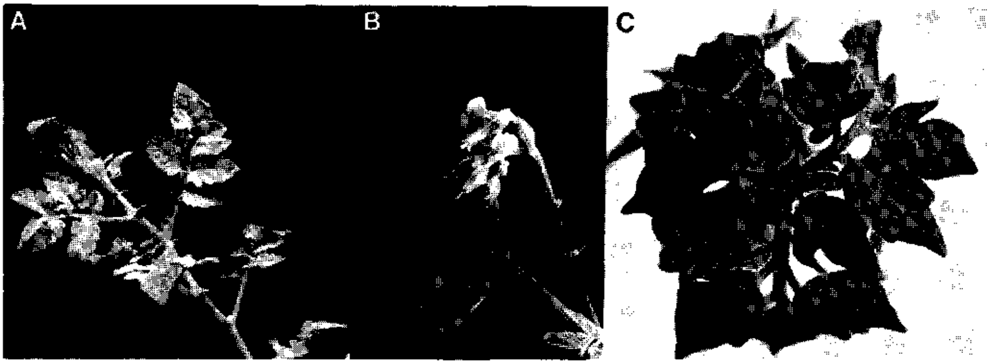

Fig. 1A-C. Morphology of leaves **(A)** and flowers **(B)** of *Lycopersicon peruvianum* (+) *Nicotiana plumbaginifolia* regenerant B3 and morphology of the *L. esculentum* (+) *N. tabacum* regenerant E3 **(C)**

Analysis of the nuclear composition of the hybrid calli

Chromosome preparations were made of root tip meristems of the two regenerants and their fusion parents (Fig. 2).

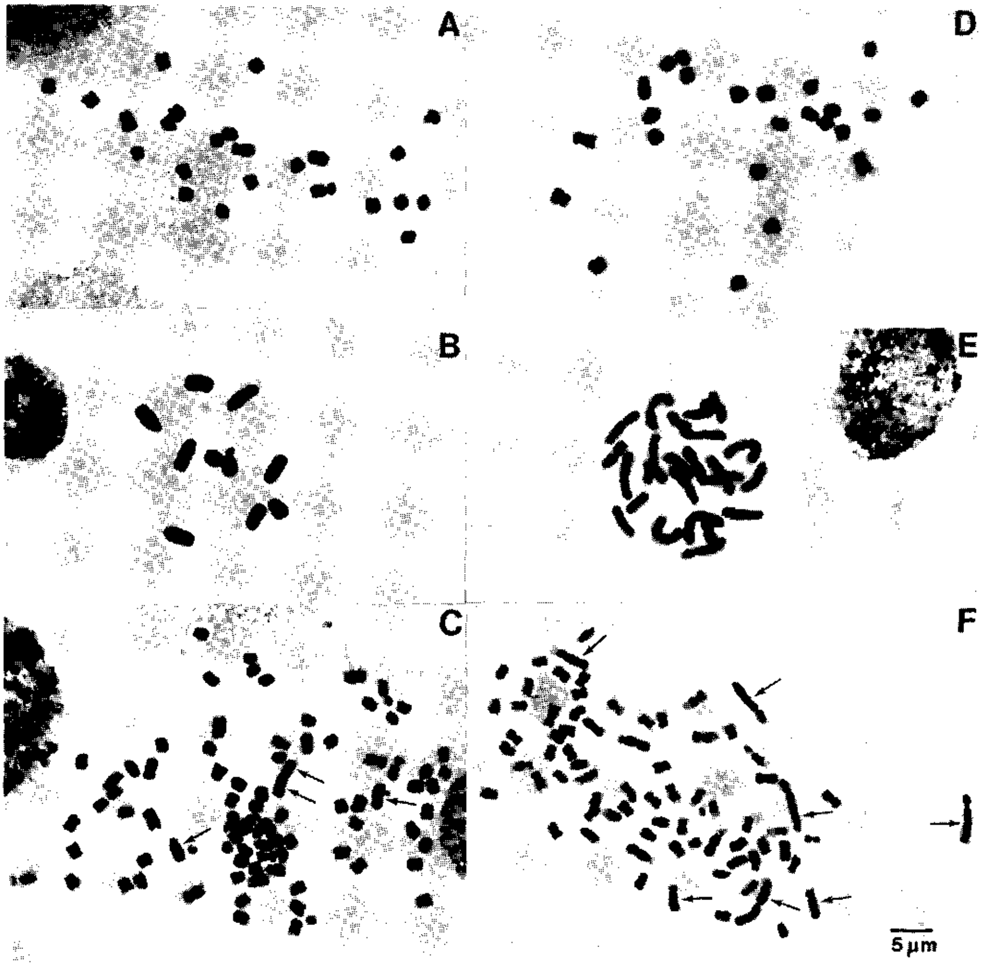

Fig. 2A-F. Chromosomes of **A**, diploid *L. peruvianum* PI128650 (2n=24); **B**, haploid *N. plumbaginifolia* (n=10); **C**, hybrid *L. peruvianum* (+) *N. plumbaginifolia* B3; **D**, diploid *L. esculentum* MsK93 (2n=24); **E**, haploid *N. tabacum* SR1 (n=24) and **F**, hybrid *L. esculentum* (+) *N. tabacum* E3. *Arrows* in **C** and **F** indicate *Nicotiana* chromosomes

Diploid *L. peruvianum* and *L. esculentum* both contain 24 small, metacentric or submetacentric chromosomes with the chromosomes 2 showing a distinct satellite (Fig. 2A, D). Haploid *N. plumbaginifolia* (Fig. 2B) has 10 (sub)telocentric chromosomes with one satellite chromosome

(chromosome 9, Mouras et al. 1986). These chromosomes are larger than the *Lycopersicon* chromosomes. This is also the case with the 24 chromosomes of haploid *N. tabacum* (Fig. 2E) which are metacentric to subtelocentric. In Fig. 2C, part of the chromosomes complement of the regenerant of hybrid B3 is shown. This hybrid has approximately 96 *L. peruvianum* chromosomes (8C) and 4-6 *N. plumbaginifolia* chromosomes. A very large metacentric chromosome was found in most cells. Most probably this chromosome is the result of a Robertsonian translocation between two telocentric *N. plumbaginifolia* chromosomes, induced by irradiation of the *Nicotiana* parent. Chromosome preparations made of the original regenerant of hybrid E3 (*L. esculentum* (+) *N. tabacum*) and of the second adventitious shoot showed approximately 72 *L. esculentum* chromosomes (6C) and 12-16 *N. tabacum* chromosomes. Preparations of the seventh adventitious shoot, however, showed a significant decrease in the number of tobacco chromosomes: the root cells of the analyzed plant contained a mean number of 66 tomato chromosomes and 7-8 tobacco chromosomes (Fig. 2F).

No attempts were made to obtain chromosome preparations of the other hybrids, of which only calli were available. The relative DNA content of each parental species in these hybrids was determined by dot blot analysis of two identical filters, each hybridized with a species-specific repetitive DNA probe, as shown in Fig. 3. The mean percentage of nuclear DNA originating from *Nicotiana* calculated for each hybrid is presented in Table 3. The total amount of DNA per nucleus of the hybrids was analyzed by flow cytometry. Frequency distributions of the nuclear DNA content of the E hybrids and their parents are shown in Fig. 4. Most hybrid calli showed one dominant G1-peak, which indicates the relative homogeneity of these calli. The broad peaks, however, signify that individual cells in a hybrid callus differed slightly from each other in DNA content. When for the amount of DNA per nucleus of diploid tomato the reported 2.0 pg (Arumuganathan and Earle 1991) is taken as a reference, in our measurements a 2C *L. peruvianum* nucleus contains 2.3 pg DNA, a 2C *N. plumbaginifolia* nucleus 4.4 pg and a 2C *N. tabacum* nucleus 8.6 pg. These values are close to those reported by Arumuganathan and Earle (1991). Similarly, the total amount of DNA per nucleus was determined for each hybrid (Table 3).

Combining the results of the dot blot analyses and the flow cytometric analyses, the absolute amount (in pg) of *Lycopersicon* DNA and of *Nicotiana* DNA per nucleus was calculated for all hybrids (Table 3). Using the aforementioned C values (amount of DNA per haploid genome) for *L. esculentum*, *L. peruvianum*, *N. plumbaginifolia* and *N. tabacum*, we also

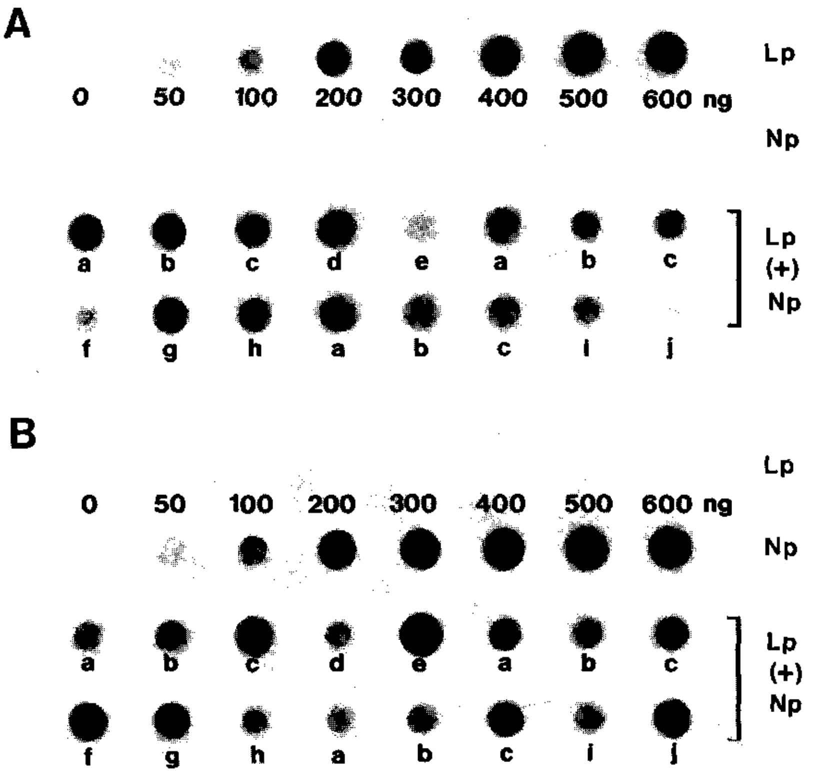

Fig. 3A,B. Dot blot analysis of hybrid calli. Concentration series of total DNA of *L. peruvianum* and of *N. plumbaginifolia* were applied to two identical dot blots in addition to 400-500 ng total DNA of hybrid A3 (**a**), A5 (**b**), A19 (**c**), A8 (**d**), A15 (**e**), A17 (**f**), A18 (**g**), A20 (**h**), A22 (**i**) and A23 (**j**). **A** blot probed with *Lycopersicon*-specific repetitive DNA probe pTHG2. **B** blot probed with pCF1, a *N. plumbaginifolia*-specific repetitive DNA probe

calculated the ploidy level of *Lycopersicon* and *Nicotiana* per hybrid (presented as C numbers) and the ratio of these ploidy levels, as a measure of symmetry (Table 3).

The calculated ploidy levels of both parents in regenerants B3 and E3 are in agreement with the results from the cytogenetic analysis. This indicates that dot blot hybridization with the probes used here, combined with flow cytometry, is a valid means of determining the nuclear composition of the hybrids. All putative hybrids tested contained nuclear DNA from both fusion parents, thus confirming the hybrid nature of these calli.

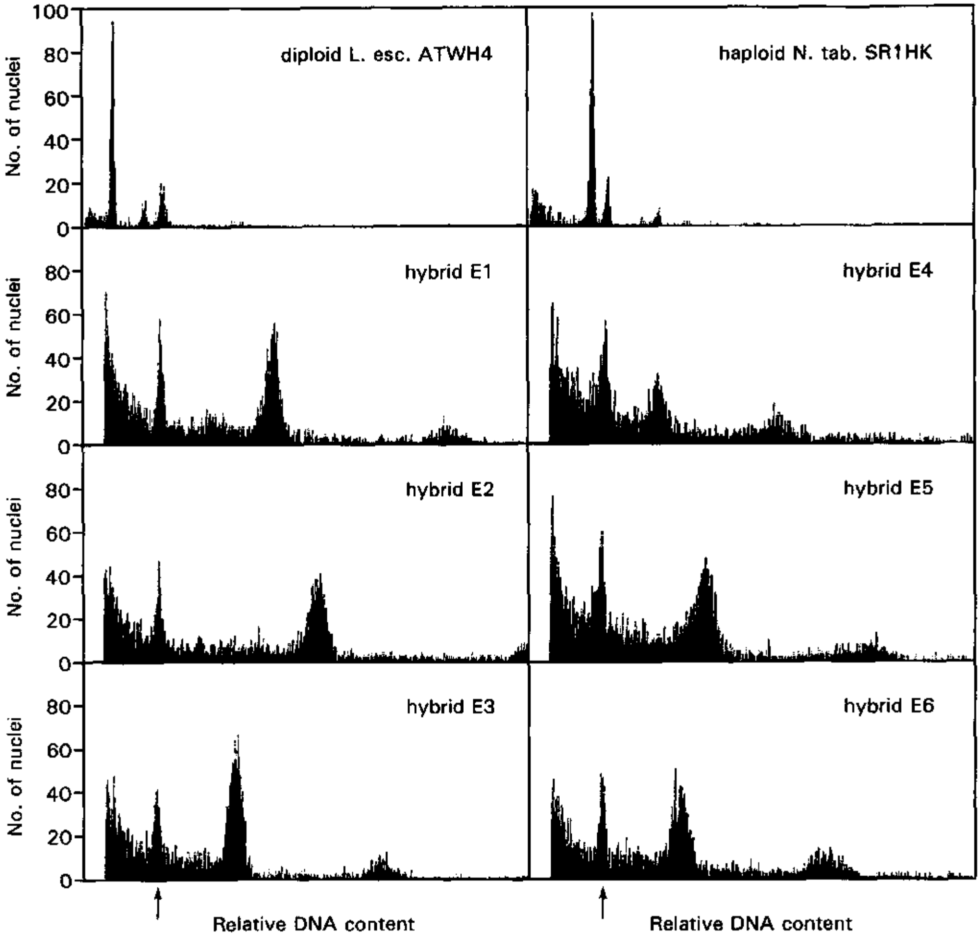

Fig. 4. Flow cytometric analysis of DNA content per nucleus of diploid *L. esculentum* ATWH4, haploid *N. tabacum* SR1HK and somatic hybrids of these species, E1-E6. *Arrows* indicate peaks of the standard, consisting of calf thymocytes

The hybrids differed greatly from each other, as shown by the wide range in percentage nuclear DNA originating from *Nicotiana* (15-69%) and the large differences in total nuclear DNA amount (ranging from 7.2 to 20.9 pg). No indications for stabilization of the genome composition around euploid chromosome numbers of one or both of the parents were found.

Table 3. Nuclear and organellar DNA composition of *Lycopersicon* (+) *Nicotiana* somatic hybrids

Genotype[a]	Regeneration[b]	Mean % N[c]	pg DNA per nucleus	*Lyc.* DNA in pg (C)[d]	*Nic.* DNA in pg(C)[d]	Ratio L/N[e]	cpDNA[f]	mtDNA fragments[g]			
								C	N	L	R
L.per.			2.3	(2)	–	–	L	3	–	5	0
N.plum.			8.8	–	(4)	–	N	3	7	–	0
A 1	–	57	13.4	5.8 (5.1)	7.6 (3.5)	1.5	N	3	7	3	0
2	–	59	18.2	7.5 (6.6)	10.8 (4.9)	1.3	N	3	7	1	0
3	–	28	10.3	7.4 (6.5)	2.9 (1.3)	5.0	L	3	3	4	3
4	–	57	ND	ND	ND	–	N	2	4	2	2
5	–	36	10.0	6.4 (5.6)	3.6 (1.6)	3.5	L	3	3	4	0
6	–	56	16.5	7.3 (6.4)	9.3 (4.3)	1.5	N	2	3	3	3
7	–	58	18.8	7.9 (6.9)	10.9 (5.0)	1.4	N	3	6	0	2
8	*	19	9.3	7.5 (6.6)	1.8 (0.8)	8.3	L	3	1	4	5
9	–	58	16.8	7.0 (6.2)	9.7 (4.5)	1.4	N	2	5	2	0
10	–	54	16.1	7.4 (6.5)	8.7 (4.0)	1.6	N	2	5	1	0
11	–	63	19.5	7.2 (6.3)	12.3 (5.6)	1.1	N	3	3	3	5
12	–	57	15.2	6.5 (5.7)	8.7 (4.0)	1.4	N	2	5	0	1
13	–	56	14.1	6.2 (5.4)	7.9 (3.6)	1.5	N	1	5	1	2
14	–	64	ND	ND	ND	–	ND	ND	ND	ND	ND
15	–	69	10.5	3.3 (2.9)	7.2 (3.3)	0.9	N	3	6	0	0
			20.9	6.5 (5.7)	14.4 (6.6)	0.9					
16	–	50	8.1	4.1 (3.6)	4.1 (1.9)	1.9	L	2	5	3	1
17	–	59	10.9	4.5 (3.9)	6.4 (3.0)	1.3	N	2	4	2	2
18	–	46	9.4	5.1 (4.4)	4.3 (2.0)	2.2	L	3	6	1	0
19	–	47	11.5	6.1 (5.4)	5.4 (2.5)	2.2	N	2	6	0	1
20	**	33	9.5	6.4 (5.6)	3.1 (1.4)	4.0	L	ND	ND	ND	ND
21	–	54	19.5	9.0 (7.9)	10.5 (4.8)	1.6	N	3	6	0	0
22	*	38	11.6	7.2 (6.3)	4.4 (2.0)	3.2	L	2	4	4	3
23	*	62	12.6	4.8 (4.2)	7.8 (3.6)	1.2	N	3	6	3	0
B 1	*	15	8.8	7.5 (6.6)	1.3 (0.6)	11.0	L	3	2	4	3
2	*	17	10.4	8.6 (7.6)	1.8 (0.8)	9.5	L	3	3	5	0
3	***	16	11.2	9.6 (8.3)	1.7 (0.8)	10.4	L	2	1	5	3
C 1	–	63	ND	ND	ND	–	N	3	4	1	1
L.esc.			2.0	(2)	–	–	L	3	–	5	0
N.plum.			8.8	–	(4)	–	N	3	7	–	0
D 1	–	41	10.9	6.4 (6.4)	4.5 (2.0)	3.2	L	3	4	3	1
2	*	22	7.2	5.6 (5.6)	1.6 (0.7)	8.0	L	2	4	4	4
			12.3	9.6 (9.6)	2.7 (1.2)	8.0					
L.esc.			2.0	(2)	–	–	L				
N.tab.			4.3	–	(1)	–	N				
E 1	*	32	9.4	6.4 (6.4)	3.0 (0.7)	9.1	L	ND	ND	ND	ND
			13.4	9.1 (9.1)	4.3 (1.0)	9.1					
2	*	36	16.7	10.7 (10.7)	6.0 (1.4)	7.6	L	ND	ND	ND	ND
3	***	36	9.6	6.1 (6.1)	3.4 (0.8)	7.6	L	ND	ND	ND	ND
4	*	38	9.2	5.7 (5.7)	3.5 (0.8)	7.1	L	ND	ND	ND	ND
5	–	36	13.4	8.6 (8.6)	4.8 (1.1)	7.8	L	ND	ND	ND	ND
6	**	37	11.3	7.1 (7.1)	4.2 (1.0)	7.1	L	ND	ND	ND	ND

[a] Hybrids obtained from fusion combinations A, B, C, D and E, described in Table 2

[b] *, hybrid showing shoot primordia; **, hybrid developing shoots; ***, hybrid developing shoots, which could subsequently be rooted

[c] Mean percentage nuclear DNA derived from the *Nicotiana* parent

[d] Absolute amount per nucleus in picograms (ploidy level in C)

[e] C value *Lycopersicon* divided by C value *Nicotiana*

[f] cpDNA, chloroplast DNA

[g] C, common; N, *Nicotiana*-specific; L, *Lycopersicon*-specific; R, new mitochondrial DNA (mtDNA) fragments

ND, not determined

Hybrids B1-B3, obtained from a fusion experiment in which the *Nicotiana* parent was irradiated, proved to be highly asymmetric, containing less than 1C *N. plumbaginifolia* DNA and more than 6C *L. peruvianum* DNA. Remarkably, from symmetric fusion experiments some highly asymmetric hybrids were also obtained, e.g. A3, A8, D2 and all six E hybrids. They all contained a low amount of *Nicotiana* nuclear DNA and a (very) large amount of *Lycopersicon* nuclear DNA. No hybrids with a small amount of *Lycopersicon* nuclear DNA were present. Only hybrid A15 had a smaller C value (i.e. a lower ploidy level) for *Lycopersicon* than for *Nicotiana*.

Analysis of the organellar DNA of the hybrid calli

The cpDNA type of the A, B, C and D hybrid calli was determined by hybridizing blots containing *Bam*HI-digested total DNA with the two cpDNA-specific probes pPCY64 and P4 (Fig. 5A). The chloroplast type determined for a hybrid with the first hybridization was in all cases confirmed by the second hybridization. The cpDNA type of the E hybrids was determined by hybridizing a blot containing *Hin*fI-digested total DNA with cpDNA probe pPCY64. The results are shown in Table 3. Each hybrid showed only cpDNA bands specific for one of the parental chloroplast types; no mixtures of fragments or rearrangements were found. Seven of the A hybrids possessed *Lycopersicon* chloroplasts and 15 contained *Nicotiana* chloroplasts. The C hybrid possessed *Nicotiana* chloroplasts. All B, D and E hybrids contained *Lycopersicon* chloroplasts.

The mtDNA composition of the A, B, C and D hybrids was analyzed by hybridizing the same blots that were used for cpDNA analysis with three mtDNA-specific probes: 26S rDNA, *Atp*9 and *Pcf* (Fig. 5B). When the 26S rDNA probe was hybridized to *Bam*HI-digested total DNA of *Lycopersicon* and of *Nicotiana*, not only mtDNA fragments carrying the 26S rRNA gene were recognized, but also a 6.0-kb *Lycopersicon* cpDNA fragment (Hause et al. 1986) and a 7.1-kb *Nicotiana* cpDNA fragment containing part of the 23S rRNA gene (see Wolters et al. 1993, Chapter 6). The cpDNA polymorphism detected by this mtDNA probe was scored in all A, B, C and D hybrids; the results were in agreement with those obtained with the cpDNA probes. The *Pcf* gene is a fused mitochondrial gene present in cytoplasmic male sterile *Petunia* (Young and Hanson 1987). The *Eco*RI/*Sal*I fragment of this gene, which was used as a probe, consists of 119 bp from the gene encoding ATP synthase subunit 9 (*atp9-1*) and 314 bp from the gene encoding cytochrome oxidase subunit II (*cox*II). Therefore, several mtDNA fragments hybridizing with the *Atp9* probe are also detected with the *Pcf* probe.

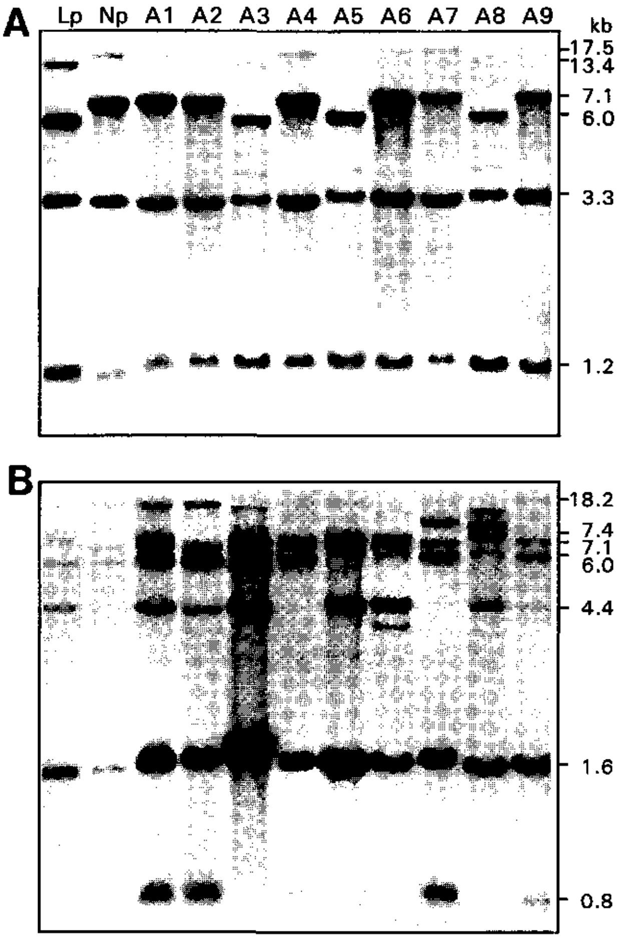

Fig. 5A,B. Analysis of chloroplast (cp) and mitochondrial (mt) DNA composition of *L. peruvianum* (+) *N. plumbaginifolia* hybrids. Autoradiograms of a blot containing *Bam*HI-digested total DNA of *L. peruvianum* (lane 1), *N. plumbaginifolia* (lane 2) and hybrids A1-A9 (lanes 3-11), hybridized with cpDNA probe P4 (**A**) and mtDNA probe *Pcf* (**B**). Sizes of bands are indicated in kb

Hybrid calli contained only *Nicotiana*-specific mtDNA fragments or a mixture of *Lycopersicon*- and *Nicotiana*-specific fragments (e.g. A1 in Fig. 5B). Many hybrid calli contained mtDNA fragments that were not present in either of the two parents, e.g. A3 and A7 in Fig. 5B. For each hybridization used in the mtDNA analysis, the numbers of common bands (bands of a particular size that are present in both parental species; C), *Nicotiana*-specific fragments (N), *Lycopersicon*-specific fragments (L) and new fragments (R) per hybrid were counted. The total number of fragments counted in the three hybridizations, excluding the cpDNA bands hybridizing with the 26S rDNA probe, is listed in Table 3 for each hybrid and for the parents. The fragments containing (part of) the *Atp9* gene were only counted once.

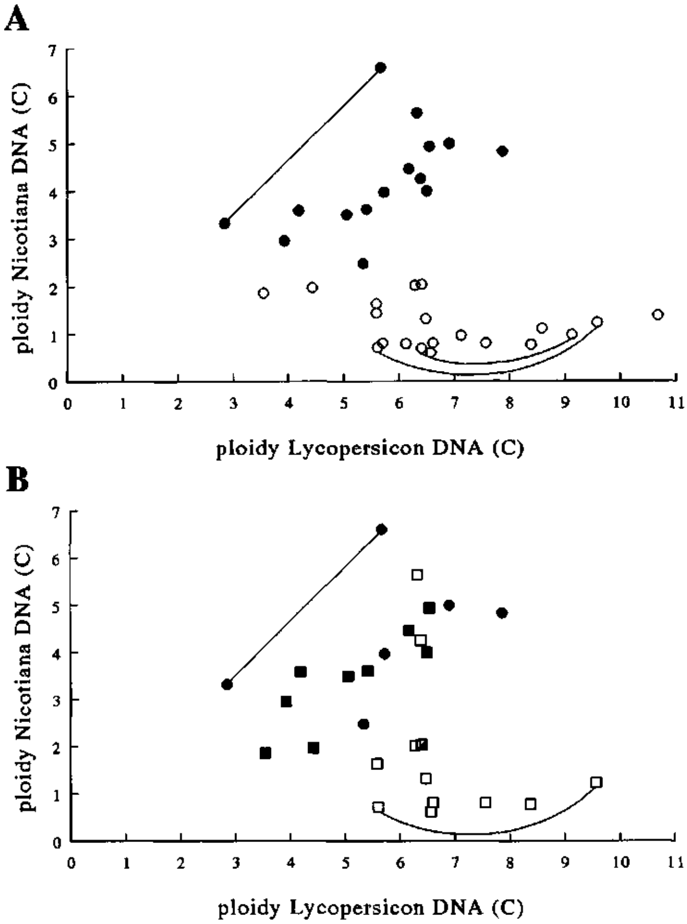

Fig. 6A,B. Correlation between nuclear DNA composition and chloroplast DNA type (**A**) or mitochondrial DNA composition (**B**) of the hybrids. Calculated ploidy level (indicated in C) of the *Lycopersicon* parent on the X-axis and of the *Nicotiana* parent on the Y-axis. In **A** ● indicates hybrids with *Nicotiana* chloroplasts, and ○ indicates hybrids with *Lycopersicon* chloroplasts. In **B** ● indicates hybrids with only *Nicotiana*-specific and new mtDNA fragments; ■ hybrids with a higher fraction of the *Nicotiana*-specific than of the *Lycopersicon*-specific mtDNA fragments; ◪ hybrids with equal fractions of *Nicotiana*-specific and of *Lycopersicon*-specific mtDNA bands and □ hybrids with a higher fraction of the *Lycopersicon*-specific than of the *Nicotiana*-specific mtDNA fragments. *Lines* connect subcalli of a hybrid with different nuclear DNA compositions

Correlation between nuclear DNA composition and organellar DNA composition

The data on genome composition of each hybrid were combined with the data on cpDNA type or on mtDNA composition in the scatter diagrams shown in Figure 6. In Fig. 6A a strong correlation is apparent between nuclear DNA composition and cpDNA type. All hybrids with more than 2C *Nicotiana* nuclear DNA possessed *Nicotiana* chloroplasts, while all hybrids with 2C or less nuclear DNA from *Nicotiana* contained *Lycopersicon* chloroplasts. In Fig. 6B a similar correlation, although not as perfect as in Fig. 6A, can be seen between nuclear DNA and mtDNA composition. Five hybrids showed only *Nicotiana*-specific (and new) mtDNA fragments; these possessed more than 2C *Nicotiana* nuclear DNA and *Nicotiana* chloroplasts. Hybrids with a higher fraction of the *Nicotiana*-specific than of the *Lycopersicon*-specific mtDNA fragments contained 2C or more *Nicotiana* nuclear DNA and most of them also had *Nicotiana* chloroplasts. The hybrids with a higher fraction of the *Lycopersicon*-specific than of the *Nicotiana*-specific mtDNA bands possessed more than 5C *Lycopersicon* nuclear DNA. All except two contained 2C or less *Nicotiana* nuclear DNA and *Lycopersicon* chloroplasts. The deviating hybrids A6 and A11 both showed three of the seven *Nicotiana*-specific and three of the five *Lycopersicon*-specific mtDNA fragments and displayed, respectively, three and five non-parental fragments.

Discussion

We have performed many fusion experiments between species of the genera *Lycopersicon* and *Nicotiana*, and ultimately obtained 35 somatic hybrid calli. These resulted from experiments involving 2.0×10^7 protoplasts. Thus the frequency of hybrid calli/protoplasts was very low: 1.8×10^{-6}. Twenty-three calli originated from a single fusion combination: *L. peruvianum* ATWH101 and *N. plumbaginifolia* R20-K10. It is not clear why only this combination gave rise to a relatively large number of hybrid calli. Both parents were also fused with other genotypes, but no hybrid calli were obtained from these fusions. Irradiation of protoplasts of the *Nicotiana* parent did not improve the yield of hybrid calli, as might be expected if an extensive elimination of donor DNA were to improve the viability of hybrids.

Although few reports on the construction of intertribal somatic hybrids mention how many protoplasts were used, the number of viable calli or plants that were obtained and that were proven to be somatic hybrids, was generally low. Krumbiegel and Schieder (1979) isolated 13 putative somatic hybrid calli of *Atropa belladonna* and *Datura innoxia* from eight fusion experiments involving 2 x 10^6 protoplasts (frequency 6.5 x 10^{-6}). De Vries et al. (1987) isolated 87 well growing hybrid cell lines out of seven fusion experiments between two *N. plumbaginifolia* genotypes and one *Solanum tuberosum* genotype, using a total number of 1.7 x 10^7 protoplasts (frequency 5.1 x 10^{-6}). Power and Chapman (1983) obtained putative somatic hybrid calli between *Petunia hybrida* (belonging to the same tribe as *Nicotiana*) and *Scopolia lurida* (belonging to the same tribe as *Lycopersicon*) at a frequency of 1 x $10^{-4}/10^{-5}$. Interestingly, in another intertribal fusion experiment between *N. tabacum* and *A. belladonna* protoplasts, Babiychuk et al. (1992) selected 447 putative hybrid colonies, most of which readily regenerated shoots. However, they did not mention the number of protoplasts involved. Probably, many intertribal fusion experiments that did not result in the production of viable hybrid calli have not been reported in the literature.

These data suggest that somatic hybrids between relatively unrelated species are only obtained at low and unpredictable frequencies. Since microscopic inspection does not indicate a lower fusion frequency as compared with protoplast fusions between more related species, the explanation for the smaller number of hybrids must be that most fusion products do not survive for a long time after fusion. Immediately after fusion, nuclei and organelles of both parents are combined and this phase seems most critical when incongruity between the nuclear and organelle genomes exists.

The presence of somatic incongruity in *Lycopersicon-Nicotiana* combinations is suggested by the observation that no true symmetric *Lycopersicon* (+) *Nicotiana* hybrids were found when the nuclear DNA composition of the hybrid calli was analyzed 2 years after fusion. The nuclei of most hybrids contained the original ploidy level of the *Nicotiana* parent or less, whereas the ploidy level of *Lycopersicon* DNA in the nuclei of these hybrids was 2-5 times that of the parent. Though irradiation of the *N. plumbaginifolia* parent caused extensive elimination of *Nicotiana* chromosomes in hybrids B1-B3, asymmetric hybrids were also obtained when the *Nicotiana* protoplasts had not been irradiated. This could indicate that viable somatic hybrid calli between species of these genera are only obtained when one *Nicotiana* protoplast is fused with two or more *Lycopersicon* protoplasts, or when one *Nicotiana* protoplast is fused with one polyploid *Lycopersicon* protoplast. Alternatively, an originally symmetric fusion product could show chromosomal instability, resulting in loss of *Nicotiana* chromosomes combined with polyploidization of the *Lycopersicon* nuclear DNA. Gilissen et al. (1992) reported that in 1-year-old somatic hybrid calli of *S. tuberosum* and *N. plumbaginifolia* the number of chromosomes from one or the other parental species dominated, indicating a selective advantage of asymmetric hybrids of either type. The simultaneous loss of chromosomes of one parent and polyploidization of the other parent is frequently observed in intertribal somatic hybrids (Binding and Nehls 1978; Krumbiegel and Schieder 1981; Potrykus et al. 1984; Pental et al. 1986) and can be considered to be a consequence of somatic incongruity (Harms 1983). The *A. belladonna* (+) *Nicotiana* spp. hybrids are an exception to this apparent rule, since both parents contributed equally to most of these hybrids and elimination was limited (Gleba et al. 1982; Babiychuk et al. 1992).

The two hybrids (B3 and E3) of which we obtained regenerants were both highly asymmetric, containing less than 1C *Nicotiana* DNA and 6-8C *Lycopersicon* DNA. This may also be an aspect of somatic incongruity; uniparental chromosome elimination, sometimes accompanied with polyploidization of the other parent might be required before morphogenetic processes are initiated. Gupta et al. (1984) could not obtain intertribal hybrid plants of *Physalis minima* and *Datura innoxia* via symmetric protoplast fusion, but they could when asymmetric fusion was performed.

In order to obtain viable somatic hybrids, not only a balance between the two parental genomes must be found, but also chloroplast segregation and mitochondrial DNA segregation and/or recombination must occur in such a way that the remaining nucleus, chloroplasts and mitochondria can function together. In our hybrids we found a strong correlation between nuclear DNA and organellar DNA composition. Most hybrids contained chloroplasts and most of the analyzed mtDNA fragments from the species that predominated in the nucleus. This strong bias, which was also present in *S. tuberosum* (+) *N. plumbaginifolia* somatic hybrid calli (Wolters et al. 1993, Chapter 6), reflects another aspect of somatic incongruity. Probably, only when rapid unilateral chromosome elimination is accompanied by elimination of organelles from the same parent is further development of the hybrid possible. As discussed for the *Solanum* (+) *Nicotiana* hybrids (Wolters et al. 1993, Chapter 6), chromosome elimination in symmetric fusions and organelle segregation are presumably both random processes, occurring simultaneously. Because of this the chance of obtaining viable hybrids with a proper combination of genophores out of the original fusion products can be expected to be relatively low. Since it is likely that the physiological stage of the isolated protoplasts determines how quickly cell division starts, and thereby the process of elimination of genophores, it is conceivable that relatively small differences between experiments are crucial and explain the variation in yield of hybrids between experiments.

We produced one highly asymmetric *Lycopersicon*-like hybrid plant which flowered during a short period, but was sterile. The effort it takes to produce one such plant is very large and the chance of transferring a desired trait from *Nicotiana* to *Lycopersicon* small even when some progeny can be obtained, since meiotic recombination is expected to be limited. In addition, the possibility of exchanging organelles is very limited or non-existent. Therefore, we feel that intertribal protoplast fusions between *Lycopersicon* and *Nicotiana* with the aim of adding useful traits to *Lycopersicon* are not recommendable.

Acknowledgements. The authors thank C. Heyting for reviewing the manuscript, W. van Cappellen for performing part of the fusion experiments and J. Wijbrandi for supervising these experiments. This research was supported by the Dutch Programme Committee on Agricultural Biotechnology (PcLB).

References

Arumuganathan K, Earle ED (1991) Nuclear DNA content of some important plant species. Plant Mol Biol Rep 9:208-218

Babiychuk E, Kushnir S, Gleba YY (1992) Spontaneous extensive chromosome elimination in somatic hybrids between somatically congruent species *Nicotiana tabacum* L. and *Atropa belladonna* L. Theor Appl Genet 84:87-91

Binding H, Nehls R (1978) Somatic cell hybridization of *Vicia faba* + *Petunia hybrida*. Mol Gen Genet 164:137-143

Bonnema AB, O'Connell MA (1992) Molecular analysis of the nuclear organellar genotype of somatic hybrid plants between tomato (*Lycopersicon esculentum*) and *Lycopersicon chilense*. Plant Cell Rep 10:629-632

Bonnema AB, Melzer JM, O'Connell MA (1991) Tomato cybrids with mitochondrial DNA from *Lycopersicon pennellii*. Theor Appl Genet 81:339-348

Dale RMK, Mendu N, Ginsburg H, Kridl JC (1984) Sequence analysis of the maize mitochondrial 26 S rRNA gene and flanking regions. Plasmid 11:141-150

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1:19-21

Derks FHM, Hakkert JC, Verbeek WHJ, Colijn-Hooymans CM (1992) Genome composition of asymmetric hybrids in relation to the phylogenetic distance between the parents. Nucleus-chloroplast interaction. Theor Appl Genet 84:930-940

Dewey RE, Schuster AM, Levings III CS, Timothy DH (1985) Nucleotide sequence of F_0-ATPase proteolipid (subunit 9) gene of maize mitochondria. Proc Natl Acad Sci USA 82:1015-1019

Dirks R, Negrutiu I, Sidorov V, Jacobs M (1985) Complementation analysis by somatic hybridisation and genetic crosses of nitrate reductase-deficient mutants of *Nicotiana* *plumbaginifolia*. Evidence for a new category of *cnx* mutants. Mol Gen Genet 201: 339-343

van den Elzen P, Lee KY, Townsend J, Bedbrook J (1985) Simple binary vectors for DNA transfer to plant cells. Plant Mol Biol 5:149-154

Gavrilenko TA, Barbakar NI, Pavlov AV (1992) Somatic hybridization between *Lycopersicon esculentum* and non-tuberous *Solanum* species of the *Etuberosa* series. Plant Sci 86:203-214

Gilissen LJW, van Staveren MJ, Verhoeven HA, Sree Ramulu K (1992) Somatic hybridization between potato and *Nicotiana plumbaginifolia* 1. Spontaneous biparental chromosome elimination and production of asymmetric hybrids. Theor Appl Genet 84:73-80

Gleba YY, Momot VP, Cherep NN, Skarzynskaya MV (1982) Intertribal hybrid cell lines of *Atropa belladonna* (x) *Nicotiana chinensis* obtained by cloning individual protoplast fusion products. Theor Appl Genet 62:75-79

Gupta PP, Schieder O, Gupta M (1984) Intergeneric nuclear gene transfer between somatically and sexually incompatible plants through asymmetric protoplast fusion. Mol Gen Genet 197:30-35

de Haas JM, Boot KJM, Haring MA, Kool AJ, Nijkamp HJJ (1986) A *Petunia hybrida* chloroplast DNA region, close to one of the inverted repeats, shows sequence homology with the *Euglena gracilis* chloroplast DNA region that carries the putative replication origin. Mol Gen Genet 81:157-161

Handley LW, Nickels RL, Cameron MW, Moore PP, Sink KC (1986) Somatic hybrid plants between *Lycopersicon esculentum* and *Solanum lycopersicoides*. Theor Appl Genet 71:691-697

Harms CT (1983) Somatic incompatibility in the development of higher plant somatic hybrids. Q Rev Biol 58:325-353

Hause B, Baldauf F, Stock K, Wasternack C, Metzlaff M (1986) Molecular analysis of mitochondrial DNA from tomato cell suspension cultures. Curr Genet 10:785-790

Jacobsen E, Reinhout P, Bergervoet JEM, de Looff J, Abidin PE, Huigen DJ, Ramanna MS (1992) Isolation and characterization of potato-tomato somatic hybrids using an amylose-free potato mutant as parental genotype. Theor Appl Genet 85:159-164

Koornneef M, Hanhart C, Jongsma M, Toma I, Weide R, Zabel P, Hille J (1986) Breeding of a tomato genotype readily accessible to genetic manipulation. Plant Sci 45: 201-208

Koornneef M, Jongsma M, Weide R, Zabel P, Hille J (1987) Transformation of tomato. In: Nevins DJ, Jones RA (eds) Tomato biotechnology. Alan R Liss, New York, pp 169-178

Krumbiegel G, Schieder O (1979) Selection of somatic hybrids after fusion of protoplasts from *Datura innoxia* Mill. and *Atropa belladonna* L. Planta 145:371-375

Krumbiegel G, Schieder O (1981) Comparison of somatic and sexual incompatibility between *Datura innoxia* and *Atropa belladonna*. Planta 153:466-470

Maliga P, Sz-Breznovits A, Márton L, Joó F (1975) Non-Mendelian streptomycin-resistant tobacco mutant with altered chloroplasts and mitochondria. Nature 255: 401-402

Marchesi ML, Castiglione S, Sala F (1989) Effect of repeated DNA sequences on direct gene transfer in protoplasts of *Nicotiana plumbaginifolia*. Theor Appl Genet 78:113-118

Melchers G, Mohri Y, Watanabe K, Wakabayashi S, Harada K (1992) One-step generation of cytoplasmic male sterility by fusion of mitochondrial-inactivated tomato protoplasts with nuclear-inactivated *Solanum* protoplasts. Proc Natl Acad Sci USA 89:6832-6836

Mettler IJ (1987) A simple and rapid method for minipreparation of DNA from tissue cultured plant cells. Plant Mol Biol Rep 5:346-349

Mouras A, Wildenstein C, Salesses G (1986) Analysis of karyotype and C-banding pattern of *Nicotiana plumbaginifolia* using two techniques. Genetica 68:197-202

Mouras A, Saul MW, Essad S, Potrykus I (1987) Localization by in situ hybridization of a low copy chimaeric resistance gene introduced into plants by direct gene transfer. Mol Gen Genet

207:204-209

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15: 473-497

Negrutiu I, Dirks R, Jacobs M (1983) Regeneration of fully nitrate reductase-deficient mutants from protoplast culture of *Nicotiana plumbaginifolia* (Viviani). Theor Appl Genet 66: 341-347

Negrutiu I, Hinnisdaels S, Mouras A, Gill BS, Gharti-Chhetri GB, Davey MR, Gleba YY, Sidorov V, Jacobs M (1989) Somatic versus sexual hybridization: features, facts and future. Acta Bot Neerl 38:253-272

Negrutiu I, Hinnisdaels S, Cammaerts D, Cherdshewasart W, Gharti-Chhetri G, Jacobs M (1992) Plant protoplasts as genetic tool: selectable markers for developmental studies. Int J Dev Biol 36:73-84

Pental D, Hamill JD, Pirrie A, Cocking EC (1986) Somatic hybridization of *Nicotiana tabacum* and *Petunia hybrida*. Recovery of plants with *P. hybrida* nuclear genome and *N. tabacum* chloroplast genome. Mol Gen Genet 202:342-347

Pijnacker LP, Ferwerda MA (1984) Giemsa C-banding of potato chromosomes. Can J Genet Cytol 26:415-419

Potrykus I, Jia J, Lazar GB, Saul M (1984) *Hyoscyamus muticus* + *Nicotiana tabacum* fusion hybrids selected via auxotroph complementation. Plant Cell Rep 3:68-71

Power JB, Chapman JV (1983) Intra- and intersubfamilial somatic hybridisation within the *Solanaceae*. In: Potrykus I, Harms CT, Hinnen A, Hütter R, King PJ, Shillito RD (eds) Protoplasts 1983. Poster Proceedings, 6th International Protoplast Symposium Basel. Birkhäuser Verlag Basel Boston Stuttgart, pp 276-277

Ratushnyak YI, Cherep NN, Zavgorodnyaya AV, Latypov SA, Borozenko IV, Rachkovskaya RI, Gleba YY (1993) Fertile asymmetric somatic hybrids between *Lycopersicon esculentum* Mill. and *Lycopersicon peruvianum* var. *dentatum* Dun. Mol Gen Genet 236:427-432

Rick CM (1982) Stock List. Rep Tomato Genet Coop 32: 3-10

Rogers SO, Bendich AJ (1988) Extraction of DNA from plant tissues. Plant molecular biology manual A6. Kluwer Academic Publ, Dordrecht, pp 1-10

Sakomoto K, Taguchi T (1991) Regeneration of intergeneric somatic hybrid plants between *Lycopersicon esculentum* and *Solanum muricatum*. Theor Appl Genet 81:509-513

Saul MW, Potrykus I (1984) Species-specific repetitive DNA used to identify interspecific somatic hybrids. Plant Cell Rep 3:65-67

Shahin EA (1985) Totipotency of tomato protoplasts. Theor Appl Genet 69: 235-240

Tabaeizadeh Z, Perennes C, Bergounioux C (1985) Increasing the variability of *Lycopersicon peruvianum* Mill. by protoplast fusion with *Petunia hybrida* L. Plant Cell Rep 4:7-11

Tabaeizadeh Z, Bergounioux C, Demarly Y (1989) *Lycopersicon* + *Petunia* somatic hybrids. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry 8. Plant protoplasts and genetic engineering I. Springer-Verlag, Berlin Heidelberg, pp 336-355

Tewes A, Glund K, Walther R, Reinbothe H (1984) High yield isolation and rapid recovery of protoplasts from suspension cultures of tomato (*Lycopersicon esculentum*). Z Pflanzenphysiol 113: 141-150

Thomas BR, Pratt D (1981) Efficient hybridization between *Lycopersicon esculentum* and *L. peruvianum* via embryo callus. Theor Appl Genet 59: 215-219

de Vries SE, Ferwerda MA, Loonen AEHM, Pijnacker LP, Feenstra WJ (1987) Chromosomes in somatic hybrids between *Nicotiana plumbaginifolia* and a monoploid potato. Theor Appl Genet 75:170-176

Wijbrandi J, van Capelle W, Hanhart CJ, van Loenen-Martinet Schuringa EP, Koornneef M (1990a) Selection and characterization of somatic hybrids between *Lycopersicon esculentum* and

Lycopersicon peruvianum. Plant Sci 70:197-208

Wijbrandi J, Wolters AMA, Koornneef M (1990b) Asymmetric somatic hybrids between *Lycopersicon esculentum* and irradiated *Lycopersicon peruvianum*. 2. Analysis with marker genes. Theor Appl Genet 80:665-672

Wolters AMA, Schoenmakers HCH, van der Meulen-Muisers JJM, van der Knaap E, Derks FHM, Koornneef M, Zelcer A (1991) Limited DNA elimination from the irradiated potato parent in fusion products of albino *Lycopersicon esculentum* and *Solanum tuberosum*. Theor Appl Genet 83:225-232

Wolters AMA, Koornneef M, Gilissen LJW (1993) The chloroplast and the mitochondrial DNA type are correlated with the nuclear composition of somatic hybrids of *Solanum tuberosum* and *Nicotiana plumbaginifolia*. Curr Genet 24:260-267

Young EG, Hanson MR (1987) A fused mitochondrial gene associated with cytoplasmic male sterility is developmentally regulated. Cell 50:41-49

Zabel P, Meyer D, van de Stolpe O, van der Zaal B, Ramanna MS, Koornneef M, Krens F, Hille J (1985) Towards the construction of artificial chromosomes for tomato. In: van Vloten-Doting L, Groot GSP, Hall TC (eds) Molecular form and function of the plant genome. Plenum Publ, New York, pp 609-624

CHAPTER 6

The chloroplast and mitochondrial DNA type are correlated with the nuclear composition of somatic hybrid calli of *Solanum tuberosum* and *Nicotiana plumbaginifolia*

A.M.A. Wolters, M. Koornneef
Department of Genetics, Wageningen Agricultural University, Dreijenlaan 2, NL-6703 HA Wageningen, The Netherlands

L.J.W. Gilissen
Centre for Plant Breeding and Reproduction Research CPRO-DLO, P.O.Box 16, NL-6700 AA Wageningen, The Netherlands

Published in: Curr Genet (1993) 24:260-267

Summary. This paper describes the analysis of chloroplast (cp) DNA and mitochondrial (mt) DNA in 21 somatic hybrid calli of *Solanum tuberosum* and *Nicotiana plumbaginifolia* by means of Southern-blot hybridization. Each of these calli contained only one type of cpDNA; 14 had the *N. plumbaginifolia* (Np) type and seven the *S. tuberosum* (St) type. *N. plumbaginifolia* cpDNA was present in hybrids previously shown to contain predominantly *N. plumbaginifolia* chromosomes whereas hybrids in which *S.tuberosum* chromosomes predominated possessed cpDNA from potato. We have analyzed the mtDNA of these 21 somatic hybrid calli using four restriction enzyme/probe combinations. Most fusion products had only, or mostly, mtDNA fragments from the parent that predominated in the nucleus. The hybrids containing mtDNA fragments from only one parent (and new fragments) also possessed chloroplasts from the same species. The results suggest the existence of a strong nucleo-cytoplasmic incongruity which affects the genome composition of somatic hybrids between distantly related species.

Introduction

Somatic hybrids of plants are more easily produced when the parents are more related; numerous hybrids between closely related species within the same genus or tribe have been described, whereas there are only a few published examples of hybrids between distantly related species (species from different subfamilies or families). Interfamily hybrids generally show extensive elimination of chromosomes of one parent (Gleba and Sytnik 1984). Chromosomes of both parents may be stably retained for a long time in intertribal hybrids, but at some stage spontaneous uniparental chromosome elimination may occur rapidly [e.g., *Arabidopsis thaliana* (+) *Brassica campestris* hybrids (Hoffmann and Adachi 1981); *Atropa belladonna* (+) *Nicotiana tabacum* hybrids (Babiychuk et al. 1992)]. In somatic hybrids between *Solanum tuberosum* and *Nicotiana plumbaginifolia* chromosomes of either one of the parents may be eliminated (biparental chromosome elimination) (de Vries et al. 1987; Gilissen et al. 1992). This elimination occurred mainly in the first 2 months after protoplast fusion (Gilissen et al. 1992).

Hybrids between distantly related species are much more difficult to obtain than somatic hybrids between closely related species, although limited quantitative data are available on this topic. No experimental data and theories have been published explaining both the direction of chromosome elimination and the low recovery rate of hybrids between distantly related species. In addition, little attention has been paid to the role of organelles in both processes. From only a few such hybrids has the chloroplast (cp) DNA type been determined, and to our knowledge no detailed analysis of the mitochondrial (mt) DNA composition of such hybrids has been published.

In this study we have analyzed the organellar DNAs of 21 somatic hybrid calli between *S. tuberosum* and *N. plumbaginifolia*. The chromosomal composition of these calli has been described previously (Gilissen et al. 1992). Chromosomes of both species can be distinguished because those of potato are metacentric or submetacentric and considerably smaller than the telocentric/subtelocentric chromosomes of *N. plumbaginifolia*. Organellar DNA types were related to the chromosomal composition of the hybrids.

Materials and methods

Plant materials. The *S. tuberosum* genotype used for fusion was the diploid ($2n=2x=24$) transformed clone 413, carrying various genetic markers (hormone autotrophy, opine synthesis, kanamycin resistance and ß-glucuronidase (GUS) activity) described by de Vries-Uijtewaal et al. (1989) and Gilissen et al. (1991). The *N. plumbaginifolia* parent was a diploid ($2n=2x=20$) wild-type clone. The production and selection of somatic hybrids and the analysis of chromosomes 2 and 12 months after fusion have been described previously (Gilissen et al. 1992). Organelle DNA analyses were performed on the 21 hybrid calli that were still available 3 years after fusion. Calli were subcultured monthly on solid MS medium supplemented with 5 mg/l NAA, 0.1 mg/l BAP and 100 mg/l kanamycin under controlled conditions of 16 h/day light (10 W/m^2, Philips TLD 36 W) at 25°C. Nine of the hybrids showed greening of the calli, but no shoot regeneration occurred on regeneration medium (Gilissen et al. 1992).

DNA isolation and organelle DNA probes. Total DNA from the fusion parents was isolated from 5 g of leaves according to Rogers and Bendich (1988). Total DNA from the somatic hybrids was isolated from 0.5 g of callus by a modified procedure of Dellaporta et al. (1983) as described in Wolters et al. (1991, Chapter 2). For the characterization of cpDNA the *Petunia* cpDNA probe pPCY64 (de Haas et al. 1986), which contains the ARS-C sequence on a 2.4-kb *Bam*HI fragment, was used. The following probes were employed for mtDNA analysis: the 14.7-kb *Bam*HI fragment containing the maize 26S rDNA gene (Iams and Sinclair 1982), the 0.45-kb

*Eco*RI-*Sal*I fragment of the *Pcf* gene of *Petunia* (Young and Hanson 1987) and the 2.2-kb *Xba*I fragment of the maize ATPase subunit 9 (*Atp9*) gene (Dewey et al. 1985). To isolate inserts from the plasmids, the right restriction fragment was cut out of an agarose gel and the DNA retrieved using the Prep-A-Gene DNA Purification kit from Bio-Rad.

DNA restriction, Southern transfer and hybridization. Five micrograms of DNA from the fusion parents were restricted with *Bam*HI, *Eco*RI, *Hin*dIII and *Hin*fI, the fragments separated on a 0.7% agarose gel and alkali-blotted onto Gene Screen Plus membrane (DuPont). This blot was used to test which probe/enzyme combinations showed polymorphisms between the two fusion parents. Subsequently 5 μg of DNA from parents and fusion products were digested with *Bam*HI or *Eco*RI, run on 0.6% agarose gels and blotted onto a Gene Screen Plus or a Hybond N^+ membrane (Amersham) respectively. Probes were radioactively labeled using the Boehringer Mannheim Random Primed DNA Labeling kit. Hybridization was performed in glass bottles using a Hybaid hybridization oven at 65°C for 16 h. The blots were rinsed to a stringency of 1 x SSC, 1% SDS in the case of Gene Screen Plus membranes or 1 x SSC, 0.5% SDS when using a Hybond N^+ membrane. After autoradiography the probe was removed according to the supplier's instructions and the membranes were re-used.

Results

Polymorphisms in organelle DNA of the fusion parents

A blot with parental DNA digested with four restriction enzymes was hybridized with the cpDNA probe pPCY64 (Fig. 1A) and mtDNA probe 26S rDNA (Fig. 1B). As shown in Fig. 1A the cpDNAs of potato and *N. plumbaginifolia* are clearly distinguishable with three of the four restriction enzymes used. With the mtDNA probe species-specific fragments are observed with all four restriction enzymes (Fig. 1B). Other mtDNA probes used showed a similar degree of divergence between the two fusion parents (data not shown). Hause et al. (1986) have shown that the 23S rDNA from spinach plastids cross-hybridizes with a tomato mtDNA fragment containing the 26S rDNA gene. Similarly, the mtDNA probe 26S rDNA hybridizes not only to mtDNA fragments, but also to cpDNA fragments containing (part of) the 23S rDNA gene. The cpDNA sequences detected are a 6.0-kb *Bam*HI fragment for *S. tuberosum* and a 7.1-kb *Bam*HI fragment for *N.*

plumbaginifolia, the 4.4-kb and 0.70-kb *Eco*RI fragments, the 1.3-kb, 1.1-kb and 0.85-kb *Hin*dIII fragments, and the 2.3-kb and 0.60-kb *Hin*fI fragments for both species [deduced from the *N. tabacum* chloroplast DNA sequence (Shinozaki et al. (1986)]. These bands are indicated with an asterisk in Fig. 1B.

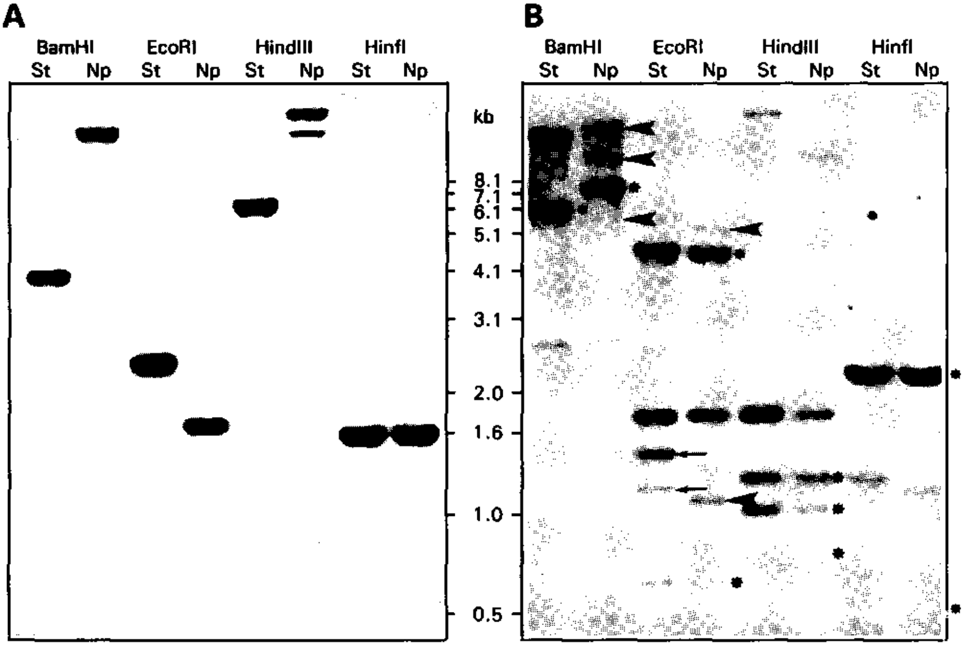

Fig. 1A,B. Polymorphisms in cpDNA and mtDNA of *S. tuberosum* and *N. plumbaginifolia*. Autoradiograms of a blot containing 5 μg total DNA of *S. tuberosum* 413 (St) and of a wild-type *N. plumbaginfolia* (Np), digested with *Bam*HI, *Eco*RI, *Hin*dIII or *Hin*fI, hybridized with the cpDNA probe pPCY64 (A) or with the mtDNA probe 26S rDNA (B). Sizes of marker bands are indicated. In **B** *S. tuberosum*-specific bands are indicated with an *arrow*, *N. plumbaginifolia*-specific bands with an *arrowhead* and chloroplast 23S rDNA fragments with an *asterisk*

Analysis of the organellar DNA of the fusion products

The cpDNA type of the 21 hybrid calli was determined by hybridization of the pPCY64 probe with blots containing *Bam*HI- or *Eco*RI-digested total DNA (Fig. 2A). Corresponding results were obtained with both restriction enzymes; these are listed in Table 1.

Table 1. Type of chloroplast DNA, number of common (C), *N. plumbaginifolia*-specific (N), *S. tuberosum*-specific (S) and new (R) mitochondrial DNA fragments and the mtDNA group of *Solanum tuberosum* (+) *Nicotiana plumbaginifolia* somatic hybrids, grouped per category [based on number of parental chromosomes, category I: ≤ 24 *S. tuberosum* (S) and > 20 *N. plumbaginifolia* (N) chromosomes; category II: > 24 S and ≤ 20 N chromosomes; category III: ≤ 24 S and ≤ 20 N chromosomes; category IV: > 24 S and > 20 N chromosomes] and of the parental species

Hybrid	Category	cpDNA	No. of mtDNA fragments				mtDNA group
			C	N	S	R	
H1	I	N	8	7	–	3	N
H5	I	N	8	8	4	1	N+s
H6[a]	I	N	7	8	1	1	N+s
H15[a]	I	N	7	9	1	–	N+s
H18[a]	I	N	8	10	–	–	N
H21[a]	I	N	8	10	–	–	N
H24[a]	I	N	8	5	2	1	N+s
H25[a]	I	N	8	6	–	–	N
H34[a]	I	N	8	7	–	–	N
H57[a]	I	N	8	6	–	3	N
H60[a]	I	N	8	8	1	1	N+s
H9	III	N	6	1	7	4	S+n
H32	III	N	8	7	5	4	N+s
H46	III	N	8	10	–	–	N
H74	III	S	8	8	4	–	N+s
H45	IV	S	6	1	8	3	S+n
H13	II	S	7	5	5	4	S+N
H30	II	S	8	2	9	9	S+n
H33	II	S	7	–	10	2	S
H36	II	S	8	–	8	9	S
H43	II	S	8	4	7	3	S+n
parents							
S.tub. 413		S	8	–	10	–	S
N.plum. wt		N	8	10	–	–	N

[a] Hybrids showing greening of calli

In this table the hybrids are grouped according to their chromosomal composition as determined 2 months after fusion (Gilissen et al. 1992). Category I hybrids contained less than 24 potato chromosomes and more than 20 *N. plumbaginifolia* (Np) chromosomes. In hybrids of category II more than 24 potato chromosomes and less than 20 Np chromosomes were present. Hybrid calli of category III contained diploid or subdiploid numbers of chromosomes from both parents and one callus contained more than the diploid number of chromosomes from both parents (category IV). Some calli consisted of two or three subpopulations of cells with very different numbers of chromosomes as a result of polyploidization of the genome of one or both parents combined with non-disjunction. Fourteen hybrids contained cpDNA fragments specific for *N. plumbaginifolia* and seven hybrids possessed *S. tuberosum*-specific cpDNA fragments; no mixture of parental fragments or recombinant fragments were observed.

We analyzed the mtDNA of the 21 hybrid calli using four enzyme/probe combinations: *Bam*HI/26S rDNA, *Eco*RI/26S rDNA, *Eco*RI/*Atp9* and *Bam*HI/*Pcf*. In the *Bam*HI/26S rDNA combination the bands hybridizing to the plastid 23S rDNA gene show a polymorphism between the two fusion parents (Fig. 1B). Thus, using this enzyme/probe combination we could also analyze the cpDNA type of the fusion products. The results were in agreement with those obtained by the *Bam*HI/pPCY64 and *Eco*RI/pPCY64 hybridizations.

Autoradiograms of the *Eco*RI/26S rDNA and the *Eco*RI/*Atp9* blots are shown in Fig. 2B and C respectively. Hybrid calli contained only *N. plumbaginifolia*-specific mtDNA fragments (e.g., H15, H18 and H21 in Fig. 2C), only *S. tuberosum*-specific fragments (e.g., H9 in Fig. 2B) or fragments from both fusion parents (e.g., H30 in Fig. 2B). Frequently, hybrid calli contained mtDNA fragments that were not present in either of the two parents. These new fragments can be the result of either intergenomic recombination (Rothenberg et al. 1985) or selective amplification of pre-existing sub-stoichiometric molecules (Small et al. 1987).

For each enzyme/probe combination used in the mtDNA analysis the numbers of common bands [bands of a particular size which are present in both parental species (C)], *Nicotiana*-specific fragments (N), *Solanum*-specific fragments (S) and new fragments (R) per hybrid, were counted. The total number of fragments counted in the four enzyme/probe combinations, excluding the cpDNA bands hybridizing with the 26S rDNA probe, is listed in Table 1 for each hybrid and for the parents. The hybrids are categorized in five groups on the basis of their mtDNA composition: one group of hybrids contained only *Nicotiana* (and new) fragments (N), the second group contained only *Solanum* (and new) fragments (S) and the three other groups

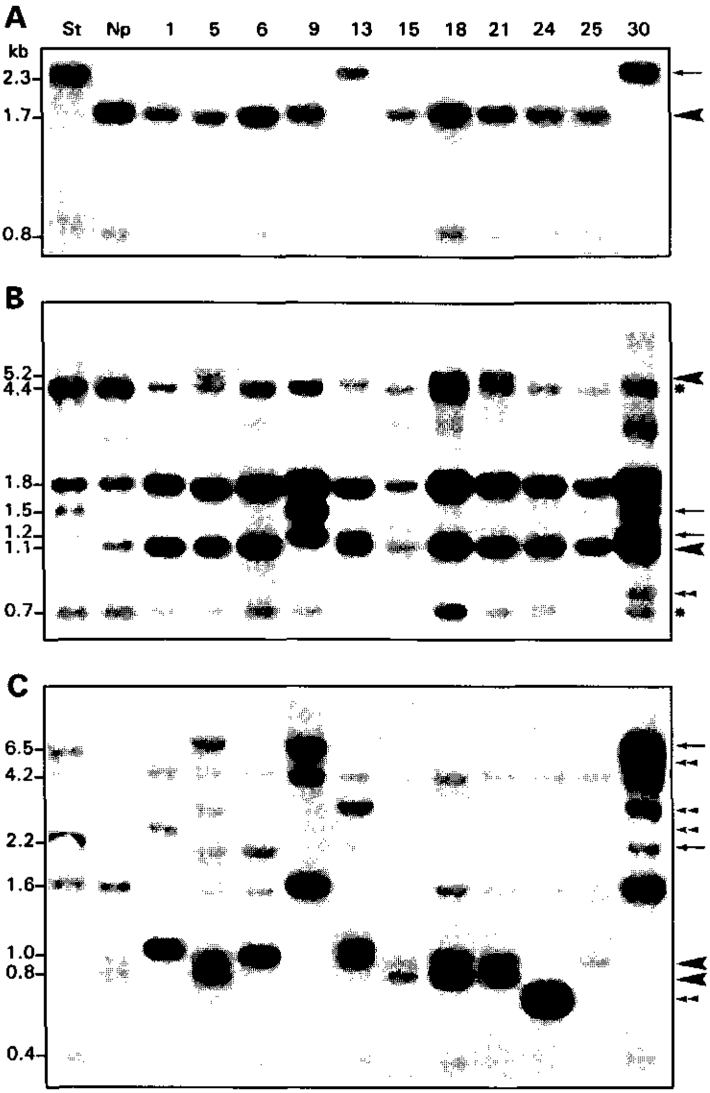

Fig. 2A-C. Analysis of chloroplast and mitochondrial DNA of *S. tuberosum* (+) *N. plumbaginifolia* somatic hybrids. Autoradiograms of a blot containing 5 μg *Eco*RI-digested total DNA of fusion parents *S. tuberosum* 413 (**St**) and wild type *N. plumbaginifolia* (**Np**) and of somatic hybrids H1-H30, hybridized with the cpDNA probe pPCY64 (**A**), the mtDNA probe 26S rDNA (**B**) or the mtDNA probe *Atp9* (**C**). *S. tuberosum*-specific bands are indicated with an *arrow*, *N. plumbaginifolia*-specific bands with an *arrowhead*, new fragments with *double arrowheads* and in **B** chloroplast 23S rDNA fragments are indicated with an *asterisk*. Sizes of fragments are indicated in kb contained fragments from both species (and new fragments), with more *Nicotiana*-specific than *Solanum*-specific fragments (N+s), with more *Solanum*-specific than *Nicotiana*-specific bands (S+n) or with equal numbers of *Nicotiana*- or potato-specific fragments (S+N). New bands were mainly found in the hybridizations with the *Pcf* and with the *Atp9* probe. In the *Bam*HI/*Pcf* assay eight new fragments were found when all hybrids are considered while in the *Eco*RI/*Atp9* assay six new bands were detected. The numbers of hybrids containing a specific new fragment in these assays are shown in Table 2. The one new band in the *Bam*HI/26S rDNA hybridization was found in six hybrids. In the *Eco*RI/26S rDNA assay one hybrid showed three novel bands and another hybrid possessed one of these new fragments. The high frequency of specific new fragments in independent hybrids may indicate the presence of "hot spots" for intra- and/or inter-molecular recombination. Such recombinationally active "hot spots" have been reported in the mtDNA of *Brassica* (Palmer and Shields 1984) and maize (Lonsdale et al. 1984), as well as in the mtDNA of somatic hybrids between *S. tuberosum* and *S. brevidens* (Kemble et al. 1986). Alternatively, cells containing specific combinations of mtDNA genes may be more viable than cells with other combinations; thus it is possible that only a small fraction of recombination products will be maintained in the hybrids.

Table 2. Number of hybrids showing specific new mitochondrial DNA bands after hybridization of *Bam*HI-digested total DNA of the hybrids with probe *Pcf*, and after hybridization of *Eco*RI-digested total DNA with *Atp9*

*Bam*HI x *Pcf*		*Eco*RI x *Atp9*	
New band	No. of hybrids	New band	No. of hybrids
R_P1	4	R_A1	4
R_P2	1	R_A2	9
R_P3	1	R_A3	3
R_P4	1	R_A4	1
R_P5	3	R_A5	2
R_P6	1	R_A6	1
R_P7	5		
R_P8	2		

Of the hybrids containing only *N. plumbaginifolia*-specific and common mtDNA fragments, H18, H21 and H46 showed all eight common bands and all ten *N. plumbaginifolia*-specific bands. Hybrids H25 and H34 have lost four and respectively three *Nicotiana*-specific fragments (Table 1) in four and respectively two of the mtDNA analyses concerning four different probe/enzyme combinations. This may indicate that subgenomic mtDNA molecules of *Nicotiana* carrying these fragments are absent or present in a very low stoichiometry in the latter hybrids.

Correlation between organelle DNA type and chromosomal composition

The hybrids analyzed in this study can be divided in two main groups on the basis of their chromosomal composition (Gilissen et al. 1992): those with predominantly *N. plumbaginifolia* chromosomes and those with predominantly *S. tuberosum* chromosomes. To assess a possible correlation between the chromosomal composition and the organelle DNA type the data on numbers of parental chromosomes were combined with the results from the chloroplast and mitochondrial DNA analyses. The resulting scatter diagrams are shown in Fig. 3 and 4. The two or three subpopulations of cells with different chromosome compositions in some hybrid calli are all included in these figures.

In Fig. 3 the chloroplast type of each hybrid is indicated. Dotted lines distinguish the categories as described by Gilissen et al. (1992). All hybrids in category I possessed *N. plumbaginifolia* chloroplasts. The hybrids in category II all possessed potato chloroplasts. Hybrids with diploid or subdiploid numbers of chromosomes from both parents (category III), or with more than the diploid number of chromosomes from both species (category IV), contained either *Nicotiana* or *Solanum* chloroplasts. Remarkably, greening calli were found only among category I hybrids (see Table 1).

When Fig. 3A and B are compared an increase in the number of chromosomes during prolonged in vitro culture is apparent in many hybrids. Four of the seven calli containing *S. tuberosum* cpDNA showed a polyploidization of potato chromosomes, while the number of *N. plumbaginifolia* chromosomes remained the same or decreased. One of the calli with potato chloroplasts (H74) showed polyploidization of chromosomes of both parents, although more extensively with respect to potato, resulting in subpopulations of cells containing twice as many *S. tuberosum* as *N. plumbaginifolia* chromosomes. Two hybrids with potato chloroplasts contained the same number of potato chromosomes 2 and 12 months after fusion, but the number of *N. plumbaginifolia* chromosomes had significantly decreased.

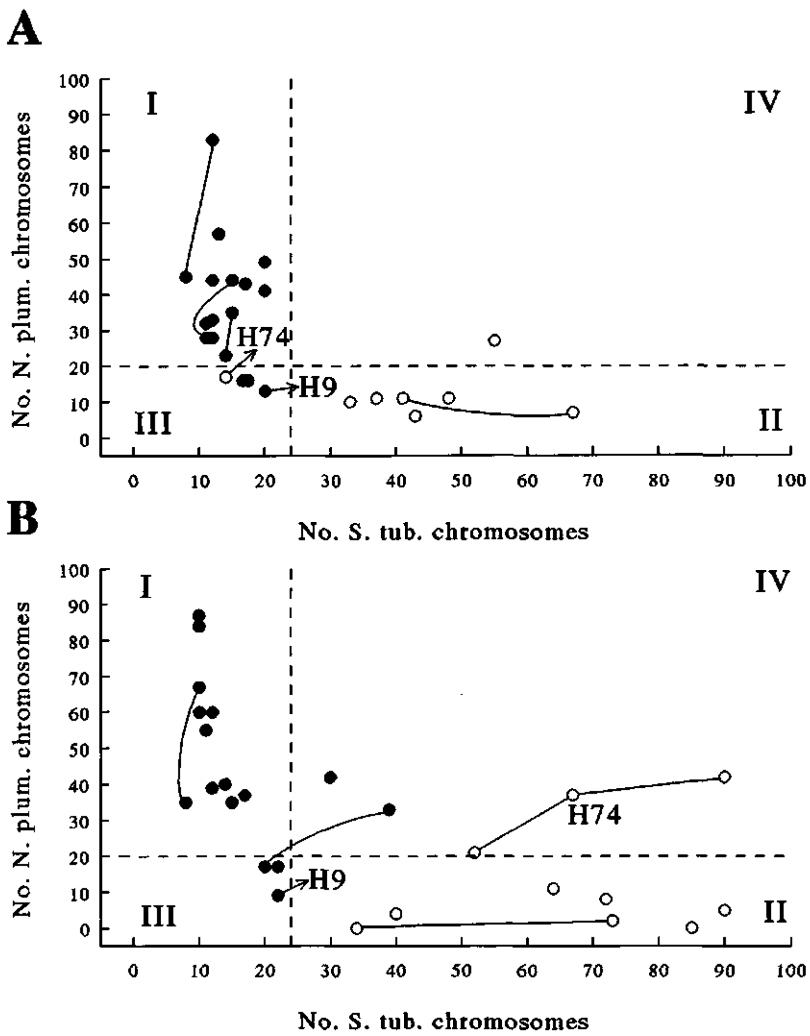

Fig. 3A,B. Scatter diagrams showing the chromosome numbers of both fusion parents in *S. tuberosum* (+) *N. plumbaginifolia* somatic hybrids containing *N. plumbaginifolia* cpDNA (●) or *S. tuberosum* cpDNA (○). In **A** chromosome numbers were counted in 2-month-old calli, in **B** chromosome numbers of 12-month-old calli are indicated. Subpopulations of cells with different chromosome numbers in one hybrid are connected with *lines*. *Dotted lines* distinguish the categories of hybrids (see text)

Ten of the fourteen calli possessing *N. plumbaginifolia* chloroplasts were stable or showed minor changes in chromosome composition. The four calli showing major changes 12 months after fusion contained two or three times the number of *N. plumbaginifolia* chromosomes and approximately the same number of potato chromosomes, when compared with the numbers present 2 months after fusion.

Table 3. Number of hybrid calli with *N. plumbaginifolia* (N) or *S.tuberosum* (S) chloroplasts in combination with a genome equivalent (GE) of *N* larger respectively smaller than the genome equivalent of S, as determined 2 and 12 months after fusion

	Two months after fusion			Twelve months after fusion		
	N cpDNA	S cpDNA	Total	N cpDNA	S cpDNA	Total
GE (N) > GE (S)	13	1	14	11	0	11
GE (N) < GE (S)	1	6	7	2	7	9
Total	14	7	21	13	7	20[a]

[a] One hybrid callus with GE (N) = GE (S) was excluded from the analysis

The association between chromosomal composition [GE (N) > GE (S) or GE (N) < GE (S), where GE is the genome equivalent, which is the number of S chromosomes divided by 12 or the number of N chromosomes divided by 10] and cpDNA type (N or S) is very significant (Table 3), both 2 months after fusion [Fisher's exact test: P = 0.00085 (Fisher 1941)] and 12 months after fusion (Fisher's exact test: P = 0.00046).

In Fig. 4 the mitochondrial DNA composition of the hybrids is indicated. Hybrids containing *N. plumbaginifolia* (and new) mtDNA fragments possessed more than 20 *N. plumbaginifolia* chromosomes and less than 24 potato chromosomes (category I), except for hybrid H46 which possessed 16 *Nicotiana* and 17 *Solanum* chromosomes 2 months after fusion and 17 *Nicotiana*/20 *Solanum* or 33 *Nicotiana*/39 *Solanum* chromosomes 12 months after fusion. The two hybrids containing only *S. tuberosum* and new mtDNA fragments possessed more than 24 potato chromosomes and less than 20 *N. plumbaginifolia* chromosomes (category II). Twelve hybrids possessed mtDNA fragments from both parents and ten of these also contained novel fragments. Hybrids with more *Nicotiana*-specific than *Solanum*-specific mtDNA bands are present in all categories except II, whereas hybrids containing more potato-specific than *Nicotiana*-specific mtDNA fragments are present in all categories except category I.

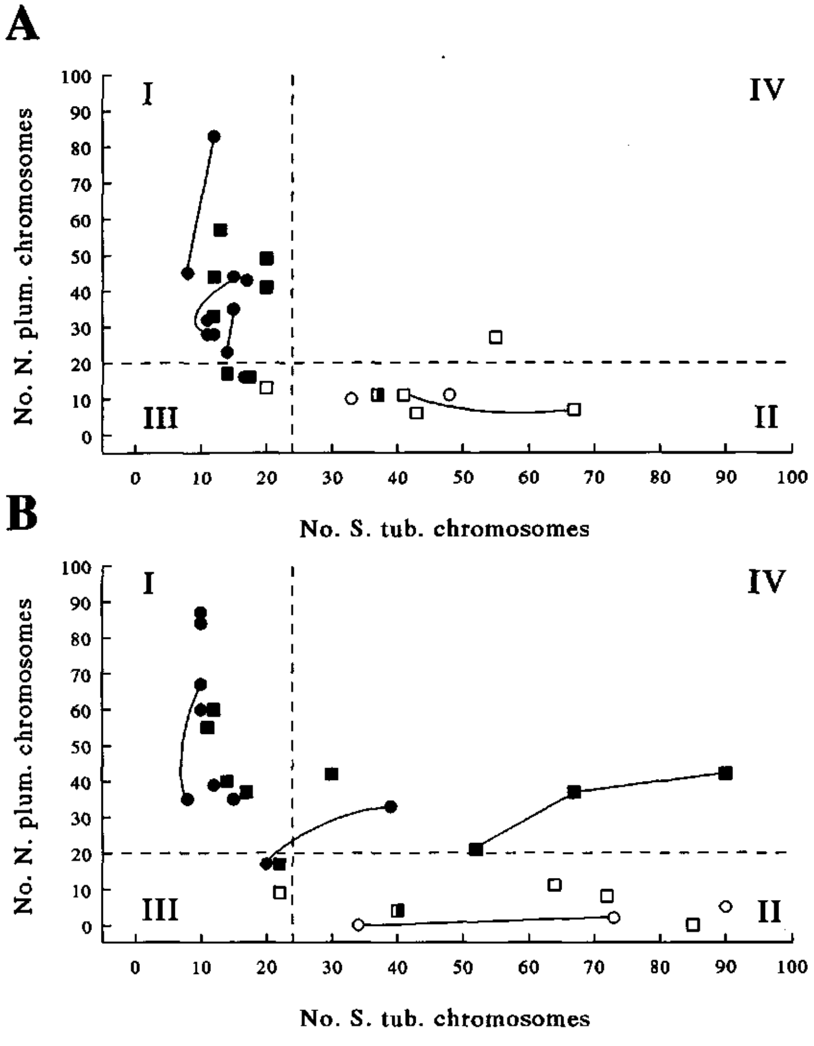

Fig. 4A,B. Scatter diagrams showing the chromosome numbers of both fusion parents in *S. tuberosum* (S) (+) *N. plumbaginifolia* (N) somatic hybrids containing N (+ new) mtDNA fragments (●), S + new mtDNA fragments (○) or N + S (+ new) mtDNA fragments, with more N than S specific fragments (N+s, ■), with more S than N specific fragments (S+n, □) or with as many N as S specific fragments (S+N, ◨). In **A** chromosome numbers in 2-month-old calli and in **B** chromosome numbers in 12-month-old calli are shown. Subpopulations of cells with different chromosome numbers in one hybrid are connected with *lines*. *Dotted lines* distinguish the categories of hybrids

Correlation between cpDNA type and mtDNA composition

A striking phenomenon that emerges from comparison of Fig. 3 and 4 is that almost all hybrids that contained exclusively or predominantly *N. plumbaginifolia* (and new) mtDNA fragments also possessed *N. plumbaginifolia* chloroplasts, whereas almost all hybrids with only or mainly *S. tuberosum* (and new) mtDNA fragments contained potato chloroplasts. To take a closer look at the correlation between chloroplast and mitochondrial DNA in the hybrids, the number of mtDNA fragments specific for one or the other fusion parent were related to the cpDNA type of each hybrid (Fig. 5). Thirteen of the fourteen fusion products containing chloroplasts of *N. plumbaginifolia* possessed more *N. plumbaginifolia-* than *S. tuberosum*-specific mtDNA fragments and seven of these had only *N. plumbaginifolia*-specific mtDNA bands. Of the seven hybrids containing *S. tuberosum* chloroplasts two showed only *S. tuberosum*-specific mtDNA bands, three had more *S. tuberosum-* than *N. plumbaginifolia*-specific mtDNA fragments, and one had equal numbers of mtDNA fragments from both parents.

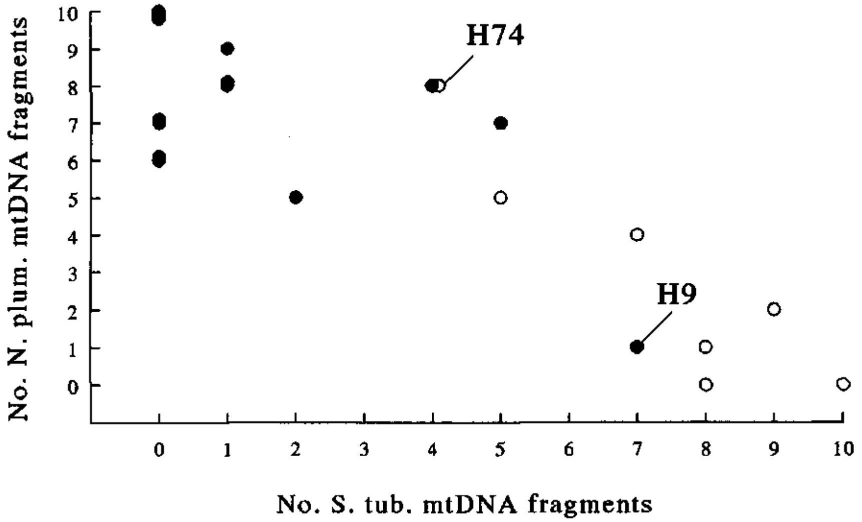

Fig. 5. Scatter diagram showing the number of *S. tuberosum* mtDNA fragments and the number of *N. plumbaginifolia* mtDNA fragments in *S. tuberosum* (+) *N. plumbaginifolia* somatic hybrids containing *N. plumbaginifolia* cpDNA (●) or *S. tuberosum* cpDNA (○)

One hybrid containing *N. plumbaginifolia* chloroplasts had more *S. tuberosum*- than *N. plumbaginifolia*-specific mtDNA fragments (H9) and one hybrid possessing *S. tuberosum* chloroplasts contained more *N. plumbaginifolia*- than *S. tuberosum*-specific mtDNA fragments (H74). Both these calli belong to category III, i.e., they contained subdiploid chromosome numbers of both parents 2 months after fusion. Twelve months after fusion H9 still belonged to this category, while H74 consisted of three subpopulations of cells, all in category IV (containing more than the diploid number of chromosomes of both parents, see Fig. 3).

Discussion

The somatic hybrids analyzed in this study show a strong correlation between chloroplast type and nuclear DNA composition: the chloroplasts in a hybrid have been contributed by the species that predominates in the nucleus. Although chromosome analyses were performed 2 years before the organelle analyses, we assume we would also have found this correlation in, for example, 1-year-old calli, because chloroplast sorting out is known to occur rapidly (Akada and Hirai 1986) and comparison of the chromosome compositions of the calli 2 and 12 months after fusion indicates that changes in time mainly involve polyploidization and a further increase in dominance of the already dominant parent.

Apparently, a viable hybrid can only be obtained if chloroplasts are combined with nuclear DNA that is predominantly derived from the same parent. This is an indication that a strong nucleo-cytoplasmic incongruity exists between *N. plumbaginifolia* and *S. tuberosum.* This phenomenon has been described for a number of species combinations (Thanh and Medgyesy 1989; Kushnir et al. 1991; Perl et al. 1991; Sundberg and Glimelius 1991; Wolters et al. 1991 (Chapter 2); Bonnema et al. 1992). Only a rare recombination event between the parental chloroplasts can overcome this incongruity, as described for a *N. tabacum* (+) *S. tuberosum* fusion product by Thanh and Medgyesy (1989).

The absence of symmetric *Nicotiana* (+) *Solanum* hybrids among the analyzed calli could be due to this proposed nucleo-cytoplasmic incongruity and elimination of a specific chloroplast type or specific chromosomes could be influenced by this incongruity. In that case the question arises: what happens first? Does a rapid unilateral chromosome elimination determine which chloroplasts have a selective advantage and thus will become the dominant type, or does a rapid chloroplast segregation determine which chromosomes have to be retained and which chromosomes can be eliminated? A complicating factor for answering this question is that both chromosome elimination and chloroplast segregation appear to be rapid processes: in our hybrid calli chromosome elimination was already grossly realized 2 months after fusion (Gilissen et al. 1992), whereas Akada and Hirai (1986) also reported that chloroplast segregation is almost complete in 2-month-old somatic hybrid calli.

Chloroplasts which segregate at random may sort out relatively quickly, because the number of chloroplasts is reduced to the number of proplastids typical of a meristematic cell after the first two divisions of a mesophyll protoplast (Thomas and Rose 1983; Rose et al. 1990). If chloroplasts sort out rapidly (Akada and Hirai 1986), it is conceivable that the remaining chloroplasts influence the direction of chromosome elimination. However, somatic hybrid calli contain only plastids and no functional chloroplasts, since photosynthesis is not required. Harada et al. (1992) have shown that rice calli containing plastid DNAs with very large deletions that render the plastids incapable of protein synthesis, are still proliferating well. This makes it unlikely that a sorted out plastid type determines the direction of chromosome elimination.

Irregularities during the first divisions of a hybrid cell can result in chromosome elimination. When chromosomes of one parent are predominantly eliminated this may cause a halt in plastid DNA replication and plastid division of that same species, because chloroplast division, cpDNA replication, transcription and translation are controlled by the nucleus (Umesono and Ozeki 1987). These plastids are then gradually diluted during subsequent cell divisions, until calli arise that have lost this plastid type.

In addition to a nucleus/chloroplast correlation we also found a nucleus/mtDNA correlation in the *S. tuberosum* (+) *N. plumbaginifolia* somatic hybrids. That this correlation is not as clear as the nucleus/chloroplast DNA correlation can be explained by the fact that the two types of mitochondria recombine easily. As in chloroplasts, the enzymes involved in mtDNA replication, transcription and processing, and translation appear to be nuclear encoded (Levings and Brown 1989). For meristematic activity and early cellular differentiation the controlled provision of energy and of many biosynthetic intermediates by mitochondria is required (Leaver 1991). Thus, in contrast with chloroplasts, functional mitochondria are probably required for growth of the hybrid calli, and the nucleus/mitochondrion interaction has to be optimal. After fusion of protoplasts of two species the two types of mitochondria will mix and recombine, but cells carrying mitochondria that can produce energy will have a selective advantage. Presumably, certain recombinant mtDNA molecules as well as unchanged parental mtDNA molecules, can be functional.

A correlation, albeit not perfect, between cpDNA type and number of species-specific mtDNA fragments was found (Fig. 5). Plastids and mitochondria do not function independently of each other. Heme, an important constituent of cytochrome and thus crucial for the fuctioning of mitochondria, is synthesised as a byproduct of the porphyrin pathway which leads to the synthesis of chlorophylls (Howe and Smith 1991). In plants the first stages of this pathway are restricted to plastids and the plastid-encoded tRNA^{Glu} plays an important role at this time (Schön et al. 1986). The rice calli with large deletions in plastid DNA studied by Harada et al. (1991, 1992) all had retained at least a small region containing the tRNA^{Glu} gene (*trn*E). Thus the function of the remaining plastid DNA may have been the production of heme for mitochondria and other organelles.

Similarly, in order to obtain functional mitochondria in somatic hybrid calli the transcription of genes such as the plastid tRNA^{Glu} gene is required. This transcription is regulated by the nucleus. In fusion products between closely related species in which all or most of the chromosomes from both parents are present, each of the two types of chloroplasts may be induced to transcribe these genes. Possibly, in hybrids between distantly related species transcription is preferentially induced in the chloroplasts and mitochondria from the fusion parent that predominates in the nucleus. Thus cosegregation of chloroplasts and mitochondria or mitochondrial DNA fragments can be explained. The observation that specific alterations of mtDNA show deleterious pleiotropic effects on chloroplast structure and function (Roussell et al. 1991) indicates that the functions of mitochondria and chloroplasts are interdependent in more than one way.

To summarize, a functional mtDNA genome is required for the growth of calli, and mitochondria can only be functional when many nuclear genes and certain chloroplast-encoded genes are present. Possibly, in symmetric hybrids between unrelated species containing nuclear DNA, chloroplasts, and mitochondria from both parents many defective organelles develop, as a result of difficulties in assembling functional protein complexes when two types of nuclear-encoded proteins and two types of organellar DNA-encoded proteins are involved. These defective organelles may cause an arrest of cell division. This would provide a selection mechanism for a "non-hybrid" genetic composition in which the origin of all genophores is identical. We suggest that, while chloroplast segregation and chromosome elimination may occur simultaneously and independently during early stages of hybrid development, there is a strong selective advantage of cells in which (certain) nuclear genes of the parent not providing the organelles are absent and that this causes the selection of hybrids with a uniparentally eliminated nuclear genome. Another way of solving the protein assembly problem can be the polyploidization of the genome of only one parent; this may cause a dilution of "wrong" nuclear-encoded components. Since only a small portion of the originally obtained somatic hybrids will meet the demands of a balanced nucleus-mitochondrion-chloroplast interaction, this possibly explains the low frequency of viable somatic hybrids between distantly related species.

Acknowledgements. The authors thank A.K. Saiedi for assistence with the organelle DNA analyses and C. Heyting for critically reading the manuscript. This work was supported by the Dutch Programme Committee on Agricultural Biotechnology (PcLB).

References

Akada S, Hirai A (1986) Studies on the mode of separation of chloroplast genomes in parasexual hybrid calli III. Random separation of two types of chloroplast genomes in a hybrid callus. Jpn J Genet 61:437-445

Babiychuk E, Kushnir S, Gleba YY (1992) Spontaneous extensive chromosome elimination in somatic hybrids between somatically congruent species *Nicotiana tabacum* L. and *Atropa belladonna* L. Theor Appl Genet 84:87-91

Bonnema AB, Melzer JM, Murray LW, O'Connell MA (1992) Non-random inheritance of organellar genomes in symmetric and asymmetric somatic hybrids between *Lycopersicon esculentum* and *L. pennellii*. Theor Appl Genet 84:435-442

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1:19-21

Dewey RE, Schuster AM, Levings III CS, Timothy DH (1985) Nuucleotide sequence of F_0-ATPase proteolipid (subunit 9) gene of maize mitochondria. Proc Natl Acad Sci USA 82:1015-1019

Fisher RA (1941) Statistical methods for research workers. Oliver & Boyd, Edinburgh, §21.02

Gilissen LJW, Sree Ramulu K, Flipse E, Meinen E, Stiekema WJ (1991) Transformation of diploid potato genotypes through *Agrobacterium* vectors and expression of T-DNA markers in root clones, regenerated plants and suspension cells. Acta Bot Neerl 40:53-61

Gilissen LJW, van Staveren MJ, Verhoeven HA, Sree Ramulu K (1992) Somatic hybridization between potato and *Nicotiana plumbaginifolia* 1. Spontaneous biparental chromosome elimination and production of asymmetric hybrids. Theor Appl Genet 84:73-80

Gleba YY, Sytnik KM (1984) Protoplast fusion. Genetic engineering in higher plants. Springer Verlag, Berlin Heidelberg New York Tokyo

de Haas JM, Boot KJM, Haring MA, Kool AJ, Nijkamp HJJ (1986) A *Petunia hybrida* chloroplast DNA region, close to one of the inverted repeats, shows sequence homology with the *Euglena gracilis* chloroplast DNA region that carries the putative replication origin. Mol

Gen Genet 202:48-54

Harada T, Sato T, Asaka D, Matsukawa I (1991) Large-scale deletions of plastid DNA in rice anther culture. Theor Appl Genet 81:157-161

Harada T, Ishikawa R, Niizeki M, Saito K (1992) Pollen-derived rice calli that have large deletions in plastid DNA do not require protein synthesis in plastids for growth. Mol Gen Genet 233:145-150

Hause B, Baldauf F, Stock K, Wasternack C, Metzlaff M (1986) Molecular analysis of the mitochondrial DNA from tomato cell suspension cultures. Curr Genet 10:785-790

Hoffmann F, Adachi T (1981) "Arabidobrassica": chromosomal recombination in asymmetric intergeneric hybrid cells. Planta 153:586-593

Howe CJ, Smith AG (1991) Plants without chlorophyll. Nature 349:109

Iams KP, Sinclair JH (1982) Mapping the mitochondrial DNA of *Zea mays*: Ribosomal gene localization. Proc Natl Acad Sci USA 79:5926-5929

Kemble RJ, Barsby TL, Wong RSC, Shepard JF (1986) Mitochondrial DNA rearrangements in somatic hybrids of *Solanum tuberosum* and *Solanum brevidens*. Theor Appl Genet 72:787-793

Kushnir S, Babiychuk E, Bannikova M, Momot V, Komarnitsky I, Cherep N, Gleba Y (1991) Nucleo-cytoplasmic incompatibility in cybrid plants possessing an *Atropa* genome and a *Nicotiana* plastome. Mol Gen Genet 225:225-230

Leaver CJ (1991) Mitochondrial genetic information. In: Huang AHC, Taiz L (eds) Molecular approaches to compartmentation and metabolic regulation. The American Society of Plant Physiologists, Rockville, pp. 48-58

Levings III CS, Brown GG (1989) Molecular biology of plant mitochondria. Cell 56:171-179

Lonsdale DM, Hodge TP, Fauron CM-R (1984) The physical map and organization of the mitochondrial genome from the fertile cytoplasm of maize. Nucl Acids Res 12:9249-9261

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

Perl A, Aviv D, Galun E (1991) Nuclear-organelle interaction in *Solanum*: Interspecific cybridizations and their correlation with a plastome dendrogram. Mol Gen Genet 228:193-200

Rogers SO, Bendich AJ (1988) Extraction of DNA from plant tissues. Plant molecular biology manual A6. Kluwer Academic Publ, Dordrecht, pp 1-10

Rose RJ, Thomas MR, Fitter JT (1990) The transfer of cytoplasmic and nuclear genomes by somatic hybridisation. Aust J Plant Physiol 17:303-321

Rothenberg M, Boeshore ML, Hanson MR, Izhar S (1985) Intergenomic recombination of mitochondrial genomes in a somatic hybrid plant. Curr Genet 9:615-618

Roussell DL, Thompson DL, Pallardy SG, Miles D, Newton KJ (1991) Chloroplast structure and function is altered in the NCS2 maize mitochondrial mutant. Plant Physiol 96:232-238

Schön A, Krupp G, Gough S, Berry-Lowe S, Kannangara CG, Söll D (1986) The RNA required in the first step of chlorophyll biosynthesis is a chloroplast glutamate tRNA. Nature 322:281-284

Shinozaki K, Ohme M, Tanaka M, Wakasugi T, Hayshida N, Matsubayasha T, Zaita N, Chunwongse J, Obokata J, Yamaguchi-Shinozaki K, Ohto C, Torazawa K, Meng BY, Sugita M, Deno H, Kamogashira T, Yamada K, Kusuda J, Takaiwa F, Kata A, Tohdoh N, Shimada H, Sugiura M (1986) The complete nucleotide sequence of the tobacco chloroplast genome. Plant Mol Biol Rep 4:111-147

Small ID, Isaac PG, Leaver CJ (1987) Stoichiometric differences in DNA molecules containing the *atpA* gene suggest mechanisms for the generation of mitochondrial genome diversity in maize. EMBO J 6:865-869

Sundberg E, Glimelius K (1991) Effects of parental ploidy level and genetic divergence on chromosome elimination and chloroplast segregation in somatic hybrids within *Brassicaceae*. Theor Appl Genet 83:81-88

Thanh ND, Medgyesy P (1989) Limited chloroplast gene transfer via recombination overcomes plastome-genome incompatibility between *Nicotiana tabacum* and *Solanum tuberosum*. Plant Mol Biol 12:87-93

Thomas MR, Rose RJ (1983) Plastid number and plastid structural changes associated with tobacco mesophyll protoplast culture and plant regeneration. Planta 158:329-338

Umesono K, Ozeki H (1987) Chloroplast gene organization in plants. Trends Genet 3:281-287

de Vries SE, Ferwerda MA, Loonen AEHM, Pijnacker LP, Feenstra WJ (1987) Chromosomes in somatic hybrids between *Nicotiana plumbaginifolia* and a monoploid potato. Theor Appl Genet 75:170-176

de Vries-Uijtewaal E, Gilissen LJW, Flipse E, Sree Ramulu K, Stiekema WJ, de Groot B (1989) Fate of introduced genetic markers in transformed root clones and regenerated plants of monohaploid and diploid potato genotypes. Theor Appl Genet 78:185-193

Wolters AMA, Schoenmakers HCH, van der Meulen-Muisers JJM, van der Knaap E, Derks FHM, Koornneef M, Zelcer A (1991) Limited DNA elimination from the irradiated potato parent in fusion products of albino *Lycopersicon esculentum* and *Solanum tuberosum*. Theor Appl Genet 83:225-232

Young EG, Hanson MR (1987) A fused mitochondrial gene associated with cytoplasmic male sterility is developmentally regulated. Cell 50:41-49

Chapter 7

General discussion

Somatic incongruity and phylogenetic divergence

Somatic hybridization is considered as a method to circumvent the sexual incompatibility barriers between different plant species and thereby to extend the range of species of which genetic information can be combined. In addition, it is seen as a method to bypass maternal inheritance of organelles. In a fusion product the two parental nuclei can either fuse or not fuse, and usually sorting out of chloroplasts and mitochondria takes place. Because of these processes at least 12 different combinations of the three genophores are possible. Since recombination between mitochondrial DNAs occurs frequently, many different mtDNA types can be formed, which greatly increases the number of possible genophore combinations. However, from this thesis, and from other publications (e.g. Sundberg and Glimelius 1991; Donaldson et al. 1993; Walters and Earle 1993), it is evident that in many somatic hybridization experiments only a small fraction of these possible combinations is actually found. When this is not a consequence of the fusion conditions or the selection method, the number of combinations may be limited because of the occurrence of somatic incongruity. Several types of somatic incongruity can be present in a heterokaryon, as is shown in Fig. 1. The nuclei of both parents may not be able to function properly together (nucleus-nucleus incongruity), chloroplasts and/or mitochondria of one parent may be unable to cooperate with the nucleus of the other parent (nucleo-cytoplasmic incongruity), and possibly, mitochondria of one species can not be functional in the presence of chloroplasts solely derived from the other species.

Nucleus-nucleus incongruity at the heterokaryon stage may lead to an inability of the nuclei to fuse and/or may prevent cell division to occur. When the nuclei do fuse, which generally is the case, a disturbed chromosome segregation and other irregularities during mitosis may result in an uneven distribution of chromosomes and sometimes in chromosome fragmentation. When this is followed by cell division, aneuploid daughter cells are obtained (Chapter 4).

Nucleo-cytoplasmic incongruity may occur when the chloroplast and/or mitochondrial DNAs of the two fusion parents differ in such a way that slightly different subunits of essential protein complexes are produced. In a somatic hybrid, the chloroplast- or mitochondrion-encoded subunits of one parent may not be able to form a functional protein complex together with subunits encoded by the nucleus of the other parent. This hybrid may then show poor growth or may not even survive.

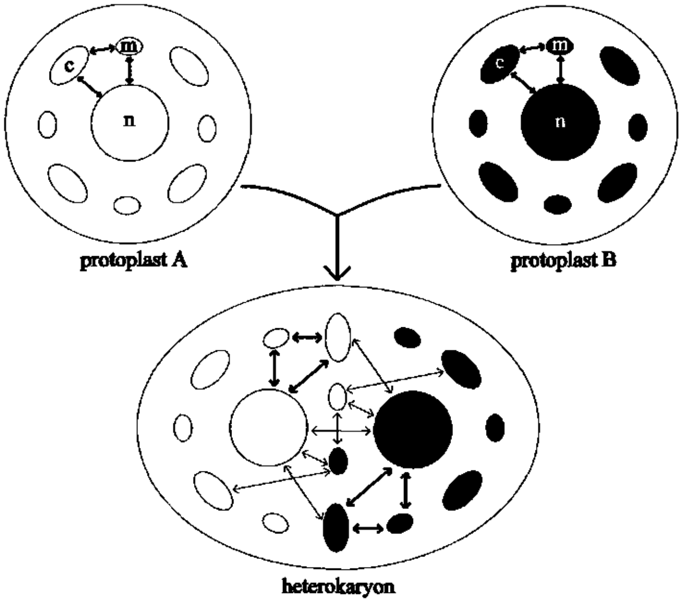

Fig. 1. Interactions between the nucleus (n), chloroplasts (c) and mitochondria (m) in parental protoplasts and in a heterokaryon. ***Solid arrows*** indicate compatible interactions, *thin arrows* indicate interactions that may or may not be functional

The sorting out of organelles, recombination of mitochondrial DNAs and irregularities during mitosis lead to genetic variation among fusion products. Tissue culture conditions may induce additional variation (somaclonal variation). When some type of incongruity is present between the two fusion parents, the genetic variation generated in the aforementioned ways will allow the selective amplification of combinations of organellar and nuclear genes that are attuned to each other.

Somatic incongruity resulting in non-viable fusion products is stronger when the fusion parents are less related. This is concluded from the observation that somatic hybrids can much more efficiently be produced between tomato and potato, which belong to the same tribe (Chapter 3), than between tomato and *Nicotiana* spp. (Chapter 5) or between potato and *Nicotiana* sp. (Chapter 6), which belong to different subfamilies.

The possible outcomes of somatic hybridization, in view of somatic incongruity, are shown schematically in Fig. 2, for closely related species (Fig. 2A), for related species (Fig. 2B) and for distantly related species (Fig. 2C). This scheme is illustrated by our experiments with *Lycopersicon*, *Solanum* and *Nicotiana* species.

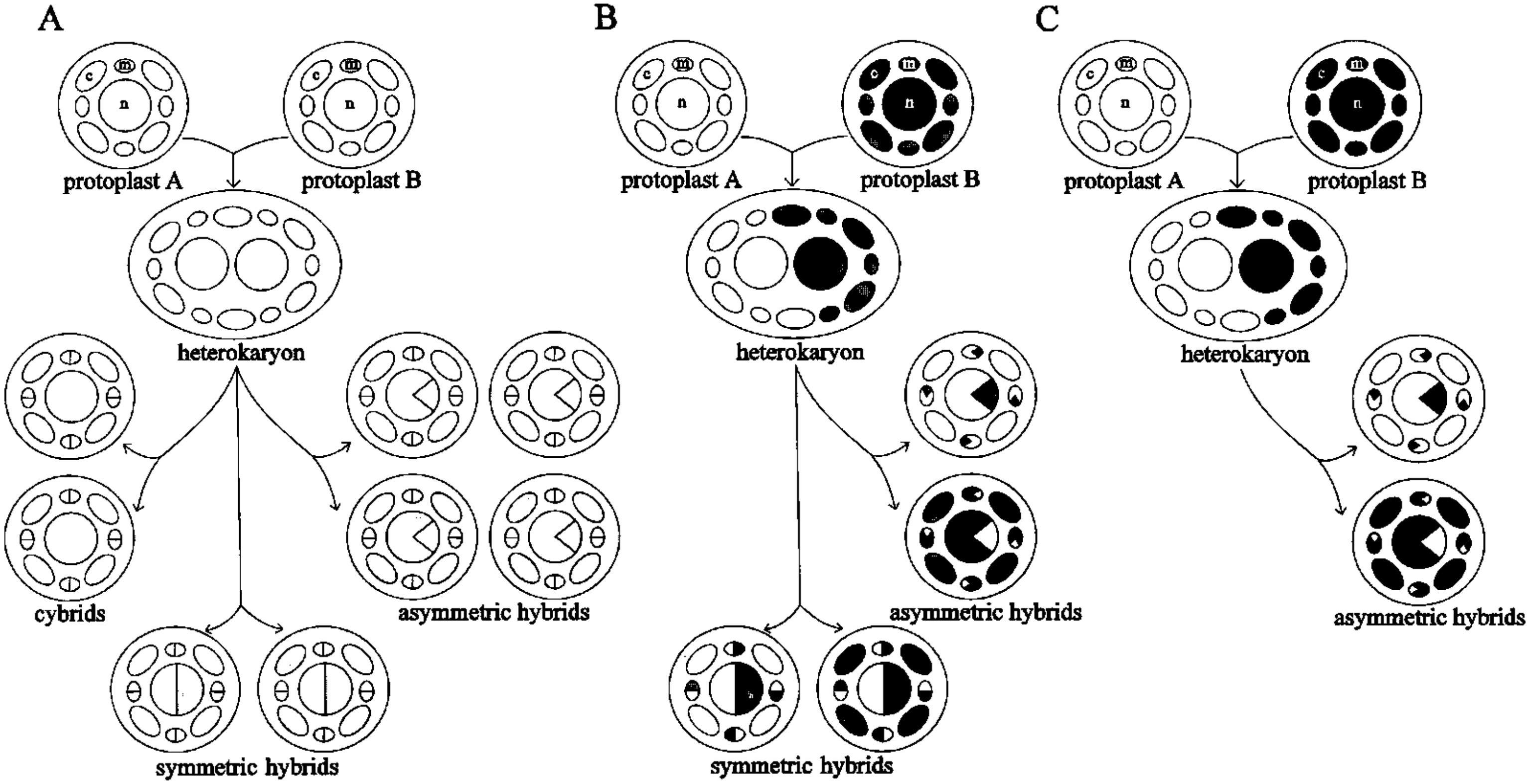

Fig. 2A-C. Possible genophore compositions of somatic hybrids resulting from heterokaryons, obtained after fusion of **A**, two closely related species, **B**, two related species, **C**, two distantly related species. **n**, nucleus; **c**, chloroplast; **m**, mitochondrion

When fusions are made between protoplasts of closely related species, e.g. intrageneric fusions within *Lycopersicon*, "cp cybrids" (cybrids combining the nucleus of one species with chloroplasts of another species) (Ratushnyak et al. 1991; Derks et al. 1992) and "mt cybrids" (cybrids combining the nucleus of one species with (a major part of) the mitochondrial DNA of another species) (Bonnema et al. 1991) can be obtained, in addition to symmetric and asymmetric hybrids (Fig. 2A).

When the fusion parents are related, but not close enough to preclude nucleo-cytoplasmic incongruity, symmetric and asymmetric somatic hybrids can be obtained, but no cybrids (Fig. 2B). This is the case when intergeneric fusions are made with *L. esculentum*, e.g. with *Solanum tuberosum*. We could not obtain true cybrids combining a tomato nucleus with potato chloroplasts (Chapter 2). In general, it seems to be difficult or impossible to produce cybrids containing a *Lycopersicon* nucleus and *Solanum* chloroplasts (Derks et al. 1992; Lefrançois et al. 1993). This is remarkable, because cp cybrids have been obtained in other intergeneric combinations, e.g. *Nicotiana tabacum* with *Petunia hybrida* (Glimelius and Bonnett 1986; Pental et al. 1986), *N. tabacum* with *Salpiglossis sinuata* (Thanh et al. 1988), and *Citrus* spp. with *Microcitrus* sp. (Vardi et al. 1989). However, in none of these examples conclusive evidence was provided that the nucleus contained only recipient DNA. On the other hand, even within the large genus *Solanum* nucleus-chloroplast incongruity between certain species was demonstrated to be present by Perl et al. (1991).

Symmetric tomato (+) potato somatic hybrids containing both tomato and potato nuclear DNA can be viable when they possess tomato chloroplasts, but also when they have potato chloroplasts. The same holds true for the mitochondria. This suggests that only the nuclear-encoded subunits of the parent from which the organelles are derived are involved in the formation of functional protein complexes in the organelle. Chloroplasts and mitochondria segregate independently in these hybrids (Chapter 3), indicating that there is no preference for a specific chloroplast-mitochondrion combination. Nucleus-nucleus incongruity does not seem to be present in tomato (+) potato somatic hybrids, up to the level of shoot regeneration. However, the inability of these hybrids to grow properly in soil in the greenhouse, and the sterility observed in all (symmetric) hybrids (Schoenmakers et al. 1993) may be an expression of nucleus-nucleus incongruity. Alternatively, these phenomena could be caused by nucleo-cytoplasmic incongruity. The aneuploidy (due to somaclonal variation) observed in many of these hybrids (Chapter 4), may be a third reason for their poor performance.

When distantly related species are fused, it may be impossible to produce cp cybrids, mt cybrids (e.g. Melchers et al. 1992) and symmetric somatic hybrids (Fig. 2C). In somatic hybrids between *Lycopersicon* and *Nicotiana* species, and between *Solanum* and *Nicotiana* species (Chapters 5 and 6) a strong nucleo-cytoplasmic incongruity exists, as is shown by the observation that the majority of these hybrids possess chloroplasts and a major part of the mitochondrial DNA from the species that predominates in the nucleus. Thus, not only the nucleus-chloroplast interaction, but also the nucleus-mitochondrion interaction, and possibly the chloroplast-mitochondrion relation, can be disturbed when the genophores of both parents show a considerable divergence. Furthermore, spontaneous elimination of chromosomes had occurred in all hybrids, often combined with an increase of the number of nuclear genomes of the parent that showed the least elimination of chromosomes. This suggests that nucleus-nucleus incongruity is present.

Probably, the requirement for mitochondrial DNA to be derived from the same parent as the major part of the nuclear DNA is less stringent than for chloroplast DNA, because recombination of mitochondrial genomes may result in a "functional" hybrid mtDNA (cf. Melchers et al. 1992). Recombined chloroplast DNA, with only a part of the cpDNA being alien, may also be functional in a cybrid, as was demonstrated by Thanh and Medgyesy (1989) for *Nicotiana/Solanum* cybrids. However, the limiting factor for chloroplast recombination is the rare occurrence of fusion of chloroplasts.

Fertility and meiotic recombination

Not only the viability, but also the degree of fertility of a somatic hybrid depends on the phylogenetic relatedness of the two fusion parents. Male sterility may result from a suboptimal nucleus-mitochondrion interaction (alloplasmic sterility). Furthermore, an irregular meiosis caused by aneuploidy, chromosome rearrangements or lack of chromosome pairing may lead to sterility of the hybrid (Chapter 4). In somatic hybrids between distantly related species incongruity between the parental nuclei may cause male and female sterility. In the case that a fusion product shows some degree of female fertility, and pollination with one of the parents results in the formation of embryos, the development of the embryos may be disturbed because of a defective endosperm or because of other post-fertilization blocks.

Many backcrosses were made with some of our triploid tomato (+) potato somatic hybrids, but no progeny was obtained (Schoenmakers et al. 1993). This seemed to be caused by a non-functional pollen-style interaction. Tomato and potato pollen tube growth was inhibited in the style of the somatic hybrid flowers. *L. pennellii* pollen tubes grew further into the style of the hybrids, but their growth was arrested near the ovules. However, somatic hybrids with different ploidy levels may differ in crossability. Jacobsen et al. (1994a,b) backcrossed several hexaploid tomato (+) potato somatic hybrids (2x tomato (+) 4x potato) with tetraploid potato. After performing embryo rescue on a large scale one progeny plant was obtained. After repeated backcrossing a second progeny plant was obtained from a different somatic hybrid, but with the same fusion parents as the one that produced the first progeny (E. Jacobsen and D.J. Huigen, pers. comm.). Thus, the possibility to obtain progeny seems to depend not only on the species combination, but also on the genotypes of the parental species and, possibly, on the genotype of the individual somatic hybrid.

When it is possible to obtain progeny, it can be questioned if meiotic recombination occurs between chromosomes of different parents. In general, genotypes are wanted that resemble one parental species, but contain some characteristics of the other parental species. For this, it is necessary that chromosome fragments of the donor species become stably integrated in the genome of the recipient. One way integration can be achieved is by intergenomic translocation. This has sometimes been observed in asymmetric somatic hybrids (Piastuch and Bates 1990; Parokonny et al. 1992), but can also occur in symmetric somatic hybrids (White and Rees 1985; Chapter 4). However, the translocation chromosome may cause meiotic irregularities, resulting in sterility of the hybrid. Therefore, meiotic recombination is preferred.

Presumably, meiotic recombination between chromosomes of different parents occurs only when the chromosomes show a high degree of homology, and therefore when the parents are relatively closely related. De Jong et al. (1993) observed pairing between homoeologous chromosomes during pachytene of hypotetraploid tomato (+) potato somatic hybrids, but at metaphase I mostly bivalents were present, occasionally with 1-3 quadrivalents and some univalents. Jacobsen et al. (1992) observed only bivalents in 13 of the 14 tetraploid tomato (+) potato somatic hybrids. Although it is likely that bivalents consist of homologous chromosomes, it cannot be excluded that some bivalents are formed between homoeologous chromosomes. By genomic *in situ* hybridization on pachytene-metaphaseI chromosomes of tomato (+) potato somatic hybrids it was shown that most bivalents consisted of homologous chromosomes, but occasionally bivalents of homoeologous chromosomes were observed (J.H. de Jong, pers. comm.).

The potential of somatic hybrids in plant breeding

Somatic hybridization can extend the range of species that can be used for improvement of a crop species, but strong limitations are present. Phylogenetic divergence of the two fusion parents plays a prominent role in determining the limitations. The possibilities for producing the different types of fusion products are given below.

mt cybrids

Cytoplasmic male sterility (CMS) can often be induced in a crop species by introduction of mitochondria from an other, not very related species via somatic hybridization (Melchers et al. 1992) or, possibly, via injection of isolated mitochondria (Verhoeven and Blaas 1992). However, for many species not only a CMS genotype is desired, but also a genotype carrying fertility restorer gene(s). It may be very difficult or impossible to produce such a restorer genotype. Therefore, the potential of alloplasmic CMS probably is limited.

cp cybrids

The possibilities of transfer of chloroplasts from one species into an other species seem to be more limited than the possibilities of transfer of mitochondrial genes. This is shown by the observation that cybrids combining a tomato nucleus with potato chloroplasts could not be obtained (Chapter 2), but cybrids combining a tomato nucleus with potato mitochondrial DNA fragments have been produced by Melchers et al. (1992). Recombination of chloroplast DNAs may extend the range of chloroplast genes than can be combined with an alien nucleus, but before recombination can be induced the chloroplast fusion barrier has to be overcome (Thanh and Medgyesy 1989). Alternatively, when a specific chloroplast-encoded trait is desired in a certain genotype, this can possibly be achieved by transformation of chloroplasts and integration of the transferred gene(s) via homologous recombination, which has recently been described (Staub and Maliga 1992; Golds et al. 1993; Maliga 1993; O'Neill et al. 1993).

Symmetric somatic hybrids

Symmetric fusion products can be produced when the parental species are relatively related. However, somaclonal variation may induce the formation of aneuploid somatic hybrids (Chapter 4). Probably, the degree of somaclonal variation is genotype-dependent, and if so, it is very important to use several different genotypes of each parent in a fusion experiment. Fertility of the resulting symmetric hybrids may be reduced when the parents are not closely related.

Asymmetric somatic hybrids

Fusion of X- or gamma-irradiated donor protoplasts with untreated recipient protoplasts often yields asymmetric hybrids showing only a limited elimination of donor DNA (Chapter 2). In addition, these hybrids often contain multiple nuclear genomes of the recipient species (Agoudgil et al. 1990; Kovtun et al. 1993; Puite and Schaart 1993; Schoenmakers et al. 1994). Possibly, microprotoplast-protoplast fusion (Ramulu et al. 1993) can be more effective for producing asymmetric hybrids. Alternatively, isolation of individual metaphase chromosomes (Arumuganathan et al. 1991; Schubert et al. 1993) and subsequent injection into the recipient nucleus may yield the desired genotype. However, stable introgression of the desired gene(s) will still be difficult to achieve. Furthermore, aneuploidy may cause an irregular morphology and an irregular meiosis, resulting in sterility.

References

Agoudgil S, Hinnisdaels S, Mouras A, Negrutiu I, Jacobs M (1990) Metabolic complementation for a single gene function associated with partial and total loss of donor DNA in interspecific somatic hybrids. Theor Appl Genet 80:337-342

Arumuganathan K, Slattery JP, Tanksley SD, Earle ED (1991) Preparation and flow cytometric analysis of metaphase chromosomes of tomato. Theor Appl Genet 82:101-111

Bonnema AB, Melzer JM, O'Connell MA (1991) Tomato cybrids with mitochondrial DNA from *Lycopersicon pennellii*. Theor Appl Genet 81:339-348

Derks FHM, Hakkert JC, Verbeek WHJ, Colijn-Hooymans CM (1992) Genome composition of asymmetric hybrids in relation to the phylogenetic distance between the parents. Nucleus-chloroplast interaction. Theor Appl Genet 84:930-940

Donaldson P, Sproule A, Bevis E, Pandeya R, Keller WA, Gleddie S (1993) Non-random chloroplast segregation in *Nicotiana tabacum* (+) *N. rustica* somatic hybrids selected by dual nuclear-encoded resistance. Theor Appl Genet 86:465-473

Glimelius K, Bonnett HT (1986) *Nicotiana* cybrids with *Petunia* chloroplasts. Theor Appl Genet 72:794-798

Golds T, Maliga P, Koop HU (1993) Stable plastid transformation in PEG-treated protoplasts of *Nicotiana tabacum*. Bio/Technology 11:95-97

Jacobsen E, Reinhout P, Bergervoet JEM, de Looff J, Abidin PE, Huigen DJ, Ramanna MS (1992) Isolation and characterization of potato-tomato somatic hybrids using an amylose-free potato mutant as parental genotype. Theor Appl Genet 85:159-164

Jacobsen E, Daniel MK, Bergervoet-van Deelen JEM, Huigen DJ, Ramanna MS (1994a) The first and second backcross progeny of the intergeneric fusion hybrids of potato and tomato after crossing with potato. Theor Appl Genet 88:181-186

Jacobsen E, de Jong JH, Kamstra SA, van den Berg PMMM, Ramanna MS (1994b) Genomic *in situ* hybridization (GISH) and RFLP analysis for the identification of alien chromosomes in the backcross progeny of potato (+) tomato fusion hybrids. Heredity (in press)

de Jong JH, Wolters AMA, Kok JM, Verhaar H, van Eden J (1993) Chromosome pairing and potential for intergeneric recombination in some hypotetraploid somatic hybrids of *Lycopersicon esculentum* (+) *Solanum tuberosum*. Genome 36:1032-1041

Kovtun YV, Korostash MA, Butsko YV, Gleba YY (1993) Amplification of repetitive DNA from *Nicotiana plumbaginifolia* in asymmetric somatic hybrids between *Nicotiana sylvestris* and *Nicotiana plumbaginifolia*. Theor Appl Genet 86:221-228

Lefrançois C, Chupeau Y, Bourgin JP (1993) Sexual and somatic hybridization in the genus *Lycopersicon*. Theor Appl Genet 86:533-546

Maliga P (1993) Towards plastid transformation in flowering plants. Trends Biotechnol 11:101-107

Melchers G, Mohri Y, Watanabe K, Wakabayashi S, Harada K (1992) One-step generation of cytoplasmic male sterility by fusion of mitochondrial-inactivated tomato protoplasts with nuclear-inactivated *Solanum* protoplasts. Proc Natl Acad Sci USA 89:6832-6836

O'Neill, C, Horváth GV, Horváth E, Dix PJ, Medgyesy P (1993) Chloroplast transformation in plants: polyethylene glycol (PEG) treatment of protoplasts is an alternative to biolistic delivery systems. Plant J 3:729-738

Parokonny AS, Kenton AY, Gleba YY, Bennett MD (1992) Genome reorganization in *Nicotiana* asymmetric somatic hybrids analysed by *in situ* hybridization. Plant J 2:863-874

Pental D, Hamill JD, Pirrie A, Cocking EC (1986) Somatic hybridization of *Nicotiana tabacum* and *Petunia hybrida*. Recovery of plants with *P. hybrida* nuclear genome and *N. tabacum* chloroplast genome. Mol Gen Genet 202:342-347

Perl A, Aviv D, Galun E (1991) Nuclear-organelle interaction in *Solanum*: Interspecific cybridizations and their correlation with a plastome dendrogram. Mol Gen Genet 228:193-200

Piastuch WC, Bates GW (1990) Chromosomal analysis of *Nicotiana* asymmetric somatic hybrids by dot blotting and in situ hybridization. Mol Gen Genet 222:97-103

Puite KJ, Schaart JG (1993) Nuclear genomic composition of asymmetric fusion products between irradiated transgenic *Solanum brevidens* and *S. tuberosum*: limited elimination of donor chromosomes and polyploidization of the recipient genome. Theor Appl Genet 86:237-244

Ramulu KS, Dijkhuis P, Famelaer I, Cardi T, Verhoeven HA (1993) Isolation of sub-diploid microprotoplasts for partial genome transfer in plants: Enhancement of micronucleation and enrichment of microprotoplasts with one or a few chromosomes. Planta 190:190-198

Ratushnyak YI, Latypov SA, Samoylov AM, Piven NM, Gleba YY (1991) Introgressive hybridization of tomatoes by 'gamma-fusion' of *Lycopersicon esculentum* Mill. and *Lycopersicon peruvianum* var. *dentatum* Dun. protoplasts. Plant Science 73:65-78

Schoenmakers HCH, Wolters AMA, Nobel EM, de Klein CMJ, Koornneef M (1993) Allotriploid somatic hybrids of diploid tomato (*Lycopersicon esculentum* Mill.) and monoploid potato (*Solanum tuberosum* L.). Theor Appl Genet 87:328-336

Schoenmakers HCH, Wolters AMA, de Haan A, Saiedi AK, Koornneef M (1994) Asymmetric somatic hybridization between tomato (*Lycopersicon esculentum* Mill.) and gamma-irradiated potato (*Solanum tuberosum* L.): a quantitative analysis. Theor Appl Genet 87:713-720

Schubert I, Dolezel J, Houben A, Scherthan H, Wanner G (1993) Refined examination of plant metaphase chromosome structure at different levels made feasible by new isolation methods. Chromosoma 102:96-101

Staub JM, Maliga P (1992) Long regions of homologous DNA are incorporated into the tobacco plastid genome by transformation. Plant Cell 4:39-45

Sundberg E, Glimelius K (1991) Effects of parental ploidy level and genetic divergence on chromosome elimination and chloroplast segregation in somatic hybrids within *Brassicaceae*. Theor Appl Genet 83:81-88

Thanh ND, Medgyesy P (1989) Limited chloroplast gene transfer via recombination overcomes plastome-genome incompatibility between *Nicotiana tabacum* and *Solanum tuberosum*. Plant Mol Biol 12:87-93

Thanh ND, Páy A, Smith MA, Medgyesy P, Márton L (1988) Intertribal chloroplast transfer by protoplast fusion between *Nicotiana tabacum* and *Salpiglossis sinuata*. Mol Gen Genet 213:186-190

Vardi A, Arzee-Gonen P, Frydman-Shani A, Bleichman S, Galun E (1989) Protoplast-fusion-mediated transfer of organelles from *Microcitrus* into *Citrus* and regeneration of novel alloplasmic trees. Theor Appl Genet 78:741-747

Verhoeven HA, Blaas J (1992) Direct cell to cell transfer of organelles by microinjection. Plant Cell Rep 10:613-616

Walters TW, Earle ED (1993) Organellar segregation, rearrangement and recombination in protoplast fusion-derived *Brassica oleracea* calli. Theor Appl Genet 85:761-769

White J, Rees H (1985) The chromosome cytology of a somatic hybrid petunia. Heredity 55:53-59

Summary

In this thesis the results are presented of somatic hybridization experiments aimed at the combination of genetic information of different solanaceous species, with an emphasis on the elucidation of the interaction between nuclei, chloroplasts and mitochondria in the obtained somatic hybrids.

In chapter 1 some factors are considered that can influence the results of somatic hybridization, in particular the phylogenetic distance between the fusion parents and somatic incongruity that may result from this. A review is given of reported somatic hybridization experiments between tomato (*Lycopersicon esculentum*) and other species from the same genus, from other genera of the same tribe, and from genera of other tribes of the *Solanaceae*. The fate of chromosomes, chloroplasts and mitochondria after fusion of two protoplasts of different species, and the possibilities of fertility of somatic hybrids on the basis of these literature data are indicated.

Chapter 2 describes the analysis of symmetric and asymmetric somatic hybrids of a cytoplasmic albino tomato and a kanamycin-resistant, ß-glucuronidase containing potato (*Solanum tuberosum*). The potato protoplasts had been irradiated with 0, 50 or 500 Gray of gamma-rays. The relative amount of potato nuclear DNA of these somatic hybrids was determined by means of dot blot hybridization with the use of species-specific repetitive DNA probes. Comparison of these relative amounts between symmetric and asymmetric hybrids revealed that the degree of irradiation-induced elimination of potato DNA was limited. Furthermore, no cybrids combining a tomato nucleus with potato chloroplasts were obtained.

Chapter 3 describes the analysis of the chloroplast and mitochondrial DNA composition of somatic hybrids of a cytoplasmic albino tomato with a monoploid potato (A7-hybrids) and of a nitrate reductase-deficient tomato with the same monoploid potato (C7-hybrids). All A7-hybrids contained potato chloroplasts. All but one of the C7-hybrids possessed either tomato or potato chloroplasts, with no preference for one parental type. The remaining C7-hybrid contained chloroplasts from both parents. The mitochondrial DNAs in the hybrids had segregated independently from the chloroplasts. No correlations were found between nuclear DNA composition and chloroplast type or mitochondrial DNA composition.

In chapter 4 mitotic and meiotic irregularities of tomato (+) potato somatic hybrids are described. The analysis of chomosome numbers of 107 somatic hybrids revealed that 79% was aneuploid. Chromosome fragments

and bridges were observed at mitotic anaphase. Genomic *in situ* hybridization performed on mitotic metaphase complements showed that one aneuploid hybrid had lost one tomato and one potato chromosome and that another aneuploid hybrid lacked one tomato chromosome arm, one complete potato chromosome and one potato chromosome arm, and contained a translocation chromosome consisting of one tomato and one potato chromosome arm. In male meiosis of three hybrids a high percentage of First Division Restitution (FDR) was observed, giving rise to unreduced gametes. However, only sterile pollen was formed by these hybrids.

In chapter 5 a detailed analysis is given of the nuclear, chloroplast and mitochondrial DNA of somatic hybrids between *L. esculentum* or *L. peruvianum* and *Nicotiana tabacum* (tobacco) or *N. plumbaginifolia*. These hybrids were difficult to produce; from a large number of fusion experiments only a relatively small number of hybrid calli was obtained. The majority of the hybrids contained the same absolute amount of *Nicotiana* DNA as the *Nicotiana* parent or (much) less, whereas the amount of *Lycopersicon* DNA per nucleus was 2-5 times that of the parental genotype. The ratio of hybrids containing *Lycopersicon* chloroplasts and hybrids with *Nicotiana* chloroplasts was 1:1. Both chloroplast type and mitochondrial DNA composition were correlated with nuclear composition; most hybrids possessed chloroplasts and mitochondrial DNA only or mainly derived from the parent predominating in the nucleus. From two hybrid calli complete plants were regenerated, which however grew weakly and were sterile.

In chapter 6 the chloroplast and mitochondrial DNA compositions of somatic hybrid calli of *S. tuberosum* and *N. plumbaginifolia* are described and related to the number of chromosomes from both parents. These hybrids contained chloroplast and mitochondrial DNA that was only or mainly derived from the parent that predominated in the nucleus; this result is comparable with the results described in chapter 5.

Chapter 7 contains a discussion on the possibilities and limitations of somatic hybridization. The results described in this thesis clearly illustrate the influence of phylogenetic relatedness of the fusion parents on the possibilities for producing cybrids, symmetric and asymmetric somatic hybrids, and the importance of organelles in somatic incongruity. Cybrids may not be viable because of nucleo-cytoplasmic incongruity, symmetric somatic hybrids that are viable may be sterile, and highly asymmetric somatic hybrids may be difficult to obtain, because irradiation-induced elimination of donor DNA is limited. Somatic hybridization can extend the range of species of which genetic information can be combined, as compared with sexual hybridization, but to a limited degree. In most cases other methods for transferring genetic information from one species to another species may be preferred.

Samenvatting

In de plantenveredeling houdt men zich bezig met het produceren van nieuwe rassen van economisch belangrijke gewassen, die zich onderscheiden van al bestaande rassen door de aanwezigheid van aanvullende gunstige eigenschappen. Zulke eigenschappen zijn bijvoorbeeld ziekte- of plaagresistenties en kwaliteitseigenschappen, zoals verbeterde kleur, vorm, smaak of houdbaarheid. Nieuwe rassen worden meestal verkregen door het maken van kruisingen tussen verschillende genotypen van een bepaald gewas, of tussen een cultuurgewas en een nauw verwante, soms wilde, soort die de gewenste eigenschap(pen) bevat. Uit de nakomelingen van deze kruisingen wordt vervolgens een nieuw, verbeterd genotype geselecteerd. Deze methode heeft echter zijn beperkingen, omdat door de aanwezigheid van kruisingsbarrières het cultuurgewas meestal slechts met een gering aantal (wilde) soorten te kruisen is. Soms komt de gunstige eigenschap alleen voor in een soort die niet met de cultuursoort te kruisen is.

In dit geval kan men proberen somatische in plaats van sexuele hybriden te maken tussen de betreffende soorten. Dit houdt in dat geïsoleerde cellen (protoplasten) van beide ouderplanten met elkaar gefuseerd worden. Op deze manier worden in principe alle eigenschappen van beide planten met elkaar gecombineerd, en wordt een "symmetrische" somatische hybride verkregen. Meestal echter wil men slechts een beperkt aantal eigenschappen van de wilde soort (de "donor"-soort) overbrengen naar het cultuurgewas (de "recipiënt"). Om dit te bewerkstelligen kunnen cellen van de donorsoort bestraald worden met Röntgen- of gamma-stralen, waardoor het kern-DNA gefragmenteerd wordt. Na fusie worden dan "asymmetrische" somatische hybriden verkregen. Soms wordt de gewenste eigenschap niet door kern-DNA bepaald, maar door organel-DNA, d.w.z. DNA van chloroplasten of van mitochondriën. In dat geval wil men alleen het bewuste organel en niet het kern-DNA van de donor overbrengen naar de recipiënt, en probeert men "cybriden" te produceren.

Technisch gezien kan men cellen van zeer onverwante soorten met elkaar fuseren, maar het verkregen fusieprodukt moet daarna wel in staat zijn tot celdeling. Uiteindelijk moet zich uit het fusieprodukt een volledige plant kunnen ontwikkelen. Om aan deze eisen te kunnen voldoen is het van belang dat de bij elkaar gebrachte kernen, chloroplasten en mitochondriën van beide fusie-ouders met elkaar kunnen samenwerken. Wanneer dit niet het geval is spreekt men van somatische incongruentie. Hierbij blijkt de verwantschap van de fusie-ouders een grote rol te spelen.

Wanneer het is gelukt na fusie een volledige plant te verkrijgen, wil men meestal dat deze fertiel is, zodat nakomelingen geproduceerd kunnen worden. Bovendien is het gewenst dat uitwisseling van genetische informatie tussen de hybride en de terugkruisingsouder kan optreden. De fertiliteit van het fusieprodukt kan echter verstoord zijn door somatische incongruentie, bijvoorbeeld doordat de interactie tussen kern en mitochondriën in het fusieprodukt niet optimaal is, maar ook door sexuele incompatibiliteit tussen de hybride en de soort waarmee men terug wil kruisen. Ook hierbij speelt de mate van verwantschap van de betrokken plantesoorten een grote rol. Deze verwantschap bepaalt in belangrijke mate de beperkingen van somatische hybridisatie. Het is dus niet eenvoudig een fertiele somatische hybride te produceren, die gebruikt kan worden voor de veredeling van een bepaald gewas.

In dit proefschrift worden de resultaten gepresenteerd van somatische hybridisatie-experimenten gericht op het combineren van genetische informatie van verschillende soorten van de Nachtschadefamilie (de *Solanaceae*), waartoe o.a. tomaat, aardappel en tabak behoren. De nadruk werd hierbij gelegd op het verkrijgen van inzicht in de interacties tussen kernen, chloroplasten en mitochondriën in de geproduceerde somatische hybriden, in relatie met de verwantschap van de beide fusie-ouders.

In hoofdstuk 1 wordt een aantal factoren beschouwd die de resultaten van somatische hybridisatie kunnen beïnvloeden, in het bijzonder de mate van verwantschap van de fusie-ouders en somatische incongruentie die hieruit kan resulteren. Een overzicht wordt gegeven van de tot nu toe gepubliceerde somatische hybridisatie-experimenten tussen tomaat (*Lycopersicon esculentum*) en andere soorten van hetzelfde geslacht, van andere geslachten van dezelfde stam, en van geslachten van andere stammen van de familie *Solanaceae*. Het lot van chromosomen, chloroplasten en mitochondriën na fusie van twee protoplasten van verschillende soorten, en de kansen op fertiliteit van de somatische hybride op basis van de reeds gepubliceerde literatuur worden in dit hoofdstuk beschreven.

In hoofdstuk 2 wordt de analyse beschreven van symmetrische en asymmetrische somatische hybriden tussen een door chloroplasten bepaalde albino mutant van tomaat en een kanamycine-resistent, ß-glucuronidase bevattend genotype van aardappel (*Solanum tuberosum*). De aardappel-ouder was bestraald met een dosis van 0, 50 of 500 Gray aan gamma-stralen. De relatieve hoeveelheid kern-DNA afkomstig van aardappel in deze somatische hybriden werd bepaald door middel van dot blot hybridisatie met behulp van soort-specifieke repetitieve DNA fragmenten. Een vergelijking van de relatieve hoeveelheden aardappel-DNA in symmetrische en asymmetrische hybriden liet zien dat de mate van door bestraling geïnduceerde eliminatie van aardappel-DNA beperkt was. Bovendien bleek dat er geen cybriden met alleen tomatekern-DNA en aardappel-chloroplasten verkregen waren.

Hoofdstuk 3 bevat een analyse van de samenstelling van het chloroplast- en het mitochondriaal DNA van twee groepen somatische hybriden tussen tomaat en aardappel. De A7-hybriden waren verkregen na fusie van een door chloroplasten bepaalde albino tomaat met een monoploïde aardappel (die slechts één volledige set aardappelchromosomen bevat); de C7-hybriden waren afkomstig van een fusie-experiment tussen een nitraatreductase-deficiënte tomaat en dezelfde aardappel-ouder als die van de A7-hybriden. Alle A7-hybriden bleken aardappelchloroplasten te bevatten, terwijl alle C7-hybriden, op één na, òf tomate- òf aardappelchloroplasten hadden, zonder duidelijke voorkeur voor een bepaald type. De overgebleven C7-hybride bevatte chloroplasten van beide ouders. Het mitochondriaal DNA was onafhankelijk van het chloroplasttype uitgesorteerd in de hybriden. Er werden geen correlaties gevonden tussen kern-DNA samenstelling aan de ene kant en chloroplasttype of mitochondriaal DNA samenstelling aan de andere kant.

In hoofdstuk 4 worden onregelmatigheden beschreven in de mitotische celdelingen, die normale dochtercellen opleveren, en de meiotische celdelingen, die zaadcellen opleveren, in somatische hybriden tussen tomaat en aardappel. Na bepaling van de chromosoomaantallen in 107 somatische hybriden bleek dat 79% van de hybriden aneuploïd was, d.w.z. enkele chromosomen meer of minder bevatte dan de som van het aantal chromosomen van beide ouders. Tijdens de mitotische en meiotische celdelingen werden chromosoomfragmenten en chromosoombruggen waargenomen. Chromosoompreparaten van een aantal hybriden werden gehybridiseerd met tomate-DNA dat was gelabeld met een fluorescerende stof, waardoor onderscheid gemaakt kon worden tussen tomaat- en aardappelchromosomen (genomische *in situ* hybridisatie). Eén aneuploïde hybride bleek zowel een tomaat- als een aardappelchromosoom verloren te hebben. Een andere aneuploïde hybride bleek een half tomate-chromosoom en anderhalf aardappel-chromosoom verloren te hebben en bovendien een translocatie-chromosoom te bevatten, bestaande uit een half tomate-chromosoom gekoppeld aan een half aardappel-chromosoom. Na de mannelijke meiose van drie somatische hybriden werd een hoog percentage zaadcellen gevonden waarin het aantal chromosomen niet gehalveerd was, zoals normaal gebeurt, maar die het volledige aantal of een afwijkend aantal chromosomen bevatten. Alleen steriel stuifmeel werd gevormd door deze hybriden.

Hoofdstuk 5 bevat een gedetailleerde analyse van het kern-, chloroplast- en mitochondriaal DNA van somatische hybriden tussen *Lycopersicon esculentum* (tomaat) of *L. peruvianum* en *Nicotiana tabacum* (tabak) of *N. plumbaginifolia*. Deze somatische hybriden konden slechts met veel moeite verkregen worden; uit een groot aantal fusie-experimenten werd slechts een relatief klein aantal hybride cellijnen verkregen. De meeste hybriden bleken een gelijke of kleinere absolute hoeveelheid *Nicotiana* kern-DNA te bevatten als de *Nicotiana* ouder, terwijl de hoeveelheid *Lycopersicon* kern-DNA in de hybriden 2 tot 5 keer zo groot was als in de *Lycopersicon* ouder. De verhouding tussen het aantal hybriden met *Lycopersicon* chloroplasten en het aantal hybriden met *Nicotiana* chloroplasten was 1:1. Zowel het chloroplast-type als de samenstelling van het mitochondriaal DNA was gecorreleerd aan de kernsamenstelling van de hybride. De meeste hybriden bevatten namelijk chloroplasten en alleen of voornamelijk mitochondriaal DNA van de ouder die overheerste in de kern. Van twee hybride cellijnen konden complete planten geregenereerd worden, die echter een zwakke groei vertoonden en steriel waren.

In hoofdstuk 6 worden de chloroplast- en mitochondriaal DNA samenstellingen van somatisch hybride cellijnen tussen *S. tuberosum* en *N. plumbaginifolia* beschreven en gerelateerd aan het aantal chromosomen van beide ouders. Deze hybriden bleken chloroplasten en mitochondriaal DNA te bezitten dat alleen of voornamelijk afkomstig was van de ouder die overheerste in de kern. Deze situatie is vergelijkbaar met die beschreven in hoofdstuk 5.

Hoofdstuk 7 bevat een algemene discussie over de mogelijkheden en beperkingen van somatische hybridisatie. De resultaten beschreven in dit proefschrift illustreren duidelijk de invloed van de mate van verwantschap van beide fusie-ouders op de mogelijkheden voor het produceren van cybriden, symmetrische en asymmetrische somatische hybriden. Cybriden kunnen soms niet geproduceerd worden door de aanwezigheid van incongruentie tussen de kern van de ene ouder en organellen van de andere ouder. Symmetrische somatische hybriden kunnen groeikrachtig zijn, maar zijn vaak steriel. Zeer asymmetrische somatische hybriden kunnen soms moeilijk te verkrijgen zijn, omdat bestraling van de donorcellen niet resulteert in de gewenste uitgebreide eliminatie van kern-DNA van de donor na fusie. Somatische hybridisatie kan het aantal soorten waartussen uitwisseling van genetische informatie mogelijk is uitbreiden, vergeleken met sexuele hybridisatie, maar slechts in beperkte mate. Echter, in de meeste gevallen verdienen andere methoden voor de overdracht van genetische informatie van de ene soort naar de andere soort de voorkeur.

Nawoord

Het proefschrift is af; alleen het nawoord rest mij nog. Vele mensen hebben mij tijdens mijn promotie-onderzoek geholpen, advies gegeven en gesteund. Op deze plaats wil ik hen allen bedanken. Ten eerste Maarten Koornneef, mijn tot promotor gepromoveerde co-promotor. Hoewel je het steeds drukker gekregen hebt, was je altijd zeer nauw bij mijn werk betrokken. Je wilde de resultaten al weten voordat ze beschikbaar waren. Je stuurde mijn onderzoek, maar liet me ook vrij om allerlei zaken uit te zoeken en te proberen. Bovendien was je steeds een wandelende/dravende Reference Manager. Wanneer ik een manuscript aan je meegaf kreeg ik het altijd snel en van nuttig commentaar voorzien terug.

Christa Heijting, bedankt voor het kritisch lezen van mijn manuscripten. Door jouw andere invalshoek stimuleerde je me mijn onderzoek in een breder perspectief te zien.

Herman Schoenmakers, mijn co-aio, jouw geweldige energie en enthousiasme werkten altijd aanstekelijk. Het was plezierig met jou samen te werken, te overleggen en te borrelen. Jelle Wijbrandi, jij hebt de basis gelegd voor ons onderzoek en fusie-experimenten opgezet waarvan ik de vruchten heb mogen plukken (nou ja, vruchten...).

Aaron Zelcer, thank you for your work at our department. You initiated the fusion experiments with albino tomato. Because of this work we became interested in the phenomenon of nucleo-cytoplasmic incongruity. Your experiments have enabled me to write my first article, with your cooperation.

Hans de Jong, bedankt voor je aanwijzingen voor, en discussies over het cytologisch werk. Verder wil ik bedanken: Corrie Hanhart, voor de vele enten en vervolgens kruisingen die je gemaakt hebt. Janny van Eden en Silvan Kamstra, voor de *in situ* hybridisaties. Henny Verhaar, voor het verzamelen en fixeren van antheren in de juiste meiose-stadia.

Annette Vergunst, Witte van Cappellen, José van der Meulen-Muisers, Esther van der Knaap, Leni Demmers-Derks, Ambro van Hoof, Frank van der Werff, José Kok, Dick Roelofs en Karim Saiedi, bedankt voor jullie bijdragen aan mijn onderzoek.

Pim Zabel, Richard Visser, Sonja Derks en Tineke Creemers-Molenaar ben ik erkentelijk voor het beschikbaar stellen van probes. Luud Gilissen, bedankt voor je *S. tuberosum* (+) *N. plumbaginifolia* calli en de waardevolle discussies over de uitkomsten van het onderzoek naar organel DNA-samenstelling van deze hybriden.

De tomatengroep van de vakgroep Moleculaire Biologie, en met name Ruud Verkerk, Rob Weide en Jan Hontelez, wil ik bedanken voor de mogelijkheid de fijne kneepjes van een aantal moleculaire technieken te komen leren aan het begin van mijn promotie-onderzoek. De sectie Moleculaire Genetica, en in het bijzonder Bert Wennekes en Theo Goossen, wil ik bedanken voor het beschikbaar stellen van protocollen en apparatuur.

Henk Kuiper, Jan Laurens, Willem Blijderveen en Gerrit van IJmeren van de Proefaccommodatie, bedankt voor jullie moeite onze planten in leven te houden.

De nog niet genoemde mensen van de sectie Botanische Genetica (Botgen): Ageeth, Ton, Karen, Wim, Isabelle, Carlos, Patty en Hetty, bedankt voor de prettige sfeer tijdens en na werktijd. Alle overige mensen van de vakgroep Erfelijkheidsleer, en alle nog niet genoemde studenten, NOPpers en gastmedewerkers die bij onze sectie gewerkt hebben, bedankt voor de gezellige koffie- en theepauzes, labfietstochten, schaatsmiddagen, volleybaltoernooien en barbecues.

De Programma Commissie Landbouw Biotechnologie (PcLB) en de Landbouwuniversiteit Wageningen ben ik zeer erkentelijk voor het financieren van dit onderzoek. Daarnaast bedank ik de stichting "Landbouw Export Bureau 1914-1918" voor de financiële bijdrage, die het uitgeven van dit proefschrift mede mogelijk heeft gemaakt.

Johan, bedankt voor je steun en ondersteuning, en de duizenden belegde boterhammen.

15 april 1994

Curriculum vitae

Anna Maria Agnes (Anne-marie) Wolters werd op 16 juni 1965 geboren in Bemmel. In 1977 ging ze naar het Canisius College-Mater Dei te Nijmegen. In juni 1983 behaalde ze het VWO-B diploma. In september van dat jaar begon Anne-marie met de studie plantenveredeling aan de toenmalige Landbouwhogeschool, nu Landbouwuniversiteit, in Wageningen. Na de propedeuse koos ze voor een biochemisch/moleculair-biologische richting binnen de plantenveredeling. Anne-marie deed gedurende 8 maanden een afstudeervak bij de vakgroep Erfelijkheidsleer op het gebied van de Somatische Celgenetica, waarbij ze werkte aan asymmetrische somatische hybridisatie tussen tomaat en *Lycopersicon peruvianum*, onder begeleiding van Drs. Jelle Wijbrandi. Vervolgens deed ze gedurende 5 maanden onderzoek in de Verenigde Staten bij Prof. Helgeson, vakgroep Plant Pathology, van de University of Wisconsin-Madison. Hier zette ze een methode op voor het isoleren van mitochondriaal DNA van somatische hybriden tussen *Solanum tuberosum* and *S. brevidens*. Terug in Nederland werkte Anne-marie 8 maanden aan een afstudeervak op de vakgroep Moleculaire Biologie onder begeleiding van Ir. Ben Scheres, waarbij ze werkte aan de karakterisering van een aantal "vroege noduline"-genen van de erwt. Op 21 november 1988 studeerde Anne-marie met lof af. Op 1 december van dat jaar begon ze als A.I.O. op de vakgroep Erfelijkheidsleer met een promotie-onderzoek aan "asymmetrische protoplastenfusie tussen onverwante soorten". Dit gebeurde onder begeleiding van Prof. Dr. Ir. Maarten Koornneef en Prof. Dr. Christa Heijting, en in samenwerking met co-A.I.O. Ir. Herman Schoenmakers. Anne-marie's resultaten van dit onderzoek staan beschreven in dit proefschrift.