Isolation and characterisation of somatic hybrids between *Lycopersicon esculentum* and *Lycopersicon peruvianum*

Isolatie en karakterisering van somatische hybriden van *Lycopersicon esculentum* en *Lycopersicon peruvianum*

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Isolation and characterisation of somatic hybrids between *Lycopersicon esculentum* and *Lycopersicon peruvianum*

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H. C. van der Plas,
in het openbaar te verdedigen
op woensdag 29 november 1989
des namiddags te twee uur in de aula
van de Landbouwuniversiteit te Wageningen

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Slioecht in rioecht

Joe deys leyt de wrâd omkere
Yn klear duwbbeld-hertigheyt.
Jou uwz Lân wer ljæve HEERE
d'Ade roune yenfadigheyt.

(G. Japicx, 1681, Friesche rymlerye)

Met dank aan de "Stichting Hendrik Nannes- en Catrijn Epes-leen" te Bolsward, die het uitgeven van dit proefschrift middels een financiële bijdrage mogelijk heeft gemaakt.

STELLINGEN

1. Genlocalisatie via asymmetrische protoplastenfusie, waarbij de protoplasten van de donor met een hoge dosis ioniserende straling behandeld worden, is niet efficient, omdat er relatief veel chromosoomherrangschikkingen plaats vinden.

   Dit proefschrift.

2. Het verdient aanbeveling om meer onderzoek te verrichten naar het proces van chromosoomeliminatie in asymmetrische fusieproducten, verkregen na bestraling van één van beide fusieouders.

3. Aneuploïdie in somatische hybriden kan leiden tot incongruentie.

4. Interspecifieke somatische hybridisatie verhoogt de genetische variatie van een gewas.

5. Als modelgewas voor celgenetisch onderzoek is de cultuurtomaat geschikter dan *Nicotiana* soorten, vanwege de goed ontwikkelde genetica bij de tomaat.

   Dit proefschrift

6. In geval van niet-groeiende of niet-regenererende fusieproducten van plantecellen, verdient het aanbeveling de term somatische incompatibiliteit te vervangen door somatische incongruentie.

   Harms CT (1983) The Quarterly Review of Biology 58:325-353.
   Hogenboom NG (1984). In: Linskens HF, Heslop-Harrison J (eds) Cellular Interactions, Springer-Verlag Berlin, pp 640-654.

7. Het feit dat de klassieke genetica bij de aardappel bijna niet ontwikkeld is, maakt dat dit gewas niet goed bruikbaar is voor fundamenteel moleculair-genetisch onderzoek.

8. Gezien de grote aantrekkingskracht van afwijkende kleuren en vormen van traditionele groenten bij de consument, verdient het aanbeveling de grote variatie binnen de tomaat wat betreft vruchtkleur en -vorm te benutten in de veredeling.

9. De definitie van genetische manipulatie door professor Barabas is onvolledig en ten dele onjuist. Gezien de grote verspreiding van de publicatie waarin zijn definitie vermeld wordt, leidt dit tot een vertekend beeld van genetische manipulatie bij een groot publiek.

   In: Vandersteen W (1987) Suske en Wiske. De woeste wespen, Standaard Uitgeverij Antwerpen, p 3.

10. Mede gezien de zorgvuldige procedures die het onderzoeksinstituut Ital gevolgd heeft alvorens een proefveld met transgene aardappels in te richten, kan gesteld worden dat 'Het Ziedende Bintje' halfgaar is.

11. Indien de huidige trend om alleen te bezuinigen door natuurlijk verloop zich doorzet en indien de migratie van Groningse biologen naar Wageningen in hetzelfde tempo doorgaat als in de afgelopen jaren, is de sluiting van het Biologisch Centrum van de Rijksuniversiteit Groningen onafwendbaar.

Stellingen bij het proefschrift van J. Wijbrandi: "Isolation and characterisation of somatic hybrids between *Lycopersicon esculentum* and *Lycopersicon peruvianum*." Wageningen, 29 november 1989.

VOORWOORD

Dit proefschrift beschrijft de resultaten van een promotie-onderzoek aangaande protoplastenfusie bij de tomaat. Dit boekje was niet verschenen zonder de hulp van vele mensen, die ik hierbij wil bedanken.
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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Partial genome transfer by somatic hybridisation

Somatic hybridisation is a technique by which nuclear genomes of different species can be combined. Interspecific somatic hybrids have been obtained of various animal cell lines (Ringertz and Savage 1976), fungi (Peberdy 1979) and plants (Glimelius 1988). Plant somatic hybrids originate from fusion of protoplasts, which are plant cells whose walls have been removed by enzymatic digestion. Somatic hybridisation is of potential interest for the improvement of crops, because it provides the possibility to circumvent sexual crossing barriers, to mix cytoplasmons of different species, and to generate novel nucleus-cytoplasm combinations (Glimelius 1988). Thus, desirable traits of a wild species can be introduced into a crop species. It is, however, equally important that undesirable traits are not transferred to the cultivated species, or that they can be removed from the hybrids. After sexual hybridisation it is often possible to achieve this by repeated backcrossing of the hybrid to the cultivated parent. In this respect somatic hybridisation has some disadvantages, since most of the fertile somatic hybrids are stable polyploids. Preferential elimination of the chromosomes of one of the parental species, like has been reported for several mammalian somatic hybrids (Ringertz and Savage 1976), rarely occurs in somatic hybrids of plants (Gleba and Sytnik 1984). The polyploid state of the hybrids may cause sexual incongruity with the cultivated parent. Crossing of an interspecific somatic hybrid to the cultivated parental species is therefore not always possible. This problem may be circumvented by asymmetric somatic hybridisation; in this case, untreated protoplasts of one species, the recipient or acceptor, are fused with protoplasts of another species, the donor, whose nuclear genome is reduced. The construction of asymmetric somatic hybrids can be done in various ways:

(i) by the use of haploid cells from a donor species. These cells can be derived either from haploid plants or from microspores in pollen tetrades (Pental and Cocking 1985). The latter technique is called gameto-somatic fusion, and has been successfully applied to *Nicotiana* and *Petunia* species (Pirrie and Power 1986; Lee and Power 1988; Pental et al. 1988). Such fusions result in allotriploid plants, in which introgression of donor genes into the recipient genome may occur by meiotic recombination and by further backcrosses to the recipient species;

(ii) another way to reduce the contribution of donor genome to that of the hybrid is the isolation of microprotoplasts from micronucleated cells (Verhoeven 1989). Micronuclei are induced by exposure of mitotic cells to a spindle toxin; protoplasts of these cells are fractionated into microprotoplasts, which contain micronuclei with one or a few chromosomes. Procedures for the isolation of microprotoplasts of *Nicotiana plumbaginifolia* have recently been developed by Verhoeven (1989);
(iii) finally, reduction of the contribution of the donor genome to that of the hybrids can also be achieved by treatment of the donor cells with high doses of ionising radiation (Röntgen- and gamma-rays), which fragmentises the chromosomes. This method was originally developed to completely eliminate the nuclear donor genome in order to obtain cybrids (fusion products that contain only the cytoplasm of the donor; Zelcer et al. 1978). However, in several cases it has been shown that partial elimination of the donor genome by irradiation of the donor cells is also possible. Fragmented chromosomes can only be stably replicated and maintained if they contain a centromere region and telomeres. If chromosome fragments lack such structures they can still be rescued by recombination with other donor or recipient chromosomes.

Asymmetric somatic hybrids can also be used for mapping of genes. This has been well established for mammalian cell hybrids. In such hybrids where preferential elimination of most of the chromosomes of one parent had occurred, the remaining donor chromosomes could be identified by cytogenetic analysis. In this way, asymmetric hybrids containing a complete rodent genome and a small number of human chromosomes in different combinations were obtained (Ringertz and Savage 1976). Another approach was to transfer directly small numbers of donor chromosomes into mammalian cells by microcell fusion; this procedure has also been used successfully for gene mapping (Lugo and Fournier 1986). Irradiation of one fusion parent has not only been used to enhance preferential elimination of chromosomes from one parent (Pontecorvo 1971), but has also been applied for regional mapping of chromosomes; thus, the linear order of different marker genes on a same chromosome (region) could be determined by measuring the frequency of the presence of these genes in a large population of hybrids (Goss and Harris 1975).

Asymmetric somatic hybrids of plants

Partial genome transfer by asymmetric somatic hybridisation has been described for several plant species. Asymmetric nuclear hybrids were isolated from cybridisation experiments, as unintended by-products (Zelcer et al. 1978; Aviv

and Galun 1980; Sidorov et al. 1981; Menczel et al. 1982, 1983; Hamill et al. 1984). Besides, asymmetric hybrids were purposely obtained from experiments in which selection was based on a nuclear encoded character of the irradiated donor. Efficient selectable markers are required to select for fusion products, because, in general, the frequencies of asymmetric hybrids obtained were much lower than those of symmetric hybrids obtained after fusion of unirradiated protoplasts. In the first reported, successful asymmetric somatic hybridisation experiment, albino carrot protoplasts were fused with irradiated parsley protoplasts; the regenerated green plant had one additional chromosome (Dudits et al. 1980). In many of the asymmetric hybridisation experiments, the selection was based on complementation of the nitrate reductase deficiency of the recipient species (e.g. Gupta et al. 1982; Gleba et al. 1988). Thus, asymmetric nuclear hybrids were selected from combinations of species differing with respect to their phylogenetic relatedness (Table 1): intrageneric, intergeneric + intrafamiliar, and interfamiliar. The applied irradiation doses ranged from 50 to 1000 Gray. The elimination of donor genome depended strongly on the

Table 1. Interspecific asymmetric somatic hybridisation experiments. Unirradiated protoplasts from a (recipient) species were fused with irradiated protoplasts from another (donor) species.

Asymmetric somatic nuclear hybrids recipient (+) donor	References
Intrageneric hybrids:	
Brassica campestris (+) *B. oleracea**	Yamashita et al. 1988
Nicotiana glauca (+) *N. langsdorffii*	Itoh and Futsuhara 1983
N. plumbaginifolia (+) *N. sylvestris**	Famelaer et al. 1989
N. plumbaginifolia (+) *N. tabacum**	Koornneef et al. 1988
N. tabacum (+) *N. paniculata*	Müller-Gensert and Schieder 1987
N. tabacum (+) *N. plumbaginifolia**	Bates et al. 1987
Solanum tuberosum (+) *S. pinnatisectum**	Sidorov et al. 1987
Intergeneric, intrafamiliar hybrids:	
Daucus carota (+) *Petroselinum hortense**	Dudits et al. 1980
Datura innoxia (+) *Physalis minima**	Gupta et al. 1984
Hyoscyamus muticus (+) *N. tabacum*	Imamura et al. 1987
N. plumbaginifolia (+) *Atropa belladonna**	Gleba et al. 1988
N. tabacum (+) *Datura innoxia*	Gupta et al. 1982
N. tabacum (+) *Physalis minima*	Gupta et al. 1982
Interfamiliar hybrids:	
N. tabacum (+) *Daucus carota**	Dudits et al. 1987
N. tabacum (+) *Hordeum vulgare**	Somers et al. 1986

* asymmetric hybrid plants obtained

combination of parents. In most of the intrageneric combinations, a limited elimination was observed, whereas in the unrelated combinations the elimination was more increased. In most of the intergeneric hybrids, one or a few chromosomes were retained, while in the interfamiliar hybrids only a few traits (Dudits et al. 1987) or even one trait (Somers et al. 1986) of the donor species could be detected. When different doses were used in the same experiment, no clear effect of the applied dose was observed on the elimination of donor genome (Gleba et al. 1988; Yamashita et al. 1988; Famelaer et al. 1989). Five different experiments have been described, where asymmetric hybrids were fertile when selfed of backcrossed to the recipient species (Somers et al. 1986; Bates et al. 1987; Dudits et al. 1987; Sidorov et al. 1987; Yamashita et al. 1988). In two other experiments backcrosses had to be rescued by embryo culture (Gleba et al. 1988; Famelaer et al. 1989).

Scope of the study

Asymmetric somatic hybrids have an important potential use for plant breeders who want to transfer mono- and multigenic traits from a more or less unrelated (donor) species to a recipient crop species. Monogenic traits of which the DNA has been cloned can be more easily transferred by other techniques, such as *Agrobacterium* transformation and direct gene transfer (Hohn and Schell 1987). To evaluate the possibilities and difficulties of somatic hybridisation, experiments were performed with a plant species that can be analysed genetically in detail, namely the cultivated tomato (*Lycopersicon esculentum*). Protoplast fusions were carried out with the tomato and its wild relative *Lycopersicon peruvianum*. These two species are difficult to hybridise sexually. Regeneration capacity from *L. peruvianum* was chosen as selectable trait and also as an example of a multigenic trait which might be desirable to transfer by somatic hybridisation. The aims of the experiments were:

(i) the isolation of somatic hybrids between both species in an efficient way and the subsequent characterisation of these plants;

(ii) the isolation of asymmetric hybrids between both species, using *L. peruvianum* as donor species;

(iii) the characterisation of these asymmetric hybrids in order to determine the degree of elimination of donor genome and, in addition, to evaluate their use for breeding purposes and mapping studies.

Lycopersicon esculentum Mill., the cultivated tomato, and
Lycopersicon peruvianum (L.) Mill., "the most variable and least exploited tomato species" (Rick 1979a)

L. esculentum and *L. peruvianum* are both members of the genus *Lycopersicon* of the family *Solanaceae*. This genus is closely related to the genus *Solanum* and consists of eight species, which are divided in the 'esculentum-complex' and the 'peruvianum-complex' (Taylor 1986). The species of the former complex can be easily crossed with *L. esculentum*, whereas the species of the latter complex, namely *L. peruvianum* and *L. chilense*, can only be crosses with great difficulty. All *Lycopersicon* species show a high degree of homology in their chromosomes, which allows meiotic recombination in species hybrids (Rick 1979b).

The cultivated tomato is an important food crop. Moreover, it is a favorite model system for genetic studies in plants, because the crop is easy to culture, has a relatively short life cycle, is highly self-fertile and is a simple diploid (2n = 2x = 24) whose chromosomes are distinguishable at the pachytene stage of meiosis (Rick and Butler 1956; Gill 1983; Rick and Yoder 1988; Hille et al. 1989). The linkage map of tomato contains more than 300 morphological, isozyme- and disease resistance markers (Mutschler et al. 1987), to which at least 300 restriction fragment length polymorphism (RFLP) markers have been added in recent years (Young and Tanksley 1989).

L. peruvianum is an outbreeding wild relative of the cultivated tomato with a high level of variability (Rick 1979). There are many accessions, which have a great number of characters desirable for tomato improvement, such as disease resistances (Rick 1982). Because of the crossing barriers between both species (Hogenboom 1972a), only a few resistances, namely against the root-knot nematode *Meloidogyne incognita* (Gilbert and McGuire 1956), *Pyrenochaeta lycopersici* (corky root; Szteyn 1962), tobacco mosaic virus (Alexander 1963), *Cladosporium* (Kerr and Baily 1964) and curly top virus (Martin 1969), could be introduced into the tomato. When *L. peruvianum* is used as pistillate parent, growth of the *L. esculentum* pollen tubes is inhibited. When *L. peruvianum* is the staminate parent, fertilisation takes place; however, the embryo soon aborts due to the degeneration of the endosperm (Barbano and Topoleski 1984). The crossing barriers were overcome by the rescue of the abortive embryo by tissue culture techniques (Smith 1944; de Nettancourt et al. 1974; Thomas and Pratt 1981) and the selection for rare *L. peruvianum* genotypes with a reduced pollen tube inhibition (Hogenboom 1972b; Rick 1983).

L. peruvianum also has desirable cell and tissue culture properties. Its

callus growth and shoot regeneration capacity are superior to those of *L. esculentum* (Zapata et al. 1977; Mühlbach 1980; Morgan and Cocking 1982). The latter species is very difficult to regenerate from protoplast and established callus cultures. An *L. esculentum* genotype with the good callus growth and superior regeneration capacity from *L. peruvianum* was obtained by classical breeding (Koornneef et al. 1986). The genetic analysis of this plant revealed that the favourable traits of *L. peruvianum* were dominant, that regeneration capacity was controlled by two genes and that the callus growth characteristics were shown to be controlled by different loci (Koornneef et al. 1987).

Somatic hybrids of Lycopersicon

Already in 1978 hybrids between tomato and potato were obtained (Melchers et al. 1978); however, these hybrids were not fertile and formed fruits nor tubers. More recently, other somatic hybrid plants between different *Lycopersicon* species and between *Lycopersicon* and *Solanum* species were obtained (Table 2). The aim of these somatic hybridisation experiments was to by-pass crossing barriers and possibly to introduce valuable agronomic traits into the tomato. Although these hybrids were vigorous plants and often did flower and set fruits, no progenies were described. Somatic hybrids between *L. esculentum* and *L. peruvianum* were also obtained; however, from 21 different fusion experiments only two hybrids were isolated, because these hybrids could only be identified as such at the plant level and not in cell culture (Kinsara et al. 1986). Asymmetric somatic hybrids of tomato have not been reported so far.

Table 2. Somatic hybridisation experiments with *Lycopersicon* species.

Fusion combination		References
L. esculentum	(+) *L. pennellii*	O'Connell and Hanson 1987
L. esculentum	(+) *L. peruvianum*	Kinsara et al. 1986
L. esculentum	(+) *S. lycopersicoides*	Handley et al. 1986 Tan 1987
L. esculentum	(+) *S. nigrum*	Guri et al. 1988 Jain et al. 1988
L. esculentum	(+) *S. rickii*	O'Connell and Hanson 1986
L. esculentum	(+) *S. tuberosum*	Melchers et al. 1978 Shepard et al. 1983
L. pennellii	(+) *L. peruvianum*	Adams and Quiros 1985 Tan 1987
L. pimpinellifolium	(+) *S. tuberosum*	Okamura 1988

Outline of the thesis

Two types of fusion experiments with protoplasts of *L. esculentum* and *L. peruvianum* were carried out. A selection strategy was developed, to obtain efficiently somatic hybrids. Selection against *L. peruvianum* was achieved by the use of kanamycin resistant *L. esculentum* genotypes and the subsequent selection for kanamycin resistant hybrids. These "symmetric" somatic hybrids were characterised cytogenetically, biochemically and morphologically (Chapter 2). Since these hybrids were fertile, their progeny could be characterised also (Chapter 3).

In the second series of protoplast fusion experiments, *L. peruvianum* was irradiated before fusion in order to achieve the transfer of only part of the genome of *L. peruvianum*. In these experiments, the effect of different doses of gamma-rays on the elimination of donor genome was analysed. All asymmetric hybrids were characterised cytogenetically and morphologically (Chapter 4). To determine the amount of transferred *L. peruvianum* genome, the asymmetric hybrids were analysed for the presence of *L. peruvianum* specific genes (Chapter 5). In addition, fifteen asymmetric somatic hybrids were characterised in detail with 30 chromosome specific RFLP markers (Chapter 6).

Finally, the significance of the somatic hybrids obtained and of the asymmetric hybridisation technique are discussed (Chapter 7).

References

Adams TL, Quiros CF (1985) Somatic hybridization between Lycopersicon peruvianum and Lycopersicon pennellii: regenerating ability and antibiotic resistance as selection systems. Plant Science 40:209-219

Alexander LJ (1963) Transfer of a dominant type of resistance to the four known Ohio pathogenic strains of tobacco mosaic virus (TMV), from Lycopersicon peruvianum to L. esculentum. Phytopathology 53:869

Aviv D, Galun E (1980) Restoration of fertility in cytoplasmic male sterile (CMS) Nicotiana sylvestris by fusion with X-irradiated N. tabacum protoplasts. Theor Appl Genet 58:121-127

Barbano PP, Topoleski LD (1984) Postfertilization hybrid seed failure in Lycopersicon esculentum x Lycopersicon peruvianum ovules. J Amer Soc Hort Sci 109:95-100

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

de Nettancourt D, Devreux M, Laneri U, Cresti M, Pacini E, Sarfatti G (1974) Genetical and ultrastructural aspects of self and cross incompatibility in interspecific hybrids between self-compatible Lycopersicon esculentum and self-incompatible L. peruvianum. Theor Appl Genet 44:278-288

Dudits D, Fejer O, Hadlaczky GY, Koncz CS, Lazar G, Horvath G (1980) Intergeneric gene transfer mediated by plant protoplast fusion. Mol Gen Genet 179:283-288

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

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

Gilbert JC, McGuire DC (1956) Inheritance of resistance to severe root knot from Meloidogyne incognita in commercial type tomatoes. Proc Am Soc Hortic Sci 68:437-442

Gill BS (1983) Tomato cytogenetics - A search for new frontiers. In: Swaminathan MS, Gupta PK, Sinha U (eds) Cytogenetics of crop plants. MacMillan India Ltd, New Delhi, pp 455-480

Gleba YY, Hinnisdaels S, Sidorov VA, Kaleda VA, Parokonny AS, Boryshuk NV, Cherup NN, Negrutiu I, Jacobs M (1988) Intergeneric asymmetric hybrids between Nicotiana plumbaginifolia and Atropa belladonna obtained by "gamma-fusion". Theor Appl Genet 76:760-766

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

Glimelius K (1988) Potentials of protoplast fusion in plant breeding programs. In: Puite KJ, Dons JJM, Huizing HJ, Kool AJ, Koornneef M, Krens FA (eds) Progress in plant protoplast research, Kluwer, Dordrecht, pp 159-168

Goss SJ, Harris H (1975) New method for mapping genes in human chromosomes. Nature 255:680-684

Gupta PP, Gupta M, Schieder O (1982) Correction of nitrate reductase defect in auxotrophic plant cells through protoplast-mediated intergeneric gene transfers. Mol Gen Genet 188:378-383

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

Hamill JD, Pental D, Cocking EC (1984) The combination of a nitrate reductase deficient nuclear genome with a streptomycin resistant chloroplast genome, in Nicotiana tabacum, by protoplast fusion. J Plant Physiol 115:253-261

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

Hille J, Koornneef M, Ramanna MS, Zabel P (1989) Tomato: a crop species amenable to improvement by cellular and molecular methods. Euphytica 42:1-23

Hogenboom NG (1972a) Breaking breeding barriers in Lycopersicon. 1. The genus Lycopersicon, its breeding barriers and the importance of breaking these barriers. Euphytica 21:221-227

Hogenboom NG (1972b) Breaking breeding barriers in Lycopersicon. 4. Breakdown of unilateral incompatibility between L. peruvianum (L.) Mill. and L. esculentum Mill.. Euphytica 21:397-404

Hohn T, Schell J (1987) Plant DNA infectious agents. Springer-Verlag, Wien New York

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

Itoh K, Futsuhara Y (1983) Interspecific transfer of only part of genome by fusion between non-irradiated protoplasts of Nicotiana glauca and X-ray irradiated protoplasts of N. langsdorffii. Jpn J Genet 58:545-553

Jain SM, Shahin EA, Sun S (1988) Interspecific protoplast fusion for the transfer of atrazine resistance from Solanum nigrum to tomato (Lycopersicon esculentum L.). In: Puite KJ, Dons JJM, Huizing HJ, Kool AJ, Koornneef M, Krens FA (eds) Progress in plant protoplast research, Kluwer, Dordrecht, pp 221-224

Kerr EA, Bailey DL (1964) Resistance to Cladosporium fulvum Cke. obtained from wild species of tomato. Can J Bot 42:1541-1554

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

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

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

Koornneef M, den Boer B, van Loenen-Martinet P, van Roggen P, Wijbrandi J (1988) Protoplast fusion of kanamycin resistant, cnx Nicotiana plumbaginifolia with streptomycin resistant N. tabacum (SR1) and the effect of irradiation of the tabacum parent. In: Puite KJ, Dons JJM, Huizing HJ, Kool AJ, Koornneef M, Krens FA (eds) Progress in plant protoplast research, Kluwer, Dordrecht, pp 287-288

Lee CH, Power JB (1988) Intra- and interspecific gametosomatic hybridisation within the genus Petunia. Plant Cell Tiss Org Cult 12:197-200

Lugo TG, Fournier REK (1986) Microcell fusion and mammalian gene transfer. In: Kucherlapati R (ed) Gene transfer. Plenum Publishing Corp, New York, pp 79-93

Martin NW (1969) Inheritance of resistance to curly top in the tomato breeding line C/F$_1$. Phytopathology 59:1040

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

Menczel L, Nagy F, Lázár G, Maliga P (1983) Transfer of cytoplasmic male sterility by selection for streptomycin resistance after protoplast fusion in Nicotiana. Mol Gen Genet 189:365-369

Morgan A, Cocking EC (1982) Plant regeneration from protoplasts of Lycopersicon esculentum Mill.. Z Pflanzenphysiol 106:97-104

Mühlbach HP (1980) Different regeneration potentials of mesophyll protoplasts from cultivated and a wild species of tomato. Planta 148:89-96

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

Mutschler MA, Tanksley SD, Rick CM (1987) Linkage maps of the tomato (Lycopersicon esculentum). Rep Tomato Genet Coop 37:5-34

O'Connell MA, Hanson M (1986) Regeneration of somatic hybrid plants formed between Lycopersicon esculentum and Solanum rickii. Theor Appl Genet 72:58-65

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

Okamura M (1988) Regeneration and evaluation of somatic hybrid plants between Solanum tuberosum and Lycopersicon pimpinellifolium. In: Puite KJ, Dons JJM, Huizing HJ, Kool AJ, Koornneef M, Krens FA (eds) Progress in plant protoplast research, Kluwer, Dordrecht, pp 213-214

Peberdy JF (1979) Protoplast fusion - A new approach to interspecific genetic manipulation and breeding in fungi. In: Ferenczy L, Farkas GL (eds) Advance in protoplast research. Pergamon Press, Oxford, pp 63-72

Pental D, Mukhopadhyay A, Grover A, Pradhan AK (1988) A selection method for the synthesis of triploid hybrids by fusion of microspore protoplasts (n) with somatic cell protoplasts (2n). Theor Appl Genet 76:237-243

Pental D, Cocking EC (1985) Some theoretical and practical possibilities of plant genetic manipulation using protoplasts. Hereditas (Suppl) 3:83-92

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

Pontecorvo G (1971) Induction of directional chromosome elimination in somatic cell hybrids. Nature 230:367-369

Rick CM (1979a) Potential improvement of tomatoes by controlled introgression of genes from wild species. Proc Conf Broadening Genet Base Crops, Wageningen, 1978. Pudoc, Wageningen, pp 167-173

Rick CM (1979b) Biosystematic studies in Lycopersicon and closely related species of Solanum. In: Hawkes JG, Lester RN, Skelding AD (eds) The biology and taxonomy of the Solanaceae. Academic Press, New York, pp 667-677

Rick CM (1982) The potential of exotic germplasm for tomato improvement. In: Vasil IK, Scowcroft WR, Frey KJ (eds) Plant improvement and somatic cell genetics. Academic Press, New York, pp 1-28

Rick CM (1983) Crossability between L. esculentum and a new race of L. peruvianum. Rep Tomato Genet Coop 33:13

Rick CM, Butler L (1956) Cytogenetics of the tomato. Adv Genet 8:267-382

Rick CM, Yoder JI (1988) Classical and molecular genetics of tomato: highlights and perspectives. Ann Rev Genet 22:281-300

Ringertz NR, Savage RE (1976) Chromosome patterns in hybrid cells. In: Cell hybrids. Academic Press, New York, pp 162-179

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

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

Sidorov VA, Zubko MK, Kuchko AA, Komarnitsky IK, Gleba YY (1987) Somatic hybridization in potato: use of gamma-irradiated protoplasts of Solanum pinnatisectum in genetic reconstruction. Theor Appl Genet 74:364-368

Smith PG (1944) Embryo culture of a tomato species hybrid. Proc Amer Soc Hort Sci 44:413-416

Somers DA, Narayanan KR, Kleinhofs A, Cooper-Bland S, Cocking EC (1986) Immunological evidence for transfer of the barley nitrate reductase structural gene to Nicotiana tabacum by protoplast fusion. Mol Gen Genet 204:296-301

Szteyn K (1982) Interspecific crosses in the genus Lycopersicon. I. Backcrosses to Lycopersicon glandulosum. Euphytica 11:149-156

Tan MMC (1987) Somatic hybridization and cybridization in some Solanaceae. Ph D Thesis, Free University Amsterdam, The Netherlands

Taylor IB (1986) Biosystematics of the tomato. In: Atherton JG, Rudich J (eds), The tomato crop. A scientific basis for improvement. Chapman and Hall, London/New York, pp 1-34

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

Verhoeven HA (1989) Induction and characterization of micronuclei in plant cells. Perspectives for micronucleus-mediated chromosome transfer. Ph D Thesis, Agricultural University Wageningen, The Netherlands.

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

Young ND, Tanksley SD (1989) Restriction fragment length polymorphism maps and the concept of graphical genotypes. Theor Appl Genet 77:95-101

Zapata FJ, Evans PK, Power JB, Cocking EC (1977) The effect of temperature on the division of leaf protoplasts of Lycopersicon esculentum and L. peruvianum. Plant Science Lett 8:119-124

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 90:397-407

CHAPTER 2

SELECTION AND CHARACTERISATION OF SOMATIC HYBRIDS BETWEEN *LYCOPERSICON ESCULENTUM* AND *LYCOPERSICON PERUVIANUM*

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Summary. Somatic hybrids of the cultivated tomato, *Lycopersicon esculentum*, and a wild species, *L. peruvianum*, were obtained by fusion of leaf protoplasts from both species in the presence of poly ethylene glycol or in an electric field. The somatic hybrids were selected on the basis of kanamycin resistance of *L. esculentum* and the plant regeneration capacity of *L. peruvianum*. Chromosome counts in root tips and the determination of the number of chloroplasts in guard cell pairs revealed that the majority of these hybrids was tetraploid (2n = 4x = 48). The remaining hybrids were at the hexaploid level with chromosome numbers between 64 and 72. The hybrid nature of the regenerated plants was confirmed by analysis of isozyme markers and by their morphology. Most hybrids did flower and set fruits and seeds after selfing. According to RFLP analysis 6 out of the 10 hexaploid hybrids contained two genomes of *L. esculentum* and four genomes of *L. peruvianum*. One of these hexaploids had genomes of two different *L. peruvianum* genotypes and was therefore considered to be derived from a triple protoplast fusion. The hexaploid plants were less fertile than the tetraploids and more resembled *L. peruvianum*.

Introduction

Somatic hybridisation is an alternative to sexual hybridisation when crossing barriers between parental species exist. By means of various cell culture techniques, protoplasts from different species can be induced to fuse with each other. Several procedures have been applied to select somatic hybrids after fusion. Somatic hybrids of *Solanum* and *Lycopersicon* species have been selected either mechanically by micromanipulation (Puite et al. 1986), by selection based on specific growth characteristics in cell culture (Gleddie et al. 1986; Handley et al. 1986), or by the identification of hybrids at the plant level (Austin et al. 1985; Shihachakr 1989; Tan 1987). Regeneration capacity that derived from one of the parents has been used as selectable marker by several authors in protoplast fusion experiments of *Nicotiana* (Maliga et al. 1977), *Petunia* (Itoh and Futsuhara 1983), *Brassica* (Terada et al. 1987) and *Lycopersicon* species (Adams and Quiros 1985; Kinsara et al. 1986).

In the present experiments, protoplasts of *Lycopersicon esculentum*, the cultivated tomato, and *L. peruvianum*, a wild tomato species with several

desirable agricultural traits (Rick 1982a), were fused. These species cannot be crossed easily with each other. When *L. esculentum* is used as staminate parent, pollen tube elongation is inhibited in the *L. peruvianum* style (unilateral stylar incompatibility or incongruity (Hogenboom 1973). When *L. peruvianum* is the pollinator, fertilisation takes place. However, the embryo soon aborts due to the degeneration of the endosperm (Barbano and Topoleski 1984). In the latter combination sexual hybrid plants were obtained by the application of tissue culture techniques such as embryo culture (Smith 1944) and embryo callus culture (Thomas and Pratt 1981). Backcrosses of such hybrids with *L. esculentum* were not possible without the application of additional tissue culture techniques (Smith 1944; Thomas and Pratt 1981).

The aim of the present experiments was the production of symmetric somatic hybrids between the two species in an efficient and reproducible manner by the development of a double selection strategy based on the dominant regeneration capacity of *L. peruvianum* (Adams and Quiros 1985; Kinsara et al. 1986; Koornneef et al. 1987a) and the dominant kanamycin resistance of *L. esculentum*. The latter selection marker had been introduced by leaf disc transformation of *L. esculentum* with *Agrobacterium tumefaciens*. The hybrids obtained were characterised on the basis of chromosome numbers, isozyme patterns, morphology, fertility and, in some cases, by the analysis of restriction fragment length polymorphisms (RFLPs).

Material and methods

Plant material

Seeds of *L. esculentum* cv. Bellina, *L. esculentum* genotype LA1182 (homozygous for recessive marker genes *sy*, *sf* (chr. 3) and *alb* (chr. 12) (Rick 1982b) and *L. peruvianum* PI128650 were kindly provided by Rijk Zwaan Seed Company (de Lier, The Netherlands), Prof. C.M. Rick (Tomato Stock Center, Davis, USA) and the Institute of Horticultural Plant Breeding (Wageningen, The Netherlands), respectively. Kanamycin resistant transformants of these genotypes, obtained after leaf disc transformation with *A. tumefaciens* containing the plasmid AGS112 (Koornneef et al. 1987b), were available and were designated ATW3001, ATW3003 (Bellina), ATW3052 (LA1182) and ATW2002 (PI128650). Four fusion combinations were made: ATW3001/-3003/-3052 (+) PI128650 and Bellina (+) ATW2002.

Isolation, fusion and culture of protoplasts

Shoot cultures were grown aseptically in glass containers at a light intensity of 30 W/m^2 (16 h), at 25 °C on medium containing MS salts (Murashige and Skoog 1962), T vitamins (Tewes et al. 1984) and 10 g/l sucrose, solidified with 9 g/l agar (shoot culture medium). Plants were kept in the dark one day before the harvest of leaflets, which then were floated in the dark at 4 °C for 24 hours on a pre-incubation medium: ½x MS salts, 1x T vitamins, 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/l benzylaminopurine (BA). Subsequently, the leaves were cut in small pieces and incubated for 16 hours in the dark at 25 °C in an enzyme solution: 10 g/l Cellulase R10 and 1.5 g/l Macerozym R10 (both enzymes from Yakult Ltd., Japan), CPW salts (Frearson et al. 1973), 100 mg/l 2[N-morpholino]-ethane-sulfonate (MES) and 137 g/l sucrose, pH 5.6. The protoplasts were separated from cell debris by filtration through

a nylon filter with a pore size of 50 μm and purified by floatation on a solution of 137 g/l sucrose + CPW salts + 100 mg/l MES by centrifugation for 5 min. at 70x *g*. The floating protoplasts were pelleted twice in W5 solution (Menczel et al. 1981) and a sample was counted in a haemocytometer to determine the concentration.

Fusion of protoplasts in poly-ethylene-glycol (PEG) was carried out as described by Menczel et al., except that 30% PEG MW 4000 instead of 40% PEG 6000 was used. In the experiments where electrofusion was used, the protoplast floatation step was carried out four times with a solution of 137 g/l sucrose without salts and MES. The layer of protoplasts was transferred to a solution of 64 g/l mannitol supplemented with 73.5 mg/l $CaCl_2 \cdot 2H_2O$ (final concentration ½-1 x 10^6 protoplasts per ml). 60μl of the protoplast suspension was transferred to a fusion chamber with a 1 mm wide gap between two parallel brass electrodes, glued in a glass Petri dish. The fusion apparatus was a combination of a function generator, a custom-built generator of direct current (D.C.) pulses and the fusion chamber. The electrofusion was performed as follows: the protoplasts were aligned in an alternating current (A.C.) field (1 Mhz) of 200 V/cm, one D.C. pulse of 2000 V/cm (25 μsec.) was applied, where after the A.C. field was slowly reduced to zero. Finally the protoplasts were transferred to a Petri dish with culture medium.

The protoplasts were cultured at a density of 1-2 x 10^5/ml in TMp medium, which is a modified TM-2 medium (Shahin 1985) containing 103 g/l sucrose and 0.5 mg/l BA (instead of zeatin riboside), in the dark at 25 °C. When the first cell divisions were observed, mostly after 3 days, the cultures were exposed to dim light and diluted (1:1 or less) once or twice a week with TMd, which is a modified TMp medium containing 68.5 g/l sucrose and 0.1 mg/l α-naphtylacetic acid (NAA). Four weeks after protoplast isolation microcalli were transferred onto TMc, which is a modified TM-3 medium (Shahin 1985) with 2.5 g/l sucrose, 36 g/l mannitol, 0.1 mg/l NAA, 0.5 mg/l BA and 8 g/l purified agar. After two weeks of culturing, we added kanamycin to the media for hybrid selection; in liquid media the final concentration of kanamycin was 50 mg/l, in TMc 100 mg/l. When the microcalli on TMc medium had developed to green calli of several millimeters in diameter, they were transferred to a shoot induction medium, which is shoot culture medium with 20 g/l sucrose (instead of 10 g/l) supplemented with 1 mg/l zeatin and 0.1 mg/l indole-acetic acid (IAA). After 4 weeks the calli were transferred to the same medium without IAA and subcultured every 4 weeks until well developed shoots were present. The shoots were rooted on shoot culture medium. After rooting the regenerants were maintained *in vitro*, tested on kanamycin resistance (100 mg/l) and transferred to soil in a heated greenhouse.

Cytogenetic analysis

Root tips were collected from greenhouse-grown plants, treated for 3-4 hours at 15-20 °C with 290 mg/l 8-hydroxyquinoline to arrest metaphase plates, and fixed in a mixture of ethanol and acetic acid (3:1) at 4 °C. The root tips were either macerated in 1N HCl for 4-5 minutes at 60 °C and then squashed in lacto-propionic orcein (Dyer 1963) or they were treated with an enzyme solution (1 g/l Cellulase RS (from Yakult, Japan) + 1 g/l Pectolyase Y23 (from Seishin, Japan) + 1 g/l Cytohelicase (from IBF, France) in 2.5 g/l Na-citrate pH 4.8) for 3 hours at 20 °C. In the latter case chromosome preparations were made by the cell spreading technique and chromosomes were stained with Giemsa, according to Pijnacker and Ferwerda (1984).

The number of chloroplasts per guard cell pair was determined in lower epiderm strips from leaves of greenhouse-grown plants as described by Koornneef et al. (1989).

Isozyme and RFLP analysis

Young leaves from greenhouse-grown plants were used for isozyme and RFLP analyses.

Acid phosphatase (APS; E.C.3.1.3.2) activity was analysed by electrophoresis of 50 μl crude extract, supplemented with 5 μl electrode buffer and 5 μl marker dye (0.5% bromophenol blue in 20% glycerol) on a 10% polyacrylamide (PAA) slab gel. The extracts were obtained by squeezing thawing leaf material, that had been

stored at -80 °C. For analysis of glutamate oxaloacetate transaminase (GOT; E.C.2.6.1.1) activity, samples were prepared according to Suurs et al. (1989) and electrophoresed on a 7.5% PAA slab gels. Electrophoresis was performed in vertical gels (Desaphor VA equipment, Desaga). The stacking gel contained 5% polyacrylamide. The electrode buffer consisted of 0.04 M tris-HCl and 0.1 M glycin, pH 8.9. After running, the gels were incubated for 15 minutes in the appropriate staining buffer with gentle shaking. Subsequently, they were incubated in the corresponding staining solutions for one of both isozymes (a few hours, gentle shaking), prepared according to Vallejos (1983).

DNA was isolated from several plants according to Dellaporta et al. (1983), digested with *Dra* I, separated by agarose gel electrophoresis, blotted onto Gene Screen PLUS (New England Nuclear) and hybridised with the tomato single copy clones TG16 and TG63 (Tanksley et al. 1988) (kindly supplied by Dr. S.D. Tanksley, Cornell University, Ithaca, USA), as described in Chapter 6.

Morphology and fertility

The following features of the plants, grown in an unheated greenhouse under Dutch summer conditions, were determined: growth habit, leaf morphology, sympodial index (= mean number of nodes between two subsequent inflorescences), presence of stipules and bracts, size of the flower parts, pollen viability, fruit and seed set. Pollen grains (at least 100 per flower) were stained with a solution of lactophenol acid fuchsin; viable pollen stained purple, non viable pollen did not stain. Observations were made on somatic hybrid plants, several *L. peruvianum* regenerants and the parental genotypes mentioned before. Also seedlings of a tetraploid plant of the tomato cultivar Moneymaker and of a tetraploid *L. peruvianum* plant, both derived from tissue culture, were used. The somatic hybrid plants were selfed and crossed with *L. esculentum* (diploid and tetraploid) and with *L. peruvianum* (diploid and tetraploid).

Results

Cell culture and plant regeneration

Upon isolation and culture with the procedures described, *L. peruvianum* protoplasts formed cell walls and grew prosperously. The cultures had to be diluted at least every 3-4 days during the first 4 weeks of culture. The resulting microcalli, that were put on the TMc-medium, developed into purple-green coloured calli. The majority of these calli produced shoots within 4 weeks on shoot induction medium; some of them even showed shoot primordia while being cultured on TMc. The shoots could easily be rooted. When kanamycin was added to the liquid culture, all cell colonies stopped growing and in cases where microcalli were put on TMc with kanamycin they became brown.

L. esculentum cells divided at a lower frequency and developed more slowly than those of *L. peruvianum*. Only half of the microcalli transferred to TMc developed into small green calli. These calli turned brown within a few weeks on shoot induction medium. No regenerated plants could be obtained by means of the described procedures.

The mixed cultures (protoplasts of both species without fusion treatment) resulted in many microcalli that appeared to be of *L. peruvianum* origin, as

judged by the growth characteristics. Self-fused *L. peruvianum* cultures did produce many microcalli also. From both culture types, shoots could be regenerated.

The fusion cultures (protoplasts of both species with fusion treatment) yielded many well growing colonies. When kanamycin was added to the liquid culture most colonies stopped growing, whereas others did not. A majority of the microcalli, that were transferred to TMc + kanamycin, developed into green calli. Most of these formed shoot primordia within 4 weeks on shoot induction medium. The shoots were characterized by broader leaflets than the *L. peruvianum* regenerants and formed roots easily. One to several shoots from 39 different putative hybrid calli (named OH2 to OH40) were rooted. Eight of the calli were derived from electrofusion treated protoplasts, while the remainder was obtained by PEG treatment. The kanamycin resistance of the putative hybrid shoots was confirmed later on by their ability to root on shoot culture medium supplemented with kanamycin.

One other putative hybrid, named OH1, was obtained after PEG-fusion of protoplasts from *L. esculentum* cv. Bellina and *L. peruvianum* ATW2002. This hybrid was selected on the basis of callus morphology (green and well-growing) and regeneration capacity.

Shoots from 33 hybrid calli and from 12 *L. peruvianum* derived calli were grown in the greenhouse and were analysed for one or more characters, together with the parental genotypes.

Cytogenetic analysis

Both parental species have chromosome numbers of 2n = 2x = 24. The chromosome numbers of 47 shoots derived from 23 different hybrid calli were determined in metaphase plates of root tip cells. Most shoots were tetraploid with the euploid number of 2n = 4x = 48 (Fig. 1A + 2); few were aneuploid at the tetraploid level. A minority of the shoots was at the hexaploid level (Fig. 1B + 2); half of these had a chromosome number slightly below 72. We found an octaploid chromosome number in one shoot derived from a cutting of a plant that we had previously determined to be a tetraploid. The few hyper-tetra/hexaploid numbers might have been obtained by the unintended counting of the macrosatellites (short arms of the chromosomes 2) as independent chromosomes.

The number of chloroplasts in the guard cell pairs can be used as an additional measure for the ploidy level in *Lycopersicon* plants (Koornneef et al. 1989). However, the relation between chloroplast number and ploidy level differs between *L. esculentum* and *L. peruvianum* (Fig. 3; Koornneef et al. 1989). The chloroplast numbers of the somatic hybrids also are shown in Fig. 3. The average chloroplast number of tetraploid hybrids (18.1), is intermediate to that of

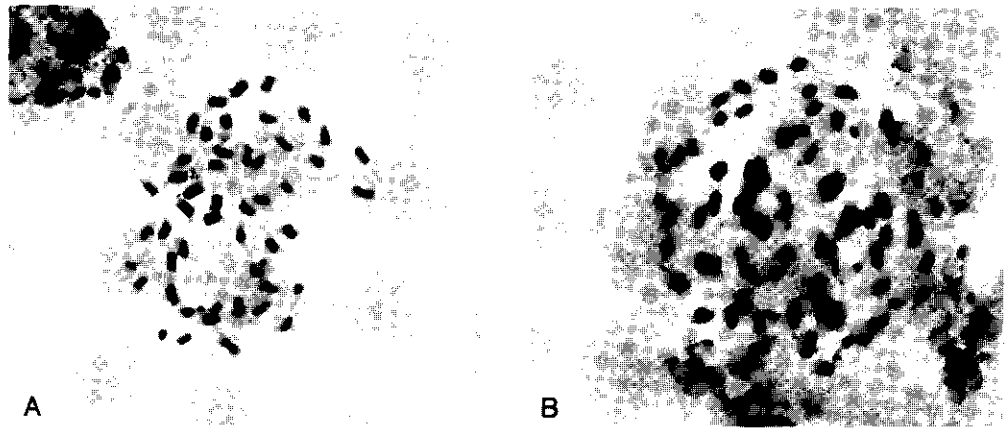

Fig. 1. Metaphase plates of root tip cells from somatic hybrids of *L. esculentum* and *L. peruvianum*. **A.** The tetraploid hybrid 0H4, 2n = 4x = 48. **B.** The hexaploid hybrid 0H14, 2n = 6x = 72.

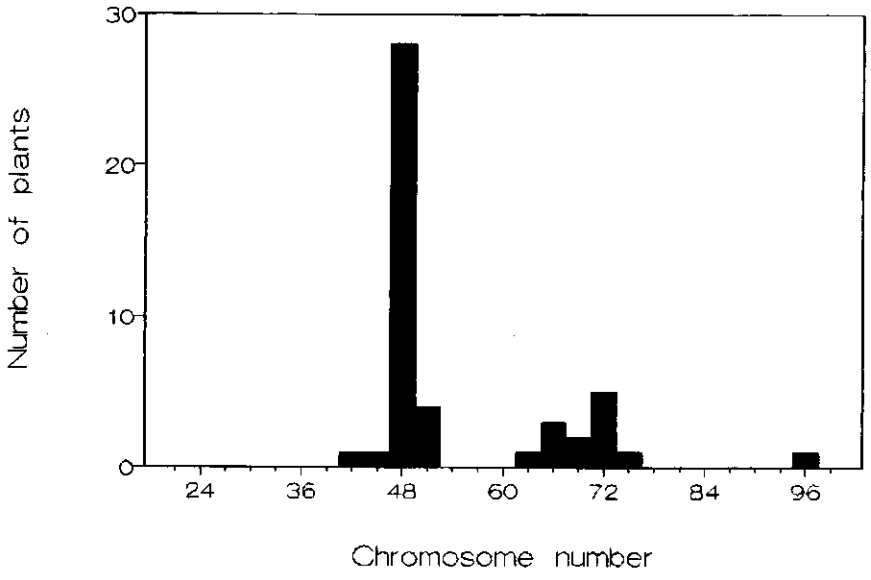

Fig. 2. Frequency distribution of average chromosome numbers of different shoots from somatic hybrid calli, derived from protoplast fusions between *L. esculentum* and *L. peruvianum*.

tetraploid *L. esculentum* and *L. peruvianum* plants and lower than that of hexaploid hybrids (24.9). However, the frequency distributions of both ploidy classes overlap, which indicates that this character does not always allow an unambiguous distinction between both ploidy groups.

We classified the ploidy level of the somatic hybrids on the basis of chromosome numbers, or, in some cases, chloroplast counts (taking <20 as tetraploid and 25-30 as hexaploid). 54 shoots regenerated from 21 hybrid calli were tetraploid, 18 shoots from 9 calli were hexaploid and 3 shoots from 2 calli were tetra- or hexaploid; the latter plants had an intermediate chloroplast number. From one callus one tetraploid and one hexaploid shoot derived. These were considered as two independent fusion products in further analyses. The fusion method used had no clear effect on the ploidy level distribution of the obtained somatic hybrids (Table 1).

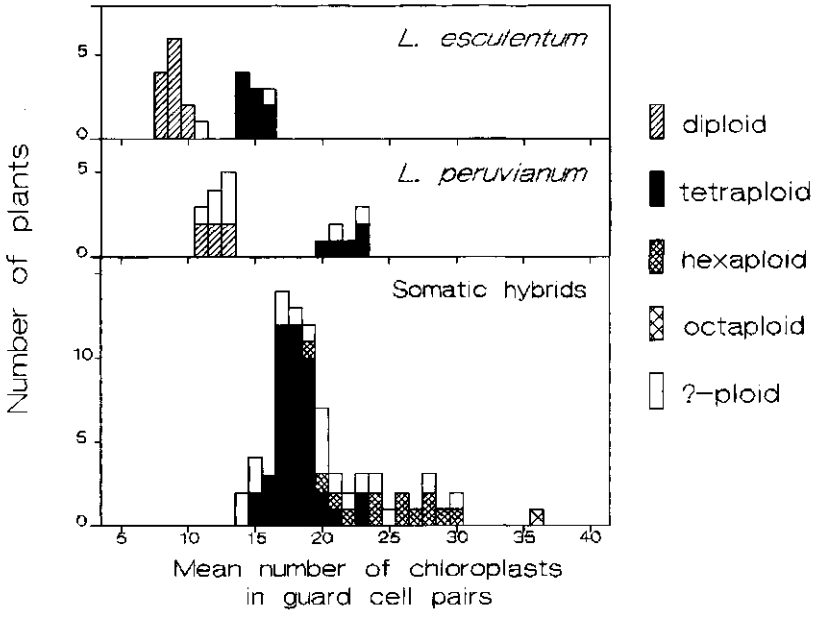

Fig. 3. Mean chloroplast number in the guard cell pairs of the leaf epiderm: upper panel, *L. esculentum* plants, that were diploid, tetraploid and of unknown ploidy level (= no chromosome counts available); middle panel, *L. peruvianum* plants, that were diploid, tetraploid and of unknown ploidy level; lower panel, Somatic hybrid (of both species mentioned above) shoots, that were tetraploid, hexaploid and of unknown ploidy level.

Table 1. The ploidy level of hybrid clones of *L. esculentum* and *L. peruvianum* for each of both fusion methods used, either PEG-fusion (Menczel et al. 1981) or electrofusion. The ploidy level was determined in shoots regenerated from the hybrid calli using chromosome counts or the mean number of chloroplasts in the guard cell pairs. Mixed means that both tetraploid and hexaploid shoots were derived from one callus.

Method	Ploidy level of hybrid clones		
	tetraploid	hexaploid	mixed
PEG-fusion	17	7	1
Electrofusion	4	2	0
Total	21	9	1

Isozyme analysis

The *Aps-1* (acid phosphatase, locus *Aps-1*; a dimeric enzyme (Rick 1983) patterns of *L. esculentum*, *L. peruvianum* and some somatic hybrids are shown in Fig. 4A. *L. esculentum* had one fast migrating band. *L. peruvianum* plants had one of two different patterns: either three bands migrating more slowly than the tomato band (pattern A) or one heavy band which migrates as fast as the tomato band (pattern B). Of the hybrid plants tested 24 had one heavy band similar to *L. peruvianum* pattern B (for instance, OH8 in Fig. 4A). Eight hybrids had an *Aps-1* pattern of 6 bands of which three were at the same position as the bands of pattern A of *L. peruvianum*, one was similar to the *L. esculentum* band, and two were "new"; OH1 in Fig. 4A is an example of this class of hybrids. We suppose that these "new" bands contain dimers of *L. peruvianum* and *L. esculentum* enzyme subunits. One of the hybrids (OH14) had this same pattern of six bands with different intensities (Fig. 4A).

Isozyme patterns of glutamate oxalate transaminase (GOT; 4 loci are known, of which at least 3 contain genes coding for dimeric enzymes (Rick 1983) are shown in Fig. 4B. *L. esculentum* had four GOT bands of which one was often very faint or absent. Again, there are two *L. peruvianum* patterns: some *L. peruvianum* plants had 5 bands, of which 2 were similar to *L. esculentum* bands (pattern A); others had 7 GOT bands (pattern B), of which 5 coincided with the bands of pattern A and 3 with the tomato bands. We analysed the GOT isozyme patterns of hybrid plants derived from 22 different calli. These hybrids had complex isozyme patterns of 9 bands (for instance, OH2 and OH14 in Fig. 4B), of which one or two did not coincide with any of the parental GOT bands; these probably represent interspecific heterodimers.

The simultaneous occurrence of isozyme bands from both parents in the putative hybrids and the occurrence of presumed interspecific heterodimer bands for *Aps-1* as well as GOT indicate the hybrid nature of the regenerated plants.

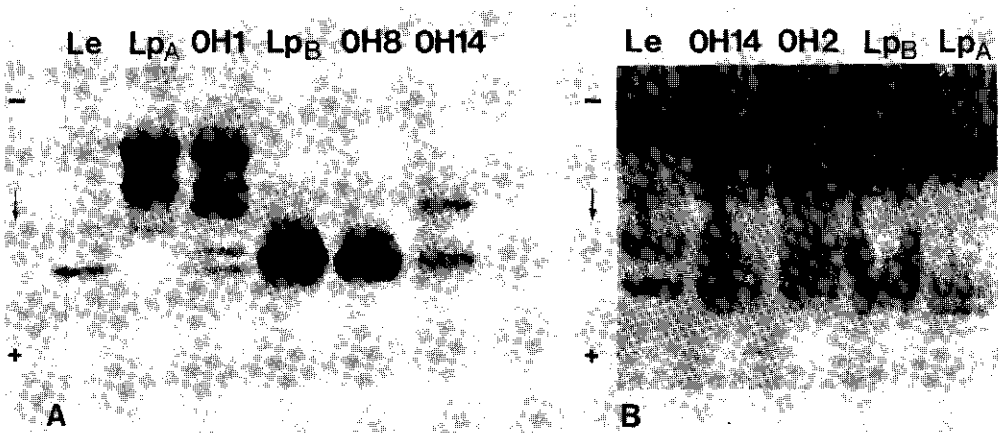

Fig. 4. A. Isozyme patterns of acid phosphatase, locus *Aps-1*, of (from left to right) *L. esculentum* cv. Bellina (Le), one *L. peruvianum* PI128650 (Lp_A), somatic hybrid OH1, another *L. peruvianum* PI128650 (Lp_B), somatic hybrid OH8, somatic hybrid OH14. **B.** Isozyme patterns of glutamate oxaloacetate transaminase of (from left to right) *L. esculentum* cv. Bellina (Le), somatic hybrid OH14, somatic hybrid OH2, one *L. peruvianum* PI128650 (Lp_B), another *L. peruvianum* PI128650 (Lp_A).

RFLP analysis

To establish the contribution of each parental genome to that of the hybrids, six hexaploid somatic hybrids and four tetraploid somatic hybrids were subjected to RFLP analysis. For this purpose we used TG16 and TG63, single copy probes located on chromosome 8 and 10, respectively, as markers. In addition, two *L. esculentum* genotypes, cv. Bellina and LA1182, and two *L. peruvianum* plants, differing in *Aps-1* and GOT patterns, were analysed with the same probes. The results are shown in Fig. 5. The RFLP patterns of the two tomato genotypes were identical, whereas those of both *L. peruvianum* plants were different. All hybrids showed a composite pattern for both RFLP probes. Five hexaploid hybrids displayed the same pattern as the tetraploid hybrids, but with the *L. peruvianum* specific band(s) being relatively more intense. We therefore tentatively conclude that these shoots contained 4 genomes of *L. peruvianum* and 2 genomes of *L. esculentum*. Hexaploid hybrid OH14 had the RFLP patterns of both *L. peruvianum* plants as well as the tomato specific band (Fig. 5B). Apparently, this hybrid arose from a fusion product of three genotypically different protoplasts. The results obtained

with hybrid OH3 were deviating: OH3 was classified as tetraploid on the basis of chromosome number and chloroplast counts. RFLP analysis of this hybrid with TG16 yielded a typical tetraploid pattern, whereas analysis with probe TG63 produced a hexaploid pattern (Fig. 5).

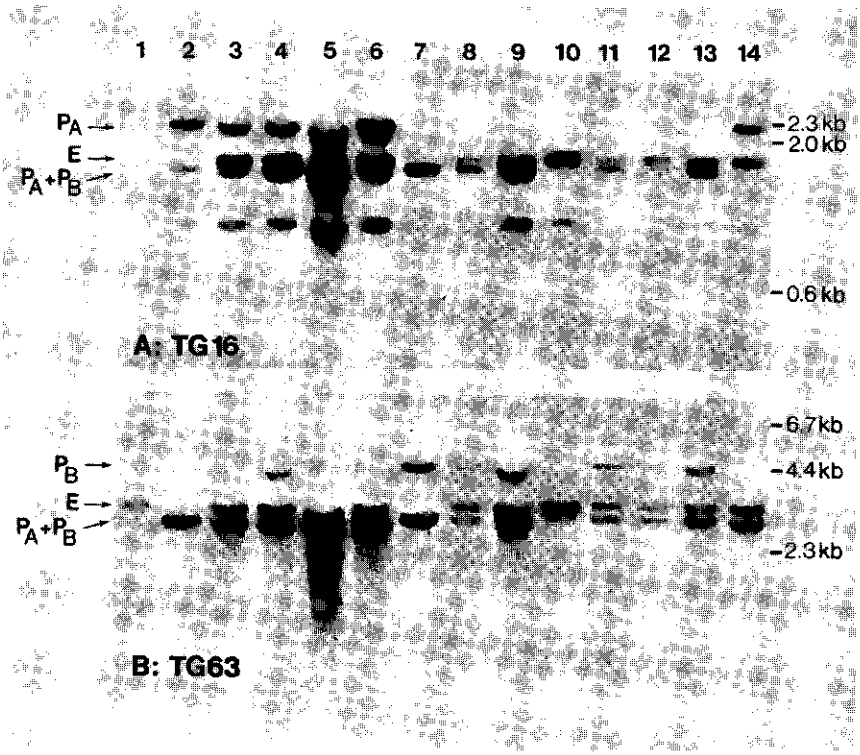

Fig. 5. Southern blot analysis of some somatic hybrids and their parents with **A.** probe TG16 and **B.** probe TG63. *Dra* I digests of the DNAs of the following genotypes were analysed: lane 1, *L. esculentum* cv. Bellina; lane 2, one *L. peruvianum* PI128650 (A); lane 3, tetraploid hybrid OH4; lane 4, hexaploid hybrid OH14; lane 5, hexaploid hybrid OH19a; lane 6, hexaploid hybrid OH27; lane 7, another *L. peruvianum* PI128650 (B); lane 8, hexaploid hybrid OH7; lane 9, tetraploid hybrid OH8; lane 10, *L. esculentum* LA1182; lane 11, hexaploid hybrid OH2; lane 12, tetraploid hybrid OH3; lane 13, hexaploid hybrid OH32; lane 14, tetraploid hybrid OH1. The parental genotypes of the hybrids were: Bellina (+) *L. peruvianum* plant A, OH4, OH19a, OH27 and OH1; Bellina (+) *L. peruvianum* plant B, OH7 and OH8; LA1182 (+) *L. peruvianum* plant B, OH2, OH3 and OH32; LA1182 (+) *L. peruvianum* plant A (+) *L. peruvianum* plant B, OH14. The species specific bands are indicated at the left side (E, *L. esculentum*; P_A, *L. peruvianum* plant A; P_B, *L. peruvianum* plant B), and the position of the *Hin* dIII fragments of phage lambda DNA at the right side.

Plant morphology

The somatic hybrids, represented by 60 shoots derived from 32 different fusion products, were very vigorously growing plants. The leaf morphology (Fig. 6) was intermediate between both parents, although in the LA1182 hybrids the leaves were more serrated than in LA1182, indicating the complementation of the recessive solanifolia (*sf*) trait of this genotype (the latter hybrids were also wild type

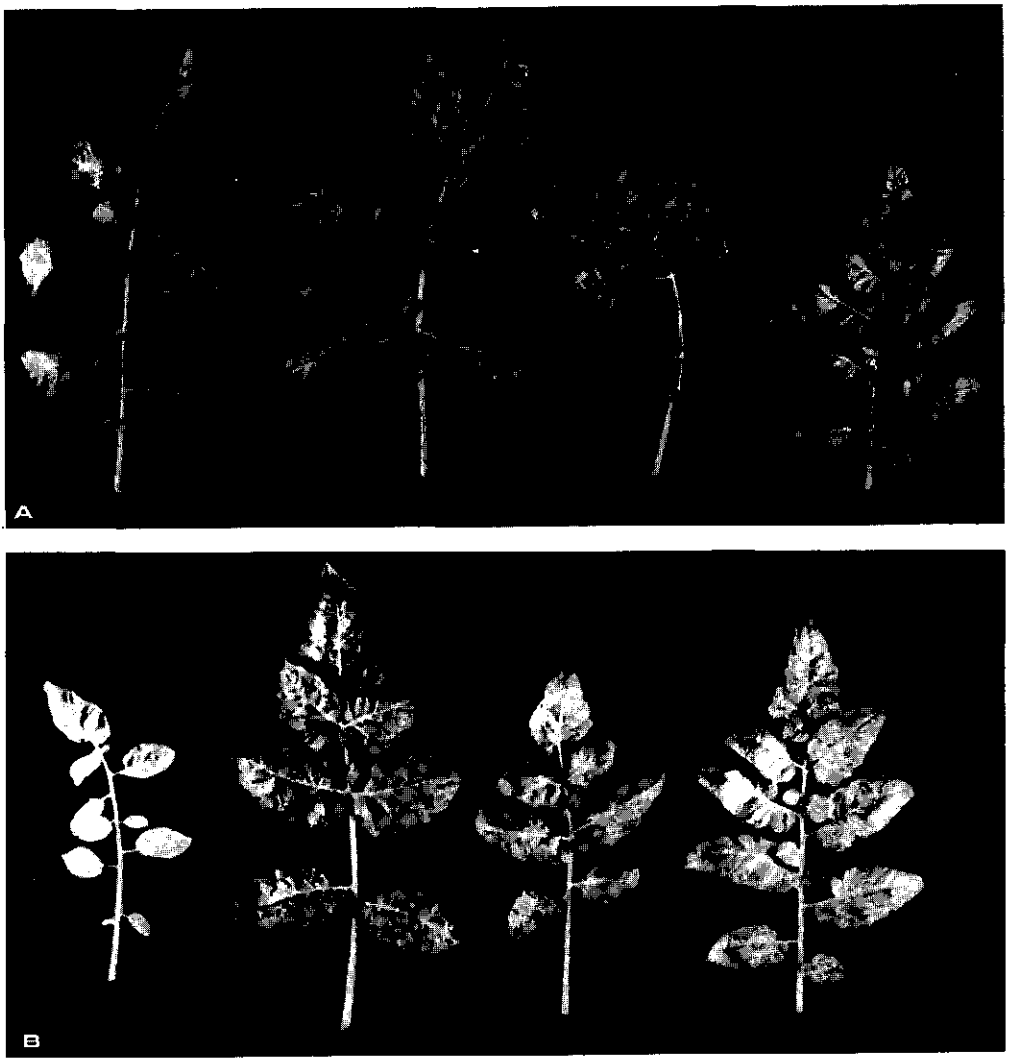

Fig. 6. A. The leaves of (from left to right) *L. esculentum* cv. Bellina, the tetraploid somatic hybrid OH36, the hexaploid somatic hybrid OH22 and *L. peruvianum* PI128650. **B.** The leaves of (from left to right) *L. esculentum* LA1182, the tetraploid somatic hybrid OH3, the hexaploid somatic hybrid OH32 and *L. peruvianum* PI128650.

for *sy*, sunny = yellow colour of young leaflets, and *alb*, albescent = white sectors). The leaves of the hexaploid hybrids were in general somewhat smaller, less incised and darker green than the leaves of the tetraploid hybrids. The sympodial index seemed to be a dominantly expressed trait derived from *L. esculentum* in the tetraploid hybrids (Fig. 7A); however, this trait was more intermediate in the hexaploid hybrids (Fig. 7A). The presence of stipules appeared to behave as a dominant *L. peruvianum* character (Fig. 7B), whereas the presence of bracts was intermediate between both parents in the somatic hybrids (Fig. 7C).

In half of the shoots variegation of grey-green spots and sectors was observed. From several hybrid calli both variegated and normal shoots were regenerated. Eleven hybrid calli formed only normal shoots. Severely variegated shoots had leaves that were smaller and irregularly shaped.

Flower morphology

Shoots from all hybrid calli, except for one hexaploid hybrid, have flowered (Table 2). The flowers of the cultivated tomato had long sepals, pale yellow petals and no protruding style. The *L. peruvianum* flowers had short sepals, dark

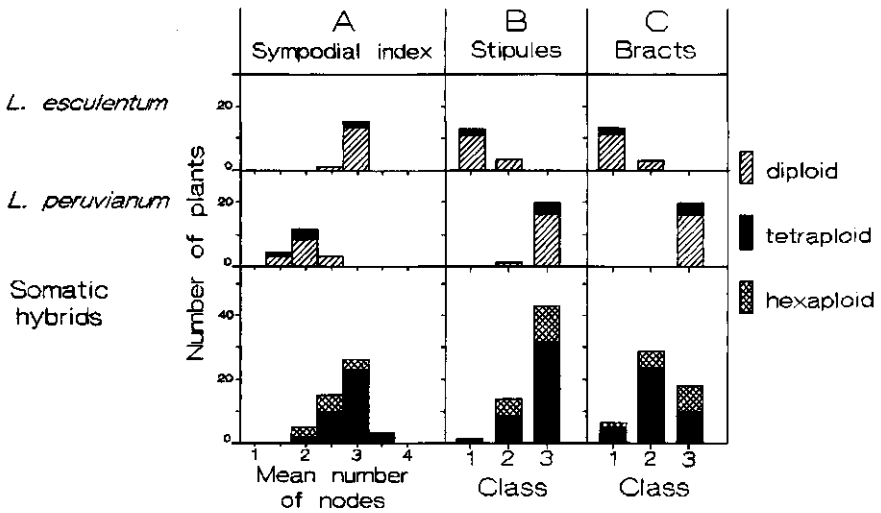

Fig. 7. Morphological characters of *L. esculentum* plants, *L. peruvianum* plants and somatic hybrids of different ploidy level. **A.** Sympodial index: mean number of nodes between two subsequent inflorescences. **B.** Presence of stipules: leaflets on the stem at the nodes. **C.** Presence of bracts: leaflets in the inflorescence at the nodes. Classification of B and C: 1 = absence, 2 = presence at a few nodes, 3 = presence at each node.

Table 2. The presence of flowers, fruits and seeds in the somatic hybrid clones (on at least one shoot of each fusion callus), divided in ploidy groups. Observations were made on greenhouse-grown plants. Seed set after controlled or spontaneous selfing, unless otherwise mentioned.

Ploidy level	Number of somatic hybrid clones			
	total	flowering	setting fruits	setting seeds
Tetraploid	20	20	20	16
Hexaploid	10	9	6	4*
Tetra- or hexaploid	2	2	2	1
Total	32	31	28	21

* 2 out of 4 only after backcrossing with tetraploid *L. peruvianum* as staminate parent

yellow petals and a protruding style. The flowers of the somatic hybrids in general had medium-sized sepals, dark yellow large petals and a slightly protruding style. The lengths of the different flower parts are given in Table 3. The sepal length of the hybrids was intermediate. The petals, the stamens and the pistil of the hybrids were larger than those of both diploid parents and comparable with those of the tetraploid *L. peruvianum* plants, except for the pistil which was longer in the latter. The hexaploid hybrids had a more exerted style than the tetraploid hybrids, because of slightly shorter stamens and a slightly longer pistil.

Table 3. Characteristics of flower parts of *L. esculentum*, *L. peruvianum* and somatic hybrids. Pollen viability was determined by lactophenol acid fuchsin staining. Each measurement was the average of at least two flowers from the same plant. 2x, diploid; 4x, tetraploid; 6x, hexaploid; SD, standard deviation.

Genotypes	Mean lengths (in millimeters ±SD)				Pollen viability (percentage ±SD)
	sepals	petals	stamens	pistil	
L. esculentum					
2x (n=11)	13.9 ±2.1	17.3 ±2.4	11.1 ±0.8	10.7 ±0.9	77.4 ±18.2
4x (n= 2)	14.4 ±0.1	17.7 ±1.0	11.8 ±0.3	9.1 ±1.3	35.7 ±32.3
L. peruvianum					
2x (n=20)	5.1 ±0.4	16.7 ±2.7	10.8 ±0.9	12.9 ±1.1	75.0 ±19.3
4x (n= 4)	5.8 ±1.0	20.3 ±3.0	13.8 ±3.2	15.1 ±3.7	44.6 ±23.8
Somatic hybrids					
4x (n=30)	9.0 ±1.3	20.7 ±2.6	13.6 ±1.2	14.0 ±1.2	50.6 ±21.9
6x (n= 8)	7.8 ±0.9	19.8 ±2.4	13.2 ±1.1	14.2 ±1.8	26.9 ±20.6

Fertility

All hybrid flowers produced pollen, although less abundant than the parental flowers. Staining with lactophenol fuchsine was used as a measure of pollen viability. As shown in Table 3, the pollen viability of the hybrids was lower than that of both diploid parents and higher than that of tetraploid plants of both parental species. The tetraploid hybrids had a higher percentage of viable pollen than the hexaploid ones.

Plants from 28 of the 31 hybrid calli which produced flowering shoots did set fruits; the 3 fruitless hybrids were hexaploids (Table 2). The fruits of the somatic hybrids were small and yellow, whereas tomato produced big red fruits and *L. peruvianum* small green ones. A very variegated hybrid plant produced irregular shaped, yellow-green variegated fruits. When the hybrid fruits contained seeds they often were slightly larger than normal *L. peruvianum* fruits.

Plants from 21 of the 28 fruit setting hybrid clones set several intermediate-sized seeds (Table 2). In most cases, the seed set resulted from controlled and spontaneous selfing. Backcrosses of the hybrids with diploid and tetraploid tomato did not result in seed set. Backcrosses with diploid *L. peruvianum* as male parent yielded a few seeds in two tetraploid hybrids, while backcrosses with pollen of a tetraploid *L. peruvianum* yielded some seeds in three hexaploids (all 2/3 *L. peruvianum* genome). The analysis of the progenies is reported in Chapter 3.

Discussion

The experiments described in this paper show that somatic hybrids of *L. esculentum* and *L. peruvianum* can be effectively selected on the basis of kanamycin resistance, introduced in the *L. esculentum* genotypes used, and the regeneration capacity of *L. peruvianum*. All regenerants that survived the selection and that were analysed biochemically and/or morphologically, proved to be hybrids. An analogous selection method was used by Adams and Quiros (1985), who obtained somatic hybrids between *L. peruvianum* and *L. pennellii* after selection for regenerating capacity of the former species and for resistance to the antibiotic G418 present in a spontaneous variant cell clone of *L. pennellii*. Kinsara et al. (1986) also isolated somatic hybrids of *L. esculentum* and *L. peruvianum*. They obtained hybrid shoots from two calli. This low yield probably was due to the absence of a selection scheme against the *L. peruvianum* parent at the cellular level. Their hybrid shoots were all hexaploid, probably due to the tetraploid origin of the suspension culture from which the *L. peruvianum* protoplasts derived. In our experiments most shoots that were

derived from hybrid calli were tetraploid (22 out of 34 hybrid clones) and the remaining hybrids were hexaploid. No obvious effect of the fusion method used on the ploidy level of the hybrids was observed.

We found two cases of aneuploidy at the tetraploid level. The only shoot of OH26 had 41 chromosomes and one shoot of OH13 had 45 chromosomes; whereas another shoot from the OH13 callus had 48 chromosomes. It is possible that spontaneous chromosome loss occurred during the latter stages of the cell culture or at the plant level. In hexaploid hybrids we observed more cases of aneuploidy. Six of the 12 hexaploid shoots (from 7 calli) counted were hypohexaploid. Also some differences between shoots of the same callus (2 out of 4 lines with more than one shoot counted) were observed. Apparently hexaploid hybrids were less stable for chromosome number than the tetraploid hybrids.

It was not possible to classify all hybrid plants as either tetraploid or hexaploid on the basis of the number of chloroplasts in guard cell pairs; this confirms the observation of Frandsen (1968) on tetraploid and hexaploid potato plants. A number of extreme deviations between chromosome number and expected chloroplast number may indicate chimerism of different tissue layers (Koornneef et al. 1989; Sree Ramulu et al. 1976).

According to the RFLP analysis, all 6 hexaploid hybrids contained one *L. esculentum* genome and two *L. peruvianum* genomes. In hexaploid OH14 the two *L. peruvianum* genomes were different; this suggests that this hybrid has originated from a triple fusion between three, genetically different protoplasts. This conclusion was further supported by the *Aps-1* isozyme pattern of OH14 (see Fig. 4A), which resembled the typical pattern of the hybrid between both *L. peruvianum* plants involved (data not shown). It is conceivable that the other 5 hexaploid hybrids also originated from triple fusion, although an alternative explanation is that the genome of these hybrids arose after doubling of the *L. peruvianum* genome either before fusion or before karyogamy. One euploid tetraploid shoot had the "tetraploid" restriction fragment pattern for one of the probes, while for the other probe, TG63, the *L. esculentum* specific band was fainter than expected. Possibly this hybrid lacked part of a *L. esculentum* chromosome 10, containing the TG63 marker (Tanksley et al. 1988).

The morphological characteristics of the somatic hybrids are similar to those described for diploid sexual hybrids of *L. esculentum* x *L. peruvianum* (de Nettancourt et al. 1974; Maheswaran et al. 1986; Smith 1944; Thomas and Pratt 1981). In general, the hexaploid somatic hybrids were somewhat less vigorous than the tetraploid hybrids. The analysed hexaploid hybrids (6 out of 10) possessed more *L. peruvianum* than *L. esculentum* genome; this explains why they resembled *L. peruvianum* more than the tetraploid hybrids in several morphological aspects.

Many hybrid shoots showed a grey-green variegation. This may be similar to what is described as silvering, whitehead or chimera, which sometimes occurs in some tomato genotypes of north-west Europe and can be induced by low temperatures (Grimbly 1986). This disorder was not observed in the *L. peruvianum* regenerants, whereas it was in some explant regenerants of tomato (data not shown). In the latter plants and in the somatic hybrids, this phenomenon was apparently induced by the tissue culture phase and not due to the hybrid nature of the plants. When the symptoms were very severe, the plant did not flower or had deformed, seedless fruits, and irregularly shaped leaves; this so-called leaf distortion is also known to be related to silvering (Grimbly 1986).

The percentage of viable pollen of the somatic hybrids (27-51%) was comparable with that of the somatic hybrids of Kinsara et al. (45%) (1986) and lower than that of some sexual hybrids (77 to 90%) (Cappadocia and Sree Ramulu 1980; Maheswaran et al. 1986). Most of the selected somatic hybrids were self-compatible: 17 out of 22 tetraploid lines and 2 out of 10 hexaploid lines. The hexaploids of Kinsara et al. (1986) were also self-compatible, whereas the diploid sexual hybrids of both species were self-incompatible (de Nettancourt et al. 1974; Maheswaran et al. 1986; Smith 1944; Thomas and Pratt 1981). This can be due to the polyploid state of the somatic hybrids. Tetraploid *L. peruvianum* plants are self-compatible too, whereas they are strictly self-incompatible as diploids. Most somatic hybrid plants set small, mostly seedless fruits. Parthenocarpy might result from an interspecific genomic combination and/or high temperatures (Stevens and Rick 1986), which were both the case in the present experiments (at summer on sunny days the temperature in the greenhouse could be above 40 °C).

The moderate fertility of the somatic hybrids could be promising for transfer of traits from *L. peruvianum* to *L. esculentum*. Although the backcrosses of the hybrids to tomato were unsuccessful, many seeds derived from selfings. If segregation for species specific traits can occur in the latter progeny, it is conceivable that some of these plants are crossable with tomato.

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References

Adams TL, Quiros CF (1985) Somatic hybridization between Lycopersicon peruvianum and L. pennellii: regenerating ability and antibiotic resistance as selection systems. Plant Science 40:209-219

Austin S, Baer MA, Helgeson JP (1985) Transfer of resistance to potato leaf roll virus from Solanum brevidens into Solanum tuberosum by somatic fusion. Plant Science 39:75-82

Austin A, Ehlenfeldt MK, Baer MA, Helgeson JP (1986) Somatic hybrids produced by protoplast fusion between S. tuberosum and S. brevidens: phenotypic variation under field conditions. Theor Appl Genet 71:682-690

Barbano PP, Topoleski LD (1984) Postfertilization hybrid seed failure in Lycopersicon esculentum x Lycopersicon peruvianum ovules. J Amer Soc Hort Sci 109:95-100

Cappadocia M, Sree Ramulu K (1980) Plant regeneration from in vitro cultures of anthers and stem internodes in an interspecific hybrid, Lycopersicon esculentum L. x L. peruvianum Mill. and cytogenetic analysis of the regenerated plants. Plant Sci Lett 20:157-166

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Molecular Biology Reporter 1:19-21

De Nettancourt D, Devreux M, Laneri U, Cresti M, Pacini E, Sarfatti G (1974) Genetical and ultrastructural aspects of self and cross incompatibility in interspecific hybrids between self-compatible Lycopersicon esculentum and self-incompatible L. peruvianum. Theor Appl Genet 44:278-288

Dyer AF (1963) The use of lacto-propionic orcein in rapid squash methods for chromosome preparations. Stain technol 38:85-90

Fish N, Karp A, Jones MGK (1988) Production of somatic hybrids by electrofusion in Solanum. Theor Appl Genet 76:260-266

Frandsen NO (1958) Die Plastidenzahl als Merkmal bei der Kartoffel. Theor Appl Genet 38:153-167

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

Gleddie S, Keller WA, Setterfield G (1986) Production and characterization of somatic hybrids between Solanum melongena L. and S. sisymbriifolium Lam. Theor Appl Genet 71:613-621

Grimbly P (1986) Disorders. In: Atherton JG, Rudich J (eds) The tomato crop. A scientific basis for improvement. Chapman and Hall, London/New York, pp 369-389

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

Guri A, Sink KC (1988) Interspecific somatic hybrid plants between eggplant (Solanum melongena) and Solanum torvum. Theor Appl Genet 76:490-496

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

Hogenboom NG (1973) Incongruity and incompatibility in intimate partner relationships. Ph.D. Thesis, Agricultural University Wageningen, the Netherlands

Itoh K, Futsuhara Y (1983) Restoration of the ability to regenerate shoots by somatic hybridization between Petunia hybrida and P. parodii. Japan J Breed 33:130-137

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

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

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

Koornneef M, van Diepen JAM, Hanhart CJ, Kieboom-de Waart AC, Martinelli L, Schoenmakers HCH, Wijbrandi J (1989) Chromosomal instability in cell- and tissue cultures of tomato haploids and diploids. Euphytica 43:179-186

Maheswaran G, Perryman T, Williams EG (1986) Use of an interspecific hybrid in identifying a new allelic specificity generated at the self-incompatibility locus after inbreeding in Lycopersicon peruvianum. Theor Appl Genet 73:236-245

Maliga P, Lázár G, Joó F, Nagy AH, Menczel L (1977) Restoration of morphogenic potential in Nicotiana by somatic hybridisation. Mol Gen Genet 57:291-296

Menczel L, Nagy F, Kiss ZR, Maliga P (1981) Streptomycin resistant and sensitive somatic hybrids of Nicotiana tabacum + Nicotiana knightiana: correlation of resistance to N. tabacum plastids. Theor Appl Genet 59:191-195

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

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

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

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

Rick CM (1982a) The potential of exotic germplasm for tomato improvement. In: Vasil IK, Scowcroft WR, Frey KJ (eds) Plant improvement and somatic cell genetics. Academic Press, New York, pp 1-28

Rick CM (1982b) Stock list Rep Tomato Genet Coop 32:3-10

Rick CM (1983) Tomato (Lycopersicon). In: Tanksley SD, Orton TJ (eds) Isozymes in plant genetics and breeding, part B. Elsevier, Amsterdam, pp 147-165

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

Sihachakr D, Haicour R, Chaput MH, Barrientos E, Ducreux G, Rossignol L (1989) Somatic hybrid plants produced by electrofusion between Solanum melongena L. and Solanum torvum Sw. Theor Appl Genet 77:1-6

Smith PG (1944) Embryo culture of a tomato species hybrid. Proc Amer Soc Hort Sci 44:413-416

Sree Ramulu K, Devreux M, Ancora G, Laneri U (1976) Chimerism in Lycopersicon peruvianum plants regenerated from in vitro cultures of anthers and stem internodes. Z Pflanzenzüchtg 76:299-319

Stevens MA, Rick CM (1986) Genetics and breeding. In: Atherton JG, Rudich J (eds) The tomato crop. A scientific basis for improvement. Chapman and Hall, London/New York, pp 35-109

Suurs LCJM, Jongedijk E, Tan MMC (1989) Polyacrylamide gradient-gel electrophoresis: a routine method for high resolution isozyme electrophoresis of Solanum and Lycopersicon species. Euphytica 40:181-186

Tan MMC (1987) Somatic hybridization and cybridization in some Solanaceae. Ph D Thesis, Free University Amsterdam, the Netherlands, pp 49-66

Tanksley SD, Miller JC, Paterson A, Bernatzky R (1988) Molecular mapping of plant chromosomes. In: Gustafson JP, Appels R (eds) Chromosome structure and function. Plenum Press, New York, pp 157-173

Terada R, Yamashita Y, Nishibayashi S, Shimamoto K (1987) Somatic hybrids between Brassica oleracea and B. campestris: selection by the use of iodoacetamide inactivation and regeneration ability. Theor Appl Genet 73:379-384

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

Vallejos CE (1983) Enzyme activity staining. In: Tanksley SD, Orton TJ (eds) Isozymes in plant genetics and breeding, part A. Elsevier, Amsterdam, pp 469-515

CHAPTER 3

ANALYSIS OF PROGENIES DERIVED FROM SOMATIC HYBRIDS BETWEEN *LYCOPERSICON ESCULENTUM* AND *LYCOPERSICON PERUVIANUM*

J. Wijbrandi, M. Posthuma, M. Koornneef

Summary. The fertility of tetraploid and hexaploid somatic hybrids between *Lycopersicon esculentum*, the cultivated tomato, and *L. peruvianum* was analysed after selfing and after backcrossing to both fusion parents. Most of the hybrids were fertile upon selfing and yielded many seeds, of which 79% germinated. These progeny plants were vigorous and often fertile after selfing. Backcrossing of the somatic hybrids with the *L. esculentum* parent did not yield any viable seeds; backcrossing with *L. peruvianum* yielded a few germinating seeds, but only if *L. peruvianum* was used as staminate parent. The plants derived from the backcross hexaploid hybrid x tetraploid *L. peruvianum* had a pentaploid chromosome number ($2n = 5x = 60$) and were vigorous, whereas the plants derived from the backcross tetraploid hybrid x diploid *L. peruvianum* grew retarded. To obtain information about the behaviour of chromosomes in the hybrids, the progenies of the selfings and backcrosses were analysed for the segregation of several traits, namely kanamycin resistance, isozyme patterns for acid phosphatase, locus *Aps-1*, and some morphological characteristics. Most of the progenies segregated for kanamycin resistance as expected on the basis of the number of loci present in the kanamycin resistant fusion parent, whereas in some progenies the fraction of kanamycin resistant plants was smaller than expected. The segregation of the *Aps-1* isozyme patterns and the variation for some of the morphological characteristics indicated a tetrasomic inheritance of at least part of the genes in the hybrids. The implications of these findings for the use of these somatic hybrids for the introgression of desirable *L. peruvianum* traits into the cultivated tomato are discussed.

Introduction

Somatic hybridisation is a technique for bringing together the genomes of sexually incongruent species. Most of the somatic hybrids that have been described are between species of the same genus or between species of related genera; some of them were completely sterile (e.g. Melchers et al. 1978; Gleddie et al. 1986), whereas other (mostly euploid) hybrids were fertile and could be selfed or backcrossed to one or both of the parental species. Examples are hybrids in the *Brassicaceae* family (Sundberg et al. 1987; Fahleson et al. 1988; Primard et al. 1988), of *Datura* species (Schieder 1980), of *Nicotiana* species (e.g. Smith et al. 1976; Evans et al. 1983; Hamill et al. 1985), of *Solanum tuberosum* and *S. brevidens* (Ehlenfeldt and Helgeson 1987), and of *L. esculentum* and *L. peruvianum* (Kinsara et al. 1986; Chapter 2). Somatic hybridisation is potentially important for crop improvement, because it allows the introduction

of desired traits from related wild species into cultivated species in cases where sexual hybridisation is not possible. However, not only the introduction of desired traits, but also the removal of undesired ones is important. This can be achieved by repeated backcrossing, but only if the polyploidised hybrids are fertile when backcrossed with the cultivated parent, and if pairing and recombination between the relevant homoeologous chromosomes occur. Homoeologous pairing has been observed in low frequencies in tetraploid hybrids of *Lycopersicon esculentum* x *L. pennellii* and *L. esculentum* x *Solanum lycopersicoides*, obtained after sexual crossing and subsequent doubling of the chromosome number by colchicine (Rick and Khush 1962; Menzel 1964).

Recently, we obtained somatic hybrids of *L. esculentum*, the cultivated tomato, and the wild species *L. peruvianum* (See Chapter 2). These hybrids grew vigorously and proved to be tetraploid or hexaploid. In the experiments described in this Chapter, we tested the fertility of the hybrids after selfing or after backcrossing to one of the parents. In addition, a provisional characterisation of the obtained progeny plants was carried out. The variation of several traits in the progeny of selfed hybrids was analysed in order to find out whether homoeologous pairing occurred during meiosis in the somatic hybrid plants.

Material and methods

The isolation and characterisation of the somatic hybrids were described earlier (Chapter 2). The hybrids that produced seeds, were classified in four groups on the basis of the parental genotypes, as shown in Table 1.

Assay for kanamycin resistance

Seeds of the somatic hybrids and the parental genotypes were surface sterilized (10 sec. 70% ethanol, 20 min. 5x diluted commercial bleach (10% NaClO), several washes in sterile water) and transferred to shoot culture medium (MS salts (Murashige and Skoog 1962), T vitamins (Tewes et al. 1984), 10 g/l sucrose) in plastic containers, in the dark. After a few days the seeds germinated and the containers were transferred to the light. Shoot tips of the seedlings were transferred to shoot culture medium supplemented with 100 mg/l kanamycin. Resistant shoots formed roots and grew, whereas sensitive shoots did not form roots and died. The remainder of the seedlings was transferred to soil in a greenhouse. In several cases, seeds of the parental genotypes were sown directly on shoot culture medium supplemented with 100 mg/l kanamycin; resistant seedlings developed normally, whereas sensitive seedlings were retarded in growth and showed poor root development.

Isozyme analysis

Leaf material from greenhouse-grown plants was used for the analysis of acid phosphatase, locus *Aps-1*. Crude extracts, which were obtained by the squeezing of thawing leaf material that had been stored at -80 °C, were electrophoresed on vertical 10% polyacrylamide slab gels (Chapter 2). Enzyme activity was determined by means of the staining technique of Vallejos (1983).

Plant characterization

Chromosome numbers of root tip cells were determined in cell spread preparations

Table 1. The parental genotypes of the somatic hybrids, from which progeny was analysed; the hybrids were subdivided on the basis of their ploidy level. The numbers of different shoots, derived from individual somatic hybrid calli, are given between parentheses. All somatic hybrids were obtained after selection for kanamycin resistance and regeneration capacity, except for the only hybrid of Bellina (+) ATW2002; the latter hybrid was selected for callus colour and regeneration capacity. The hexaploid somatic hybrids all contained a diploid genome of *L. esculentum* and a tetraploid genome of *L. peruvianum*.

L. esculentum	*L. peruvianum*	Somatic hybrids	
		tetraploid	hexaploid
cv. Bellina-ATW3001**	PI128650	5 (9)	1 (1)
cv. Bellina-ATW3003**	PI128650	7 (13)	2 (2)
LA1182*-ATW3052**	PI128650	2 (2)	1 (2)
cv. Bellina	PI128650-ATW2002**	1 (1)	0

* tomato genotype, homozygous recessive for *sy*, *sf* (chr. 3), and *alb* (chr. 12) (Rick 1982).

** transgenic plants containing the neomycin phosphotransferase II gene, which causes resistance to the antibiotic kanamycin (Koornneef et al. 1987).

(Chapter 2). In addition, growth habit and leaf morphology were analysed, and sympodial index (= mean number of nodes between two subsequent inflorescences), size of the different flower parts, pollen viability, fruit and seed set were determined. Pollen was stained in a solution of lactophenol acid fuchsin to establish their viability; at least 100 pollen grains per flower were scored.

Results

Progeny of the somatic hybrids

The somatic hybrids were selfed and crossed with diploid and tetraploid plants of *L. esculentum* and *L. peruvianum*, both as pistillate and staminate parents (Table 2). The selfings, either spontaneous or controlled, yielded many normal-looking seeds in most of the somatic hybrids (See Table 2 in Chapter 2). The crosses with *L. esculentum* as pistillate parent yielded fruits that contained several to many abortive seeds. The backcrosses to *L. peruvianum* only succeeded when the somatic hybrids were used as pistillate parent. 75% to 90% of the seeds germinated (Table 2).

Segregation of kanamycin resistance

Table 3 shows the segregation ratios of kanamycin resistance/sensitivity for the offspring of the kanamycin resistant fusion parents (*L. esculentum* and

Table 2. The results of the crosses with the somatic hybrids (SH) of *L. esculentum* (*Le*) and *L. peruvianum* (*Lp*), and the frequency of germination of the produced seeds. The ploidy level of the plants is given between brackets. The number of different shoots, derived from the individual somatic hybrid calli, are given between parentheses. One cross represents one pollinated flower. The 828 seeds of "SH[4x] selfed" were derived from 17 hybrid clones (27 different shoots); the seeds from 15 (23) were tested. The seeds of "SH[4x] x *Lp*[2x]" were derived from 3 clones (3 shoots), "SH[4x] x *Lp*[4x]" from 2(2), "SH[6x] selfed" from 2 (3) and "SH[6x] x *Lp*[4x]" from 3 (3).

Crosses ♀ x ♂	No. of hybrids (shoots)	No. of crosses	No. of fruits	No. of seeds	Germinating seeds
Le[2x] x *Lp*[2x]	-	9	4	2 *	0%
Le[2x] x *Lp*[4x]	-	1	1	0	
Lp[2x] x *Le*[2x]	-	52	0		
Lp[4x] x *Le*[2x]	-	9	0		
SH[4x] selfed	21 (45)	ND	333	828	79% #
SH[4x] x *Le*[2x]	21 (40)	264	7	0	
SH[4x] x *Le*[4x]	12 (15)	53	0		
SH[4x] x *Lp*[2x]	18 (24)	71	32	4 **	75%
SH[4x] x *Lp*[4x]	14 (19)	37	4	2 **	0%
Le[2x] x SH[4x]	16 (21)	50	18	0	
Le[4x] x SH[4x]	9 (10)	30	7	1 *	0%
Lp[2x] x SH[4x]	10 (13)	26	1	0	
Lp[4x] x SH[4x]	2 (2)	3	0		
SH[6x] selfed	9 (14)	ND	32	37	84% ##
SH[6x] x *Le*[2x]	7 (11)	71	3	0	
SH[6x] x *Le*[4x]	6 (7)	25	0		
SH[6x] x *Lp*[2x]	6 (6)	17	0		
SH[6x] x *Lp*[4x]	5 (6)	10	3	20	90%
Le[2x] x SH[6x]	5 (6)	14	2	0	
Le[4x] x SH[6x]	3 (3)	10	1	0	
Lp[2x] x SH[6x]	1 (1)	2	0		

ND not determined, because majority of fruits was set spontaneously
* very small flat seeds
** flat seeds
\# 376 seeds were tested
\## 32 seeds were tested

L. peruvianum) and of the somatic hybrids having one kanamycin resistant parent (either *L. esculentum* or *L. peruvianum*; Table 1). The resistant parents were obtained by leaf disc transformation (Koornneef et al. 1987) of *L. esculentum* cv. Bellina, of *L. esculentum* LA1182 and of the *L. peruvianum* accession PI128650. The segregation ratio for the progeny of the selfed kanamycin resistant *L. esculentum* genotype ATW3001 is 3 resistant : 1 sensitive, which is consistent with one hemizygous kanamycin resistance locus in this genotype. Accordingly, the segregation ratio for the progeny of the selfed tetraploid hybrids descending from this genotype is also 3:1. The progeny obtained by selfing of ATW3003 also showed a ratio of 3:1. However, the ratio for the progeny of the selfed tetraploid hybrids descending from ATW3003 deviated significantly from 3:1; the

Table 3. Segregation ratios of kanamycin resistance in seedlings of the original ATW-plants and of the somatic hybrids that had a ATW-plant as parent. The ATW-plants of *L. esculentum* (*Le*), ATW3001, ATW3003 and ATW3052, were selfed, whereas the *L. peruvianum* (*Lp*) ATW2002 was crossed with a kanamycin sensitive plant. The somatic hybrids (SH) were selfed or backcrossed with *L. peruvianum*. The ploidy level of the plants is given between brackets.
Km^R, kanamycin resistant; Km^S, kanamycin sensitive.

Crosses	Kanamycin resistant parental genotype			
	ATW3001 $Km^R:Km^S$	ATW3003 $Km^R:Km^S$	ATW3052 $Km^R:Km^S$	ATW2002 $Km^R:Km^S$
Le[2x]^R selfed	32:8 *	36:13*	-	
Lp[2x]^R x *Lp*[2x]^S				63:18#
SH[4x]^R selfed	92:37*	73:41**	4:0	33:1 ##
SH[4x]^R x *Lp*[2x]^S	1:0	-	-	-
SH[6x]^R selfed	-	2:0	12:8	
SH[6x]^R x *Lp*[4x]^S	1:2	2:4	4:4	

R kanamycin resistant plant
S kanamycin sensitive plant
* segregation ratio does not deviate significantly from 3:1 ($\chi^2 < \chi^2(1, 0.95) = 3.84$); consistent with the presence of one locus of the kanamycin resistance (NPT II) gene
** segregation ratio deviates significantly from 3:1 ($\chi^2 = 7.31$)
\# segregation ratio does not deviate significantly from 3:1; consistent with two unlinked NPT II loci
\#\# segregation ratio does not deviate significantly from 15:1; consistent with two unlinked NPT II loci

fraction of kanamycin resistant plants was smaller than expected (Table 3). The testcross of the kanamycin resistant *L. peruvianum* parent ATW2002 yielded a progeny with a segregation ratio of 3 resistant : 1 sensitive, which indicates the presence of two unlinked hemizygous kanamycin resistance loci in this genotype. The observed ratio of 33:1 in the progeny obtained by selfing of the single somatic hybrid descending from ATW2002 is in agreement with this (Table 3). We could only harvest a few seedless fruits from ATW3052; the segregation ratios of the progeny of the hybrids descending from this genotype is consistent with the presence of one hemizygous kanamycin resistance locus in these hybrids.

Analysis of acid phosphatase-1

The different *Aps-1* isozyme patterns of the analysed genotypes are represented in Fig. 1 (See also Chapter 2): *L. esculentum* had one fast migrating band (pattern E); *L. peruvianum* had two different patterns, either three slow migrating bands (pattern A) or one band similar to the *L. esculentum* band, but much darker (pattern B); the somatic hybrids had, depending on their *L. peruvianum* parent, either one band like pattern B or a pattern of six bands which can be interpreted as a combination of the *L. esculentum* band, pattern A

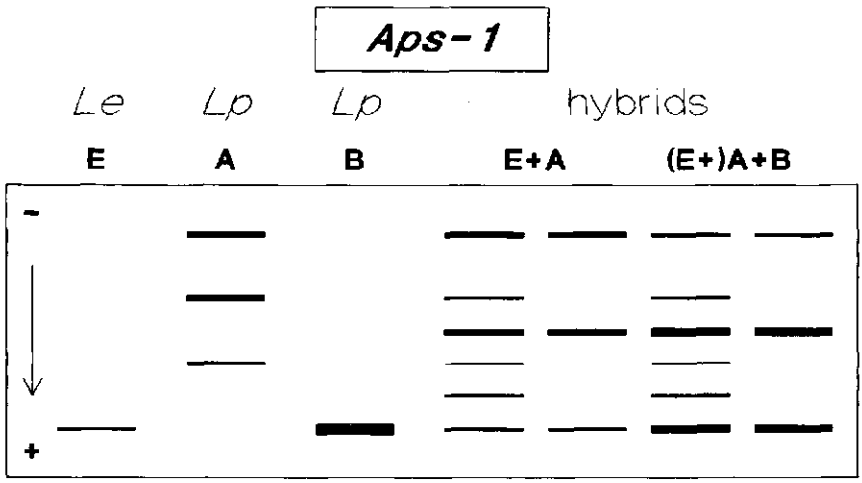

Fig. 1. Representation of the different patterns of *acid phosphatase-1* found in *L. esculentum* (*Le*), *L. peruvianum* (*Lp*), their somatic hybrids and the sexual progeny of the latter. *Le* has one band, pattern E, while *Lp* has two different patterns, either three bands (pattern A) or one heavy band (pattern B). Four different hybrid patterns were distinguished: (i) combination of E and A, resulting in 6 bands; (ii) combination of E and part of A, resulting in 3 bands; (iii) combination of E + A + B or A + B, resulting in 3 strong and 3 weak bands; (iv) combination of E + part of A + B or part of A + B, resulting in 3 strong bands. Note that patterns E and B differ only in intensity of the band and that the combination of E and B in a hybrid is not different from pattern B.

Table 4. Isozyme patterns of acid phosphatase, locus *Aps-1*, in sexual progenies of *L. esculentum* (*Le*), *L. peruvianum* (*Lp*) and their somatic hybrids (SH); the ploidy level of the selfed or (back)crossed plants is given between square brackets. The isozyme patterns of the parents are shown between parentheses. See Fig. 1 for explanation of the patterns. The second column gives the designations of the individual somatic hybrid OH-plants; the numbers indicate independent hybrid clones and the lower case letters independent shoots from the same clone.

Crosses (*Aps-1*)	Hybrid plants	*Aps-1* pattern of progeny			
		Le(E)	hybrid	*Lp*(A)	*Lp*(B)
Le[2x](E) selfed	-	12			
Lp[2x](A) x *Lp*[2x](B)	-		31		
SH[4x](EA) selfed	OH4a,b,c,d,e; OH16a; OH17a; OH18a; OH19c	0	101	4 *	
SH[4x](EB) x *Lp*[2x](?)	OH6a; OH10c		1		1
SH[6x](EAB) selfed	OH14a,b		15		4
SH[6x](EAB) x *Lp*[4x](B)	OH14b		5		3

* Segregation ratio does not deviate significantly from 1:34:1; $\chi^2 = 3.35 < \chi^2(2, 0.95) = 7.82$

of *L. peruvianum* and two intermediate bands. In several cases two bands of pattern A and one intermediate band were lacking in the hybrid pattern; hybrids of both *L. peruvianum* types also showed patterns of six or three bands.

The segregation of *Aps-1* patterns among the progenies is given for each ploidy level and parental pattern type (Table 4). The offspring of the parental genotypes had patterns as expected. In 4 out of 105 tested progeny plants of selfed tetraploid somatic hybrids having a hybrid pattern of 6 bands, we found the *L. peruvianum* specific pattern A. The 101 other progeny plants had a hybrid pattern. Although we did not find the *L. esculentum* specific pattern, the ratio of *Aps-1* patterns in this progeny did not deviate significantly from a segregation ratio of 1:34:1 (Table 4), which was expected in case of a tetrasomic inheritance of *Aps-1* in these hybrids.

The isozyme patterns of two plants, each of which resulted from a backcross of a tetraploid hybrid (pattern B) with a diploid *L. peruvianum* plant were also analysed: one had pattern B, whereas the other showed a hybrid pattern (Table 4). The latter pattern indicates that at least one of both plants derived from a backcross and not from an unintended selfing; the progeny of the selfed tetraploid hybrids with pattern B all also had pattern B (43 plants tested; data not shown).

We also analysed the segregation of the *Aps-1* pattern in the progeny of hexaploid hybrid OH14, which probably contains one diploid genome of *L. esculentum*, one of *L. peruvianum* (pattern A) and one of *L. peruvianum* (pattern B) (Chapter 2). Most of the progeny plants resulting from selfing of this hybrid had a hybrid pattern, while a few had pattern B (Table 4). As expected, the progeny from the backcross of OH14 with a tetraploid *L. peruvianum* (pattern B), showed a larger proportion of pattern B than the progeny derived from selfing (Table 4).

Morphology

The progeny plants of the selfed hybrids and of the backcrosses "SH[6x] x *Lp*[4x]" were very vigorous with large leaves and flowers, similar to the somatic hybrids. The expected pentaploid chromosome number of these backcross plants was confirmed by chromosome counts of 5 plants (at least one plant of each three crosses) out of 15 grown in the greenhouse; all had 60 chromosomes. Two plants derived from the cross "SH[4x] x *Lp*[2x]" grew slowly and had small leaves and small flowers with very exerted styles.

In many of the somatic hybrids variegation of grey-green spots was observed, which resembled the disorder called chimera or silvering, occurring in glasshouse tomatoes (Grimbly 1984). The progenies of several of these hybrids showed the same symptoms (Table 5); this indicates a genetical basis of the disorder.

Table 5. The presence of grey-green variegation, "chimera", in progeny plants of somatic hybrids (SH) of *L. esculentum* and *L. peruvianum* (*Lp*); the ploidy level of the selfed or (back)crossed plants is given between brackets. The individual somatic hybrid plants (OH) are added; the numbers indicate independent hybrid clones and the lower case letters independent shoots from the same clone. The symptoms of "chimera" in the hybrids are indicated; severe symptoms are the presence of large grey sectors and irregularly shaped leaves.
+, "chimera" symptoms; -, no symptoms.

Crosses of somatic hybrids	"Chimera" symptoms	"Chimera" seedlings	
		+	-
SH[4x] selfed:			
OH1b; OH4c,e; OH6c; OH8a,d; OH13a; OH15b; OH16a; OH18a,b; OH19c; OH24c; OH31a; OH36a	no	0	160
OH4a; OH10b; OH17a; OH24a	moderate	4	47
OH4b; OH25a,c	severe	39	0
SH[4x] x *Lp*[2x]:			
OH10c	no	0	1
OH6a	severe	1	0
SH[6x] selfed:			
OH14a,b	moderate	2	18
OH19a	severe	2	0
SH[6x] x *Lp*[4x]:			
OH7c; OH14b; OH27a	moderate	6	9

For a few characters, e.g. the sympodial index (s.p.i.), we observed more variation among the progeny of the tetraploid somatic hybrids than among these hybrids themselves (Fig. 2A). The means of the s.p.i. of the progeny plants (2.83) did not differ significantly from that of the hybrids (2.86), but the variances (σ^2; 0.364 and 0.118, respectively) did (variance ratio test: $F = 3.08 > F(65, 38, 0.95) = 1.65$). A similar result was obtained for the sizes of the flower parts; e.g. the average ratios of the lengths of sepals and petals (Fig. 2B) was 0.43 ($\sigma = 0.06$) for the tetraploid somatic hybrids and 0.44 ($\sigma = 0.11$) for the progeny obtained by selfing of these hybrids. The variances of this ratio were significantly different for both experimental groups ($F = 2.65 > F(56, 30, 0.95) = 1.75$)

Fertility

The average pollen viability of the progeny plants obtained by selfing of the somatic hybrids was similar to that of these hybrids, namely 30% to 50% (See also Chapter 2). Only the tetraploid progeny plants set mature fruits (Table 6). The small, often parthenocarpic fruits were usually yellow; one plant had pink-yellow fruits. Three progeny plants had fruits with much purple pigmentation,

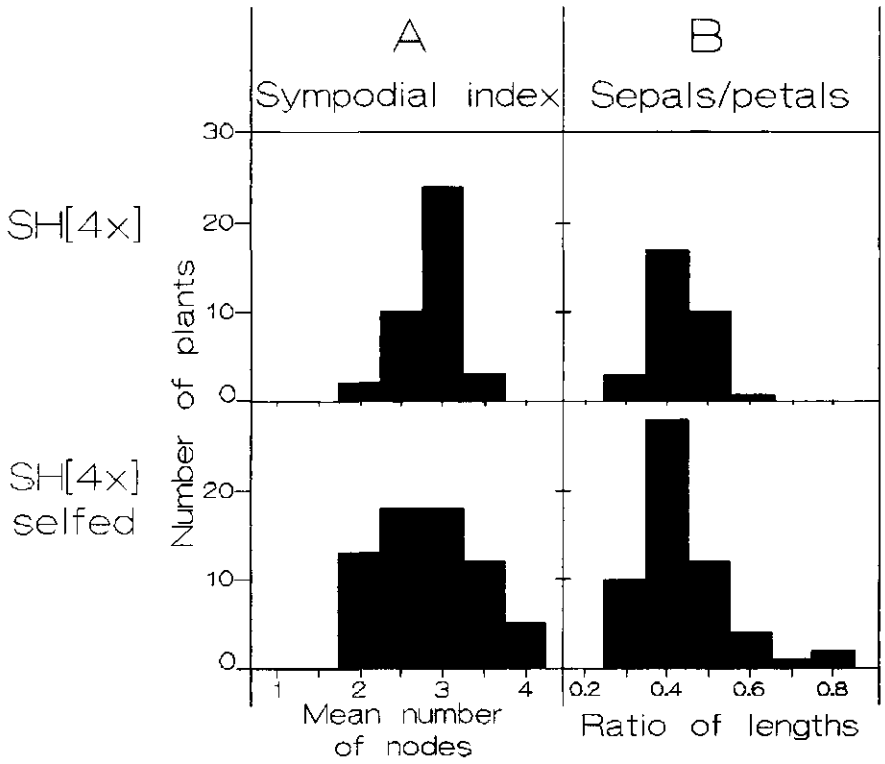

Fig. 2. Distribution of some morphological characteristics among tetraploid somatic hybrids (SH[4x]) of *L. esculentum* and *L. peruvianum* and their progeny after selfing (SH[4x] selfed). **A.** Sympodial index (= mean number of nodes between two subsequent inflorescences). **B.** Ratio between the lengths of sepals and petals.

Table 6. The presence of flowers, fruits and seeds in the progeny of somatic hybrids of *L. esculentum* and *L. peruvianum*. The somatic hybrids (SH) had been selfed or backcrossed with *L. peruvianum* (*Lp*); the ploidy level of the parental plants is given between brackets. Observations were made on greenhouse-grown plants. The fruit and seed yield (after selfing) of the "SH[4x] selfed" progeny was 194 and 348, respectively.

Progeny plants	Total	Flowering (+buds)	Setting fruits (+immature)	Setting seeds
SH[4x] selfed	70	68	24 (+13)	23
SH[4x] x *Lp*[2x]	2	2	0	
SH[6x] selfed	8	4 (+3)	0 (+1)	
SH[6x] x *Lp*[4x]	15	12 (+2)	0	

like *L. peruvianum* fruits. Trusses with many fruits (> 10) were observed on one plant. Twenty-three progeny plants set seeds, in general after spontaneous

selfing. Backcrosses with diploid *L. esculentum* as staminate parent were not successful.

Discussion

One of the factors that are important for the successful application of somatic hybridisation to the improvement of crops is the fertility of the hybrids when they are selfed or backcrossed to the cultivated parent. The results presented in this paper show that somatic hybrids between *L. esculentum* and *L. peruvianum* are fertile when selfed. Backcrosses of tetraploid hybrids as pistillate parent to diploid *L. peruvianum* did succeed, whereas those to diploid *L. esculentum* did not. The backcrosses of tetraploid hybrids as staminate parents to diploid *L. peruvianum* did not succeed, while those to diploid *L. esculentum* only yielded abortive seeds. These results are in agreement with those obtained by Soost (1958) and Szteyn (1962), who isolated allotriploid sexual hybrids from crosses of tetraploid *L. esculentum* with diploid *L. peruvianum*. They found that crosses of these allotriploids as pistillate parents to diploid *L. peruvianum* yielded viable offspring, but those to diploid *L. esculentum* did not. However, Szteyn (1962) found that if the allotriploids were used as staminate parents, viable offspring could be obtained from crosses with diploid *L. esculentum*. Allodiploids of *L. esculentum* x *L. peruvianum* can also be backcrossed to *L. esculentum* as pistillate parent (Ancora et al. 1981). We found that the backcrosses of the tetraploid hybrids to *L. esculentum* as pistillate parent yielded several fruits with many abortive seeds. It is quite possible that if such backcrosses are repeated on a larger scale, viable triploid offspring will be obtained. Subsequent backcrosses of these triploids to diploid *L. esculentum* are then probably relatively easily obtained (Rick and Butler 1956).

Another important factor is the occurrence of homoeologous pairing and recombination in the somatic hybrids. Our results provide some indications for tetrasomic inheritance of several traits in the hybrids. The variances of the s.p.i. and of the ratio of sepal and petal lengths was significantly larger among the progeny of the selfed tetraploid hybrids than among the hybrids themselves. With respect to other traits, such as *Aps-1* and fruit colour, phenotypes were present among the progeny of the selfings that were absent among the parental hybrids. With respect to the morphological characteristics, these results can be attributed at least in part to heterozygosity of the parents of the hybrids: *L. esculentum* cv. Bellina is an F_1 hybrid and *L. peruvianum* is a very variable, outbreeding species. But this explanation does not hold for the segregation of *Aps-1* isozyme patterns and for the appearance of purple pigmented fruits in the

progeny of the selfed hybrids. We therefore tentatively conclude that at least *Aps-1*, but probably other markers as well, have a tetrasomic inheritance in the hybrids. This implies that the relevant homoeologous chromosomes probably paired in the tetraploid somatic hybrids. A more detailed analysis of the segregation of monogenic inherited markers, and of the behaviour of chromosomes at metaphase I in the hybrids is required to support this conclusion, however.

The kanamycin resistance marker (or NPT II locus) was present as one or two unlinked hemizygous loci in one of the parents of the hybrids. This marker segregated as expected among the progeny of most of the selfed or backcrossed kanamycin resistant hybrids. However, if tetraploid hybrids descending from ATW3003 (with one hemizygous kanamycin resistance locus) were selfed, the ratio of resistant to sensitive progeny differed significantly from 3:1 and was closer to 2:1. This deviating ratio can be explained by the more frequent transmission of the corresponding *L. peruvianum* chromosome than of the *L. esculentum* chromosome containing the NPT II locus. Deviating segregation ratios of several traits were also observed in F_2 generations of sexual hybrids of tomato and *L. pennellii* (Bernatzky and Tanksley 1986; Gadish and Zamir 1987) and of tomato and *L. chilense* (Rick 1963), all in favour of characters of the wild species. In backcross progenies of tomato species hybrids deviating segregation ratios in either direction have been observed (Rick 1963, 1969; Vallejos and Tanksley 1983; Rick et al. 1988). The phenomenon was ascribed to differential survival of the male gametes or to differential zygotic lethality (Rick 1963, 1969; Gadish and Zamir 1987). Both explanations are conceivable for our results.

The good fertility of some progeny plants derived from selfed tetraploid somatic hybrids and the assumed tetrasomic inheritance of some traits, is an indication that such plants might be used for introgression of *L. peruvianum* specific characters into the cultivated tomato. This probably can be achieved more efficiently by the pre-selection of fertile progeny plants with some *L. esculentum* specific traits and the subsequent crossing of these progeny plants with *L. esculentum*.

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References

Ancora G, Saccardo F, Cappadocia M, Sree Ramulu K (1981) Backcross progenies from Lycopersicon esculentum L. x hybrid (L. esculentum x L. peruvianum Mill.). Z Pflanzenzüchtg 87:153-157

Bernatzky R, Tanksley SD (1986) Majority of random cDNA clones correspond to single loci in the tomato genome. Mol Gen Genet 203:8-14

Ehlenfeldt MK, Helgeson JP (1987) Fertility of somatic hybrids from protoplast fusions of Solanum brevidens and S. tuberosum. Theor Appl Genet 73:395-402

Evans DA, Bravo JE, Kut SA, Flick CE (1983) Genetic behaviour of somatic hybrids in the genus Nicotiana: N. otophora + N. tabacum and N. sylvestris + N. tabacum. Theor Appl Genet 65:93-101

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

Gadish I, Zamir D (1987) Differential zygotic abortion in an interspecific Lycopersicon cross. Genome 29:156-159

Gleddie S, Keller WA, Setterfield G (1986) Production and characterization of somatic hybrids between Solanum melongena L. and S. sisymbriifolium Lam.. Theor Appl Genet 71:613-621

Grimbly P (1986) Disorders. In: Atherton JG, Rudich J (eds), The tomato crop. A scientific basis for improvement. Chapman and Hall, London/New York, pp 369-389

Hamill JD, Pental D, Cocking EC (1985) Analysis of fertility in somatic hybrids of Nicotiana rustica and N. tabacum and progeny over two sexual generations. Theor Appl Genet 71:486-490

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

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, Inc, New York, pp 169-178

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

Menzel MY (1964) Preferential chromosome pairing in allotetraploid Lycopersicon esculentum-Solanum lycopersicoides. Genetics 50:855-862

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

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

Primard C, Vedel F, Mathieu C, Pelletier G, Chèvre AM (1988) Interspecific somatic hybridization between Brassica napus and Brassica hirta (Sinapsis alba L.). Theor Appl Genet 75:548-552

Rick CM (1963) Differential zygotic lethality in a tomato species hybrid. Genetics 48:1497-1507

Rick CM (1969) Controlled introgression of chromosomes of Solanum pennellii into Lycopersicon esculentum: segregation and recombination. Genetics 62:753-768

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

Rick CM, Butler L (1956) Cytogenetics of the tomato. Adv Genet 8:267-382

Rick CM, Chetelat RT, DeVerna JW (1988) Recombination in sesquidiploid hybrids of Lycopersicon esculentum x Solanum lycopersicoides and derivatives. Theor Appl Genet 76:647-655

Rick CM, Khush GS (1962) Preferential pairing in tetraploid tomato species hybrids. Genetics 47:979-980

Schieder O (1980) Somatic hybrids of Datura innoxia Mill. + Datura discolor Bernh. and of Datura innoxia Mill. + Datura stramonium L. var. tatula L. II. Analysis of progenies of three sexual generations. Mol Gen Genet 179:387-390

Smith HH, Kao KN, Combatti NC (1976) Interspecific hybridization by protoplast fusion in Nicotiana. Confirmation and extension. J Heredity 67:123-128

Soost RK (1958) Progenies from sesquidiploid F_1 hybrids of Lycopersicon esculentum and L. peruvianum. J Heredity 49:208-213

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

Szteyn K (1962) Interspecific crosses in the genus Lycopersicon. I. Backcrosses to Lycopersicon glandulosum. Euphytica 11:149-156

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

Vallejos CE (1983) Enzyme activity staining. In: Tanksley SD, Orton TJ (eds) Isozymes in plant genetics and breeding, part A. Elsevier, Amsterdam, pp 469-515

Vallejos CE, Tanksley SD (1983) Segregation of isozyme markers and cold tolerance in an interspecific backcross of tomato. Theor Appl Genet 66:241-247

CHAPTER 4

ASYMMETRIC SOMATIC HYBRIDS BETWEEN *LYCOPERSICON ESCULENTUM* AND IRRADIATED *LYCOPERSICON PERUVIANUM*

I. CYTOGENETICS AND MORPHOLOGY

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Summary. Asymmetric somatic hybrids of *Lycopersicon esculentum* and *Lycopersicon peruvianum* were obtained by fusion of leaf protoplasts from both species after irradiation of protoplasts or leaf tissue of *L. peruvianum* with 50, 300 or 1000 Gy of gamma-rays. These irradiation doses were sufficient to abolish the growth of the *L. peruvianum* protoplasts. The hybrids were selected on their ability to regenerate plants; this regeneration capacity derived from *L. peruvianum*. All asymmetric hybrid plants were aneuploid. The ploidy level, the morphology as well as the regeneration rate were analysed in relation to the irradiation dose, applied to *L. peruvianum*. After a low dose (50 Gy) most hybrids had near-triploid chromosome numbers, whereas after a high dose (300 or 1000 Gy) most hybrids had near-pentaploid numbers. The morphology of the asymmetric hybrids was intermediate between that of *L. esculentum* and symmetric somatic hybrids of both species (obtained without irradiation treatment), and approached the morphology of *L. esculentum* more after a high dose of irradiation. The asymmetric hybrids regenerated more slowly than the symmetric hybrids, and regeneration proceeded more slowly after a high dose than after a low dose of irradiation. The high dose hybrids also grew more slowly, flowered less and set fruits less than the low dose hybrids. No seeds could be obtained from any asymmetric hybrid.

Introduction

The transfer of desirable traits from wild into cultivated species is a method to improve crops. For this purpose, interspecific sexual or somatic hybrids may be constructed. The formation of sexual hybrids is limited to closely related species. Symmetric somatic hybrids, obtained by fusion of untreated protoplasts of different species, can be more easily made in some combinations. However, species hybrids, both sexual and somatic, contain many unwanted traits of the wild species besides the desired ones, and are often sterile. Fertility is required to perform several backcrosses with the cultivated species to remove unwanted characters of the wild species. These problems may be circumvented by asymmetric somatic hybridisation. By this procedure protoplasts of one species, the recipient, are fused with inactivated (mostly by Röntgen- or gamma-irradiation) protoplasts of another species, the donor. The donor genome will be fragmented and the asymmetric hybrids will contain the complete genome of the recipient species and a small part of the donor genome. The advantages of this

procedure could be that hybrids arise with few unwanted donor traits, and that fewer backcrosses of the asymmetric hybrids are required to get rid of these. Several asymmetric hybrids were obtained in other studies (Chapter 1). The fraction of donor genome that was transferred varied from one or a few traits (e.g. Dudits et al. 1987), one or a few chromosomes (e.g. Gupta et al. 1984) to many chromosomes (e.g. Gleba et al. 1988). Fertile asymmetric hybrids were reported in several cases and irrespective of the amount of transferred donor genome (Chapter 1).

We are interested in the transfer of traits of the wild tomato species *Lycopersicon peruvianum* to the cultivated tomato, *Lycopersicon esculentum*. *L. peruvianum* has many desirable characters for tomato improvement (Rick 1982a), but only a few of these (e.g. *Mi*- and TMV-resistance) have been introduced into the cultivated tomato, because sexual hybrids of both species are very difficult to obtain (Taylor 1986). In the present experiments we obtained asymmetric somatic hybrids by fusing *L. esculentum* protoplasts with irradiated *L. peruvianum* protoplasts. The hybrids were selected for the dominant regeneration capacity character of *L. peruvianum* (Koornneef et al. 1987a; Chapter 2). Our purpose was to analyse the cytogenetics, morphology and fertility of the regenerated hybrids in relation to the irradiation dose, applied to *L. peruvianum*.

Materials and methods

Plant materials

As recipient (*Lycopersicon esculentum*) the Dutch hybrid cultivar Bellina (kindly provided by Rijk Zwaan Seed Company, de Lier, The Netherlands) and the genotypes LA291, LA1164, LA1166, LA1182, LA1189, LA1444 and LA1665 from the Tomato Genetics Stock Center in Davis, USA (kindly provided by Prof. C.M. Rick) were used. The latter genotypes allow observations on the complementation of monogenic morphological mutant phenotypes by the donor (Chapter 5). Plants from the *Lycopersicon peruvianum* accession PI128650 (received from the Institute of Horticultural Plant Breeding, Wageningen, The Netherlands) were used as donor. Kanamycin resistant plants of *L. peruvianum* and *L. esculentum* cv. Bellina were also available; these had been obtained by leaf disc transformation with *Agrobacterium tumefaciens* containing the plasmid AGS112 (Koornneef et al. 1987b). Kanamycin resistance in *L. esculentum* was used to select for symmetric somatic hybrids (Chapter 2); kanamycin resistant *L. peruvianum* was used to monitor the loss of the resistance in the asymmetric somatic hybrids (Chapter 5).

Fusion combinations

The protoplast fusion experiments are listed in Table 1. *L. peruvianum* was irradiated with gamma rays from a ^{60}Co source at a dose rate of approximately 2000 Gray/hour at the Pilot-Plant for Food Irradiation, Wageningen (The Netherlands). Three different doses were used: 50, 300 or 1000 Gy (= 5, 30 or 100 kRad). Putative hybrids that resulted from these fusions, are indicated as 5H, 30H and 100H, respectively. Symmetric hybrids, which resulted from fusions of unirradiated protoplasts, are indicated as 0H-hybrids. The irradiation was carried out either to protoplasts, suspended at 1-3 x 10^5/ml in W5 solution (Menczel et al. 1981) one to three hours before fusion, or to leaflets one day before fusion.

Table 1. The genotype of the recipient species *L. esculentum* and the irradiation treatment of the donor species *L. peruvianum*, involved in the protoplast fusion experiments. The LA-genotypes are multiple marker lines, homozygous recessive for morphological marker genes (Rick 1982b). The protoplast fusion method used is given for each combination; M = method according to Menczel et al. (1981), CMS = method according to Negrutiu et al. (1986). The number of calli, which did regenerate shoots, is given in the right most column.

Recipient	Irradiation of donor		Number of experiments	Fusion method	Regenerating calli
	material	dose (Gy)			
cv. Bellina	protoplasts	0	1*	M	11
cv. Bellina	protoplasts	50	3	M	53
cv. Bellina	protoplasts	300	3	M	25
cv. Bellina	protoplasts	1000	3	M	13
LA291	protoplasts	300	4	M/CMS	31
LA1164	protoplasts	300	1	M	16
LA1166	leaflets	300	2	M	14
LA1182	leaflets	300	1	M	8
LA1182	protoplasts	300	3	M	12
LA1189	leaflets	300	1	M	3
LA1189	protoplasts	300	2	M	7
LA1444	protoplasts	300	2	CMS	9
LA1665	leaflets	300	4	M	13
LA1665	protoplasts	300	1	M	1

* this experiment was used to determine the regeneration frequency, as shown in Fig. 1; the recipient (ATW3003) was kanamycin resistent to select against the unirradiated donor species; only a sample of 14 calli was tested on shoot induction medium

Cell culture

The isolation and culture of protoplasts are described in Chapter 2. The pretreatment of leaf material, in the dark and on pre-incubation medium, was omitted in several experiments without negative effects. Protoplast fusion was carried out either according to Negrutiu et al. (1986; the CMS method) or according to Menczel et al. (1981), except that 30% PEG 4000 was used instead of 40% PEG 6000 (Table 1). The ratio of *L. esculentum* to *L. peruvianum* protoplasts during fusion was 1:1 to 2:1. In each fusion experiment 1 x 10^6 to 3 x 10^6 protoplasts were involved. In each experiment, cultures of the parental protoplasts, both separate and mixed, were started in parallel with the fusion cultures (i.e. cultures of mixed protoplasts of recipient and donor, after fusion treatment); in some experiments also donor protoplasts were subjected to a fusion treatment. Rooting of regenerated shoots was induced on shoot culture medium (MS salts (Murashige and Skoog 1962), T vitamins (Tewes et al. 1984), 10 g/l sucrose), without hormones or supplemented with 0.1 mg/l indole-butyric-acid.

The regeneration capacity of a number of asymmetric hybrids was tested as described by Koornneef et al. (1987a).

Plant characterisation

Chromosome numbers of root tip cells were determined in squash preparations or by means of a slightly modified procedure of Pijnacker and Ferwerda (1984; see also Chapter 2). Several morphological characters distinguishing both species as well as fertility were monitored as described in Chapter 2. As controls served diploid and tetraploid plants of the parental species and symmetric somatic hybrids of both species, derived from fusion experiments without irradiation treatment.

Results

Cell culture and plant regeneration

With the culture procedures employed, calli could be obtained from Bellina protoplasts. These calli did not regenerate shoots on shoot induction medium. The LA genotypes differed somewhat in their protoplast culture responses. Most of them showed limited cell division and very few microcalli developed. However, growth of these *L. esculentum* calli seemed to be improved in cultures where they were mixed with irradiated *L. peruvianum* protoplasts, which apparently behaved as feeder-cells. In the case of LA1182 a tetraploid plant with the LA1182 phenotype could be regenerated from such a mixed culture.

The *L. peruvianum* protoplasts that were irradiated with 50, 300 or 1000 Gy of gamma rays, formed cell walls. Some of these cells strongly increased in size, while some others divided once or twice. In one out of 12 experiments where *L. peruvianum* protoplasts, irradiated with 300 Gy, were subjected to a fusion treatment, we obtained three calli; these did not regenerate plants.

The fusion cultures yielded many calli of which several formed shoots. Within each experiment there was a large variation in callus as well as shoot morphology. Fig. 1 shows the time course of shoot formation by asymmetric hybrid calli, resulting from fusions where different irradiation doses were applied to the donor protoplasts. The rate of shoot regeneration as well as the fraction of calli that could form shoots decreased with the irradiation dose. The

Table 2. Characteristics of different hybrid calli, that regenerated shoots: root formation of shoots (*in vitro*), establishment in soil in the greenhouse (at least one month), flowering, fruit and seed set. All hybrids were obtained after fusion of protoplasts from *L. esculentum* with protoplasts from *L. peruvianum*, which had not been irradiated (0H) or irradiated with 50, 300 or 1000 Gray of gamma-rays (5H, 30H or 100H, respectively) before fusion.

Hybrids	Regenerating calli	Root formation	Greenhouse plants	Flowers	Fruits	Seeds
0H	ND	40	32*	31	28	21
5H	53	38	27	21	13	0**
30H	139	63	32	15	5	0***
100H	13	9	3	1	1	0

ND not determined
* not all rooted hybrids were transferred to soil
** 5 with abortive seeds
*** 1 with abortive seeds

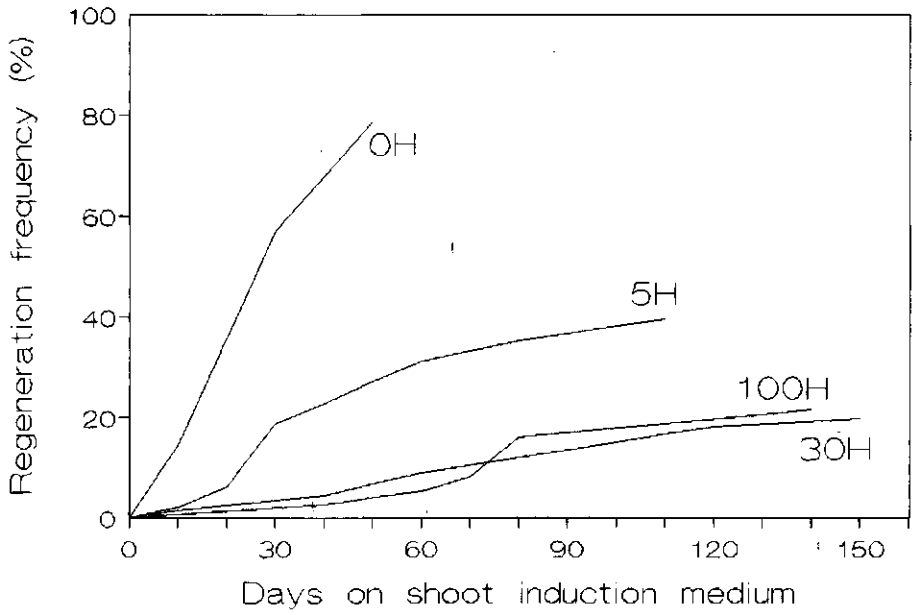

Fig. 1. Regeneration frequency, i.e. the percentage of calli tested that formed shoots on shoot induction medium. The calli were derived from cultures of mixed, fusion-treated protoplasts of *L. esculentum* cv. Bellina and *L. peruvianum* PI128650-ATW2002, which latter were irradiated with 50, 300 or 1000 Gray of gamma-rays (5H, 30H, 100H; 48, 66, 37 calli tested, respectively) and of *L. esculentum* cv. Bellina-ATW3003 and unirradiated *L. peruvianum* PI128650 (0H; 14 calli tested). The 0H-hybrid calli derived from a fusion experiment of a kanamycin resistant Bellina and a *L. peruvianum* sensitive to this antibiotic, so that selection against the *L. peruvianum* parent was possible.

regeneration efficiency of the symmetric somatic hybrids (0H in Fig. 1) is similar to that of unirradiated *L. peruvianum* (data not shown).

The characteristics of the putative hybrid calli that regenerated shoots, with respect to root formation, establishment in soil, flowering and seed set, are shown in Table 2 for each irradiation dose. The 5H-hybrids could be transferred to soil more efficiently than the 30H- and 100H-hybrids.

Only one out of 14 5H-hybrid plants tested could not regenerate shoots from established callus (Table 3), whereas 3 out of 6 30H-hybrids did not. The controls reacted as expected (Koornneef et al. 1987a); *L. esculentum* was non-responsive, whereas all tested *L. peruvianum* plants and 0H-hybrids showed plant regeneration from established callus cultures.

Table 3. Regeneration capacity, measured as shoot formation on shoot induction medium of established callus cultures, which were induced on leaf explants of *L. esculentum*, *L. peruvianum* and somatic hybrids. The number of independent hybrid calli, from which the tested plants derived, is given between parentheses. The hybrids were obtained after fusion of *L. esculentum* protoplasts with *L. peruvianum* protoplasts, which had been unirradiated (0H) or irradiated with 50, 300 or 1000 Gray of gamma-rays (5H, 30H or 100H, respectively) before fusion. +, callus regenerating; -, callus not regenerating.

Plants	Regeneration capacity	
	+	-
L. esculentum	0	4
L. peruvianum	7	0
0H-hybrids	2 (2)	0
5H-hybrids	14 (9)	1 (1)
30H-hybrids	6 (5)	3 (3)
100H-hybrids	1 (1)	0

Cytogenetics

The parental species both have 2n = 2x = 24, relatively small chromosomes. The *L. peruvianum* chromosomes are homoeologous to the *L. esculentum* chromosomes (Rick and Butler 1956). Most of the symmetric somatic hybrids between these species had a chromosome number of 48 or 72 (2n = 4x and 6x, respectively; Chapter 2). The chromosome numbers of the asymmetric somatic hybrids varied from 29 to 85, as presented in Fig. 2 and 3. The majority of the 5H-hybrids had chromosome numbers below the tetraploid level (shoots from 13 out of 21 hybrid calli), whereas the 30H- and the 100H-hybrids usually were above the tetraploid level (shoots from 17 out of 25 30H-calli and both 100H-calli). A few hybrids even exceeded the hexaploid level (shoots from one 5H- and three 30H-hybrid calli). The counting of the chromosomes was hampered by their small size. In some cases very small particles, most probably centric chromosome fragments, were observed (e.g. Fig. 2A). In general the chromosome number of each shoot varied slightly, within and between the analysed roots. In several cases larger differences were observed, mostly occurring between different roots. In these cases chromosome numbers differed by ten to twenty. Normally the shoots from one fusion callus had similar chromosome numbers. Also when shoots of different subclones of the same hybrid callus were present, they mostly (4 out of 5 calli) had similar chromosome numbers. However, one hybrid, 30H28, deviated: four well-looking shoots from one subclone had 41 and a fifth had 70 chromosomes, whereas the only shoot from the other subclone probably had 29 chromosomes (only two cells counted); the shoots with 29 and 70 chromosomes grew worse than the four other shoots derived from the same callus.

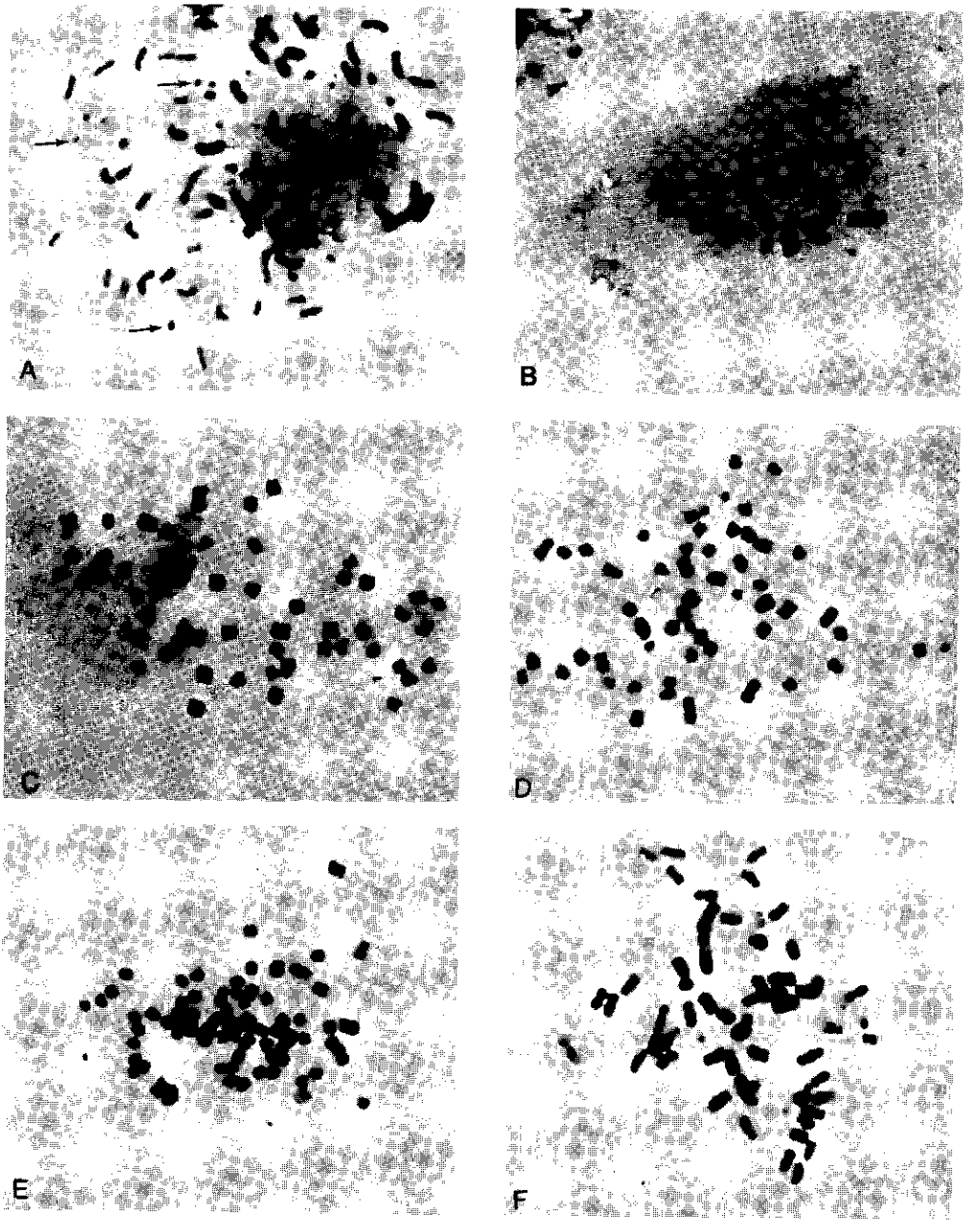

Fig. 2. Metaphase plates of root tip cells from asymmetric somatic hybrids of *L. esculentum* and irradiated *L. peruvianum*. **A.** 5H24 (irradiation dose of 50 Gy was applied), 2n = about 66; notice the small particles which probably are centric chromosome fragments (arrows). **B.** 5H15, 2n = 43. **C.** 5H19, 2n = 57. **D.** 30H46 (dose of 300 Gy), 2n = 56. **E.** 30H28, 2n = 70. **F.** 100H3 (dose of 1000 Gy), 2n = 57.

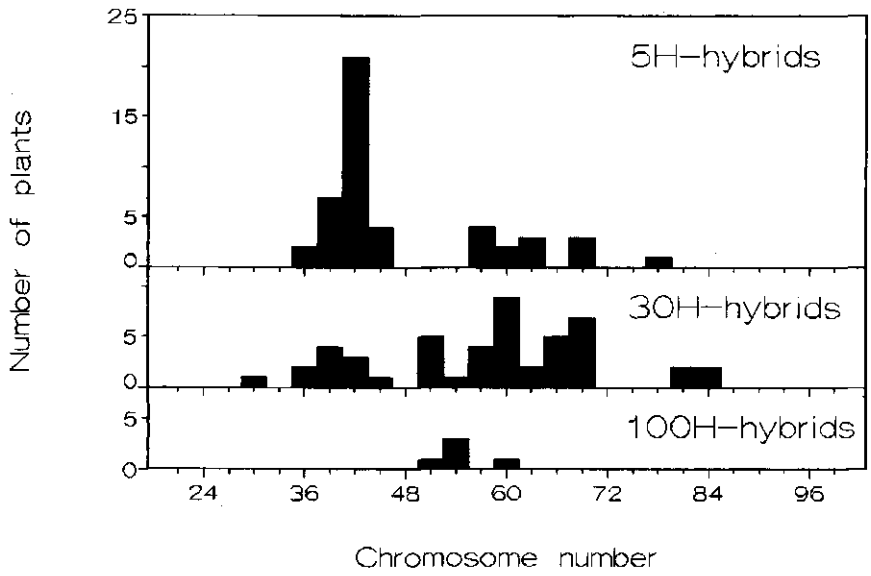

Fig. 3. Frequency distribution of average chromosome numbers of different shoots from asymmetric somatic hybrids of *L. esculentum* and *L. peruvianum* (the donor species). Upper panel: 5H-hybrids (donor irradiated with 50 Gy); middle panel: 30H-hybrids (300 Gy); lower panel: 100H-hybrids (1000 Gy).

Plant morphology

The asymmetric hybrid plants were less vigorous than both parental species and the symmetric hybrids. Especially the 30H- and 100H-hybrids were very retarded in growth and often died after transfer to soil. The leaves of the asymmetric hybrids differed much in colour (from light- to grey-green), shape and size (Fig. 4). Generally the leaves were smaller than those of the parental species (Fig. 5A). Leaves of the 30H- and 100H-hybrids mostly were smaller than those of the 5H-hybrids. Usually the asymmetric hybrids had leaves with deep incisions, similar to *L. esculentum*, and secondary leaflets, as *L. esculentum* and the symmetric hybrids. No stipules were present on the stems of the 30H-hybrids and only a few small ones on the stems of the 5H-hybrids (Fig. 5B); the same holds for the presence of bracts (leaflets in the inflorescences; Fig. 5C). The sympodial index (i.e. the mean number of nodes between two subsequent inflorescences) of the asymmetric hybrids in general was three, similar to that of the cultivated tomato and the symmetric hybrids.

A grey-green variegation was observed in 29 out of 51 5H-shoots and 13 out of 45 30H-shoots. Some shoots were very affected and had small irregular leaves.

Fig. 4. The leaves of twelve different asymmetric somatic hybrids, *L. esculentum* cv. Bellina (left) and *L. peruvianum* PI128650 (right). All hybrids were obtained after protoplast fusion of *L. esculentum* cv. Bellina with *L. peruvianum*, which was irradiated with 50 Gray of gamma-rays before fusion.

Nine 5H-hybrid calli (33% of total) and 11 30H-calli (55%) produced plants that were not variegated. The same leaf variegation has also been observed in many symmetric hybrids (Chapter 2) and could be similar to the disorder, named silvering, white head or chimera (Grimbly 1984), which occurs in some European tomato cultivars.

Flower morphology

A majority of the greenhouse-grown 5H-hybrids and a minority of the greenhouse-grown 30H- and 100H-hybrids formed flowers (Table 2). The asymmetric hybrids flowered less abundantly than the parents and the symmetric hybrids. The corolla of the 5H-hybrid flowers were in general dark yellow, like that of *L. peruvianum* and the symmetric hybrids, whereas most 30H-hybrids and the only flowering 100H-

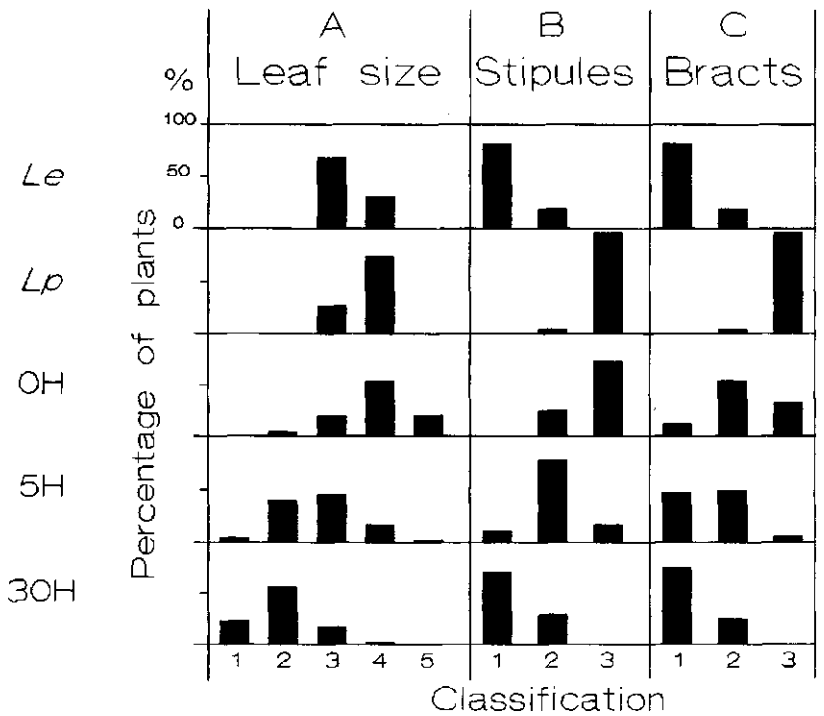

Fig. 5. Frequency distribution of some morphological characters of *L. esculentum* (*Le*; 16 plants tested), *L. peruvianum* (*Lp*; 22 tested), symmetric somatic hybrids of both species (0H; 60 tested) and asymmetric somatic hybrids of both species, in which occasion *L. peruvianum* was irradiated before fusion with 50 and 300 Gray of gamma-rays (5H and 30H; 41 and 34 shoots tested, respectively).
A. Leaf size: An arbitrary classification. 1 = very small leaves (<2 cm) to 5 = very large leaves (>50 cm). **B.** Presence of stipules: Leaflets on the stem at the nodes. **C.** Presence of bracts: Leaflets in the inflorescence. Classes of B + C: 1 = absence; 2 = presence at a few nodes; 3 = presence at each node.

hybrid had pale yellow flowers, such as *L. esculentum* ones. The size of the flower parts as well as the ratios of the sizes of different flower parts varied strongly between hybrids (Fig. 6A + 6B). The average size of the flowers of the 5H-hybrids was similar to that of the parental species, whereas the 30H-hybrids had smaller flowers. The relative sepal length of the asymmetric hybrids usually was intermediate between *L. esculentum* and the symmetric hybrids. In most plants of both asymmetric hybrid classes the pistil was smaller than the stamens; this was also observed in tetraploid *L. esculentum* plants (Table 3 in Chapter 2).

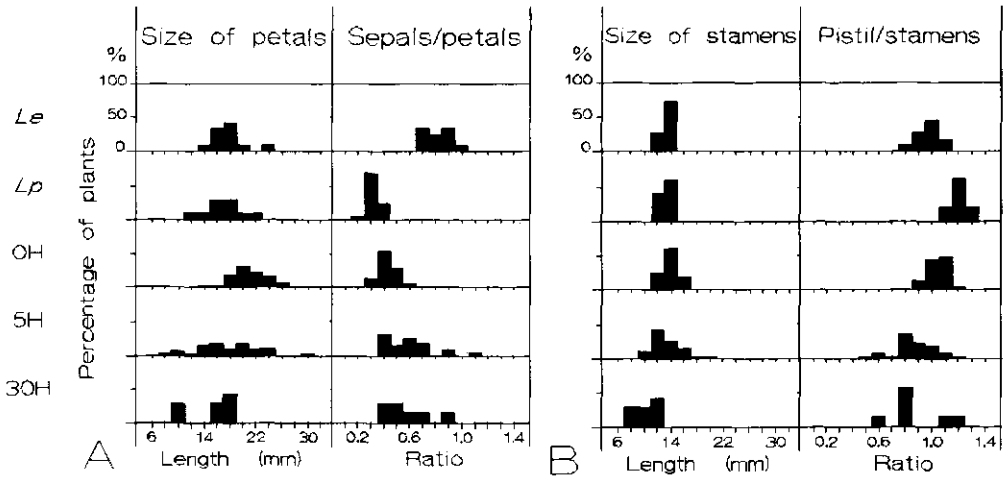

Fig. 6. Frequency distribution of some sizes of flower parts of *L. esculentum* (*Le*; 12 plants tested), *L. peruvianum* (*Lp*; 20 tested), symmetric somatic hybrids of both species (0H; 39 tested) and asymmetric somatic hybrids of both species, in which occasion *L. peruvianum* was irradiated before fusion with 50 and 300 Gray of gamma-rays (5H and 30H; 28 and 7 shoots tested, respectively).
A. Size of the petals and relative length of the sepals (with respect to the petal length). **B.** Size of the stamens and relative length of the pistil (with respect to the stamen length). Each measurement was the average of at least two flowers from the same plant.

Fertility

The flowers of the asymmetric somatic hybrids produced no or small amounts of pollen which were not viable, as tested with lactophenol acid fuchsin staining.

Several asymmetric hybrids set fruits (Table 2) with a diameter from a half to a few centimeters. Usually they were smaller than *L. peruvianum* fruits (1-2 cm) and much smaller than tomato fruits (>3 cm). Also the colour of the fruits differed between hybrids (Table 4). Most fruits were orange, which is intermediate between the fruit colour of the symmetric hybrids (yellow) and of the cultivated tomato (red).

No seed set was obtained in the asymmetric hybrids after selfing or backcrossing with the parental species (Table 2); the symmetric hybrids, on the contrary, did set seeds. In some fruits of five 5H-hybrids and one 30H-hybrid very small, abortive seeds were observed.

Table 4. The colour of the fruits obtained from different asymmetric somatic hybrids. The hybrids were obtained after protoplast fusion of *L. esculentum* with *L. peruvianum*, which was irradiated with 50, 300 or 1000 Gray of gamma-rays (5H, 30H or 100H, respectively) before fusion. Each observation was done on one to several shoots from one hybrid callus.

Plants	Fruit colour		
	yellow	orange	red
5H-hybrids	5	6	1
30H-hybrids	0	4	1
100H-hybrids	0	1	0

Discussion

The present experiments show that asymmetric somatic hybrid plants can be derived from protoplast fusions between *L. esculentum* and irradiated *L. peruvianum*. Selection of these hybrids was possible on the basis of the regeneration capacity derived from the irradiated species. The asymmetric hybrid nature could be confirmed by several morphological characteristics, which were intermediate between the symmetric somatic hybrids (Chapter 2) and *L. esculentum*, and by the analysis of specific marker genes (Chapter 5). However, the irradiation of *L. peruvianum* before fusion did not have the desired effect, namely elimination of the donor genome to such an extent, that only a small fraction was conserved in the asymmetric hybrids.

All asymmetric hybrids were aneuploid. From the cytogenetic and morphological analysis, we tentatively conclude that most asymmetric hybrids contained one, two or three diploid *L. esculentum* genomes, supplemented with one partial diploid genome of *L. peruvianum*. For 15 asymmetric hybrids this was confirmed by RFLP analysis (Chapter 6). The 5H-hybrids, of which most had one diploid *L. esculentum* genome, had on the average 16 presumed *L. peruvianum* chromosomes (range: 5 to 22); the 30H-hybrids, which in most cases probably contained a tetraploid genome of *L. esculentum*, had a mean of 13 (range: 2 to 22) *L. peruvianum* chromosomes; and both 100H-hybrids probably had a tetraploid *L. esculentum* genome and, on the average, 7 (range: 4 to 12) *L. peruvianum* chromosomes. Although there is no clear correlation between the irradiation dose and elimination of the *L. peruvianum* genome, the 30H-hybrids resembled *L. esculentum* more than the 5H-hybrids. This is not necessarily due to the elimination of *L. peruvianum* chromosomes, but can also be ascribed to the presence of a larger number of *L. esculentum* genomes in most 30H-hybrids. Anyway, a relatively large fraction of the *L. peruvianum* genome

is retained, even in the 100H-hybrids. Limited chromosome elimination, together with a weak dose effect, was also observed in asymmetric somatic hybrids of other species. Retention of the donor chromosomes ranged from 11% to 90% (of a diploid donor genome) in *Nicotiana plumbaginifolia* (recipient) (+) *Atropa belladonna* (donor) hybrids (100-1000 Gy; Gleba et al. 1988), 8%-75% in *N. plumbaginifolia* (+) *N. sylvestris* hybrids (100-1000 Gy; Famelaer et al. 1989) and 25%-100% in *Brassica oleracea* (+) *B. campestris* hybrids (100-800 Gy; Yamashita et al. 1989). In all those cases chromosome rearrangements and/or deleted donor chromosomes were reported. In some of the *L. esculentum* (+) *L. peruvianum* asymmetric hybrids reported here, we observed fragments. It was often difficult to identify fragments with certainty, because of the small size of the tomato chromosomes. However, RFLP analysis, applied to 15 hybrids, clearly indicated the presence of incomplete chromosomes in all tested plants (Chapter 6).

The asymmetric 5H-hybrids were more viable than the 30H- and 100H-hybrids, irrespective whether shoot regeneration, root formation or morphological characteristics were used as criteria, whereas all asymmetric hybrids were less viable than the symmetric hybrids. A possible explanation for this is the unbalanced genome of the asymmetric hybrids. It has been reported that in *L. esculentum* only primary trisomics and some monosomics are viable (Rick and Butler 1956), whereas aneuploidy is tolerated better by primitive tomatoes and species hybrids of the tomato than in the cultivated tomato (Soost 1958; Rick and Notani 1961; Györffy and Mako 1963). In general the asymmetric somatic hybrids were near the triploid or pentaploid level; the 5H-hybrids usually had a diploid *L. esculentum* genome, whereas most 30H- and 100H-hybrids presumably had a tetraploid recipient genome. In other asymmetric hybridisation experiments that resulted in limited chromosome elimination (Gleba et al. 1988; Famelaer et al. 1989; Yamashita et al. 1989), the recipient genome in the asymmetric hybrids usually was around tetraploid or hexaploid. An explanation of this could be that cells with polyploidised recipient genomes, which may have arisen during the tissue culture phase, better tolerate additional and abnormal chromosomes (rearrangements and fragments).

The variation in chromosome number within each of the asymmetric hybrids might indicate instability at the plant level. Probably rearranged and incomplete chromosomes tend to get lost more frequently. The observed loss of regeneration capacity in established callus cultures of some 30H-hybrids might be explained this way. An other explanation is elimination of this trait in the established callus cultures.

The asymmetric somatic hybrids were not fertile. Some fruits contained small abortive seed-like structures. Embryo rescue techniques were applied two to four weeks after pollination (selfing or backcross with *L. esculentum* pollen). In two

cases callus was induced on tiny, prematurely isolated seeds; one of these formed shoot primordia. However, these calli might also descend from maternal tissue.

The limited elimination of donor chromosomes is a serious drawback for the application of asymmetric hybrids in plant breeding, because several backcrosses to the recurrent parent are still required to get rid of the unwanted donor traits. Crosses will be impossible or at least hampered strongly by the sterility and the polyploidy of these hybrids. The sterility is probably due to the cytogenetic aberrations, such as aneuploidy and rearrangements. The latter were more frequent after higher irradiation doses (Chapter 6). Despite the fact that several tens of asymmetric hybrids were obtained and analysed, none of the plants had just one or two chromosomes above the diploid level, which probably would have allowed backcrossing to the diploid recipient. If the polygenic nature of the selectable donor traits (both callus growth and regeneration characteristics) is the main cause of this limited elimination, the use of simpler selectable markers, such as antibiotic resistance or alleles complementing auxotrophic mutations, would help to overcome the problem. If not, other ways to enhance elimination of donor chromosomes should be looked for or very large populations of asymmetric hybrids have to be evaluated.

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References

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

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

Gleba YY, Hinnisdaels S, Sidorov VA, Kaleda VA, Parokonny AS, Boryshuk NV, Cherup NN, Negrutiu I, Jacobs M (1988) Intergeneric asymmetric hybrids between Nicotiana plumbaginifolia and Atropa belladonna obtained by "gamma-fusion". Theor Appl Genet 76:760-766

Grimbly P (1986) Disorders. In: Atherton JG, Rudich J (eds), The tomato crop. A scientific basis for improvement. Chapman and Hall, London/New York, pp 369-389

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

Györffy B, Mako J (1963) Two aneuploid progenies from a sesquidiploid tomato hybrid after uncontrolled pollinations. TGC Rep 13:36-37

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

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

Menczel L, Nagy F, Kiss ZR, Maliga P (1981) Streptomycin resistant and sensitive somatic hybrids of Nicotiana tabacum + Nicotiana knightiana: correlation of resistance to N. tabacum plastids. Theor Appl Genet 59: 191-195.

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

Negrutiu I, De Brouwer D, Watts JW, Sidorov VI, Dirks R, Jacobs M (1986) Fusion of plant protoplasts: a study using auxotrophic mutants of Nicotiana plumbaginifolia Viviani. Theor Appl Genet 72:279-286

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

Rick CM (1982a) The potential of exotic germplasm for tomato improvement. In: Vasil IK, Scowcroft WR, Frey KJ (eds), Plant improvement and somatic cell genetics. Academic Press, New York, pp 1-28

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

Rick CM, Butler L (1956) Cytogenetics of the tomato. Adv Genet 8:267-382

Rick CM, Notani NK (1961) The tolerance of extra chromosomes by primitive tomatoes. Genetics 46:1231-1235

Soost RK (1958) Progenies from sesquidiploid F_1 hybrids of Lycopersicon esculentum and L. peruvianum. J Heredity 49:208-213

Taylor IB (1986) Biosystematics of the tomato. In: Atherton JG, Rudich J (eds), The tomato crop. A scientific basis for improvement. Chapman and Hall, London/New York, pp 1-34

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

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

CHAPTER 5

ASYMMETRIC SOMATIC HYBRIDS BETWEEN *LYCOPERSICON ESCULENTUM* AND IRRADIATED *LYCOPERSICON PERUVIANUM* II. ANALYSIS WITH MARKER GENES

J. Wijbrandi, A.M.A. Wolters, M. Koornneef

Summary. Asymmetric somatic hybrids of *Lycopersicon esculentum* and *Lycopersicon peruvianum* were analysed for the retention of genes and alleles, specific for *L. peruvianum*. The hybrids were obtained by fusion of protoplasts from *L. esculentum* with those of *L. peruvianum* (the donor), which had been irradiated before fusion with 50, 300 or 1000 Gy of gamma-rays. The retention of three different types of genes or alleles was analysed: (1) The gene coding for kanamycin resistance, which is dominant and had been introduced in most of the *L. peruvianum* donor plants by transformation. It was present at one locus in 16 *L. peruvianum* donor plants and at two loci in one donor plant. (2) The genes coding for acid phosphatase, locus *Aps-1*, and glutamate oxaloacetate transaminase (GOT); different alleles of these genes are co-dominant, and were detected by isozyme analysis. (3) Eighteen single gene morphological markers, for which most of the *L. esculentum* genotypes used were homozygous recessive.
Kanamycin resistance from the donor plants with one locus was retained in about 50% of the asymmetric 30H-hybrids (the donor was irradiated with 300 Gy). *L. peruvianum* specific alleles of *Aps-1* and GOT were present in at least 70% of the hybrids; the retention of donor alleles was lower in 30H- than in 5H-hybrids (donor irradiated with 50 Gy). On the average, 74% of the *L. peruvianum* specific alleles (one or both) of the morphological markers were detected in the 30H-hybrids. Several of the *L. esculentum* genotypes used were homozygous recessive for two morphological markers on the same chromosome. In 36% of the 30H-hybrids derived from them, only one of these markers was complemented by the *L. peruvianum* allele. This is an indication for frequent breakage of the *L. peruvianum* chromosomes. Several hybrid calli regenerated genotypically different shoots.
On the whole, this analysis confirms the conclusion from the cytogenetic and morphological analysis of these asymmetric hybrids, namely that irradiation prior to fusion eliminated the *L. peruvianum* genome only to a limited extent.

Introduction

Partial genome transfer by asymmetric somatic hybridisation, which involves fusion of protoplasts of a recipient species with irradiated protoplasts of a donor species, has been described for several plants. The transferred donor genome varied from a few traits (e.g. Dudits et al. 1987) to many chromosomes (e.g. Gleba et al. 1988). The amount of transferred donor genome was often assessed on the basis of chromosome counts. This is not always an accurate estimation, because discrimination between the species specific chromosomes and identification of incomplete chromosomes are not always possible.

In Chapter 4 we described the cytogenetic and morphological analysis of a series of asymmetric hybrids of *L. esculentum* (the cultivated tomato) and *L. peruvianum* (a wild species; the donor). These hybrids appeared to contain still a relatively large number of *L. peruvianum* chromosomes. In most of the asymmetric hybrids, the chromosome number was around the triploid or pentaploid level; probably, the genome of these hybrids consisted of a diploid or tetraploid genome from *L. esculentum* and several chromosomes from *L. peruvianum*. The amount of donor chromosomes varied and did not correlate strongly with the irradiation dose applied to the *L. peruvianum* protoplasts (50, 300 and 1000 Gray, respectively). To study the elimination of genetic material of the donor species more in detail, the same asymmetric hybrids were analysed for transfer of single gene markers. This kind of analysis can be performed very well in the tomato, because of its detailed linkage map. The map consists of more than 300 morphological, isozyme- and disease resistance genes (Mutschler et al. 1987) and at least 300 RFLP markers (Young and Tanksley 1989). We tried to estimate the fraction of the *L. peruvianum* genome that was conserved in the asymmetric somatic hybrids by analysing the retention of three types of genes or alleles:

(i) the neomycin phosphotransferase II (NPT II) gene, which causes resistance to the antibiotic kanamycin. This is a dominant gene, which had been introduced into the nuclear genome of most of the *L. peruvianum* donor plants by transformation;

(ii) the isozyme markers acid phosphatase (*Aps-1*) and glutamate oxaloacetate transaminase (GOT);

(iii) single gene determined morphological markers, present in most of the tomato genotypes used.

Material and methods

The genotypes of *L. esculentum*, the recipient species, were the Dutch hybrid cultivar Bellina (kindly provided by Rijk Zwaan Seed Company, de Lier, The Netherlands) and seven multiple marker lines (Table 1) from the Tomato Genetics Stock Center in Davis, USA (kindly provided by Prof. C.M. Rick). Plants from the *L. peruvianum* accession PI128650 (received from the Institute of Horticultural Plant Breeding, Wageningen, The Netherlands) were used as donor. Seventeen kanamycin resistant *L. peruvianum* plants, obtained by leaf disc transformation with *A. tumefaciens* containing the plasmid pAGS112 (Koornneef et al. 1987b) were available; these plants were designated ATW2001 to ATW2027. The isolation of the asymmetric somatic hybrids was described in Chapter 4. The asymmetric hybrids were designated according to the irradiation dose applied to *L. peruvianum* before protoplast fusion: 5H-, 30H- and 100H-hybrids, which were irradiated with a dose of 50, 300 and 1000 Gy of gamma-rays, respectively.

Kanamycin resistance assays

To determine the number of NPT II loci in the independent *L. peruvianum* transformants, they were backcrossed as pistillate parent to wild type *L. peruvianum*. The resulting seeds were decontaminated by treatment for 10" in

Table 1. The multiple marker lines of *L. esculentum* used in the present study. The morphological marker genes are given with their chromosomal location (Rick 1982). * indicates a character which was used in the analysis of the asymmetric somatic hybrids of *L. esculentum* (recipient) and *L. peruvianum* (donor).

Tomato genotype		Markers [chromosome arm]
LA291		*ms-2* (=male sterile) [2L]
	*	*hl* (=hairless, no large trichomes) [11S]
	*	*a* (=anthocyaninless) [11L]
LA1164	*	*var* (=variabilis, leaves emerge yellow) [7S]
	*	*not* (=notabilis, leaves wilting) [7L]
	*	*ah* (=anthocyaninless) [9L]
	*	*marm* (=marmorata, leaves marbled white-green) [9L]
LA1166	*	*clau* (=clausa, leaves subdivided) [4S]
	*	*di* (=divergens, stems slender and whitish) [4L]
		icn (=incana, leaves with whitish margins) [10S]
		ag (=anthocyanin gainer, laminae abaxially pigmented) [10L]
LA1182	*	*sy* (=sunny, leaves emerge yellow) [3S]
	*	*sf* (=solanifolia, leaflets entire and concave) [3L]
	*	*alb* (=albescent, strong white-green variegation) [12S]
		mua (=multifurcata, dull green interveinal chlorosis) [12L]
LA1189	*	*yv* (=yellow virescent, leaves emerge yellow) [6L]
	*	*c* (=potato leaf, fewer leaf segments) [6L]
LA1444	*	*af* (=anthocyaninless) [5S]
		tf (=trifoliate, leaves 3-segmented) [5S]
		wv (=white virescent, leaves emerge white) [2L]
		d (=dwarf) [2L]
LA1665	*	*dgt* (=diageotropica, stems and roots diageotrophic) [1L]
	*	*l* (=lutescent, leaves yellowing) [8S]
	*	*al* (=anthocyanin loser, pigmented only at nodes later) [8L]

70% ethanol and 20' in 5x diluted commercial bleach (10% NaClO), and washed several times in sterile water. The seeds were transferred to plastic containers with shoot culture medium (Chapter 2) containing 100 mg/l kanamycin and incubated in the dark. After a few days the seeds germinated and were transferred to light. Two weeks later the seedlings were scored for growth (=resistance).

To determine the kanamycin resistance in calli, derived from protoplast cultures of a *L. esculentum* genotype and one of the *L. peruvianum* ATW-plants (with and without fusion treatment), we sliced the calli and subcultured one part on the callus medium TMc (Chapter 4) and transferred the other part to TMc with 100 mg/l kanamycin. Kanamycin sensitive calli turned brown on the latter medium. To test kanamycin resistance of somatic hybrid plants, cuttings of hybrid shoots were transferred to shoot culture medium supplemented with 100 mg/l kanamycin. Sensitive shoots formed no roots and bleached after a few weeks.

Southern blot analysis

DNA was isolated from several plants according to Dellaporta et al. (1983), digested with *Dra* I, separated by agarose gel electrophoresis, blotted onto Gene Screen PLUS (New England Nuclear) and hybridised with a probe for the NPT II gene; as probe was used the *Cla* I-*Sal* I fragment, which contained the NOS-promoter and the structural gene, of the T-region of plasmid pAGS112 (kindly provided by Dr. P.J.M. van den Elzen, MOGEN Leiden, The Netherlands). The hybridisation procedure is described in Chapter 6.

Isozyme analysis

Leaf material from greenhouse-grown plants was used for the analysis of acid phosphatase, locus *Aps-1*, and glutamate oxaloacetate transaminase (GOT), loci *Got-1*, *Got-2*, *Got-3* and *Got-4*; the positions of these loci on the linkage map of tomato are shown in Fig. 1. Crude extracts and extracts prepared according to Suurs et al. (1989), respectively, were electrophoresed on vertical polyacrylamide slab gels (Chapter 2). Enzyme activity was stained according to Vallejos et al. (1983).

Morphological markers

Fig. 1 shows the position of the recessive morphological markers, used in the present study, on the linkage map of *L. esculentum*. The *L. esculentum* genotypes that were used for the fusions, were homozygous for two to four of these markers located on one to two different chromosomes (Table 1). The asymmetric hybrids, preferably greenhouse-grown plants, were assayed for the phenotype of the relevant markers to determine the retention of the dominant *L. peruvianum* wild type alleles. The presence of some markers could not be assayed, because they were typical hypocotyledon markers (e.g. *ag*) or because the hybrid and aneuploid nature of the hybrids interfered with the expression of the mutant phenotype.

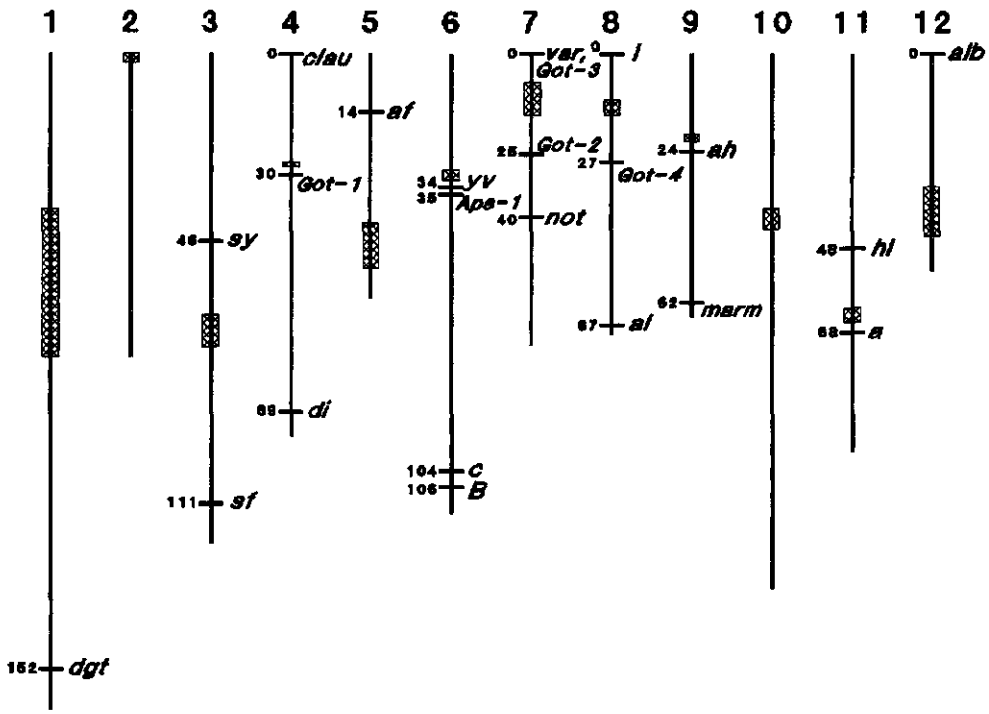

Fig. 1. Linkage map of the morphological and isozyme markers used in the analysis of the asymmetric somatic hybrids. The shaded areas indicate the centromeres. All positions are according to Mutschler et al. (1987).

Results

Number of kanamycin resistance loci in L. peruvianum plants

The segregation ratios of kanamycin resistance in the testcross of *L. peruvianum* ATW-plants with wild type *L. peruvianum* are given in Table 2. Sixteen ATW-plants showed a ratio of 1:1, which indicates the presence of one NPT II locus, whereas one plant, ATW2002, had a ratio of 3:1, which indicates that NPT II genes were inserted at two different unlinked loci. This was confirmed by Southern blot analysis with the NPT II gene as probe (Fig. 2): ATW2002 showed two distinct fragments; the tested kanamycin resistant hybrids of Bellina (+) ATW2002, namely one symmetric and ten asymmetric hybrids, had either one or both of these bands; the bands segregated independently. The only tested kanamycin sensitive hybrid of the same parental genotypes, 5H10, had no such band(s).

Table 2. Segregation of kanamycin resistance in the testcross of kanamycin resistant *L. peruvianum* PI128650 plants (ATW2001 to ATW2011, ATW2014 to ATW2016, ATW2020, ATW2021 and ATW2027) as female parent with wild type *L. peruvianum* PI128650, and among regenerating 30H-hybrid calli, which derived from protoplast fusions between *L. esculentum* and 300 Gy gamma-irradiated kanamycin resistant *L. peruvianum*. The assays were performed on media supplemented with 100 mg/l kanamycin. The calli were assayed before regeneration occurred.
Km^R, kanamycin resistant; Km^S, kanamycin sensitive.

L. peruvianum genotype	Segregation ratio in testcross		Ratio in 30H-calli
	Km^R:Km^S	[χ^2(1:1)]*	Km^R:Km^S
ATW2001	29:35	[0.56]	1:3
ATW2003	35:29	[0.56]	1:0
ATW2004	32:32	[0.00]	9:3
ATW2005	73:53	[3.17]	6:2
ATW2006	41:53	[1.53]	2:1
ATW2007	31:33	[0.06]	1:0
ATW2008	36:27	[1.29]	3:0
ATW2009	33:32	[0.02]	7:5
ATW2010	33:31	[0.06]	3:0
ATW2011	31:33	[0.06]	4:1
ATW2014	59:66	[0.39]	5:3
ATW2015	31:33	[0.06]	1:3
ATW2016	29:34	[0.40]	1:1
ATW2020	29:33	[0.26]	13:1
ATW2021	31:41	[1.39]	5:3
ATW2027	32:32	[0.00]	3:2
			total 65:28
ATW2002	47:17	[14.06]**	

* segregation ratio does not deviate significantly from 1:1, if $\chi^2 < \chi^2(1, 0.95) = 3.84$; consistent with the presence of one NPT II locus

** segregation ratio does not deviate significantly from 3:1 ($\chi^2 = 0.08$); consistent with two unlinked NPT II loci

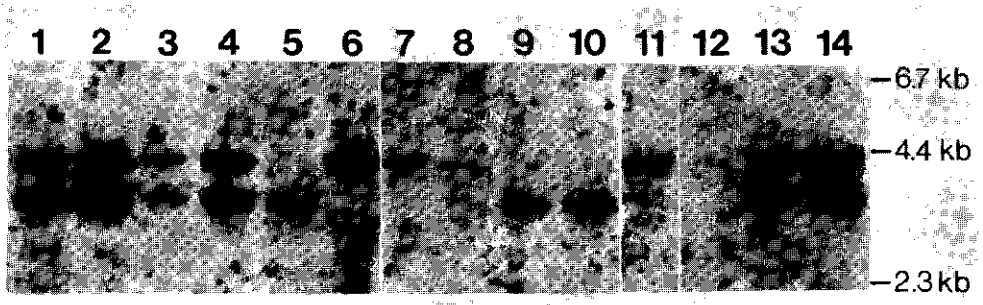

Fig. 2. Southern blot analysis of some somatic hybrids and their parents with a probe of the NPT II gene. *Dra* I digests of the following genotypes were analysed: lane 1, 5H2; lane 2, 5H3; lane 3, 5H5; lane 4, 5H7; lane 5, 5H13; lane 6, 5H16; lane 7, 5H28; lane 8, 5H10; lane 9, 30H1; lane 10, 30H3; lane 11, 30H7; lane 12, *L. esculentum* cv. Bellina; lane 13, 0H1; lane 14, *L. peruvianum* PI128650-ATW2002. All hybrids derived from protoplast fusions between *L. esculentum* cv. Bellina and ATW2002. The latter was either unirradiated (symmetric hybrid 0H1), irradiated with 50 Gy of gamma-rays (asymmetric hybrids 5H2 to 5H28) or irradiated with 300 Gy of gamma-rays (asymmetric hybrids 30H1 to 30H7) before fusion. The position of the *Hin* dIII fragments of phage lambda DNA is indicated at the right.

Retention of kanamycin resistance loci in asymmetric hybrids

The ratio of kanamycin resistant to kanamycin sensitive regenerating calli derived from fusions of *L. esculentum* and irradiated *L. peruvianum* containing one NPT II locus, is shown in Table 2 (right column). Seventy percent of the tested calli (65 out of 93) were kanamycin resistant. The assay was repeated for shoots that had regenerated from 12 resistant 30H-calli; only a minority of the 30H-hybrids were tested at the plant level, because of the very low growth rate of many of these hybrids and because we preferred to transfer the shoots to the greenhouse. Shoots from 7 of these 12 resistant calli were still resistant, whereas the shoots from 4 resistant calli were kanamycin sensitive. From another resistant hybrid callus one resistant and one sensitive shoot was regenerated. So, in one third of the 30H-hybrids that were kanamycin resistant at the callus level, the resistance was lost in the shoots. Assuming that the chance to loose this trait was the same in all resistant 30H-hybrids, we estimate that 46 percent (≈43 out of 93) of the 30H-hybrids had retained the kanamycin resistance from *L. peruvianum*.

The retention of kanamycin resistance in nine 30H-hybrids (all nine tested at the plant level) derived from ATW2002 (with two NPT II loci), was higher: all shoots from eight hybrids were resistant and those from one hybrid were sensitive.

Isozyme analysis

Both parental species, *L. esculentum* and *L. peruvianum*, had different isozyme patterns (Fig. 3) for *Aps-1*, a locus which encodes acid phosphatase-1, a dimeric enzyme (Rick 1983). In the asymmetric somatic hybrids, three different patterns were found (Fig. 3): one pattern resembled the tomato pattern with one fast migrating band; another pattern had six bands, all bands from both species and two novel, presumed heterodimeric ones; the third pattern consisted of the single band from tomato, one of the *L. peruvianum* specific bands and one presumed interspecific heterodimeric band. The latter pattern indicates the presence of a single allele of the *L. peruvianum Aps-1* in the hybrid. The number of asymmetric hybrids which have lost or retained *Aps-1* alleles from the donor species are shown in Table 3. Most of the hybrids contained alleles from the donor species. A larger fraction of the higher dose hybrids (30H and 100H) than of the low dose (5H) hybrids lacked *L. peruvianum* alleles. For two hybrid calli, differences between the separate shoots were observed. We could not determine the number of retained donor alleles, because the hybrid pattern of six bands can result from one or two *L. peruvianum* alleles (data not shown).

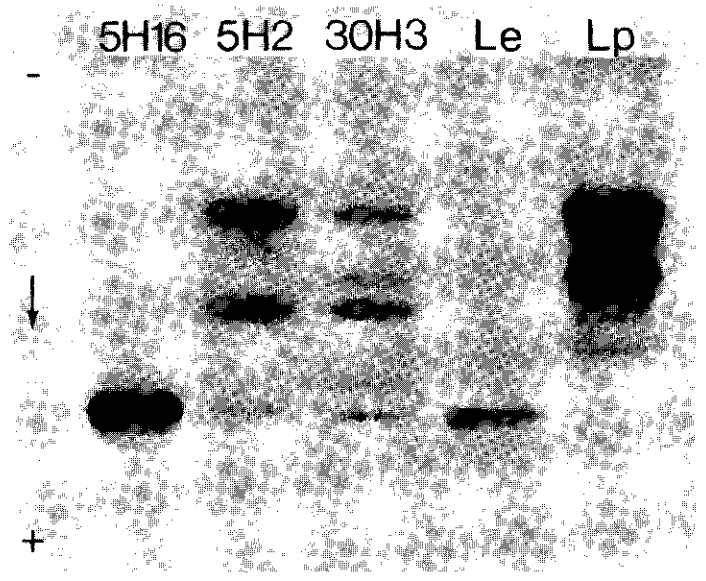

Fig. 3. Isozyme patterns of acid phosphatase, locus *Aps-1*, of (from left to right) asymmetric somatic hybrids 5H16, 5H2 and 30H3, *L. esculentum* cv. Bellina (Le) and *L. peruvianum* PI128650 (Lp). The asymmetric hybrids derived from protoplast fusions between *L. esculentum* and *L. peruvianum*, irradiated with 50 Gy (5H2 and 5H16) or 300 Gy (30H3) of gamma-rays.

Table 3. The presence of *L. peruvianum* specific and hybrid bands in isozyme patterns of asymmetric somatic hybrids of *L. esculentum* and *L. peruvianum*, irradiated with 50, 300 and 1000 Gy of gamma-rays (5H-, 30H- and 100H-hybrids, respectively). The tested isozymes were acid phosphatase, locus *Aps-1*, and glutamate oxaloacetate transaminase (GOT). The GOT bands were assayed in two parts: the two slow migrating bands and the three fast migrating bands (see Fig. 4). "mixed" means that the assayed bands were absent in one shoot from a hybrid callus, whereas these bands were present in another shoot from the same callus. The number of hybrids in which not all *L. peruvianum* and hybrid bands were present, is indicated between parentheses.

Genotypes	*Aps-1*		
	absent	present	mixed
5H-hybrids	1	23 (5)	0
30H-hybrids	2	7	1
100H-hybrids	1	0	1 (1)

Genotypes	"Slow" GOT bands		
	absent	present	mixed
5H-hybrids	1	7	0
30H-hybrids	7	14 (8)	3 (2)
100H-hybrids	0	2 (2)	0

Genotypes	"Fast" GOT bands		
	absent	present	mixed
5H-hybrids	1	6 (4)	1 (1)
30H-hybrids	2	20 (8)	2 (1)
100H-hybrids	2	0	0

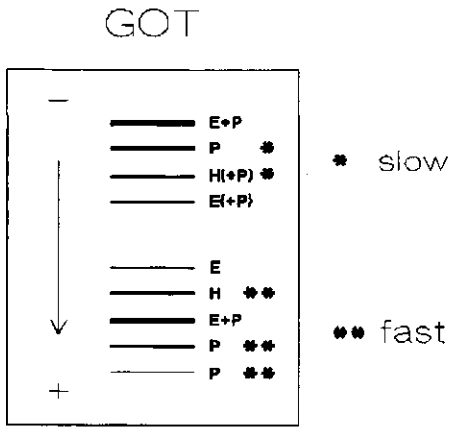

Fig. 4. Representation of the isozyme pattern of glutamate oxaloacetate transaminase, found in symmetric somatic hybrids between *L. esculentum* and *L. peruvianum*. At the right side are indicated the *L. esculentum* specific (E), the *L. peruvianum* specific (P) and hybrid specific (H) bands. The latter two types of bands were divided in slow migrating (*) and fast migrating (**).

For glutamate oxaloacetate transaminase (GOT), four loci on three different chromosomes (Fig. 1) are known; at least 3 are dimeric enzymes (Rick 1983). Isozyme analysis of the cultivated tomato shows four GOT bands, while that of *L. peruvianum* shows 5 or 7 bands, of which 2 or 3 were at the same position as tomato bands (see Fig 4B in Chapter 2). A typical hybrid pattern, as expressed in symmetric somatic hybrids (Chapter 2), contained 9 bands and is represented in Fig. 4. We could not assess which bands correlated with each of the loci. Therefore, the *L. peruvianum* specific and hybrid bands were divided in slow and fast migrating, because these segregated independently. The presence of those bands in the asymmetric hybrids was determined (Table 3). As in the case of *Aps-1*, most hybrids had retained donor alleles. Some hybrid calli had regenerated shoots that differed with respect to the retained donor bands. The loss of slow migrating GOT bands was dose dependent. The fast bands were retained more often and the frequency of their loss was not clearly dose dependent. Probably, products from more than one locus are present in the fast migrating bands. Four hybrids had a complete hybrid pattern of 9 bands and the patterns of only two hybrids resembled the *L. esculentum* pattern. The latter hybrids do not necessarily lack all *L. peruvianum* specific alleles, because several *L. peruvianum* bands comigrate with *L. esculentum* bands on the polyacrylamide gels.

Morphological analysis

For the analysis of the retention of *L. peruvianum* alleles of morphological marker genes, we had used *L. esculentum* genotypes that were homozygous recessive for such genes as recipient (Table 1); *L. peruvianum* carries dominant alleles for all morphological marker genes involved. The complementation of the recessive *L. esculentum* alleles in the asymmetric 30H-hybrids is given in Table 4. In the majority of these hybrids, the recessive markers were complemented by *L. peruvianum* specific alleles. Several hybrid calli (7 out of 102) regenerated shoots which differed with respect to the retention of *L. peruvianum* alleles.

The marker *b*, which is co-dominant and present in all *L. esculentum* genotypes used, is included in Table 4. The corresponding gene of *L. peruvianum* is *B* (= Beta, increase of β-carotene and reduction of lycopene). The red colour (caused by a high level of lycopene) of *L. esculentum* fruits indicates absence of *B*. The symmetric hybrids, containing complete genomes of both parental species, had yellow fruits (Chapter 2). The asymmetric hybrids set yellow, orange or red fruits (see Table 4 in Chapter 4). So, at least one *B* allele is present in the plants that set yellow and orange fruits; this was the case in 92% (11 out of 12) of the 5H-hybrids and 80% (4 out of 5) of the 30H-hybrids.

Table 4. Complementation of marker genes in asymmetric somatic hybrids of *L. esculentum* and 300 Gy gamma-irradiated *L. peruvianum*. The table shows the number of hybrids having retained *L. peruvianum* allele(s) of each of a series of marker genes. "absent" means that no complementation was observed in any of the analysed shoots; "present" means that complementation was observed in all analysed shoots; "mixed" means that complementation was observed in part of the analysed shoots of a hybrid. Observations were made on greenhouse-grown plants, derived from 71 hybrid calli, and on shoots *in vitro*. The latter shoots were assayed only for clearly scorable phenotypes.

Marker gene	Chromosome position	Corresponding *L. peruvianum* allele(s) in asymmetric hybrids		
		absent	present	mixed
dgt	1-152	1	1	
sy	3- 46	4	4	
sf	3-111	4	6	
clau	4- 0	1	1	
di	4- 89	0	2	1
af	5- 14	0	1	
yv	6- 34	0	2	
c	6-104	3	0	
b *	6-106	1	4	
var	7- 0	0	4	
not	7- 40	0	2	1
l	8- 0	0	1	
al	8- 67	1	5	
ah	9- 24	4	6	
marm	9- 62	0	3	1**
hl	11- 48	1	12	2
a	11- 68	6	9	
alb	12- 0	1	5	2***
	total	27	68	7

* *b*, low β-carotene and high lycopene
** a few sectors of one plant were *marm*
*** one shoot *in vitro* of each hybrid was *alb*

Each of the *L. esculentum* multiple marker lines contained two markers on a single chromosome (Table 1). Therefore, it was possible to determine whether a given *L. peruvianum* chromosome was transferred completely by analysing both markers. The frequency of complementation of no, one or both of these genes is shown in Table 5 (*Aps-1* and *B* data included). In 36% of the plants complementation of only one gene was observed. This suggests the presence of fragments or incomplete, deleted chromosomes of *L. peruvianum* in these asymmetric hybrids.

Table 5. Complementation of *L. esculentum* marker genes located on a same chromosome by corresponding *L. peruvianum* alleles. Observations were carried out on asymmetric somatic hybrids of *L. esculentum* and *L. peruvianum* irradiated with 300 Gy. Only those plants were included where both markers could be scored unambiguously. The gene symbols supplemented with $^+$ indicate the presence of the wild type gene derived from *L. peruvianum* in the asymmetric hybrids. *Aps*E means an *Aps-1* isozyme pattern with only the *L. esculentum* specific band, and *Aps*P means that *L. peruvianum* specific and hybrid bands were also present; *b* means observation of red fruits indicating only *L. esculentum* specific alleles to be present, while *B* means the presence of the *L. peruvianum* specific allele(s), because of the observation of orange fruits.

Chromosome	Phenotypic observations				Single complementation
3	*sy sf* 3	*sy sf*$^+$ 1	*sy*$^+$ *sf* 0	*sy*$^+$ *sf*$^+$ 4	12½%
4	*clau di* 0	*clau di*$^+$ 1	*clau*$^+$ *di* 0	*clau*$^+$ *di*$^+$ 1	50 %
6	*yv c* 0	*yv c*$^+$ 0	*yv*$^+$ *c* 2*	*yv*$^+$ *c*$^+$ 0	100 %
6	*Aps*E *b* 1	*Aps*E *B* 0	*Aps*P *b* 0	*Aps*P *B* 4	0 %
7	*var not* 0	*var not*$^+$ 0	*var*$^+$ *not* 1**	*var*$^+$ *not*$^+$ 2	33 %
8	*l al* 0	*l al*$^+$ 0	*l*$^+$ *al* 0	*l*$^+$ *al*$^+$ 1	0 %
9	*ah marm* 0	*ah marm*$^+$ 2***	*ah*$^+$ *marm* 0	*ah*$^+$ *marm*$^+$ 2	50 %
11	*hl a* 0	*hl a*$^+$ 2#	*hl*$^+$ *a* 3##	*hl*$^+$ *a*$^+$ 3	62½%
	4	12		17	12/33= 36 %

* one of both also was *Aps*P c (hybrid 30H37)
** concerning one subclone, other subclone was *var*$^+$*not*$^+$ (hybrid 30H36)
*** only sectors of one shoot, other shoots were *ah marm* (hybrid 30H36)
\# one subclone of one hybrid, other subclone was *hl*$^+$*a*$^+$ (hybrid 30H33)
\#\# one subclone of one hybrid, other subclone was *hl a* (hybrid 30H22)

Discussion

The results presented in this paper show that a large amount of *L. peruvianum* genome is retained in asymmetric somatic hybrids of *L. esculentum* and irradiated *L. peruvianum*. This is in agreement with the relatively large number of donor chromosomes, observed in these hybrids (Chapter 4). From the isozyme analysis

of the hybrids, it appears that the higher dose hybrids (30H and 100H) retained less *L. peruvianum* specific alleles than the lower dose hybrids (5H). This agrees with the observation that the higher dose hybrids resemble *L. esculentum* more than the low dose hybrids in general morphological appearance (Chapter 4).

The limited elimination of donor genome in our asymmetric somatic hybrids is in contrast with asymmetric hybrids of other species, which had retained only one or a few donor chromosomes (Bates et al. 1987; Dudits et al. 1980; Gupta et al. 1984), or even one or a few traits (Dudits et al. 1987; Somers et al. 1986). The limited elimination in the tomato asymmetric hybrids can be a consequence of the unintended selection for good callus growth, because this trait is better in *L. peruvianum* than in *L. esculentum*, is multi-genic and not linked to regeneration capacity (Koornneef et al. 1987a). Another explanation for the limited elimination could be the lack of somatic incongruity. In the cases with much elimination, relatively unrelated species (from different genera or families) were fused. No symmetric hybrids could be obtained from these species. Therefore, if somatic incongruity occurs, only hybrids with a minimal amount of donor genome can survive. When related species were fused, the elimination of donor genome was often limited (e.g. Famelaer et al. 1989; Yamashita et al. 1988). Those species were somatic congruent, because also symmetric hybrids could be obtained. An exception are some of the asymmetric hybrids of the related species *Nicotiana tabacum* (+) *N. plumbaginifolia* (donor), that contained only one donor chromosome (Bates et al. 1987).

Kanamycin resistance was retained in 46 percent of the 30H-hybrids, that derived from a donor parent with one NPT II locus. This percentage is an average, obtained from the analysis of 16 independent kanamycin resistant *L. peruvianum* genotypes. If the NPT II gene integrates randomly in the *L. peruvianum* genome, this would imply that any allele of the donor genome is retained in about 50% of the hybrids. In contrast with the single kanamycin resistance allele, the isozyme and morphological markers were represented by two alleles in the *L. peruvianum* donor genome. When a hybrid isozyme pattern or complementation of a mutant phenotype was observed, either one or two donor alleles were present. The average chance that a certain allele is retained, can be deduced from the number of hybrids which had lost both homologous alleles. The isozyme markers (Table 3) and morphological markers (Table 4) were distributed more or less at random over the genome. The frequences of loss of both donor alleles of a given locus in the 30H-hybrids was, on the average, 21% to 30% ("mixed" is considered as lost in the latter). An individual *L. peruvianum* allele is thus lost with a frequency of 0.21 to 0.30 = 46% to 55%, and is therefore retained with a frequency of 45% to 54%. These frequencies agree well with the frequency of retention of the kanamycin resistance alleles.

We frequently observed that shoots, which derived from a same hybrid callus, differed with respect to one or more or the analysed markers, namely in two of the 38 tested 5H-hybrids (5%), 10 of the 63 30H-hybrids (16%) and one of the 9 100H-hybrids (11%). Apparently, the higher dose hybrids showed this phenomenon more often. The segregation occurred most probably in the hybrid callus. Segregation at the plant level was shown in one hybrid shoot containing sectors in the leaf that were *marmorata*, the phenotype of the recipient LA1164 (Table 4). Somatic segregation has also been observed in asymmetric hybrid calli and plants of *Nicotiana plumbaginifolia* (+) (irradiated) *N. sylvestris* for several isozymes (Famelaer et al. 1989).

Because the complementation of only one of two genes on the same chromosome occurred rather frequently (Table 5), a large fraction of the asymmetric somatic hybrids must have contained incomplete chromosomes of *L. peruvianum*. In each fusion combination this could be analysed for only one or two chromosomes. Even when both markers were complemented by *L. peruvianum* alleles, this may have resulted from the retention of two incomplete chromosomes. Therefore, we conclude that the high dose of gamma irradiation (300 Gy) induced many breaks in the *L. peruvianum* chromosomes. This was confirmed by the RFLP analysis of 7 30H-hybrids, in which, on the average, at least 12 of the 18 retained donor chromosomes were incomplete (Chapter 6).

The asymmetric somatic hybrids were selected on regeneration capacity. This trait of *L. peruvianum* is supposed to be governed by two unlinked, dominant genes (Koornneef et al. 1987a). Theoretically, it should have been possible to locate these regeneration genes in our experiments, because of the availability of marker genes for each of the chromosomes. Genes of *L. peruvianum* linked to the regeneration genes, should be present in the asymmetric hybrids. However, we were not able to locate these genes. This could have been caused by: (i) the presence of many *L. peruvianum* chromosomes in the asymmetric hybrids; (ii) the small number of asymmetric hybrids that had lost any given gene; (iii) the high frequency of breakage of the donor chromosomes, so that only closely linked genes could be used for this analysis. Nevertheless, we can state that the regeneration genes are not closely linked to *sy*, *sf* (both mapped on chromosome 3), *hl* and *a* (both chromosome 11), because both *L. peruvianum* alleles of these genes were absent in a large fraction of the asymmetric hybrids.

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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

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Molecular Biology Reporter 1:19-21

Dudits D, Fejer O, Hadlaczky GY, Koncz CS, Lazar G, Horvath G (1980) Intergeneric gene transfer mediated by plant protoplast fusion. Mol Gen Genet 179:283-288

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

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

Gleba YY, Hinnisdaels S, Sidorov VA, Kaleda VA, Parokonny AS, Boryshuk NV, Cherup NN, Negrutiu I, Jacobs M (1988) Intergeneric asymmetric hybrids between Nicotiana plumbaginifolia and Atropa belladonna obtained by "gamma-fusion". Theor Appl Genet 76:760-766

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

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

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

Mutschler MA, Tanksley SD, Rick CM (1987) Linkage maps of the tomato (Lycopersicon esculentum). Rep Tomato Genet Coop 37:5-34

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

Rick CM (1983) Tomato (Lycopersicon). In: Tanksley SD, Orton TJ (eds) Isozymes in plant genetics and breeding, part B. Elsevier, Amsterdam, pp 147-165

Somers DA, Narayanan KR, Kleinhofs A, Cooper-Bland S, Cocking EC (1986) Immunological evidence for transfer of the barley nitrate reductase structural gene to Nicotiana tabacum by protoplast fusion. Mol Gen Genet 204:296-301

Suurs LCJM, Jongedijk E, Tan MMC (1989) Polyacrylamide gradient-gel electrophoresis: a routine method for high resolution isozyme electrophoresis of Solanum and Lycopersicon species. Euphytica 40:181-186

Vallejos CE (1983) Enzyme activity staining. In: Tanksley SD, Orton TJ (eds) Isozymes in plant genetics and breeding, part A. Elsevier, Amsterdam, pp 469-515

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

Young ND, Tanksley SD (1989) Restriction fragment length polymorphism maps and the concept of graphical genotypes. Theor Appl Genet 77:95-101

CHAPTER 6

ASYMMETRIC SOMATIC HYBRIDS BETWEEN *LYCOPERSICON ESCULENTUM* AND IRRADIATED *LYCOPERSICON PERUVIANUM* III. ANALYSIS WITH RESTRICTION FRAGMENT LENGTH POLYMORPHISMS

J. Wijbrandi, P. Zabel, M. Koornneef

Summary. The genome composition of asymmetric somatic hybrids, obtained by fusion of leaf protoplasts from *Lycopersicon esculentum* and gamma-irradiated leaf protoplasts from *L. peruvianum*, was characterised by Southern blot analysis with 29 RFLP markers and a rDNA probe. Eight "low dose hybrids" and seven "high dose hybrids" (irradiation dose 50 Gy and 300 Gy, respectively) were analysed. By densitometric scanning of the autoradiographs, the number of alleles for each locus of the component species was established. In general, elimination of alleles from the irradiated *L. peruvianum* donor genome was limited and ranged from 17% to 69%. Three *L. peruvianum* loci, located on chromosome 2, 4 and 7, respectively, were present in each asymmetric hybrid, which may suggest linkage to the regeneration capacity trait which was used in selecting the asymmetric hybrids. The loss of donor genome was dose dependent. Low dose hybrids contained more alleles, loci and complete chromosomes from *L. peruvianum* than high dose hybrids, whereas the high dose hybrids contained more incomplete chromosomes. In most hybrids some *L. esculentum* alleles were lost. The amount of ribosomal DNA from *L. peruvianum* retained in the asymmetric hybrids varied strongly: in one hybrid amplification had occurred, whereas in others *L. peruvianum* rDNA was either absent or (in)completely present. The amount of rDNA from *L. esculentum* was decreased in most asymmetric hybrids. The possible implications of these results for the use of asymmetric hybrids in plant breeding are discussed.

Introduction

Various strategies have been developed for combining the genetic information of two plant species which can not be hybridised sexually. While DNA transformation techniques are particularly designed to allow the introduction of defined but small-sized DNA fragments into a recipient species, protoplast fusion and related cellular techniques are the obvious means for transferring large chromosomal segments or even complete genomes (for a review concerning tomato, see Hille et al. 1989). However, in most protoplast fusion experiments, only a limited number of traits from the donor species is of interest and elimination of the bulk of the donor genome has to be achieved. In mammalian somatic cell hybrids, preferential loss of chromosomes from one parent species often occurs spontaneously (Ringertz and Savage 1976). In plant somatic cell hybrids, however, chromosome elimination is limited and, in general, does not concern specifically the

chromosomes of one of the component species (Gleba and Sytnik 1984). In an attempt to decrease the amount of donor genome, several investigators have irradiated the donor protoplasts before fusion with Röntgen- or gamma-rays, so as to induce loss of entire chromosomes or chromosome segments. In some cases, 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. 1988; Gleba et al. 1988; Imamura et al. 1987; Müller-Gensert and Schieder 1987; Yamashita et al. 1989).

In the present paper we have analysed the effects of irradiation of *L. peruvianum* on the elimination of the *L. peruvianum* genome in *L. esculentum* (+) *L. peruvianum* somatic hybrids. For this purpose *L. peruvianum* protoplasts were irradiated before fusion with either a low or a high dose of gamma-rays, and the asymmetric somatic hybrids were selected on the basis of the superior regeneration capacity of *L. peruvianum*. Previously, evidence has been presented showing that the regeneration capacity trait of *L. peruvianum* is dominant (Adams and Quiros 1985; Kinshara et al. 1986; Koornneef et al. 1987a; Wijbrandi et al. 1988) and likely to be controlled by a limited number of genes (Koornneef et al. 1987a). By means of 30 RFLP markers of known chromosomal location, the genome constitution of a number of asymmetric hybrids was established.

Materials and methods

Plant material

Somatic hybrid plants were obtained from independent protoplast fusion events (Wijbrandi et al. 1988). The parental genotypes of the hybrids are shown in Table 1. Seeds of the tomato cultivar Bellina, the LA-genotypes and the *L. peruvianum* accession PI128650 were kindly provided by Rijk Zwaan Seed Company (de Lier, The Netherlands), Prof. C.M. Rick (Tomato Genetics Stock Center, Davis, USA) and the Institute of Horticultural Plant Breeding (Wageningen, The Netherlands), respectively. The *L. peruvianum* parent was irradiated before fusion with a dose of either 50 Gray ("low dose hybrids", designated as 5H) or 300 Gray ("high dose hybrids", 30H), using a ^{60}Co source at a dose rate of about 2000 Gray/hour (at the Pilot-Plant for Food Irradiation, Wageningen, The Netherlands). The symmetric somatic hybrid 0H1 (2n = 4x), *L. esculentum* cv. Bellina, and *L. peruvianum* PI128650-ATW2002 were used as controls in DNA analysis.

Hybridisation probes

The following tomato single copy clones were used: (i) the cDNA clones CD1, CD14, CD15, CD27, CD41, CD50, CD56, CD59 (Bernatsky and Tanksley 1986) and "Adh2" (pCB25E6, containing a part of the Adh-2 coding region; Chase and Williams 1986, see also van Daelen et al. 1989). (ii) the genomic clones TG8, TG9, TG16, TG20, TG27, TG30, TG31, TG34, TG37, TG42, TG43, TG44, TG45, TG50, TG54, TG61, TG62, TG63, TG68, TG69 (Tanksley et al. 1988) and "Adh1" (pTAdh-1, a genomic 1.8 kb fragment cloned in the *Eco* RI-*Sal* I site of pTZ18R). The CD- and TG-clones were kindly supplied by Dr. S.D. Tanksley, Cornell University, Ithaca (USA), pCB25E6 was a gift from Dr. Th. Chase Jr., Rutgers University, New Jersey (USA), and pTAdh-1 from Dr. E. Lifschytz, Technion-Israel Institute of Technology, Haifa

Table 1. The parental genotypes of the somatic hybrids and the dose of gamma-irradiation applied to the donor.

Hybrid	Recipient (*L. esculentum*)	Donor (*L. peruvianum*)	Dose (Gray)
0H1	cv. Bellina	PI128650-ATW2002*	0
5H2, 5H3, 5H5, 5H7, 5H13, 5H16, 5H28	cv. Bellina	PI128650-ATW2002*	50
5H22	cv. Bellina	PI128650	50
30H1, 30H3, 30H7	cv. Bellina	PI128650-ATW2002*	300
30H6, 30H12, 30H27	LA 1182**	PI128650-ATW2003*	300
30H26	LA 291***	PI128650-ATW2001*	300

* transgenic plants containing a kanamycin resistance gene (see Koornneef et al. 1987b).
** tomato genotype, homozygous recessive for *sy*, *sf* (chr. 3), and *alb* (chr. 12) (Rick 1982).
*** tomato genotype, homozygous recessive for *hl* and *a* (chr. 11) (Rick 1982).

(Israel). A pea genomic clone for 45S rRNA, "R45S" (pBsλR1), was a gift from Dr. J.P. Nap, Research Institute Ital, Wageningen (The Netherlands). The map positions of the clones used in the hybrid analysis, are shown in Fig. 1. Except for the ribosomal clone, the insert of each clone was excised from the vector with the appropriate restriction endonucleases and separated from the vector on a 1% agarose gel. DNA fragments were radiolabeled with [^{32}P]dATP by means of the random-priming method (Feinberg and Vogelstein, 1983).

Plant DNA analysis

Total DNA was extracted from leaves (fresh or freeze dried) of plants grown in the greenhouse, as described by Dellaporta et al. (1983). DNA samples (2.5 μg) were digested to completion with *Eco* RI, *Eco* RV, *Hin* dIII, *Dra* I or *Pst* I (5 hours at 37 °C in buffers according to Maniatis et al. 1982), separated by electrophoresis on 0.7% agarose gels and transferred to Gene Screen PLUS (New England Nuclear) by means of an alkaline blotting procedure (Reed and Mann 1985). Hybridisation in 10% dextran sulphate, 1% SDS and 1M NaCl, at 65 °C, was carried out for 18 hours under conditions as recommended by the manufacturer of the membrane. After hybridisation, blots were washed under stringent conditions: three 5 min.-washes in 2xSSC at 20 °C, two 20 min.-washes in 2xSSC + 1% SDS at 65 °C, two 30 min.-washes in 0.1xSSC + 1% SDS at 65 °C, and two 15 min.-washes in 0.1xSSC at 20 °C. Subsequently, the blots were exposed to X-ray film (Kodak X-Omat AR or Konica H7A) at -80 °C with intensifying screens. After autoradiography, blots were deprobed in 0.4N NaOH at 42 °C according to the manufacturer. The blots could thus be used at least four times.

To obtain quantitative data on the relative intensity of bands derived from both parents, autoradiographs were scanned with a LKB Ultrascan XL Laser Densitometer connected to an Olivetti M24 personal computer, which was equipped with the LKB 2400 GelScan XL software. The following calculations were made: (i) the relative number of *L. peruvianum* and *L. esculentum* specific alleles for a given probe in each asymmetric hybrid was determined as the ratio of the absorption values (peak areas) of the relevant bands on the autoradiographs. This ratio was divided by the corresponding ratio in the symmetric hybrid 0H1 that was used as a reference (=1);

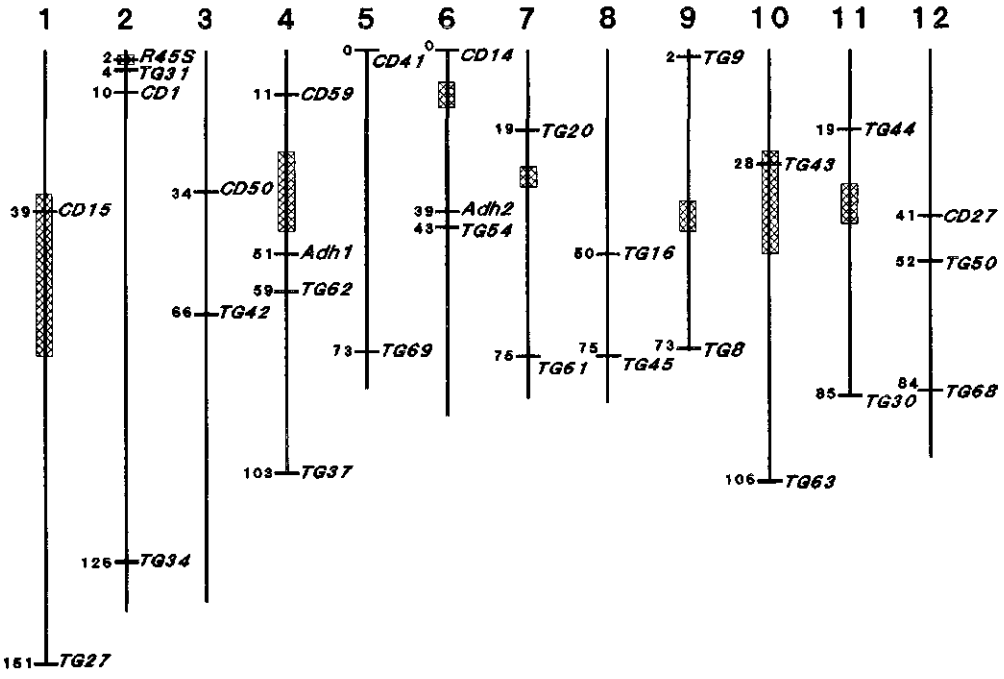


Fig. 1. Linkage map of the RFLP markers used in the analysis of the asymmetric somatic hybrids. The shaded areas indicate the centromere regions. All positions are according to Young and Tanksley (1989).

(ii) the ratio of the absorption values of the *L. esculentum* specific band in each asymmetric hybrid and that in the symmetric hybrid OH1 was determined for a given probe. If all *L. esculentum* genomes in the asymmetric hybrid were complete, this ratio should be the same for each probe that was hybridised to the same blot; the use of the ratios from one blot excluded possible variations in the amount of DNA loaded/transferred from each hybrid. Deviating ratios for individual probes in an asymmetric hybrid were therefore interpreted as deviations in the amount of *L. esculentum* specific DNA/alleles.

Results

Restriction fragment length polymorphisms (RFLPs) between L. esculentum and L. peruvianum

To establish probe-restriction enzyme combinations which reveal polymorphisms between the two parents, Southern blots of DNA from *L. esculentum*, *L. peruvianum* and OH1, a symmetric somatic hybrid between both species, were hybridised to each of the RFLP markers mentioned in the Materials and methods section. Each probe was tested on DNA digested with the restriction enzymes *Eco* RI, *Hin* dIII or

Dra I; in addition, 13 and 22 probes were tested on *Eco* RV- and *Pst* I-digests, respectively (Table 2). For each of the enzymes tested, approximately a third of the probes showed a "useful" polymorphism in that each of the two parents displayed one or more unique bands. Approximately an other third of the probes revealed a polymorphism, referred to as "not useful", with only one species

Table 2. Restriction fragment length polymorphisms (RFLPs) between *L. esculentum* and *L. peruvianum*. Plant DNA was digested with *Eco* RI, *Eco* RV, *Hin* dIII, *Dra* I or *Pst* I, fractionated by agarose gel electrophoresis, transferred to Gene Screen PLUS and hybridised with DNA from the clones indicated. + = "useful" RFLP, both parents have (a) unique band(s); +/- = "not useful" RFLP, only one of the parents has a unique band; - = no RFLP; nd = not determined. The right column (total) indicates whether a "useful" RFLP was found for each probe with the restriction enzymes tested. The lower panel shows the totals of "useful", "not useful" and no RFLPs for each restriction enzyme.

Probe	*Eco* RI	*Eco* RV	*Hin* dIII	*Dra* I	*Pst* I	Total
TG8	+/-	nd	+	+	+	+
TG9	-	nd	+	-	nd	+
TG16	+/-	+/-	+/-	+	-	+
TG20	+/-	nd	+	+/-	-	+
TG27	+	nd	+	+/-	+	+
TG30	+/-	nd	+	+/-	nd	+
TG31	+	nd	-	-	-	+
TG34	+/-	nd	+	+/-	-	+
TG37	+/-	nd	+/-	+	-	+
TG42	+	+/-	+/-	+/-	+/-	+
TG43	+/-	+	+/-	+/-	-	+
TG44	+	-	+/-	-	-	+
TG45	-	-	-	+	-	+
TG50	+/-	nd	+/-	+	+	+
TG54	+/-	nd	+/-	+	+/-	+
TG61	+/-	+	-	+/-	+/-	+
TG62	-	+	-	-	-	+
TG63	+	nd	-	+	-	+
TG68	+	-	+	-	nd	+
TG69	+	+	+/-	+/-	nd	+
CD1 *	-	+	+/-	+	nd	+
CD14	-	nd	+	+	nd	+
CD15	+	nd	+	+	+	+
CD27	+	nd	-	+	-	+
CD41	+/-	+	+/-	+/-	+/-	+
CD50	-	+	+	+/-	nd	+
CD56	+/-	nd	+/-	-	nd	+/-
CD59	+	nd	+/-	-	nd	+
"Adh1"	+/-	nd	+/-	+/-	+	+
"Adh2"	+	+	+	+	nd	+
"R45S"	-	nd	-	+	-	+
+	11	8	11	13	5	30
+/-	13	2	13	11	4	1
-	7	3	7	7	12	0

* excision of the insert of CD1 with *Pst* I yielded three fragments of 0.5 kb, 1.0 kb and 1.8 kb, respectively; the 1.0 kb fragment was used in the hybridisation experiments.

containing one or more specific bands. By testing the five enzymes mentioned above, a polymorphism was found for each of the 31 probes, 30 of which showed at least one "useful" RFLP (Table 2; the map positions of the "useful" clones are shown in Fig. 1). Seven (TG8, TG34, TG63, TG68, TG69, CD50 and "Adh1") out of the 31 probes produced a hybridisation signal with DNA from *L. peruvianum* that was markedly weaker than with *L. esculentum* DNA. As expected, the symmetric somatic hybrid OH1 showed the composite pattern for all clones tested.

Analysis of asymmetric somatic hybrids

Southern blots of appropriate restriction enzyme digests of DNA from eight "low dose hybrids" (5H-hybrids), seven "high dose hybrids" (30H-hybrids) and the controls *L. esculentum*, OH1 and *L. peruvianum* were hybridised with 29 single copy clones and a rDNA clone. With respect to the number of *L. peruvianum* specific fragments hybridising, two classes of restriction patterns were distinguished, the single and multi-fragment patterns, respectively. As an example of the first class, hybridisation patterns of two clones located on chromosome 2, TG31 and TG34, are shown in Fig. 2A and 2B. Both clones produced a simple RFLP pattern with each species exhibiting a single, unique fragment. The *L. esculentum* specific TG31 and TG34 bands were present in all hybrids, as was the case with the *L. peruvianum* specific TG34 band. In contrast, the *L. peruvianum* specific TG31 band was absent in four "high dose hybrids". The second class, the multi-fragment patterns, was represented by eleven probes (TG27, TG62, TG37, CD41, CD14, 'Adh2', TG20, TG16, TG30, TG44 and TG50) and showed a pattern such as exemplified by clone TG50 in Fig. 2C: one band present in both parental species, one *L. esculentum* specific band and two major *L. peruvianum* specific bands. The asymmetric hybrids surveyed for the presence of the *L. peruvianum* TG50 alleles, were found to contain either none, one, or two bands, which indicates heterozygosity for the TG50 locus in *L. peruvianum* and loss of specific alleles in the hybrids.

For all probes, a marked variation in the intensity of the hybridisation signal between *L. peruvianum* and *L. esculentum* specific bands was observed among the asymmetric hybrids. To quantify these differences, the autoradiographs were scanned with a densitometer. The ratio of the band intensities in the symmetric hybrid was used as a reference. As an example of such an analysis, the densitographs of the TG31 patterns of four asymmetric hybrids are shown in Fig. 3. The ratio of the band intensities from both parents, as calculated from the peak areas within one lane, was (*L. esculentum* : *L. peruvianum*) 1:1 (hybrid 5H22), 2:1 (30H3), 4:1 (30H1) and x:0 (30H6). Similarly, these ratios, which can be interpreted as relative numbers of *L. peruvianum* alleles contained within the hybrids, were calculated for all clones hybridising to the fifteen asymmetric

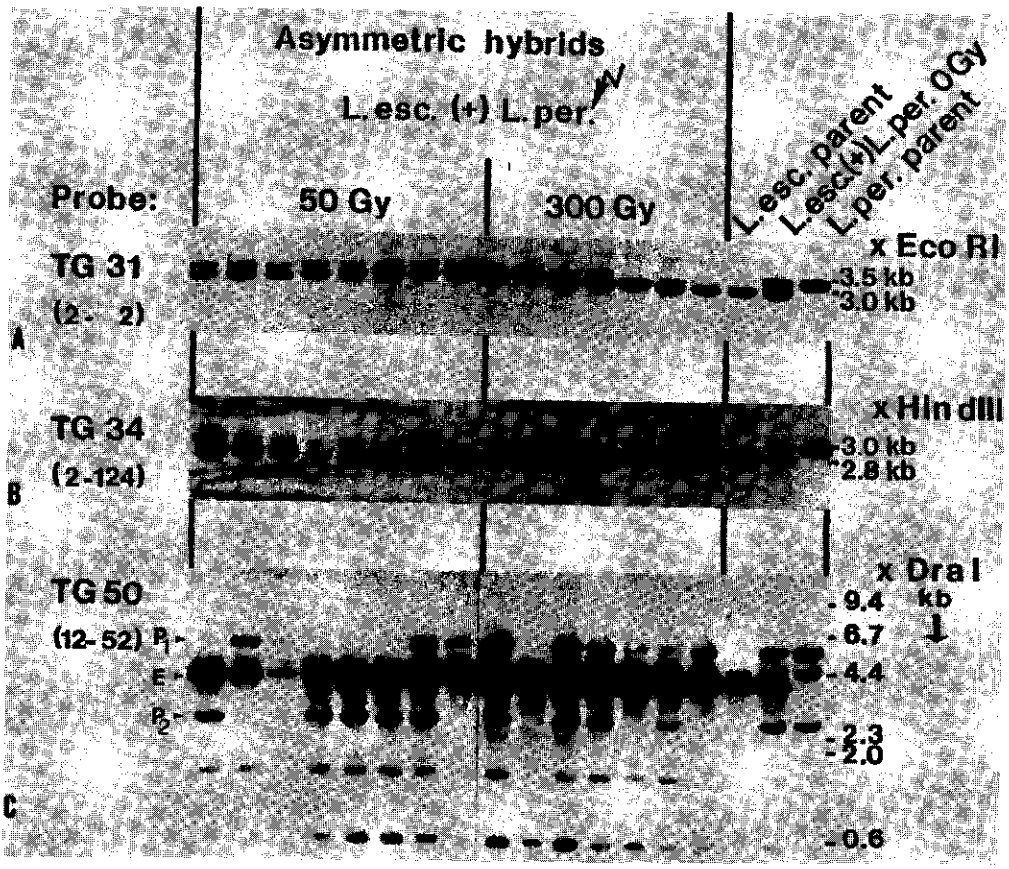
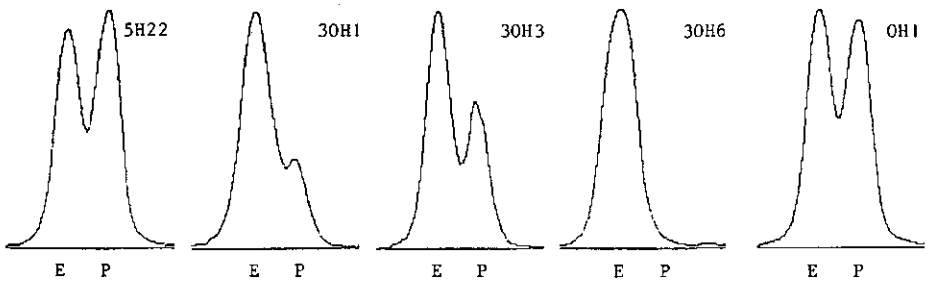


Fig. 2. Survey for the presence of the *L. peruvianum* specific alleles of TG31, TG34, and TG50 in asymmetric hybrids by Southern blot analysis. DNA from eight low dose hybrids (5H2, 5H3, 5H5, 5H7, 5H13, 5H16, 5H22 and 5H28; lanes 1-8), seven high dose hybrids (30H1, 30H3, 30H6, 30H7, 30H12, 30H26 and 30H27; lanes 9-15), *L. esculentum* cv. Bellina (lane 16), symmetric hybrid 0H1 (lane 17) and *L. peruvianum* PI128650-ATW2002 (lane 18) was digested with the restriction enzymes indicated, electrophoresed, blotted and hybridised with the clone indicated.
A. Hybridisation with clone TG31. A single fragment was detected in the respective parents. The size of the fragments is indicated at the right.
B. Hybridisation with clone TG34. A single fragment was detected in the respective parents. The size of the fragments is indicated at the right.
C. Hybridisation with clone TG50. P_1 and P_2 at the left indicate the two major *L. peruvianum* specific bands and E the *L. esculentum* specific band. The position of the *Hin* dIII fragments of phage lambda DNA is indicated at the right.

hybrids. Most hybrids showed ratios of either x:0, 4:1, 2:1 or 1:1. One hybrid (30H12) showed ratios of x:0, 6:1 and 3:1, suggesting a higher number of *L. esculentum* alleles for each locus than in the other hybrids. In a few cases (TG61 in hybrid 5H7, TG16 and TG45 in hybrid 5H28) a ratio of 1:2 was found, indicating the presence of two *L. peruvianum* alleles for each *L. esculentum* allele. In some other cases (TG43 in hybrids 30H3, 30H7 and 30H27; TG9, TG8, CD27 and TG50 in hybrid 30H12) ratios higher than 4:1 (or 6:1) were found, suggesting total elimination of these *L. peruvianum* alleles in part of the plant.

Thus, by determining the relative number of a series of *L. peruvianum* specific alleles, we could assess the genetic make-up of each asymmetric hybrid. Because the chromosome number, as counted in metaphase plates of root tip cells, was known for each hybrid, we could also deduce the absolute number of each allele (Table 3, 4 and 5). As expected, the low dose hybrids contained more loci and alleles from *L. peruvianum* than the high dose hybrids (Table 3). This



Hybrid	Peak areas			Factor	E/P x factor	Allele ratio
	E	P	E/P			(E:P)
0H1	2.70	2.59	1.04	0.96	1	1:1
5H22	2.43	2.55	0.95		0.91	1:1
30H1	2.82	0.63	4.48		4.29	4:1
30H3	1.67	0.91	1.84		1.76	2:1
30H6	5.27	0	∞		∞	x:0*

* x is an integer > 0

Fig. 3. Densitographs of *Eco* RI-digested DNA from the low dose hybrid 5H22, the high dose hybrids 30H1, 30H3, 30H6 and the symmetric hybrid 0H1, probed with TG31. The autoradiograph shown in Fig. 2A, upper panel, was scanned (left side of each scan corresponds to the low molecular weight region of the autoradiograph) and the species specific peak areas (P, *L. peruvianum*; E, *L. esculentum*) were calculated. The ratio of the peak areas was determined (E/P) and normalised by a correction factor deduced from the ratio of the peak areas of 0H1, which was used as a reference (E/P = 1), These ratios are shown in the table below the densitographs; the ratios of alleles in the asymmetric hybrids were determined from these corrected values.

Table 3. The amount of *L. peruvianum* genome (number of loci, alleles and chromosomes) in the "low dose"- and "high dose" asymmetric hybrids, as determined by autoradiographic analysis and densitometric scanning of Southern blots hybridised with 29 single copy clones (29 RFLPs = 29 loci = 58 alleles). A chromosome was considered complete when for each locus of the chromosome the same number of alleles was found.

	Low dose hybrids (n=8)	High dose hybrids (n=7)
Loci (n=29) *	27.3 ± 3.4 (94%)	20.4 ± 4.3 (70%)
Alleles (n=58)	38.6 ± 7.8 (67%)	30.6 ±11.2 (53%)
Complete chromosomes *	13.4 ± 2.8	7.1 ± 4.1
Incomplete chromosomes *	4.9 ± 1.6	11.4 ± 3.2
Lost chromosomes	5.8 ± 3.6	6.9 ± 3.9

* significant difference between the two groups of hybrids, as tested with Student's t test ($P < 0.01$).

difference was also obvious at the chromosome level; a chromosome was considered complete when for each locus of the chromosome the same number of alleles was present. Low dose hybrids contained more complete *L. peruvianum* chromosomes than high dose hybrids, whereas the high dose hybrids contained a higher number of incomplete chromosomes. Surprisingly, the number of lost *L. peruvianum* chromosomes was low and virtually the same for both hybrid types (Table 3). In general, the chromosome numbers derived from the RFLP mapping data approximated the number of chromosomes as determined by counting metaphase plates of root tips (Table 4). In ten out of fifteen asymmetric hybrids, the total number of complete chromosomes from *L. esculentum* and *L. peruvianum* as determined by RFLP analysis was somewhat lower than the number of counted chromosomes, whereas the total number of complete plus incomplete chromosomes was somewhat higher.

In most hybrids, one to several *L. esculentum* specific alleles were lost (determined as described in Materials and methods section), as shown in Table 4. Except for two low dose hybrids (5H3 and 5H5), no euploid number of complete *L. esculentum* chromosomes was found among the asymmetric hybrids. Four hybrid plants had lost one to three complete *L. esculentum* chromosomes, either by missing one out of two (5H28, chromosome 8), one out of four (5H16, chromosomes 3, 4 and 9; 30H7, chromosome 9), one out of six (30H12, chromosome 3) or two out of six (30H12, chromosome 9) homologous chromosomes.

Table 4. The number of *L. esculentum* (E) and *L. peruvianum* (P) chromosomes in 8 low dose asymmetric hybrids (5H2 to 5H28) and 7 high dose asymmetric hybrids (30H1 to 30H27), as determined by RFLP analysis with 29 single copy clones and by chromosome counting in metaphase plates of root tip cells. The chromosome numbers of the parental species and a symmetric hybrid (0H1) are listed below. The total number of presumed complete chromosomes, indicated as "c", and the total number of incomplete chromosomes, "+inc" between parentheses, of each genotype are given as well.

Genotype	Chromosome number							
	RFLP analysis							metaphases
	complete		incomplete		lost			
	E	P	E	P	E	P	total c(+inc)	2n
5H2	23	13	1	8	0	3	36(+9)	37-40
5H3	24	14	0	4	0	6	38(+4)	37-41
5H5	24	16	0	4	0	4	40(+4)	39-43
5H7	20	13	4	4	0	7	33(+8)	41-43
5H13	45	13	3	6	0	5	58(+9)	59-63
5H16	45	7	0	4	3	13	52(+4)	56-58
5H22	23	16	1	6	0	2	39(+7)	39-45
5H28	23	14	0	6	1	4	37(+6)	35-42
30H1	45	6	3	15	0	6	51(+18)	56-62
30H3	44	7	4	8	0	9	51(+12)	54-59
30H6	44	4	4	7	0	13	48(+11)	50-58
30H7	45	6	2	11	1	7	51(+13)	55-61
30H12	62	6	7	12	3	7	68(+19)	76-85
30H26	43	16	5	14	0	0	59(+19)	64-70
30H27	43	7	5	11	0	6	50(+16)	68-70
L. esculentum	24	0	0	0	0	0	24(+0)	24
0H1	24	24	0	0	0	0	48(+0)	48
L. peruvianum	0	24	0	0	0	0	24(+0)	24

To reveal a possible linkage of these loci with regeneration capacity, which trait was used to select the asymmetric hybrids, the presence of 30 individual *L. peruvianum* loci among the fifteen asymmetric hybrids was determined (Table 5). Only three loci, on chromosome 2 (TG34), 4 (CD59) and 7 (TG61), respectively, were present in all hybrids. None of the loci was absent from all hybrids.

We also analysed the representation of individual *L. peruvianum* chromosomes in the asymmetric hybrids. The results for chromosome 6 are shown in Fig. 4. With the exception of hybrid 5H16, all hybrids contained *L. peruvianum* specific alleles of chromosome 6 (Fig. 4). In ten hybrids all *L. peruvianum* loci tested were present, whereas in the remaining hybrids one or more of the loci were missing. Hybrids 30H3, 30H6 and 30H7 provided evidence for rearrangements of *L. peruvianum* loci. In 30H3 and 30H7, the *L. peruvianum* TG54 alleles were present, while on the other hand Adh2 alleles were lacking. Because Adh2 is located more closely to the centromere and chromosome fragments without centromere are not stably maintained, the chromosomal fragment bearing the *L. peruvianum* TG54 alleles must have been translocated to another chromosome or to a centromere-containing fragment. Similarly, in hybrid 30H6, the Adh2 and TG54 alleles from *L. peruvianum* were retained, but not the *Aps-1* and CD14 loci which are more proximal to and on either side of the centromere of chromosome 6

Table 5. Distribution of 30 *L. peruvianum* loci among 15 asymmetric hybrids, as deduced from the *L. peruvianum* specific bands on Southern blots following hybridisation with 29 single copy probes and one repeat probe. Between parentheses is given the chromosome on which each probe (= molecular marker) is located.

Present in no. of asymmetric hybrids	Molecular marker (chromosome)
15 hybrids	TG34 (2); CD59 (4); TG61 (7)
14 hybrids	TG54 (6); TG16 (8); TG8 (9); TG43 (10); TG63 (10); TG44 (11); TG30 (11); TG50 (12)
13 hybrids	R45S (2); Adh1 (4); TG20 (7)
12 hybrids	CD15 (1); TG27 (1); CD50 (3); TG37 (4); Adh2 (6); CD27 (12)
11 hybrids	TG31 (2); CD1 (2); TG42 (3); TG62 (4); CD41 (5); CD14 (6); TG45 (8)
10 hybrids	TG69 (5); TG68 (12)
9 hybrids	TG9 (9)

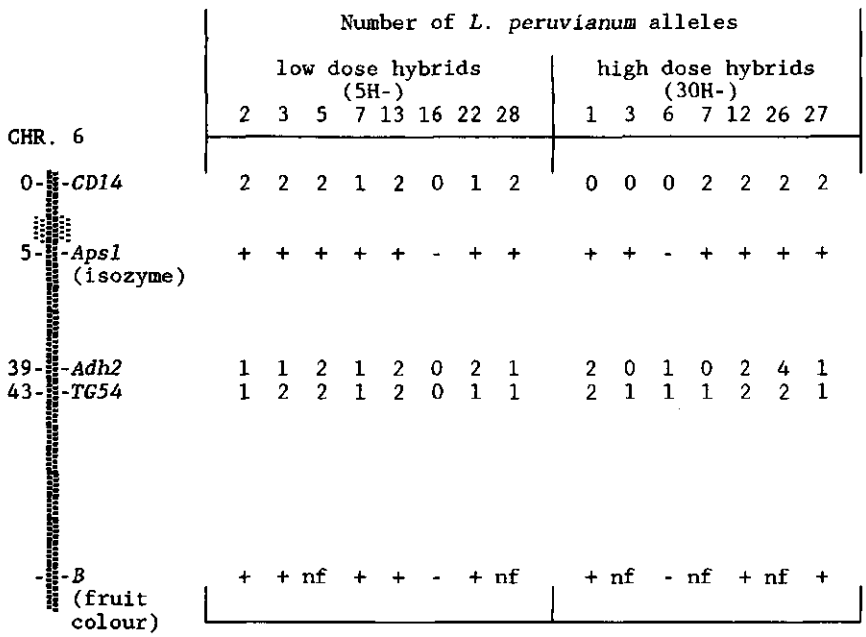


Fig. 4. Survey for the presence of *L. peruvianum* specific alleles of five loci located on chromosome 6 in eight low dose hybrids and seven high dose hybrids. The number of *L. peruvianum* alleles corresponding to the loci *CD14*, *Adh2* and *TG54* was determined by RFLP analysis. The presence (+) or absence (-) of the acid phosphatase-1 locus (*Aps1*) and the β-carotene locus (*B*) of *L. peruvianum* was detected by isozyme analysis and observation of the colour of the fruits (if present), respectively; nf, no fruits observed. The light shaded area indicates the centromere region. Map positions are according to Young and Tanksley (1989); the position of *B* is near the end of the long arm, at 106 cM on the classical map (Mutschler et al. 1987). Note the presence of the *Adh2* allele and the *TG54* allele in hybrid 30H6, while *CD14*, *Aps1* and *B* are absent.

(Fig. 4). These phenomena were not typical for loci on chromosome 6 of *L. peruvianum*. Similar results were obtained for other *L. peruvianum* chromosomes, where more than two probes were used (see Fig. 1).

Analysis of ribosomal DNA in the asymmetric hybrids

The ribosomal RNA genes (rDNA) of tomato are arranged in tandem repeating units at a single locus on the short arm of chromosome 2 (Dobrowolski et al. 1989; Ganal et al. 1988; Vallejos et al. 1986; Zabel et al. 1985) and represent approximately 3% of the genome. Ribosomal DNA was included in our analysis to determine whether the rDNAs of both species were present as complete sets in the asymmetric hybrids. We found a useful polymorphism between the *Dra* I-digestion patterns of the rDNA of *L. esculentum* and *L. peruvianum* (Table 2). In

addition to common bands at 1.8 and 4.0 kb, *L. esculentum* and *L. peruvianum* showed unique bands at 3.8 kb and 4.6 kb, respectively (lanes 16 and 18 in Fig. 5). The total fragment lengths of 9.6 kb (*L. esculentum*) and 10.4 kb (*L. peruvianum*) compares well with the repeating unit length of 8.6 to 11.0 kb described for *Lycopersicon* species (Dobrowolski et al. 1989; Vallejos et al. 1986).

Except for two low dose hybrids (5H3 and 5H28), all asymmetric hybrids contained the *L. peruvianum* specific 4.6 kb fragment. The ratio between *L. peruvianum* and *L. esculentum* alleles, as calculated from the peak areas of the characteristic bands, ranged from 0 to 2.1 (see Fig. 5). Values larger than 1.0 suggest that either amplification of *L. peruvianum* specific or loss of *L. esculentum* specific rDNA sequences had occurred. In order to quantify the

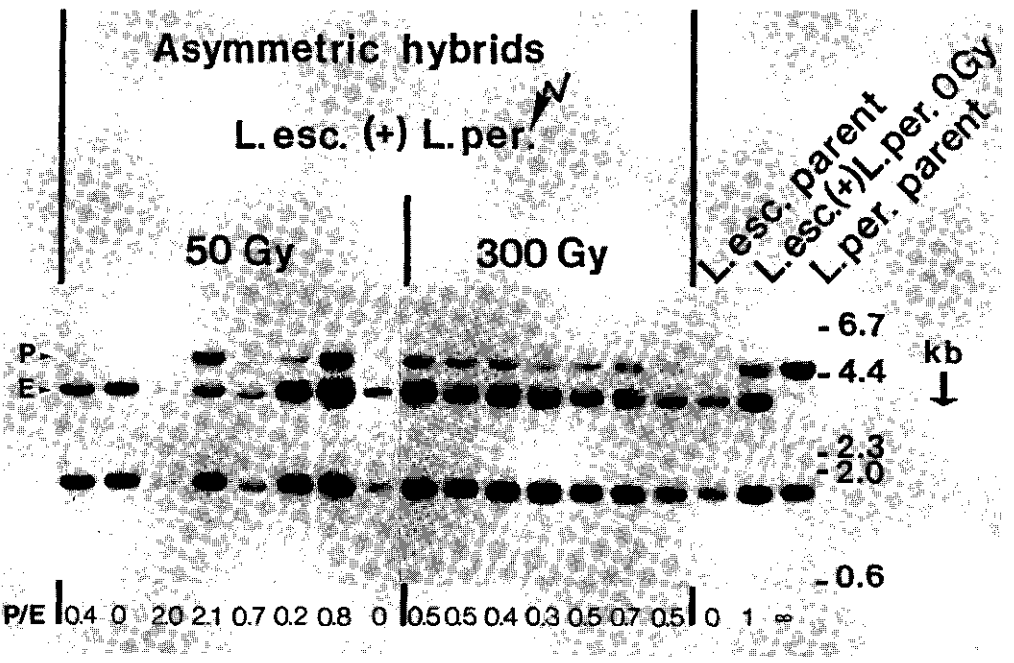


Fig. 5. *L. peruvianum* specific ribosomal DNA sequences in asymmetric hybrids. DNA from eight low dose hybrids (5H2, 5H3, 5H5, 5H7, 5H13, 5H16, 5H22, 5H28; lanes 1-8), seven high dose hybrids (30H1, 30H3, 30H6, 30H7, 30H12, 30H26, 30H27; lanes 9-15), *L. esculentum* cv. Bellina (lane 16), symmetric hybrid 0H1 (lane 17) and *L. peruvianum* PI128650-ATW2002 (lane 18) was digested with *Dra* I, electrophoresed, blotted and hybridised with pBsλR1, a ribosomal DNA clone from pea. P and E at the left indicate the *L. peruvianum* and *L. esculentum* specific band, respectively. Below each lane the ratio between the *L. peruvianum* alleles and the *L. esculentum* alleles is given (P/E) as calculated from the peak areas of densitometric scans (see Fig. 3). The ratio in the symmetric hybrid 0H1 was used as reference (P/E = 1). The position of the *Hin* dIII fragments of phage lambda DNA is indicated at the right.

amount of *L. esculentum* rDNA in each hybrid, we determined the densitometric absorption value of the *L. esculentum* specific band relative to the same band in the symmetric hybrid 0H1 (see Materials and methods section), assuming that the symmetric hybrid 0H1 contained the complete sets of rDNA repeats from both parents. The calculations revealed a large variation in the amount of *L. esculentum* specific rDNA in the asymmetric hybrids (Table 6). Among the six low dose hybrids containing a diploid *L. esculentum* genome (5H2, 5H3, 5H5, 5H7, 5H22 and 5H28), three (5H3, 5H5 and 5H22) had the expected two sets of rDNA repeats from *L. esculentum*, whereas the other three had a lower number. Eight hybrids (5H13, 5H16, 30H1, 30H3, 30H6, 30H7, 30H26 and 30H27) contained a tetraploid genome of *L. esculentum*, but their *L. esculentum* specific rDNA content was lower than the expected four sets and ranged from 0.6 to 3.2. The plant with a hexaploid *L. esculentum* genome (30H12) also had a lower number of rDNA sets

Table 6. The composition of the ribosomal DNA in the asymmetric hybrids, 8 low dose hybrids (5H2 to 5H28) and 7 high dose hybrids (30H1 to 30H27). The amount of each parental rDNA is given in sets; one set corresponds to one complete rRNA coding region located on one chromosome 2. The number of sets of *L. esculentum* was determined by the ratio of the densitometric absorption values of the *L. esculentum* band in each asymmetric hybrid with that in the symmetric hybrid 0H1. This ratio was divided by the ratios determined after hybridising the same blot with the single copy clones TG50 and TG54; the difference between the ratios obtained with TG50 and TG54 was less than 10% for each asymmetric hybrid. It was assumed that 0H1 contained two complete rDNA sets from each species. The amount of *L. peruvianum* sets was deduced from the P/E ratio in Fig. 5. The numbers of complete (c) plus incomplete (inc) chromosomes 2, based on RFLP analysis with three chromosome 2 specific single copy probes, are given between brackets for each genotype.

Genotype	Number of rDNA sets [c + inc chr. 2]			
	L. esculentum		*L. peruvianum*	
5H2	1.2	[2+0]	0.5	[1+1]
5H3	1.8	[2+0]	0.0	[1+0]
5H5	2.1	[2+0]	4.2	[1+0]
5H7	1.2	[1+1]	2.5	[1+0]
5H13	0.6	[4+0]	0.4	[1+1]
5H16	3.2	[4+0]	0.8	[1+0]
5H22	2.2	[2+0]	1.9	[2+0]
5H28	1.2	[2+0]	0.0	[1+1]
30H1	3.1	[3+1]	1.6	[0+1]
30H3	2.6	[4+0]	1.3	[0+2]
30H6	2.3	[4+0]	1.0	[0+1]
30H7	2.8	[4+0]	1.0	[1+0]
30H12	4.2	[5+1]	2.0	[0+1]
30H26	2.9	[4+0]	2.1	[0+2]
30H27	2.4	[3+1]	1.2	[0+1]
0H1	2	[2+0]	2	[2+0]

than expected.

The amount of *L. peruvianum* specific rDNA (expressed in sets of repeats) was calculated from the *L. peruvianum/L. esculentum* ratio shown in Fig. 5 and found to range from 0 to 4.2. In various hybrids (5H5, 5H7, 30H1 and 30H12) the number of rDNA sets was higher than the number of chromosomes 2 (complete and incomplete); this suggests either preferential retention or amplification of the *L. peruvianum* rDNA repeating units. In hybrid 5H5, the *L. peruvianum* rDNA was clearly amplified.

Discussion

RFLPs between L. esculentum and L. peruvianum

In this paper we have shown that RFLP analysis is an effective means of characterizing, qualitatively as well as quantitatively, the genome composition of asymmetric somatic hybrids obtained after fusion of protoplasts of the divergent species *L. esculentum* and *L. peruvianum* (See also Young et al. 1988). Using only a limited number of restriction enzymes, we could readily establish diagnostic RFLPs for 30 of the 31 molecular markers tested. These RFLPs allow each chromosome of the respective *Lycopersicon* species to be identified in the hybrid by at least two markers. In conjunction with studies by Young et al. (1988), which show that a monogenic trait residing on a small *L. peruvianum* DNA segment introgressed into *L. esculentum* can be recognised in the *L. esculentum* DNA background by virtue of RFLPs associated with the specific target gene, we conclude that, in principle, any *L. peruvianum* sequence, introduced into *L. esculentum* should be identifiable by RFLP analysis.

While the level of variability, as detected by RFLPs, is high among *Lycopersicon* species, *L. esculentum* genotypes show few polymorphisms (Helentjaris et al. 1985). This has been attributed to the inbred character of *L. esculentum*. Because *L. peruvianum* is an outbreeding species, we expected a high level of RFLPs in this species, and this was indeed what we found. The *L. peruvianum* plant used in the present experiments, which was derived from an accession that was maintained by sibmating a limited number of plants for many generations, was heterozygous for the isozyme acid phosphatase-1 (*Aps-1*; Chapter 2) and for 11 of the 30 DNA probes tested. Besides, with 20 out of 22 probes polymorphisms were detected in another individual from the same population (data not shown); in addition, two isozymes (*Aps-1* and glutamate oxaloacetate transaminase) showed different patterns for both plants (Chapter 2). These observations agree with those of Young et al. (1988), who described RFLPs for two loci between several individuals of the same accession of *L. peruvianum*.

Genomic constitution of the asymmetric somatic hybrids

One of the major conclusions of the present experiments is that asymmetric somatic hybrids obtained by fusion of *L. esculentum* protoplasts with gamma-irradiated *L. peruvianum* protoplasts and selected for the regeneration trait of *L. peruvianum*, contain a substantial amount of the donor genome. Elimination of the *L. peruvianum* genome was limited in the 15 asymmetric hybrids tested: 17% to 69% of the donor alleles, as determined by RFLP analysis, and 13% to 88% of the donor chromosomes, as determined by counts of metaphase plates, was eliminated. Similar results have been reported for asymmetric somatic hybrids of other plant species. Thus, on the basis of chromosome numbers, elimination of the donor genome was found to range from 10% to 89% in *Nicotiana plumbaginifolia* (+) *Atropa belladonna* hybrids (recipient (+) donor; 100-1000 Gy; Gleba et al. 1988), 40%-92% in *N. plumbaginifolia* (+) *N. sylvestris* hybrids (500 and 1000 Gy; Famelaer et al. 1989) and 0%-75% in *Brassica oleracea* (+) *B. campestris* hybrids (100-800 Gy; Yamashita et al. 1989). In all those cases, chromosome rearrangements and/or deleted donor chromosomes were observed.

To account for the limited elimination of the *L. peruvianum* genome in the *L. esculentum* (+) *L. peruvianum* asymmetric hybrids, several explanations are conceivable:

(i) chromosome breaks were repaired before and/or after fusion. Repair of single-stranded breaks within one hour of irradiation has been shown to occur in carrot protoplasts following treatment with 200 Gy of gamma irradiation (Howland et al. 1975);

(ii) hybrid cells with a high proportion of donor genome were better balanced (around the tri-, penta- or hepta-ploid level) and therefore grew better than cells with a low proportion of donor genome;

(iii) in selecting the fusion products, we were biased to good callus growth, a multi-genic trait from *L. peruvianum* which is not linked to regeneration capacity (Koornneef et al. 1987a).

The amount of donor DNA detected in the fusion products was dependent on the dose of irradiation applied, though the differences between the high- and low dose hybrids were not dramatic. Low dose hybrids contained more loci, alleles and complete chromosomes from *L. peruvianum* than high dose hybrids (Table 3), while the latter class contained more incomplete *L. peruvianum* chromosomes (probably due to a higher frequency of chromosome breaks). For plant asymmetric somatic hybrids, it is assumed that the donor genome is retained as fragments bearing a centromere and that exchange events are rare, since the chromosomes of both parents are not mixed in the first divisions (Hinnisdaels et al. 1988). Our results, however, provide substantial evidence showing that chromosome fragments generated by irradiation were involved in inducing rearrangements, like

translocations:
(i) as chromosome fragments without a centromere are not stably maintained in the absence of rearrangements, *L. peruvianum* loci located at the ends of chromosomal arms are expected to be found less frequently in asymmetric hybrids following fragmentation by irradiation than loci more proximal to the centromere. No relation was found, however, between the elimination of loci/alleles from *L. peruvianum* and their chromosomal map position. Hybrid 30H6, for example, was missing the *L. peruvianum* Aps-1 and CD14 alleles which are located at either side of the centromere of chromosome 6, but did contain the Adh-2 and TG54 alleles residing more distal from the centromere (Fig. 4). Besides, telomeres are necessary for stable maintenance of chromosome fragments as well (Blackburn and Szostak 1984);
(ii) the total number of complete plus incomplete chromosomes as estimated by RFLP analysis usually was larger than the number counted in metaphase plates (Table 4);
(iii) in a few cases, amplification of alleles had occurred as shown by the Adh2 alleles on chromosome 6 in hybrid 30H26 (Fig. 4) and the locus TG50 on chromosome 12 in hybrid 30H1 (upper band lane 9, Fig. 2C).
In most of the hybrids, also a few alleles of the recipient *L. esculentum* genome were absent and in some cases even complete chromosomes (Table 4). Maybe, exchange events with *L. peruvianum* chromosomes or chromosome fragments had taken place shortly after fusion, followed by elimination of translocated parts. Another explanation for the loss of *L. esculentum* chromosomes and fragments might be that rearrangements, such as structural chromosome mutations and non-disjunction, occurred at the tissue culture phase (Pijnacker et al. 1986; Lee and Phillips 1988; Evans et al. 1989).

Ribosomal DNA in the asymmetric hybrids

In the present study a large variation in the amount of ribosomal DNA was detected in the asymmetric somatic hybrids. Calculations were based on the assumption that the parental species did not vary in their rDNA content and that the symmetric somatic hybrid 0H1, which was used as a reference, contained the complete sets of rDNA from both species. Variation in the amount of rDNA has so far not been reported for *Lycopersicon* genotypes, although variation was demonstrated in a few individuals of populations following regeneration from tissue culture (potato, Landsmann and Uhrig 1985; triticale, Brettell et al. 1986); however in another study, plants regenerated from tissue culture showed no variation (carrot and artichoke, Hase et al. 1982). On the other hand, in symmetric somatic hybrids of *Solanum tuberosum* and *S. phureja*, the nucleolar

chromosomes of the latter species were either eliminated or had rearranged satellites (Pijnacker et al. 1987). Variation in rDNA was also found in symmetric hybrids of *L. esculentum* and *Solanum lycopersicoides* (Moore and Sink 1988): the amount of rDNA (with respect to total DNA) ranged from 28% to 106% relative to the sexual hybrid between these species; in 6 out of 12 somatic hybrids this amount was not significantly different from that in the sexual hybrid. In these experiments, the *S. lycopersicoides* protoplasts derived from a suspension culture; suspension cultures are known to accumulate various chromosomal aberrations (e.g. Pijnacker et al. 1986). We assumed that in our reference plant, the symmetric hybrid OH1, no significant changes had occurred in the rDNA contribution of either parent, because the parental protoplasts derived from leaf tissue, which is expected to be stable at the chromosome level, and because of the short tissue culture phase involved in generating this hybrid.

In our asymmetric hybrids, the amount of rDNA ranged from 25% to 158% as compared to that in the symmetric hybrid OH1. Even if the symmetric hybrid OH1 had deviating amounts of ribosomal DNA, still the large variation in the asymmetric hybrids is striking. The rDNA contribution of *L. esculentum* in 12 out of 15 asymmetric hybrids analysed, was decreased relative to the number of copies of chromosome 2 from *L. esculentum*. The *L. peruvianum* rDNA was absent in 2 hybrids, whereas it was present in various amounts, irrespectively of the copy number of *L. peruvianum* chromosomes 2, in the other hybrids. The observed variation might be caused by the irradiation treatment (in case of *L. peruvianum* rDNA), the tissue culture conditions (Lee and Phillips 1988; Evans et al. 1989) and/or the interspecific genome combination (Pijnacker et al. 1987).

Linkage of molecular markers to regeneration capacity

Originally, it was anticipated that gamma-irradiation of the *L. peruvianum* protoplasts would lead to elimination of the bulk of the donor genome and identification of chromosomal markers associated with the regeneration trait. However, elimination of the *L. peruvianum* genome was rather limited. All markers representing the 12 chromosomes of *L. peruvianum*, were recovered in at least 9 out of 15 asymmetric hybrids surveyed. Since only a limited number of genes is known to be involved in regeneration as shown by classical genetic analysis (Koornneef et al. 1987a), this result suggests that extensive chromosomal elimination by irradiation did not occur and/or that *L. peruvianum* loci, which are not associated with the regeneration trait, were retained on their own (incomplete) chromosomes or by translocation to other chromosomes. Three *L. peruvianum* loci, TG34 (chromosome 2), CD59 (chromosome 4) and TG61 (chromosome 7), were present in all asymmetric hybrids; this suggests linkage of these loci to those which are essential for regeneration.

In general, even with the rather limited number of asymmetric hybrids surveyed, our studies cast serious doubts on asymmetric hybridisation as a useful means of introducing desirable traits into a recipient species. The aneuploid character of the fusion products and the numerous chromosomal rearrangements involved - phenomena which both are known to cause sterility - render the hybrid plants inaccessible to backcrossing (Chapter 4).

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References

Adams TL, Quiros CF (1985) Somatic hybridization between Lycopersicon peruvianum and Lycopersicon pennellii: regenerating ability and antibiotic resistance as selection systems. Plant Science 40:209-219

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

Bernatzky R, Tanksley SD (1986) Toward a saturated linkage map in tomato based on isozymes and random cDNA sequences. Genetics 112:887-898

Blackburn E and Szostak J (1984) The molecular structure of centromeres and telomeres. Ann Rev Biochem 53:163-194

Brettell RIS, Pallotta MA, Gustafson JP, Appels R (1986) Variation at the Nor loci in triticale derived from tissue culture. Theor Appl Genet 71:637-643

Chase T, Williams BG (1986) Characterization of complete cDNA for tomato Adh2. Abstract Tomato Biotechnology Symposium, Univ of California, Davis, August 20-22

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Molecular Biology Reporter 1:19-21

Dobrowolski B, Glund K, Metzlaff M (1989) Cloning of tomato nuclear ribosomal DNA, rDNA organization in leaves and suspension-cultured cells. Plant Science 60:199-205

Dudits D, Fejer O, Hadlaczky GY, Koncz CS, Lazar G, Horvath G (1980) Intergeneric gene transfer mediated by plant protoplast fusion. Mol Gen Genet 179:283-288

Evans DA (1989) Somaclonal variation. Genetic basis and breeding applications. Trends in Genetics 5:46-50

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

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6-13

Ganal MW, Lapitan NLV, Tanksley SD (1988) A molecular and cytogenetic survey of major repeated DNA sequences in tomato (Lycopersicon esculentum). Mol Gen Genet 213:262-268

Gleba YY, Hinnisdaels S, Sidorov VA, Kaleda VA, Parokonny AS, Boryshuk NV, Cherup NN, Negrutiu I, Jacobs M (1988) Intergeneric asymmetric hybrids between Nicotiana plumbaginifolia and Atropa belladonna obtained by "gamma-fusion". Theor Appl Genet 76:760-766

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

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

Hase Y, Hase A, Tanifuji S (1982) Constancy of rDNA content during dedifferentiation of carrot and Jerusalem artichoke explants. Plant Cell Physiol 23:323-331

Helentjaris T, King G, Slocum M, Siedenstrang C, Wegman S (1985) Restriction fragment polymorphisms as probes for plant diversity and their development as tools for applied plant breeding. Plant Mol Biol 5:109-118

Hille J, Koornneef M, Ramanna MS, Zabel P (1989) Tomato: a crop species amenable to improvement by cellular and molecular methods. Euphytica 42:1-23

Hinnisdaels S, Negrutiu I, Jacobs M, Sidorov V (1988) Plant somatic cell hybridizations: evaluations and prospectives. Newsletter IAPTC July 1988:2-10

Howland GP, Hart RW, Yette ML (1975) Repair of DNA strand breaks after gamma-irradiation of protoplasts isolated from cultured wild carrot cells. Mutation Res. 27:81-89

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

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

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

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

Landsmann J, Uhrig H (1985) Somaclonal variation in Solanum tuberosum detected at the molecular level. Theor Appl Genet 71:500-505

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

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning. Cold Spring Harbor Laboratories. Cold Spring Harbor, New York

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

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

Mutschler MA, Tanksley SD, Rick CM (1987) Linkage maps of the tomato (Lycopersicon esculentum). Rep Tomato Genet Coop 37:5-34

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, Hermelink JHM, Ferwerda MA (1986) Variability of DNA content and karyotype in cell cultures of an interdihaploid Solanum tuberosum. Plant Cell Reports 5:43-46

Reed KC, Mann DA (1985) Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res 13:7207-7221

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

Ringertz NR, Savage RE (1976) Gene mapping and gene complementation analysis. In: Cell hybrids. Academic Press, New York, pp 224-244

Tanksley SD, Miller JC, Paterson A, Bernatzky R (1988) Molecular mapping of plant chromosomes. In: Gustafson JP, Appels R (eds) Chromosome structure and function. Plenum Press, New York, pp 157-173

Vallejos CE, Tanksley SD, Bernatzky R (1986) Localization in the tomato genome of DNA restriction fragments containing sequences homologous to the rRNA (45s), the major chlorophyll a/b binding polypeptide and the ribulose bisphosphate carboxylase genes. Genetics 112:93-105

Van Daelen RAJ, Jonkers JJ, Zabel P (1989) Preparation of megabase-sized tomato DNA and separation of large restriction fragments by field inversion gel electrophoresis (FIGE). Plant Mol Biol 12:341-352

Wijbrandi J, Vos JGM, Koornneef M (1988) Transfer of regeneration capacity from Lycopersicon esculentum to L. peruvianum by protoplast fusion. Plant Cell Tissue Organ Culture 12:193-196

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

Young ND, Tanksley SD (1989) Restriction fragment length polymorphism maps and the concept of graphical genotypes. Theor Appl Genet 77:95-101

Young ND, Zamir D, Ganal MW, Tanksley SD (1988) Use of isogenic lines and simultaneous probing to identify DNA markers tightly linked to the Tm-2a gene in tomato. Genetics 120:579-585

Zabel P, Meyer D, van de Stolpe O, van der Zaal B, Ramanna RS, 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 Press, New York, pp 609-624

CHAPTER 7

GENERAL DISCUSSION

The experiments presented in this thesis show that the favourable plant regeneration capacity of *L. peruvianum* is an efficient selectable marker in protoplast fusion experiments; this confirms the results of Adams and Quiros (1985) and Kinsara et al. (1986). Symmetric somatic hybrids were easily obtained. These plants were vigorous and produced fertile progenies upon selfing. The asymmetric somatic hybrids were more difficult to obtain, less vigorous and very variable. They were characterised in detail by chromosome specific markers. Especially the RFLP analysis was very useful, because each donor chromosome could be detected by at least two markers. Such a detailed analysis of somatic hybrids has not been reported before. The analysis revealed that a large number of *L. peruvianum* chromosomes, which were often incomplete, was retained in the asymmetric hybrids.

Application of somatic hybrids of tomato in breeding programs ?

The tetraploid symmetric somatic hybrids of both *Lycopersicon* species used were self compatible; their progeny showed some segregation towards both fusion parents (Chapter 3). No backcrosses of these hybrids to tomato did succeed. However, it seems feasible that a plant sexually congruent with *L. esculentum* can be found among the large population of progeny plants derived from the selfed tetraploid hybrids or among a next generation of these plants, because of the observed variation.

The asymmetric somatic hybrids obtained in the present study were sterile (Chapter 4). The reasons are most probably the aneuploidy of these plants and the assumed frequent occurrence of rearrangements. Besides, these hybrids retained a lot of chromosomes from the donor. When very large numbers of asymmetric hybrids are assayed, maybe some highly asymmetric hybrids and/or fertile plants can be found.

Somatic mapping by asymmetric hybridisation ?

It was anticipated that highly asymmetric hybrids, which contained a limited number of more or less undamaged donor chromosomes, could arise from the

described experiments. In that case, the genes coding for the regeneration capacity of *L. peruvianum* could be mapped to particular chromosomes and to particular chromosomal regions. However, the amount of retained donor chromosomes was large (Chapter 4, 5 and 6). In addition, the irradiation treatment generated many fragmented chromosomes, which probably induced rearrangements of chromosomes, also involving *L. esculentum* chromosomes (Chapter 5 and 6). Incorporation of donor fragments in the recipient genome might be established by *in situ* hybridisation with species specific repetitive DNA probes, if these might become available for *Lycopersicon* species. In such a manner, integration of *Nicotiana plumbaginifolia* fragments in *N. tabacum* chromosomes has been demonstrated for asymmetric hybrids of these species (Piastuch and Bates 1988). The asymmetric hybrids between *L. esculentum* and *L. peruvianum* showed some instability with respect to the chromosome numbers counted in root tip cells (Chapter 4) and with respect to the retention of *L. peruvianum* specific traits in different shoots from a same hybrid clone (Chapter 5). This hampers somatic mapping by asymmetric hybridisation after fragmentation of the donor genome by high doses of ionising radiation. On the other hand, regional mapping (i.e. mapping of traits in certain small chromosome regions) should be feasible in plant asymmetric hybrids as has already been shown for mammalian cell hybrids (Goss and Harris 1975). For this purpose, one needs a sufficient number of marker genes to define specific regions and one has to test many hybrids. The mapped RFLP markers of the tomato (Tanksley et al. 1988) are appropriate for such studies.

The only statement that can be made about the localisation of the "regeneration genes" of *L. peruvianum* is that these genes are probably not mapped to chromosomes 1, 3, 5 and 11, because markers specific for these chromosomes were lost in a large fraction of the asymmetric hybrids (Chapter 5 and 6). An alternative method for mapping these "regeneration genes" to particular chromosomes, is the analysis of sexual populations that segregate for this trait (Koornneef et al. 1987). This analysis can be performed with the abundantly available morphological and biochemical markers (isozyme and RFLP) of tomato.

Elimination of chromosomes in the somatic hybrids

The symmetric somatic hybrids between *L. esculentum* and *L. peruvianum* rarely lost chromosomes spontaneously (Chapter 2). In contrast, all asymmetric hybrids between these species had lost chromosomes, as indicated by the aneuploid chromosome numbers and the loss of *L. peruvianum* traits (Chapter 4, 5 and 6).

This clearly shows that high doses of ionising radiation cause elimination of chromosomes. However, no highly asymmetric hybrids with only a few donor traits or chromosomes were obtained. Most of the hybrids had retained at least half of a diploid *L. peruvianum* genome. Several explanations are conceivable for this limited elimination:

(i) the selectable marker used is not appropriate for the selection of highly asymmetric hybrids. Regeneration capacity is important only in the last stage of the cell culture phase. Markers that act as early as possible after protoplast fusion, such as recessive auxotrophic mutations in the recipient (e.g. nitrate reductase deficiency) or resistances to antibiotics introduced by transformation in the donor species, might be more effective for the selection of highly asymmetric hybrids;

(ii) the choice of parental species may not have been suitable to obtain highly asymmetric hybrids. The donor species *L. peruvianum* was for all cell culture traits superior to the recipient *L. esculentum*. The selection on the basis of several favourable *L. peruvianum* genes, which code for callus growth characteristics and regeneration capacity, may have lead to the retention of large numbers of donor chromosomes;

(iii) the chromosome numbers tend to be more or less balanced around the triploid or pentaploid level. There might be selection against aneuploid chromosome numbers that deviate too much from these numbers. In addition, the somatic congruity between the two species can imply a lack of selection against high numbers of donor chromosomes.

To verify or exclude these possible explanations, fusion experiments might be performed in which unirradiated protoplasts of *L. peruvianum* are fused with irradiated protoplasts of a kanamycin resistant *L. esculentum*. The hybrids have to be selected for kanamycin resistance in an early stage of the cell culture phase. If the obtained asymmetric hybrids would contain few traits or chromosomes of *L. esculentum*, this would support explanations (i) and (ii). If the elimination of *L. esculentum* chromosomes is limited, explanation (iii) is more likely.

Other methods to achieve chromosome elimination

High doses of gamma-rays were not sufficient, despite the frequent breakage of *L. peruvianum* chromosomes, to eliminate the bulk of the donor genome in the presented experiments. Moreover, the chromosome lesions probably induced chromosome rearrangements. Other methods, which have hardly been tested in plants, to get highly asymmetric hybrids could be tested:

(i) fragmentation by premature chromosome condensation (PPC). This is induced in protoplasts of interphase cells when they are fused with protoplasts of cells in metaphase (Szabados and Dudits 1980);
(ii) fragmentation of the donor genome by chemical agents, for example Acridin Orange (Pedersen et al. 1988) and BUdR (5-bromodeoxyuridine; used in mammalian cell hybrids, Pontecorvo 1971). Both chemicals are incorporated into DNA, photosensitise the DNA and therefore cause chromosome breaks when the cells are exposed to light;
(iii) gameto-somatic fusion and subsequent backcrossing (Pental et al. 1988). Haploid microspore protoplasts, which are isolated from young pollen tetrads, are fused with protoplasts of diploid somatic cells. The resulting triploid hybrids have to be backcrossed to achieve further elimination of the donor chromosomes; this prerequisite limits the method to more or less congruent combinations. Gameto-somatic fusion has been tried in several experiments of the present study (data not shown). Microspore protoplasts could be isolated from small flower buds of *L. peruvianum* and were fused with mesophyll protoplasts of *L. esculentum*. However, the fusion products as well as unfused cells in the same culture did not grow. Apparently, the presence of microspore protoplasts inhibited cell growth;
(iv) microprotoplast-protoplast fusion (Verhoeven 1989). The microprotoplasts, containing one or a few chromosomes or fragments, are isolated from micronucleated cells, which are induced by treatment with an anti-mitotic drug. This method is attractive, because elimination is achieved before fusion.
The methods (iii) and (iv) probably also suffer less from chromosome rearrangements.

Thus, although highly asymmetric somatic hybrids of *Lycopersicon* species have not yet been constructed, there are still several procedures available that could be tried to achieve this.

References

Adams TL, Quiros CF (1985) Somatic hybridization between Lycopersicon peruvianum and Lycopersicon pennellii: regenerating ability and antibiotic resistance as selection systems. Plant Science 40:209-219

Goss SJ, Harris H (1975) New method for mapping genes in human chromosomes. Nature 255:680-684

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

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

Pedersen HC, Larsen AB, Vamling K, Keimer B (1988) Inactivation of sugarbeet protoplasts using Acridin Orange, an agent for late selection of fusion products. In: Puite KJ, Dons JJM, Huizing HJ, Kool AJ, Koornneef M, Krens FA (eds) Progress in plant protoplast research, Kluwer, Dordrecht, pp 265-266

Pental D, Mukhopadhyay A, Grover A, Pradhan AK (1988) A selection method for the synthesis of triploid hybrids by fusion of microspore protoplasts (n) with somatic cell protoplasts (2n). Theor Appl Genet 76:237-243

Piastuch WC, Bates GW (1988) Genetic analysis of asymmetric hybrids using species-specific repetitive DNA probes. In: The second international congress of plant molecular biology, Jerusalem, Nov 13-18. Book of abstracts, no. 194

Pontecorvo G (1971) Induction of directional chromosome elimination in somatic cell hybrids. Nature 230:367-369

Szabados L, Dudits D (1980) Fusion between interphase and mitotic plant protoplasts. Induction of premature chromosome condensation. Exp Cell Res 127:442-446

Tanksley SD, Miller JC, Paterson A, Bernatzky R (1988) Molecular mapping of plant chromosomes. In: Gustafson JP, Appels R (eds) Chromosome structure and function. Plenum Press, New York, pp 157-173

Verhoeven HA (1989) Induction and characterization of micronuclei in plant cells. Perspectives for micronucleus-mediated chromosome transfer. Ph D Thesis, Agricultural University Wageningen, the Netherlands.

SUMMARY

Several desirable traits, such as disease resistances, have been introduced from more or less related wild *Lycopersicon* species, into the cultivated tomato, *L. esculentum*, by classical breeding techniques. Somatic hybridisation by protoplast fusion can enhance the germplasm pool available for tomato breeding, because this procedure allows to by-pass sexual crossing barriers. Especially the technique of asymmetric hybridisation, by which untreated recipient protoplasts are fused with donor protoplasts of which the major part of the genome has been eliminated, for instance by irradiation, might be very useful. The resulting asymmetric hybrids will contain only a small fraction of the donor genome, and, therefore, the number of backcrosses required to eliminate undesired donor traits might be relatively small; it might also be possible to breed at the (near) diploid level with such hybrids. Asymmetric somatic hybrids might also be useful for chromosome mapping. In this thesis several aspects of somatic hybridisation that are important for the application of this technique to the improvement of the tomato, were analysed in detail.

The efficient isolation of symmetric somatic hybrids between *L. esculentum* and the wild species *L. peruvianum* by means of a double selection strategy is described in Chapter 2. The symmetric hybrids were selected on the basis of kanamycin resistance of *L. esculentum* and the superior regeneration capacity of *L. peruvianum*. These hybrids were very vigorous plants; most of them had a tetraploid chromosome number of 48 ($2n = 4x$). A minority of the hybrids was at the hexaploid level with chromosome numbers from 64 to 72. The hybrid nature of all regenerated plants was confirmed by analysis of isozyme markers, by their intermediate morphology and, in some cases, by the analysis of restriction fragment length polymorphisms (RFLPs). According to RFLP analysis 6 hexaploid hybrids that were tested all contained one diploid genome of *L. esculentum* and two diploid genomes of *L. peruvianum*. One of these hexaploids had genomes of two different *L. peruvianum* genotypes and was therefore considered to be derived from a triple protoplast fusion. The hexaploid plants resembled *L. peruvianum* more than the tetraploids did. The hybrids did flower and set fruits.

The fertility of the tetraploid and hexaploid somatic hybrids was analysed after selfing and after backcrossing to each of both fusion parents (Chapter 3). Most of the somatic hybrids, especially the tetraploids, were fertile upon selfing and yielded many seeds, of which 79% germinated. These progeny plants were vigorous and often fertile after selfing. Backcrossing of the somatic hybrids with the *L. esculentum* parent did not yield any viable seeds;

backcrossing with *L. peruvianum* yielded a few germinating seeds, but only if *L. peruvianum* was used as staminate parent. The plants derived from the backcross hexaploid hybrid x tetraploid *L. peruvianum* had a pentaploid chromosome number (2n = 5x = 60) and were vigorous, whereas the plants derived from the backcross tetraploid hybrid x diploid *L. peruvianum* grew retarded. To obtain information about the behaviour of chromosomes in the hybrids, the progenies of the selfings and backcrosses were analysed for the segregation of several traits, namely kanamycin resistance, isozyme patterns for acid phosphatase, locus *Aps-1*, and some morphological characteristics. Most of the progenies segregated for kanamycin resistance as expected on the basis of the number of loci present in the kanamycin resistant fusion parent, whereas in some progenies the fraction of kanamycin resistant plants was smaller than expected. The segregation of the *Aps-1* isozyme patterns and the variation for some of the morphological characteristics among the progeny plants indicated a tetrasomic inheritance of at least part of the genes in the hybrids.

Asymmetric somatic hybrids between *L. esculentum* and *L. peruvianum* were selected on the basis of the regeneration capacity of *L. peruvianum* (Chapter 4). Selection against growth of *L. peruvianum* was effected by lethal doses (50, 300 or 1000 Gray) of gamma-irradiation applied to this species before protoplast fusion. The asymmetric hybrids needed a longer regeneration time than the symmetric hybrids, and showed a large variation in callus morphology, plant morphology and chromosome numbers. All asymmetric hybrid plants were aneuploid. After a low dose (50 Gy) most hybrids had a near-triploid chromosome number, whereas after a high dose (300 or 1000 Gy) most of the hybrids were near pentaploid. In spite of the aneuploid chromosome numbers, many plants grew vigorously, flowered and, in some cases, set fruits. In general the morphology of the asymmetric hybrids was intermediate between that of *L. esculentum* and symmetric somatic hybrids of both species, and approached the morphology of *L. esculentum* more after a high dose of irradiation. The high dose hybrids also grew more slowly, flowered and set fruits less than the low dose hybrids. No viable seeds could be obtained from any asymmetric hybrid.

The asymmetric hybrids were also analysed for the retention of specific genes and alleles of the donor *L. peruvianum* (Chapter 5). About 50% of the asymmetric 30H-hybrids (*L. peruvianum* parent irradiated with 300 Gy) had retained the kanamycin resistance trait from donor plants with one hemizygous resistance locus. *L. peruvianum* specific alleles of the isozyme markers *Aps-1* and glutamate oxaloacetate transaminase were present in at least 70% of the hybrids; the retention of donor alleles was lower in 30H-than in 5H-hybrids (donor irradiated with 50 Gy). On the average, 74% of the *L. peruvianum* specific alleles (one or

both) of 18 morphological markers, which were located on 10 of the 12 tomato chromosomes, were detected in the 30H-hybrids. It was estimated that each allele of a given marker was, on the average, present in half of the 30H-hybrids. In 36% of the 30H-hybrids, only one of both recessive morphological markers that were located on a same *L. esculentum* chromosome was complemented by the corresponding *L. peruvianum* allele. This is an indication for frequent breakage of the *L. peruvianum* chromosomes. Several hybrid calli regenerated genotypically different plants, which suggested that some segregation occurred in these calli before shoot regeneration.

Fifteen asymmetric hybrids, eight 5H- and seven 30H-hybrids, were analysed with 29 RFLP markers (Chapter 6); each tomato chromosome was represented by at least two such markers. Retention of alleles from the irradiated *L. peruvianum* donor genome in the asymmetric hybrids ranged from 31% to 83%. On the average, 67% of the 58 alleles of the 29 RFLP loci was present in the 5H-hybrids and 53% in the 30H-hybrids. The 5H-hybrids contained more complete *L. peruvianum* chromosomes, as determined by the retention of RFLP alleles of the loci on one chromosome of the donor, than the 30H-hybrids, whereas the 30H-hybrids contained more incomplete chromosomes; this indicated a more frequent breakage of *L. peruvianum* chromosomes in the 30H-hybrids. In most hybrids some *L. esculentum* alleles were lost. Three *L. peruvianum* loci, located on chromosome 2, 4 and 7, respectively, were present in each asymmetric hybrid, which may suggest linkage to the regeneration capacity trait which was used in selecting the asymmetric hybrids. The asymmetric hybrids were also analysed with a probe for ribosomal DNA (rDNA). The amount of rDNA from *L. peruvianum* retained in the hybrids varied strongly: in one hybrid amplification had occurred, whereas in others *L. peruvianum* rDNA was either absent or (in)completely present. The amount of *L. esculentum* specific rDNA was decreased in most asymmetric hybrids.

The analyses of the asymmetric somatic hybrids showed that irradiation before fusion eliminated the *L. peruvianum* genome only to a limited extent. In addition, the hybrids were sterile despite their ability to flower and set fruits. It can be concluded therefore that application of these hybrids in breeding programs is very restricted. However, fertile progeny plants derived from selfed symmetric hybrids might be used for this purpose, because segregation for *L. esculentum* specific traits was observed. The use of the asymmetric hybrids for somatic mapping is not favourable, because of the limited elimination of the donor genome, the large number of incomplete donor chromosomes and the probable presence of rearranged chromosomes. It is possible that highly asymmetric hybrids can be obtained by the use of other selectable markers or other procedures to induce elimination of the donor genome.

SAMENVATTING

DE ISOLATIE EN KARAKTERISERING VAN SOMATISCHE HYBRIDEN VAN *LYCOPERSICON ESCULENTUM* EN *LYCOPERSICON PERUVIANUM*

Bij de veredeling van de tomaat, *Lycopersicon esculentum*, wordt gebruik gemaakt van verwante, wilde *Lycopersicon* soorten om gunstige eigenschappen, zoals ziekteresistenties, uit deze soorten via kruisingen over te brengen naar de cultuurtomaat. Een alternatieve methode om gewenste eigenschappen uit andere soorten over te dragen is somatische hybridisatie of protoplastenfusie. Hierbij worden protoplasten, ongeslachtelijke cellen (bijvoorbeeld uit bladweefsel) waarvan de celwand verwijderd is, tot fusie aangezet. Fusieproducten worden vervolgens opgekweekt tot callusweefsel, van waaruit planten geregenereerd kunnen worden. Eén van de voordelen van deze techniek is dat hybriden verkregen kunnen worden van niet-kruisbare soorten. Een nadeel is dat de ontstane somatische hybriden de complete aantallen chromosomen van beide soorten bevatten, waardoor er, zo dit al mogelijk is, vele terugkruisingen nodig zijn met de gecultiveerde soort om alle ongewenste eigenschappen van de wilde soort te verwijderen. Asymmetrische protoplastenfusie, waarbij de cellen van de ene soort bestraald zijn met een hoge dosis gamma- of Röntgen-straling, zou dit nadeel kunnen omzeilen. De chromosomen van de bestraalde cellen worden gefragmenteerd, waardoor deze snel geëlimineerd worden in een fusieproduct. Deze asymmetrische somatische hybriden zouden minder terugkruisingen nodig hebben. In dit proefschrift zijn experimenten beschreven waarin wordt nagegaan wat de mogelijkheden en moeilijkheden zijn van de overdracht van eigenschappen uit een wilde soort naar de cultuurtomaat met behulp van protoplastenfusie. De volgende aspecten, die van belang zijn voor de toepassing van de protoplastenfusie bij de veredeling van de tomaat zijn in detail onderzocht:

(i) het ontwikkelen van een doeltreffende selectiemethode voor de isolatie van somatische hybriden;

(ii) het isoleren van asymmetrische somatische hybriden waarbij de wilde soort bestraald wordt voor de fusie;

(iii) het karakteriseren van de hybriden om te bepalen wat de bijdrage is van de (bestraalde) wilde soort aan het genoom van de hybriden.

Als wilde soort werd *Lycopersicon peruvianum* gebruikt. Deze is zeer moeilijk kruisbaar met de tomaat en heeft vele voor de veredeling waardevolle

eigenschappen. Bovendien heeft *L. peruvianum*, in tegenstelling tot de tomaat, een zeer goed regeneratievermogen; dit is het vermogen planten te regenereren uit protoplasten- en calluscultures. Deze eigenschap werd gebruikt in de beschreven experimenten om fusieproducten tussen beide soorten te selecteren.

Hoofdstuk 2 beschrijft de efficiënte isolatie van symmetrische somatische hybriden van *L. esculentum* en *L. peruvianum* via een dubbel selectiesysteem. Hybriden werden geselecteerd op grond van resistentie tegen het antibioticum kanamycine (een eigenschap van *L. esculentum*) en het vermogen van *L. peruvianum* om uit celkweek planten te regenereren. De geregenereerde planten waren zeer groeikrachtig en de meerderheid had een tetraploïd chromosoomaantal (2n = 4x = 48); een aantal planten waren (hypo)hexaploïd met 2n = (64-)72. De hybride aard van de planten bleek uit hun intermediaire morfologie en werd aangetoond met behulp van isozymanalyse en voor sommige planten m.b.v. analyse van restrictiefragment-lengte-polymorfismes (RFLP's). Volgens deze RFLP-analyse bevatten de 6 geteste hexaploïde hybriden één diploïd genoom van *L. esculentum* en twee diploïde genomen van *L. peruvianum*. Eén van deze hexaploïden had genomisch materiaal van twee verschillende *L. peruvianum* genotypen; deze hybride moet ontstaan zijn uit een fusie van drie verschillende protoplasten. De hexaploïde hybriden leken morfologisch iets meer op *L. peruvianum* dan de tetraploïde hybriden. De somatische hybriden bloeiden volop en vormden vruchten.

De vruchtbaarheid van de tetraploïde en hexaploïde somatische hybriden werd onderzocht via zelfbevruchtingen en terugkruisingen met beide fusie-ouders (Hoofdstuk 3). De meeste hybriden, vooral de tetraploïden, vormden veel zaden na zelfbevruchtingen; de zaden waren levensvatbaar (79% kieming). De nakomelingen hieruit waren zeer groeikrachtig en vormden vaak zaden na zelfbevruchting. De terugkruisingen van de somatische hybriden met *L. esculentum* leverden geen levensvatbare zaden op. De terugkruisingen met *L. peruvianum* leverden enkele levensvatbare zaden op mits *L. peruvianum* als stuifmeelplant werd gebruikt. De planten afkomstig van de terugkruising hexaploïde hybride x tetraploïde *L. peruvianum* hadden een pentaploïd chromosoomaantal (2n = 5x = 60) en waren groeikrachtig, terwijl de planten uit de terugkruising tetraploïde hybride x diploïde *L. peruvianum* vertraagd groeiden. De uitsplitsing van enkele eigenschappen werden bij de nakomelingen geanalyseerd om informatie te krijgen over het chromosoomgedrag in de somatische hybriden. De nakomelingen van de zelfbevruchte tetraploïde somatische hybriden vertoonden uitsplitsing voor isozympatronen van zure fosfatase (locus *Aps-1*) en gaven een grotere variatie voor enkele morfologische kenmerken te zien dan de somatische hybriden; dit wijst op tetrasome vererving van tenminste *Aps-1*.

De isolatie van asymmetrische somatische hybriden van *L. esculentum* en *L. peruvianum* was gebaseerd op selectie voor regeneratievermogen (Hoofdstuk 4). Selectie tegen *L. peruvianum* werd uitgevoerd door deze te bestralen voor protoplastenfusie met lethale doses (50, 300 of 1000 Gray) gamma-straling. De asymmetrische hybriden hadden meer tijd nodig voor plantregeneratie dan de symmetrische hybriden, en vertoonden een grote variatie in callusmorfologie, plantmorfologie en chromosoomaantallen. Alle asymmetrische hybriden waren aneuploïd; na een lage dosis (50 Gy) hadden de meeste hybriden chromosoomaantallen rondom het triploïde niveau, terwijl na een hoge dosis (300 of 1000 Gy) de chromosoomaantallen van de meeste planten zich rondom het pentaploïde niveau bevonden. Ondanks de aneuploïdie waren veel planten vitaal, bloeiden en sommige vormden vruchten. De algemene morfologie van de asymmetrische hybride planten was intermediair tussen die van de tomaat en die van de symmetrische hybriden. Na een hoge dosis leken de hybriden meer op *L. esculentum*, groeiden slechter, bloeiden minder en vormden minder vruchten dan na een lage dosis bestraling. De kruisingen met en zelfbevruchtingen van de asymmetrische hybriden leverden geen levensvatbare zaden op.

De asymmetrische somatische hybriden werden ook geanalyseerd op aanwezigheid van genen en allelen van de donor *L. peruvianum* (Hoofdstuk 5). Ongeveer 50% van de asymmetrische 30H-hybriden, welke ontstaan waren na fusie van *L. esculentum* en *L. peruvianum*, bestraald met 300 Gy en hemizygoot voor één copie van het kanamycine-resistentie-gen, bezat kanamycine-resistentie. In minstens 70% van de asymmetrische hybriden waren *L. peruvianum* specifieke allelen van de isozymen *Aps-1* en glutamaat oxaalacetaat transaminase aanwezig; de 30H-hybriden (donor bestraald met 300 Gy) bezaten minder van deze allelen dan de 5H-hybriden (donor bestraald met 50 Gy). Gemiddeld 74% van de 18 recessieve morfologische eigenschappen, die van *L. esculentum* afkomstig waren en die gelocaliseerd zijn op 10 van de 12 tomatechromosomen, waren gecomplementeerd door de corresponderende *L. peruvianum* allelen (één of beide) in de 30H-hybriden. Volgens berekening is elk willekeurig donor allel van een bepaalde isozym- of morfologische eigenschap gemiddeld in de helft van de 30H-hybriden aanwezig. In 36% van de 30H-hybriden was maar één van beide op hetzelfde chromosoom gelegen morfologische eigenschappen gecomplementeerd door een corresponderend *L. peruvianum* allel; dit wijst op het vaak voorkomen van breuken in de donor chromosomen. Uitsplitsing voor bepaalde eigenschappen moet opgetreden zijn in de asymmetrische hybride klonen voor regeneratie optrad, omdat verscheidene klonen genotypisch verschillende scheuten vormden.

In Hoofdstuk 6 wordt van vijftien asymmetrische hybriden (acht 5H- en zeven 30H-hybriden) een zeer gedetailleerde analyse beschreven met 29 RFLP-merkers. Elk tomatechromosoom is vertegenwoordigd met twee tot vier van dergelijke

merkers. 31% tot 83% van de *L. peruvianum* specifieke allelen van deze merkers was aanwezig in de asymmetrische hybriden. Gemiddeld was 67% van de 58 allelen van de 29 RFLP-loci aanwezig in de 5H-hybriden en 53% in de 30H-hybriden. In de 30H-hybriden werden meer incomplete *L. peruvianum* chromosomen gevonden dan in de 5H-hybriden; kennelijk bevatten de van *L. peruvianum* afkomstige chromosomen in de 30H-hybriden meer breuken dan die in de 5H-hybriden. De meeste hybriden hadden ook enkele *L. esculentum* specifieke allelen verloren. Drie RFLP-loci van *L. peruvianum*, gelocaliseerd op de chromosomen 2, 4 en 7, waren in elke asymmetrische hybride aanwezig; mogelijk zijn deze loci gekoppeld aan de *L. peruvianum* specifieke genen voor het regeneratievermogen, waarop de hybriden geselecteerd waren. De asymmetrische hybriden werden ook geanalyseerd met een RFLP-probe voor het ribosomale DNA (rDNA). De asymmetrische hybriden verschilden onderling sterk wat betreft de hoeveelheid *L. peruvianum* specifieke rDNA in hun genoom. In het genoom van één hybride was de relatieve hoeveelheid groter dan in het *L. peruvianum* genoom; in dat van andere hybriden ontbrak het *L. peruvianum* specifieke rDNA geheel, of de hoeveelheid was kleiner dan of even groot als in het *L. peruvianum* genoom. De hoeveelheid *L. esculentum* specifiek rDNA was in de meeste hybriden iets afgenomen.

Uit de resultaten blijkt dat de eliminatie van *L. peruvianum* chromosomen vrij beperkt was in de asymmetrische somatische hybriden. Ondanks het feit dat vele asymmetrische hybriden in staat waren te bloeien, is er geen fertiele plant verkregen. Daarom zijn deze hybriden van een zeer beperkte waarde voor veredelingsdoeleinden. Echter de vruchtbare nakomelingen van de zelfbevruchte tetraploïde symmetrische hybriden zouden bruikbaar kunnen zijn vanwege de waargenomen uitsplitsing voor enkele tomate-eigenschappen. Een andere mogelijke toepassing van asymmetrische hybriden is het localiseren van genen door koppeling van de selectie-eigenschap met andere reeds gelocaliseerde genen aan te tonen. Het is niet aan te raden om de hier beschreven asymmetrische hybriden voor dit doel te gebruiken omdat de eliminatie van donor chromosomen beperkt was, er een groot aantal incomplete chromosomen van de donor aanwezig was en er waarschijnlijk ook herrangschikkingen van de chromosomen opgetreden waren. Met andere selectiemethoden of met andere technieken om eliminatie te bevorderen, zouden misschien sterker asymmetrische hybriden verkregen kunnen worden.

CURRICULUM VITAE

Jelle Wijbrandi werd geboren op 26 maart 1959 in Bolsward. Na het behalen van het VWO-diploma aan het Bogerman College te Sneek, begon hij in 1977 met de studie Biologie aan de Rijksuniversiteit Groningen. In december 1981 werd het kandidaatsdiploma B5b (2^e hoofdvak bodemkundige geologie) behaald. De doctoraalfase omvatte het hoofdvak Celgenetica en de bijvakken Plantenfysiologie en Plantensystematiek. In het kader van het hoofdvak werden gedurende twee maanden experimenten uitgevoerd op het 'Rothamsted Experimental Station' in Harpenden (Groot-Brittannië). Eind augustus 1985 haalde hij het doctoraalexamen. Per 1 september van hetzelfde jaar werd hij aangesteld als assistent-onderzoeker bij de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO; voorheen ZWO), op een 3-jarig onderzoeksproject gesteund door de Stichting voor Biologisch Onderzoek in Nederland (BION, werkgemeenschap GEFYBO). Het onderzoek werd uitgevoerd onder leiding van dr. ir. M. Koornneef op de vakgroepen Erfelijkheidsleer en Moleculaire Biologie van de Landbouwuniversiteit Wageningen. Eind oktober 1989 treedt hij in dienst als celbioloog bij het plantenbiotechnologiebedrijf Keygene te Wageningen.