SOMATIC HYBRIDIZATION BETWEEN *LYCOPERSICON ESCULENTUM* AND *SOLANUM TUBEROSUM*

SOMATISCHE HYBRIDISATIE TUSSEN *LYCOPERSICON ESCULENTUM* EN *SOLANUM TUBEROSUM*

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SOMATIC HYBRIDIZATION BETWEEN *LYCOPERSICON ESCULENTUM* AND *SOLANUM TUBEROSUM*

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ABSTRACT

This thesis describes an analysis of the possibilities and limitations of asymmetric somatic hybridization between tomato and potato. For this, nitrate reductase-deficient tomato mutants were isolated and characterized. It was shown that two of these mutations could be used as selectable markers in somatic hybridization experiments with potato. Allotriploid somatic hybrids between diploid tomato and monoploid potato were isolated from fusion experiments with a nitrate reductase-deficient mutant of tomato and with an albino tomato mutant. Asymmetric somatic hybrids between hygromycin resistant tomato and gamma irradiated, kanamycin resistant potato were isolated on media that contained both antibiotics. Although many hybrid calli were obtained, no viable plants could be regenerated. Tomato and potato protoplasts were also used to study the effect of gamma irradiation on karyogamy in heterokaryons, DNA fragmentation, DNA repair and DNA synthesis.

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STELLINGEN

1. De synthese van nitraatreductase bij de tomaat wordt bepaald door minstens één gen dat codeert voor het apo-enzym en minstens vier genen die coderen voor het co-enzym of voor genprodukten die betrokken zijn bij de aanhechting hiervan aan het apo-enzym.
   Dit proefschrift

2. De bestaande mutanten van de tomaat met een sterk verminderde nitraatreductase aktiviteit zijn vanwege hun niet transgene karakter geschikter voor somatische hybridisatie met aardappel dan transgene tomaten die kanamycine- of hygromycine-resistent zijn.
   Dit proefschrift

3. Gamma bestraling van aardappel-protoplasten vóór fusie met protoplasten van tomaat leidt tot eliminatie van aardappel-DNA in fusieprodukten. Sterk asymmetrische en fertiele somatische hybriden zijn hiermee echter zeer moeilijk te verkrijgen.
   Dit proefschrift

4. Het verdient aanbeveling om meer onderzoek te verrichten naar alternatieve methoden om de eliminatie van donor-DNA in asymmetrische protoplastenfusies te bewerkstelligen.

5. Sierteeltgewassen die voor bepaalde eigenschappen transgeen zijn zullen door de consument eerder geaccepteerd worden dan voedingsgewassen die voor vergelijkbare eigenschappen transgeen zijn.

6. Het opkweken van transformanten in transformator-huisjes is ten strengste verboden zonder toestemming van de VCOGEM.

7. Van een Assistent In Opleiding kan men geen veren plukken.

8. Mensen kunnen goed ziek worden van computer-virussen.

9. Sommige oplaadbare batterijen bevatten kip.

10. Het aanvragen van subsidie voor innoverend onderzoek wordt meer en meer werk voor specialisten.

Stellingen behorende bij het proefschrift "Somatic hybridization between *Lycopersicon esculentum* and *Solanum tuberosum*" door Herman C.H. Schoenmakers, in het openbaar te verdedigen op maandag 11 oktober 1993, te Wageningen.

VOORWOORD

Dit proefschrift beschrijft de beste resultaten van een vierjarig promotie-onderzoek "De overdracht van genetische informatie door middel van asymmetrische protoplastenfusie". Aan dit onderzoek is door veel mensen een bijdrage geleverd. Niet altijd werd het gewenste doel bereikt maar wel steeds waren de werkzaamheden onontbeerlijk voor het inzicht in de problematiek. In het bijzonder bedank ik hierbij mijn promotor Maarten Koornneef en mijn co-promotor Christa Heyting voor hun vakkundige begeleiding en dito correcties van de vele concept manuscripten. Mijn mede-assistent in opleiding (AIO) Anne-marie Wolters bedank ik hierbij voor de plezierige samenwerking en voor de hulp bij de moleculaire en cytogenetische analyse van de somatische hybriden. Corry Hanhart bedank ik voor het bijspringen in de weefselkweek. De doctoraalstudenten Dianne van der Kop, Witte van Cappellen, Annette Vergunst, Carolien Zijlstra, Lonne Gerritsen, Sjaan van Agtmaal, Sjoukje Dijkstra, José Muisers, Ambro van Hoof, Coert Engels, Joukje Buitenveld, Esther van der Knaap, Aat Vogelaar, Arend van Silfhout, Carianne de Klein en A.Karim Saiedi; de stagiaires Ed Nobel, Brigitte Bakker en Aveline van Doorn; de gastmedewerkers Aaron Zelcer, Gosia Gaj en Danhua Zhu, en de Na-doctoraal Onderzoeks Project (NOP)-ers Jos van Boxtel, Anita de Haan en Leni Demmers-Derks waren allen direct bij het onderzoek betrokken. Het gaat te ver om hier ieders bijdrage te schetsen. Ik moet er niet aan denken dat ik alles zelf had moeten doen - hartelijk bedankt voor jullie hulp. Verder bedank ik alle collega's van "onze" vakgroep, van de vakgroepen Plantenveredeling, Moleculaire Biologie en Toxicologie en van het Centrum voor Plantenveredelings- en Reproduktie Onderzoek te Wageningen voor overleg, hulp en samenwerking. Michel Caboche et Isabelle Chérel, "Je vous remercie pour Versailles et l'I.N.R.A.".

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Zonder jullie allen was het nooit gelukt !

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1 Somatic hybridization

The exploitation of genetic variation, present in alien species of a crop plant, is often hampered by crossing barriers between those species and the crop plant. The introgression of desired traits, like disease resistances, interesting metabolites and growth and harvest characteristics, from wild species into a cultivated species that belongs to the same genus can sometimes be accomplished by means of interspecific hybridization and subsequent recurrent backcrossing with the crop species. Crosses between species of different genera, and the subsequent combination of desired traits from phylogenetically distant species normally fail.

In principle, it is possible to combine the genetic information of any two plant species by means of somatic or parasexual hybridization (Carlson 1972; Gleba and Sytnik 1984). Cell walls can be removed from somatic cells and the protoplasts of the two species can be fused. Subsequently, hybrid cells may start to divide. The hybrid cell clumps can ultimately develop into somatic hybrid plants. However, these so-called symmetric somatic hybrids, which mostly carry the sum of both nuclear genomes in combination with organellar genomes of either species, are seldom desirable from a plant breeding point of view. Usually one tries to improve a crop species with only one or a few traits from another species. In terms of somatic hybridization this means that the transfer of only a small part of the genetic information of a donor (wild) protoplast to a recipient (crop) protoplast and the elimination of the other part are intended. Somatic hybrids that contain the complete genome of a recipient species and part of the genome of a donor species are called asymmetric.

It is of major importance for plant breeding to analyze the possibilities and limitations of asymmetric somatic hybridization. A large pool of interesting and valuable traits is present in alien species. Yet, the technique of asymmetric protoplast fusion has shown to be troublesome and unpredictable. Usually, too many donor traits are retained in the fusion products and regeneration and fertility of asymmetric hybrids is limited or absent. This thesis describes some investigations of the possibilities and limitations of asymmetric somatic hybridization.

1.2 Partial genome transfer by asymmetric somatic hybridization

Apart from the fact that most symmetric somatic hybrids are not desirable from a plant breeding point of view (see 1.1), experimental data suggest that symmetric hybrids of wide species combinations are extremely difficult to obtain. In some wide somatic hybrids chromosome elimination resulted in an improved regeneration (Gleba and Hoffmann 1979; Hoffmann and Adachi 1981; Dudits et al. 1979; Pental et al. 1986). These observations suggested that deliberate attempts to induce directed chromosome elimination might improve regeneration and fertility of wide somatic hybrids (Gleba and Hoffmann 1979).

Asymmetric somatic hybrids can be constructed by means of gamma or X-irradiation of donor protoplasts prior to fusion with recipient protoplasts; transplantation of isolated donor nuclei in recipient protoplasts; fusion of donor microprotoplasts with recipient protoplasts; fusion of haploid donor protoplasts with diploid recipient protoplasts and by means of various transformation techniques.

Pontecorvo (1971) showed that gamma- or X-irradiation could direct chromosome elimination in animal somatic hybrids with a high efficiency. The ionizing radiation damages the nuclear DNA. Chromosomes of the cell type that was irradiated prior to fusion, were always the ones that were eliminated from the fusion products. Directed donor chromosome elimination by irradiation, when first applied to plant protoplasts, resulted in the formation of cybrids (cytoplasmic hybrids), in which the irradiated nuclear donor genome was completely removed and the donor chloroplasts were retained (Zelcer et al. 1978; Aviv and Galun 1980; Sidorov et al. 1981; Menczel et al. 1982, 1983; Hamill et al. 1984). When low irradiation doses were used, it was possible to withhold some donor chromosomes (Menczel et al. 1982; Koornneef et al. 1988). In these experiments the selection of cybrids was based on traits that were encoded by chloroplast genes of the donor.

In many of the experiments that aimed at the isolation of asymmetric somatic hybrid plants, the selection of fusion products was based on a character that was encoded by a nuclear gene of the irradiated donor. This selection was necessary because the frequency of viable asymmetric fusion products in the fusion mixture was low. Interspecific, intergeneric and

interfamiliar asymmetric somatic hybrids that were obtained with an irradiated donor, are listed in table 1. Many investigators described a troublesome recovery of fusion products, limited elimination of donor DNA, high aneuploidy, strong polyploidization, poor growth and regeneration, distorted morphology of asymmetric somatic hybrids and, in case when plants could be obtained, lack of fertility. For interspecific fusions, symmetric combinations often were fertile while asymmetric combinations displayed sterility or a low fertility. Furthermore, most of these asymmetric hybrids exhibited an intermediate phenotype, associated with a limited chromosome elimination. In those few intergeneric and interfamiliar fusions, where viable and fertile plants were obtained, the hybrids were highly asymmetric and resembled the recipient parent. For several asymmetric hybrids it was shown that part of the chromosomes were made up from DNA of both species (Bates et al. 1987; Hinnisdaels et al. 1991; Piastuch and Bates 1990; Parokonny et al. 1992). This indicates that chromosome fragments of the donor can be rescued by their direct introgression into the recipient genome. Homoeologuous meiotic recombination and the introgression (by subsequent backcrosses) of the asymmetric hybrid with the recipient have not been described thus far.

It is not clear why many asymmetric hybrid plants show a limited elimination of donor traits, especially if one takes into account that in cybridization experiments with interspecific combinations, complete elimination is often found. In hybrids between animal cells, the irradiation causes an efficient elimination of donor chromosomes and highly asymmetric hybrids can be obtained routinely. Furthermore, animal cell hybrids often show a spontaneous elimination of chromosomes of one species (Ringertz and Savage 1976). In plants, strong spontaneous elimination has been observed mainly in hybrids between relatively distantly related species (Gillisen et al. 1992a,b; Wolters et al. 1993) but not in intrageneric combinations. In addition, Wolters et al. (1991) and Derks et al. (1992) have suggested that part of the nuclear DNA of the donor is also retained to establish functional donor chloroplasts in the fusion product. Unlike animal cells and animal cell hybrids, many plant cells and plant cell hybrids can form highly differentiated and organized structures. The regeneration of plant cells requires the expression of a large part of the genetic information of the cells that were isolated from organs.

Table 1. Asymmetric somatic hybridization experiments between plant species: recipient protoplasts were fused with irradiated donor protoplasts. In most cases, the selection criterion was a nuclear (N) or chloroplast (C) encoded trait. The described regeneration (R) and fertility (F) are indicated with + = yes and/or — = no.

Recipient	(+) Irradiated donor	Selection criterion	R	F	References
Interspecific combinations					
Brassica campestris	(+) *Brassica oleracea*	N	+		Yamashita et al. 1988
Brassica napus	(+) *Brassica carinata*	N	+	+	Sjödin & Glimelius 1989
Brassica napus	(+) *Brassica juncea*	N	+	+	Sjödin & Glimelius 1989
Brassica napus	(+) *Brassica nigra*	N	+		Sjödin & Glimelius 1989
Brassica napus	(+) *Brassica nigra*	N	+		Sacristan et al. 1989
Lycopersicon esculentum	(+) *Lycopersicon pennellii*	N			O'Connell & Hanson 1985, 1987
Lycopersicon esculentum	(+) *Lycopersicon pennellii*	N	+	—	Wachocki et al. 1991
Lycopersicon esculentum	(+) *Lycopersicon pennellii*	N	+	+	Melzer and O'Connell 1992
Lycopersicon esculentum	(+) *Lycopersicon peruvianum*	N	+	—	Wijbrandi et al. 1990a,b,c
Lycopersicon esculentum	(+) *Lycopersicon peruvianum*	N	+	—	Ratushnyak et al. 1991
Lycopersicon esculentum	(+) *Lycopersicon peruvianum*	N	+	+	Ratushnyak et al. 1993
Nicotiana glauca	(+) *Nicotiana langsdorffii*	N	—	—	Itoh & Futsuhara 1983
Nicotiana plumbaginifolia	(+) *Nicotiana sylvestris*	N	+		Famelaer et al. 1989
Nicotiana plumbaginifolia	(+) *Nicotiana tabacum*	N	+		Koornneef et al. 1988
Nicotiana tabacum	(+) *Nicotiana paniculata*	N	—	—	Müller-Gensert & Schieder 1987
Nicotiana tabacum	(+) *Nicotiana plumbaginifolia*	N	+		Bates et al. 1987
Nicotiana tabacum	(+) *Nicotiana repanda*	N	+	+	Bates et al. 1990
Solanum tuberosum	(+) *Solanum pinnatisectum*	N	+		Sidorov et al. 1987
Solanum tuberosum	(+) *Solanum brevidens*	N	+		Xu et al. 1993
Solanum tuberosum	(+) *Solanum brevidens*	N	—	—	Puite et al. 1993
Intergeneric combinations					
Daucus carota	(+) *Petroselinum hortense*	N	+	+	Dudits et al. 1980
Datura innoxia	(+) *Physalis minima*	N	+		Gupta et al. 1984
Hyoscyamus muticus	(+) *Nicotiana tabacum*	N	—	—	Imamura et al. 1987
Lycopersicon esculentum	(+) *Solanum tuberosum*	C	—	—	Wolters et al. 1991
Lycopersicon esculentum	(+) *Solanum commersonii*	C	+	—	Derks et al. 1992
Lycopersicon esculentum	(+) *Solanum etuberosum*	C	+	—	Derks et al. 1992
Lycopersicon esculentum	(+) *Solanum nigrum*	C	—	—	Derks et al. 1992
Lycopersicon esculentum	(+) *Nicotiana plumbaginifolia*	N	+	—	Wolters et al. 1993
Nicotiana plumbaginifolia	(+) *Atropa belladonna*	N	+	+	Gleba et al. 1988
Nicotiana tabacum	(+) *Datura innoxia*	N	—	—	Gupta et al. 1982
Nicotiana tabacum	(+) *Physalis minima*	N	—	—	Gupta et al. 1982
Nicotiana	(+) *Petunia*	N	+		Hinnisdaels et al. 1991
Interfamiliar combinations					
Nicotiana tabacum	(+) *Daucus carota*	N	+	+	Dudits et al. 1987
Nicotiana tabacum	(+) *Hordeum vulgare*	N	+	+	Somers et al. 1986

The proliferation and regeneration of plant cell hybrids requires a proper collaboration of the genes and gene products of both species. Animal cell hybrids are unable to differentiate and probably do not need a similar high degree of fine tuning of the two genomes to proliferate. Furthermore, the cell cycle of plant cells is much longer than that of animal cells. Whereas in animal cell hybrids the chromosomes of the species with the longest cell cycle are normally eliminated, the differences in replication rate of chromosomes may be relatively smaller in plant cell hybrids. These factors might explain the limited elimination in many somatic plant cell hybrids.

In plants, no information is available on the effect of gamma irradiation on karyogamy in heterokaryons. The effect of irradiation on DNA breakage and DNA repair in plants has been described concisively by Howland et al. (1975), Tano and Yamaguchi (1976) and Hall et al. (1992a,b) whereas the effect of irradiation on subsequent DNA synthesis has not been described. In animal cells the effects of ionizing irradiation on DNA breakage, DNA repair and DNA synthesis have been studied thoroughly (e.g. review by George and Cramp 1987).

Apart from asymmetric protoplast fusion with an irradiated donor, various other procedures, in which asymmetric hybrids were obtained, have been described. The transplantation of isolated plant cell nuclei in protoplasts by means of fusion between protoplasts and nuclei (first described by Potrykus and Hoffmann 1973) also resulted in fusion products with a partially deleted donor genome (Saxena et al. 1986; Saxena and King 1988). However, because distantly related species (nuclei of *Brassica nigra* and *Vicia hajastana* and protoplasts of *Datura inoxia*) were used in these experiments, it is not known whether the technique itself or a spontaneous chromosome elimination upon fusion caused the asymmetry.

Techniques like the fusion of donor microprotoplasts, that contain micronuclei with one or a few chromosomes (Verhoeven 1989), with recipient protoplasts (Ramulu et al. 1992) and micro-injection of isolated chromosomes (de Laat et al. 1989) or micronuclei theoretically yield highly asymmetric hybrids with only a small amount of donor DNA. However, these procedures are technically complicated and require cultures of the donor with a synchronized cell cycle.

Another way to reduce the contribution of the donor genome to that of the somatic hybrid is to use an unirradiated haploid instead of the normally used

irradiated diploid donor protoplasts for the fusion. Haploid protoplasts can either be of somatic or gametic origin. Theoretically, fusions between a diploid recipient and a haploid donor result in allotriploid somatic hybrids, in which introgression of donor traits into the recipient genome may occur by meiotic recombination and by recurrent backcrosses to the recipient species (Lee and Power 1988; Pental et al. 1988; Pirrie and Power 1986). In many plant species, including tomato (Rick and Butler 1956), triploids are more easy to backcross with diploids than tetraploids. In the latter hybrids endosperm/embryo unbalance often causes embryo abortion.

Finally, interesting monogenic traits from wild plant species, once molecularly cloned, can be transferred by various transformation techniques. By means of these techniques an intended trait, without linked traits from the donor organism, can be added to a crop. Since there is a rapid progress in the molecular cloning of agronomically important genes, it can be expected that "molecular breeding" will grow to maturity in the near future.

To summarize, asymmetric somatic hybridization is a potentially important but troublesome plant breeding procedure. At present, the various processes that finally yield useful asymmetric somatic hybrid plants are not clear. The purpose of this thesis is to gain better insight in the possibilities of obtaining asymmetric hybrids.

Our efforts were concentrated on the efficient selection of fusion products and on the use of haploid or gamma irradiated donor protoplasts to produce asymmetric hybrids with the recipient. Furthermore, we studied the early effects of gamma irradiation on DNA breakage, DNA repair, *de novo* DNA synthesis and on karyogamy in fusion products.

1.3 Selection of fusion products

The selection of hybrid fusion products from a fusion mix, that also contains non-fused cells and fusion products of protoplasts derived from one parent only, can be carried out at the level of the cell, of the callus and of the plant. Early selection of hybrid fusion products, shortly after the fusion treatment, is attractive because it increases the efficiency of the procedures. Especially in asymmetric somatic hybridization experiments the frequencies at

which fusion products can be obtained, are low and the selection at the cellular level is essential. Apart from the mechanical selection of fusion products, also genetic markers, that are expressed at the cellular level, are used. Glimelius et al. (1978) were the first investigators that used auxotroph, nitrate reductase deficient *Nicotiana* genotypes in protoplast fusion experiments. Genetic complementation of the auxotrophy by the donor wild type nitrate reductase alleles allowed an efficient selection of the symmetric hybrids at the cellular level. Koornneef et al. (1988) and Famelaer et al. (1989) used the nitrate reductase-deficiency in asymmetric somatic hybridization experiments. These investigators fused auxotroph, nitrate reductase-deficient, recipient protoplasts with gamma irradiated wild type donor protoplasts. However, auxotrophs are available only for a limited number of plant species (Gleba and Shlumukov 1990). This limitation also holds for other mutations that can be used as a basis for hybrid selection, such as dominant mutations that cause antibiotic resistance and mutations that cause resistance against amino-acid analogues and herbicides (Maliga et al. 1975; Bourgin 1978; Sato et al. 1988; De Vries et al. 1987). Dominant resistances against kanamycin (Bevan et al. 1983) and hygromycin (Waldron et al. 1985) can also be obtained by transformation of plants with bacterial genes. Although these antibiotic resistance genes are generally applicable as cell selectable markers, their use is often limited by legal restraints to the use of transgenic plants.

Universal recipient hybridizers have been constructed by the combination of a recessive and a dominant selection marker in one fusion parent. Hamill et al. (1984) and Koornneef et al. (1988) selected *Nicotiana* species that were nitrate reductase deficient and resistant to an antibiotic, simultaneously. Only fusion products, that were both complemented for the nitrate auxotrophy and resistant to the antibiotic were able to proliferate.

Summarizing, selection markers that are expressed at the cellular level, are important for the isolation of asymmetric hybrids. From the moment of selection, attention can be focused on processes in fusion products. In our experiments we used nitrate reductase-deficiency (Chapters 3 and 4), a cytoplasmic albino mutation (Chapter 4) and antibiotic resistances (Chapter 5) as selection markers.

1.4 Hybrids of *Lycopersicon* and *Solanum*.

In order to analyze the possibilities and limitations of asymmetric somatic hybridization as a plant breeding technique, we performed our studies with a model system. Protoplast fusions were carried out between the tomato (*Lycopersicon esculentum* Mill.) as the recipient and potato (*Solanum tuberosum* L.) as the donor. Both cultivated species are important food crops and they are member of the subfamily *Solaneae* of the family *Solanaceae*. Both species have the same basic chromosome number (x=12).

The tomato is a favorite model plant for genetic studies (Rick and Yoder 1988; Hille et al. 1989). The plant is easy to culture, has a short life cycle of roughly five months, is highly self-fertile and is a simple diploid (2n=2x=24) whose chromosomes are distinguishable at the pachytene stage of meiosis. Some of the tomato chromosomes can also be identified at mitosis (Rick and Butler 1956; Ramanna and Prakken 1967; Gill 1983). The linkage map of tomato contains over 300 morphological, isoenzyme and disease resistance markers (Tanksley and Mutschler 1990), to which over 1,000 restriction fragment length polymorphism (RFLP) markers have been added in recent years (Tanksley et al. 1992). Koornneef et al. (1987) isolated genotypes of tomato with improved cell and tissue culture traits.

The potato is cultivated as a tetraploid (2n=4x=48), but diploid and mono(ha)ploid genotypes are available. The tetraploid crop is self-fertile whereas the dihaploid is usually selfincompatible. The propagation is normally vegetative. Karyotyping is possible (Pijnacker and Ferweda 1984) and an RFLP map, largely colinear with tomato, is available (Tanksley et al. 1992). As for tomato (Zabel et al. 1985), potato specific repeat probes have been isolated (Visser et al. 1988).

The tomato (*L. esculentum*) has successfully been crossed with all other *Lycopersicon* species, although some combinations required embryo rescue. Furthermore, sexual hybrids have been obtained between tomato and *Solanum lycopersicoides* respectively *S. rickii*, which are the *Solanum* species that are most related to tomato. These intergeneric crosses also needed embryo rescue (Rick et al. 1986). Tomato and potato cannot be cross-hybridized.

The first somatic hybrids between tomato and potato were described by Melchers et al. (1978). These symmetric hybrids, called "pomatoes", formed flowers, parthenocarpic fruits and slightly thickened rhizomes. However, all hybrids were sterile. More recently, other somatic hybrid plants between

Lycopersicon and *Solanum* species were constructed (listed in Table 2). The aim of these somatic hybridization experiments was to by-pass crossing barriers and to introduce valuable agronomic traits from *Solanum* species into the tomato or vice versa.

We characterized our symmetric and asymmetric hybrids between tomato and potato by means of isoenzyme analysis (Chapters 3, 4 and 5), karyotyping (Chapter 4), RFLP analysis of chloroplast DNA (Chapter 5) and with the tomato and potato specific repeat probes (Chapter 5).

Table 2. Somatic hybridization between *Lycopersicon* and *Solanum* species

Fusion combination		References
L. esculentum	(+) *S. acaule*	Melchers et al. 1992
L. esculentum	(+) *S. commersonii*	Derks et al. 1992
L. esculentum	(+) *S. etuberosum*	Derks et al. 1992
L. esculentum	(+) *S. lycopersicoides*	Handley et al. 1986
		Levi et al. 1988
		Li and Sink 1992
		Tan 1987
L. esculentum	(+) *S. muricatum*	Sakomoto and Taguchi 1991
L. esculentum	(+) *S. nigrum*	Guri et al. 1988
		Jain et al. 1988
L. esculentum	(+) *S. rickii*	O'Connell & Hanson 1986
L. esculentum	(+) *S. tuberosum*	Melchers et al. 1978
		Shepard et al. 1983
		Wolters et al. 1991
		Schoenmakers et al. 1992
		Jacobsen et al. 1992
L. pimpinellifolium	(+) *S. tuberosum*	Okamura 1988

1.5 Outline of the thesis

In this thesis, I describe how we have analyzed some of the problems of asymmetric somatic hybridization. For the protoplast fusion experiments we used plant genotypes that allowed efficient selection of fusion products at the cellular level. We isolated and characterized nitrate reductase deficient (NAR) mutants of tomato (Chapter 2), because of legal restraints to the use of transgenic plants. The nitrate auxotrophy of two "leaky" NAR mutants was sufficiently expressed at the level of the cell, to allow its use in somatic

hybridization experiments with potato as a selection marker (Chapter 3). We fused protoplasts of one of these "leaky" NAR mutants and of a cytoplasmic albino tomato with protoplasts of monoploid potato and recovered triploid somatic hybrids (Chapter 4). Apart from the non-transgenic fusion parents, tomato and potato were transformed with hygromycin and kanamycin resistance respectively, with the aid of *Agrobacterium tumefaciens*. We used hygromycin resistant tomato and kanamycin resistant potato genotypes in protoplast fusion experiments. The potato (donor) was gamma irradiated prior to fusion with tomato. We were able to select asymmetric fusion products on the basis of the transferred monogenic trait kanamycin resistance of potato. Correlations were determined between the elimination of potato DNA, the presence of tomato or potato chloroplasts, the ploidy level, the regeneration capacity and the presence or absence of the isoenzymes Malate deHydrogenase and Glutamate Oxaloacetate Transferase in the fusion products (Chapter 5). Because many investigators found a strong retainment of donor DNA in asymmetric somatic hybrids, we decided to study the direct effects of gamma irradiation on single and double strand DNA breakage, on DNA repair and on DNA synthesis after gamma irradiation in tomato and potato protoplasts. Furthermore, we studied karyogamy in heterokaryons to explain the low frequencies at which asymmetric fusion products usually are obtained (Chapter 6). Finally, I discuss the possibilities and limitations of asymmetric somatic hybridization as a plant breeding procedure (Chapter 7).

1.6 Published work

The data, presented in Chapters 1 through 7 have been or will be published in:

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

Schoenmakers HCH, Koornneef M, Alefs SJHM, Gerrits WFM, Kop D vd, Chérel I, Caboche M (1991) Isolation and characterization of nitrate reductase-deficient mutants in tomato (*Lycopersicon esculentum* Mill.) Mol Gen Genet 227: 458-464

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

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CHAPTER 2

ISOLATION AND CHARACTERIZATION OF NITRATE REDUCTASE-DEFICIENT MUTANTS IN TOMATO (*Lycopersicon esculentum* Mill.)

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Summary. Five nitrate reductase deficient mutants of tomato were isolated from an M_2 population after ethylmethanesulphonate (EMS) seed treatment by means of selection for chlorate resistance. All mutations were monogenic and recessive and complementation analysis revealed that they were non-allelic. Biochemical and molecular characterization of these mutants showed that four of them are cofactor mutants while one is an apo-enzyme mutant.

Introduction

Biochemically well defined mutants are relatively rare in higher plants. Among the best characterized are nitrate reductase deficient (NAR) mutants, which have been isolated both at the level of the cell and of the plant (Wray 1986). The enzyme nitrate reductase (NR), which catalyses the reduction of nitrate to nitrite, is a complex enzyme containing Flavine Adenine Dinucleotide (FAD), cytochrome b_{557} and molybdenum cofactor (MoCo) as prosthetic groups of a homodimeric structure (Kleinhofs et al. 1985; Chérel et al. 1990). NAR mutants have been used to analyse the genetics and molecular biology of nitrate assimilation, and in combination with molecular techniques to study the regulation of genes involved in nitrogen metabolism (Crawford et al. 1986; Galangau et al. 1988). An attractive aspect of the NR system is the expression of the different genotypes at the level of the cell: NAR mutants are resistant to chlorate, whereas they cannot grow on medium containing nitrate as sole nitrogen source. This implies that depending on the growth medium one can select for either the mutant or the wild type phenotype. This property makes the NAR mutants a useful tool in studies in mutagenesis and in somatic hybridization experiments. The efficient cell selection system and the availability of the cloned NR structural gene (Vaucheret et al. 1989) has allowed the cloning of an endogenous transposon from NR apo-enzyme (*nia*) mutants, isolated from an unstable tobacco genotype (Grandbastien et al. 1989).

NAR mutants have been studied extensively in *Nicotiana* species where at least seven different complementation groups have been identified and characterized genetically as well as biochemically (Gabard et al. 1987, 1988).

Thus far well defined biochemical mutations in tomato are limited to isozyme loci (Mutschler et al. 1987). NAR mutants will provide a new set of biochemical mutants of the tomato, which can be used to study nitrate assimilation in this species. In addition the mutations will provide, because of their selectable

phenotype in cells, useful markers for cloning of endogenous transposons and for studies on somatic hybridization. This report describes the isolation and characterization of five NAR mutants selected on the basis of chlorate resistance at the seedling stage.

Materials and methods

Mutagenic treatment of seeds.
Mutant selection was performed on the progeny of plants grown from mutagenized seeds of the homozygous genotypes GT (a tomato mosaic virus-resistant pure line, kindly provided by Deruiterseeds, Bleiswijk, The Netherlands) and cv. Moneymaker (MM). To induce mutations, seeds were submerged in a freshly prepared, unbuffered 60 mM ethylmethanesulphonate (EMS, Eastman Kodak Company USA, 7830) solution in the dark for 24 h at 25° C. After rinsing with tap water M_1 plants were grown from these seeds in an unheated greenhouse in Dutch summer conditions. Seeds were harvested from groups of ten fertile M_1 plants each, from the first cluster upwards.

Selection for chlorate resistance.
M_2 seeds, from the selfed M_1 progeny, were germinated at 25° C in the dark in 500 ml translucent plastic containers, with a perforated lid for gas exchange, containing 100 ml vermiculite and 50 ml of a mineral solution consisting of 53.8 mM KCl, 3 mM $CaCl_2.2H_2O$, 1.5 mM $MgSO_4.7H_2O$, 1.25 mM KH_2PO_4, sequestrene and trace-elements according to Murashige and Skoog (1962). After 4 days the containers were transferred to the light (Philips TLD 36W, intensity 4 W/m^2) and 10 ml of 10 mM $KClO_3$ was added per container to a final concentration of 1.67 mM $KClO_3$. Chlorate resistant plants were selected 4 to 7 days later. Resistant seedlings were transferred to a new container in which 5 mM ammonium succinate had been added to the medium described above.

Growth conditions and genetic characterization.
Chlorate resistant mutants were transferred to soil or grafted onto GT root stocks in the case of "extreme" mutants that were not able to grow in soil. All mutants could be crossed with wild type. Wild type / mutant segregation ratios were determined by counting the number of wild type versus chlorate resistant seedlings, as tested in the container system described above, or by growing

plants in soil, watered with tap water, and screening for the wild type versus the NAR seedling phenotype. Since the extreme mutants were very difficult to hybridize, complementation analysis of these mutants was based on crosses between mutant x wild type F_1 hybrids and the presence or absence of mutant phenotypes in the progeny of these crosses.

Growth of callus on nitrate containing medium.
Hypocotyls of 18 day old *in vitro* seedlings were put on a callus inducing solidified R3B medium. This medium contains salts according to Murashige and Skoog (1962), vitamins according to Tewes et al. (1984), 100 mg/l myo-inositol, 30 g/l sucrose, 2 mg/l 1-naphthylacetic acid (NAA), 1 mg/l 6-benzylaminopurine (BAP) and 0.8 g/l agar. pH was adjusted to 5.8 before autoclaving. Relative growth rates were calculated from the increase in weigth of the hypocotyls 17 days later.

Determination of nitrate content.
For determination of nitrate + nitrite concentrations leaf tissue was frozen and stored at -80° C. For each determination 0.2 - 0.5 g of frozen leaf material was thawed in a test tube and 3 ml of cold demineralized water was added; the mix was then homogenized on ice by means of a blender. After centrifugation at 2500 g for 5 min, supernatants were collected and subjected to dialysis in a continuous-flow analysis system (Technicon autoanalyzer) to separate ions from interfering substances like colloids and coloured organic compounds. Ions from the extracts passed through the membrane and were taken in an understream of 0.19 M NH_4Cl, adjusted to pH 6 with NH_3. The nitrate was then reduced to nitrite by means of copper-coated cadmium. Next sulfanylamide, H_3PO_4 and n-1-naphthyl-ethylenediamine were added to final concentrations of 13 mM, 0.68 M and 1.2 mM respectively and after mixing for 10 min absorption was measured at 550 nm.

In vivo *tests for NR activity.*
100 mg of leaf tissue was cut into pieces with a razor blade and put in a test tube containing 2 ml of an incubation buffer derived from a buffer described by Jaworsky (1971). This consisted of 0.1 M potassium phosphate buffer pH 7.5, 40 mM KNO_3, 3 μM chloramphenicol and 2.5% (v/v) n-propanol. Incubation was carried out at 28° C in a water bath for 1 h. After this, 1 ml of the incubation mixture was transferred to a new test tube and 0.5 ml of 58 mM

sulfanylamide in 1 M HCl and 0.5 ml of 0.54 mM n-1-naphthyl-ethylenediamine were added and mixed. After 10 min absorption was measured at 540 nm in an Ultrospec-K automatic spectrophotometer.

Extraction and purification of nitrate reductase.
Soluble proteins were extracted from samples of 5 g each of leaves, stored frozen at -80° C. The proteins were precipitated with $(NH_4)_2SO_4$ as described by Chérel et al. (1986). These preparations were used as such for immunochemical as well as enzymatic tests.

Immunochemical tests for NR.
We estimated the antigenicity of the mutant NR proteins by a two-sites ELISA using the monoclonal antibody (MAb) ZM 96(9)25, raised against the maize NR apo-enzyme, as coating reagent, and a rabbit polyclonal antiserum raised against maize NR as second antibody (Chérel et al. 1985, 1986). Binding of the polyclonal antiserum was detected according to Chérel et al. (1985, 1986) by incubation in an anti rabbit-IgG-alkaline phosphatase conjugate, followed by a phosphatase assay. The O.D. at 405 nm was measured 15 min later. The ELISA reactivity was calculated from the distance between the titration curve of the mutant and the wild type curve.

In vitro *tests for NR activity.*
Nitrate reduction with, respectively, reduced β-nicotinamide-adenine dinucleotide (NADH), reduced methylviologen (MV), reduced flavine mononucleotide ($FMNH_2$) and reduced bromophenolblue (BPB) as electron donors was measured as described by Chérel et al. (1990) in $(NH_4)_2SO_4$ precipitates of plant extracts. The cytochrome *c* reductase (CcR) activity was measured according to Wray and Filner (1970). As the protein extracts may contain NADH-CcR activities that are not linked to NR, the NR associated NADH-CcR activity was estimated according to Chérel et al. (1990) by the difference between the total CcR activity of the extract measured in the presence of a control monoclonal antibody 42(22), which does not cross-react with tomato NR, and the residual activity after inhibition by a saturating amount of the monoclonal antibody ZM 96(9)25.

Polyacrylamide gel electrophoresis (PAGE) of xanthine dehydrogenase (XDH).
Analysis of XDH was performed by PAGE according to Mendel and Müller

(1976). For this test 0.5 g fresh leaf material was ground in 0.5 ml of 0.05 M TRIS-HCl, 20% (v/v) glycerol and 1% (v/v) β-mercaptoethanol solution, pH 6.8. After centrifugation in an Eppendorf centrifuge for 5 min at maximum speed the supernatants were subjected to electrophoresis.

RNA extraction and Northern blotting.
RNA was isolated according to Galangau et al. (1988) from samples of 2 g each of leaves which had been stored at -80° C. For Northern blot analysis 8 μg of RNA per sample was electrophoresed on an agarose gel and blotted onto nitrocellulose (Thomas 1980). The blot was probed with the cloned tomato *nia* gene (Daniel-Vedele et al. 1989).

Results

Mutant selection

Preliminary tests with wild type seedlings in the plastic containers showed that the nitrogen source affected both the growth and tolerance to chlorate (Hofstra 1977; Fig.1). Medium without nitrate was chosen for selection of chlorate resistant seedlings because the visual effect of the chlorate treatment was more pronounced on this than on medium with nitrate.

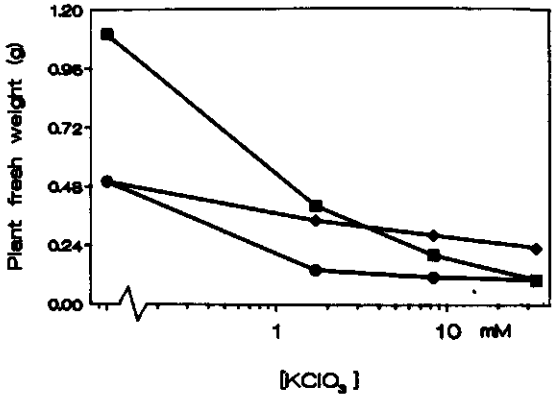

Fig.1. Relationship between nitrogen supply and resistance to chlorate of 4 day old wild type tomato seedlings. —●— : no nitrate from the water supply, —■— : 10 mM KNO_3, —◆— : no nitrate + 5 mM ammonium succinate, Chlorate concentrations are on a logarithmic scale.

Approximately 9900 M_2 seeds from 165 different M_2 lots (combining seeds harvested from 10 different M_1 plants) were tested for chlorate resistance. Chlorate resistant seedlings were observed in 7 different M_2 lots (Fig.2); in 5 cases plants could be grafted successfully onto root stocks. Mutation frequencies for chlorate resistance were approximately 0.4% for both MM and GT. MM gave rise to M_2 chlorate resistant mutants A29 and A57; GT gave rise to M2 mutants C3, C31, and C42.

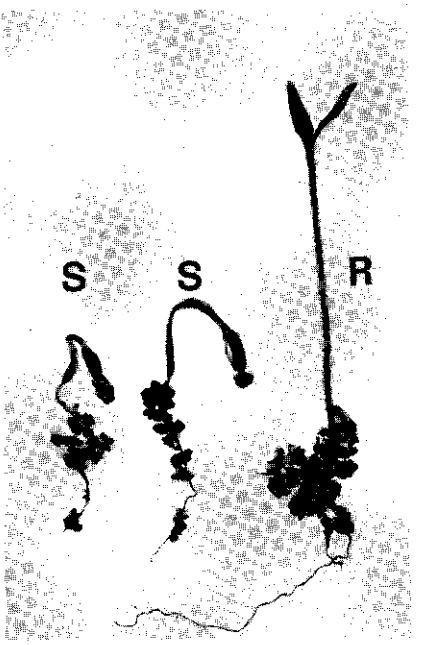

Fig.2. Chlorate sensitive (S) and resistant (R) tomato plants from the GT x C3 F_2 at the time of screening (7 days after the addition of chlorate).

Scions of the mutants A57, C3 and C42 soon showed chlorosis which gradually developed into necrosis starting at the tips of the leaves. Cuttings (side shoots) from these scions did not grow in soil. The poor growth and seedset of these mutants, even after grafting, made it very difficult to maintain these mutants either as scions or seeds. However the flowers could be used to pollinate wild type plants. Cuttings from the chlorate resistant seedlings A29 and C31 rooted and grew well in soil. When grown in soil, A29 seedlings developed a mild chlorosis and growth was somewhat reduced. Later in development the differences between A29 and wild type became less obvious. C31 seedlings and mature plants are phenotypically identical to wild type under these conditions.

Genetic analysis

For all five chlorate resistant mutants Table 1 shows the summed segregation ratios of wild type and mutant phenotype in F_2 populations derived from wild type x mutant crosses. For four of the mutations we found that the F_2 populations always had segregation ratios that were consistent with a 3:1 ratio of wild type and mutant phenotype; the corresponding chlorate resistance trait can be considered as monogenic and recessive. However most of the F_2 populations derived from MM x A57 crosses had segregation ratios which deviated significantly from 3:1 (data not shown); in the F_2 populations the summed segregation ratio was not consistent with 15:1 either [$X^2_1(15:1) = 28.56$, $P(X^2_1(15:1) < 0.01$] and thus this result cannot be explained by the homozygosity of A57 for two recessive chlorate resistance mutations. We assume that the segregation ratios of these F_2 population are distorted because NAR seeds did not germinate well. Germination of F_2 seeds from MM x A57 crosses was low (about 50%) in this analysis. A reduced transmission of the mutant allele through pollen could also be the reason for this shortage of mutants.

Table 1. Summed segregation ratios in F_2 populations derived from crosses of MM x A29, MM x A57, GT x C3, GT x C31 and GT x C42

Genotype	Wild type	Mutant	$X^2_1(3:1)$	$P[X^2_1(3:1)]$
A29[a)]	168	57	0.01	0.97
A57[b)]	458	62	47.43	<0.01
C3 [b)]	175	57	0.02	0.82
C31[a)]	88	32	0.18	0.74
C42[b)]	117	45	0.67	0.46

a) based on chlorate selection
b) based on the "lethal" phenotype.

Non-allelism of all mutants is inferred from the exclusive presence of plants, phenotypically similar to wild type, in the progeny of either mutant x mutant (A29 x C31; A29 x C42; C31 x C3), mutant x [F_1 mutant x wild type] or

reciprocal crosses (A29 x A57; A29 x C3; A29 x C42; C31 x A57; C31 x C42; C42 x A57; C42 x C3), or [F_1 mutant x wild type] x [F_1 mutant x wild type] (A57 x C3; C3 x C42) crosses. For this complementation analysis 15 - 183 seedlings per F_2 population were scored.

Complementation between A29 and C31, both mutations with less extreme phenotypes was confirmed by the wild type levels of in vitro NR activity in the F_1 hybrid and by the segregation of chlorate sensitive seedlings in the F_2 population (data not shown).

Growth of callus on nitrate containing medium.

Hypocotyl explants of A57, C3 and C42 formed hardly any callus and turned brown within 1 week after transfer to R3B medium. A29 and C31 formed callus but not as much as wild type (Fig.3).

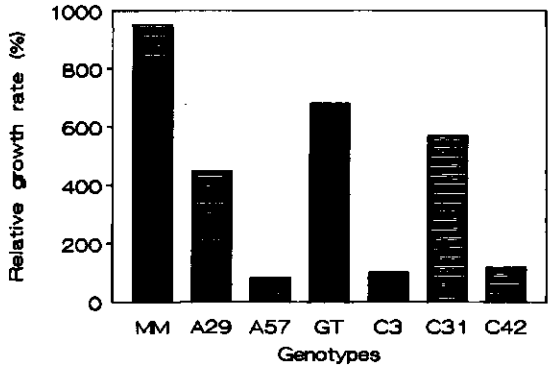

Fig.3. Relative growth rate (percentage fresh weight increase) of hypocotyl explants of chlorate resistant mutants and wild types on R3B medium determined after 17 days.

Determination of nitrate content.

The nitrate content was determined in soil grown plants of MM, A29, GT and C31, and in grafted plants of C3 and C42 (Table 2). In all mutants tested nitrate is over-accumulated in leaf cells; this suggests that they are also able to take up chlorate. Therefore we do not consider these mutants as "uptake mutants", which are resistant to chlorate because of their inability to take chlorate up in their cells.

Table 2. Nitrate content of fresh leaf material of 3 week old MM, A29, GT and C31 seedlings and C3 and C42 grafted plants.

Genotype	NO_3^-content (μmol/g leaf)	Standard deviation (n-1)	Number of plants
MM	9.8	3.0	11
A29	144.2	29.8	12
GT	18.9	6.4	9
C3	148.9		1
C31	28.2	8.3	8
C42	66.6		1

In vivo *tests for NR activity.*

Three of the tested mutants do not have detectable *in vivo* NR activity (Table 3) which confirms the nature of the mutants. However, *in vivo* NR activity did not seem significantly reduced in A29 and C31.

Table 3. In vivo nitrate reductase (NR) activity.

Genotype	NR activity (u/g)
MM	24
A29	19
A57	0
GT	48
C3	0
C31	43
C42	0

One unit is defined as the amount of enzyme necessary to reduce 1 nmol nitrate per minute at 28° C.

Because A29 and C31 grow in soil, and thus yield larger amounts of plant material, we could analyse them in more detail. To minimize background mutation effects we analysed F_3 lines (derived from MM x A29 and GT x C31 crosses) which were homozygous for chlorate resistance and did not show any other visually detectable phenotypes.

Immunochemical tests for NR.

The MAb ZM 96(9)25 cross-reacted strongly with both MM and GT extracts. The titration curves of A29 and C31 do not only show a shift to the right, but also a (slightly) less steep slope (Fig.4). In addition, the curve of C31 shows a lower plateau. Therefore we conclude that the epitopes (for the MAb and/or the polyclonal antiserum) of the antigen (the NR) involved in the ELISA test are (slightly) altered, especially in the case of C31. This might be due to conformational alterations of the dimer, a monomeric state or degradation of the apo-enzyme, perhaps related to a monomeric state. Because of the assumed alterations of the antigen it is possible that the protein content has been underestimated from these ELISA curves. For A29 only roughly 15% of the antigen was present in leaves compared with its wild type, MM; for C31 this was roughly 25% compared to its wild type GT.

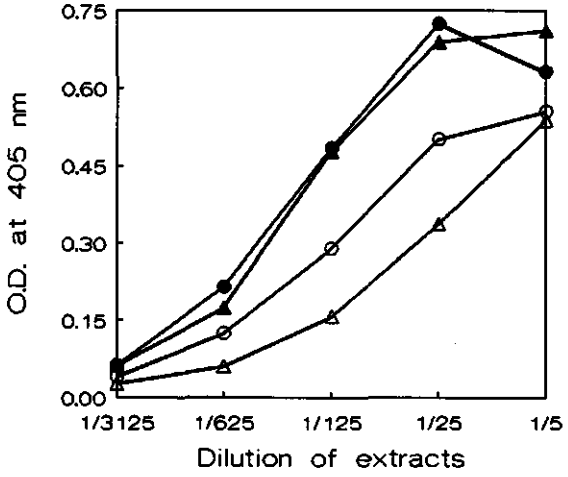

Fig.4. Two-sites ELISA titration curves of NR apo-enzyme in leaf extracts of MM —▲— , A29 —△— , GT —●— , C31 —○— with monoclonal antibody (MAb) ZM 96(9)25. The optical density at 405 nm was measured in the wells.

In vitro *tests for NR.*

To determine the properties of NR in the A29 and C31 mutants *in vitro* tests for NR were carried out. NR accepts electrons for nitrate reduction not only from NADH but also from a variety of artificial electron donors (Hoarau et al. 1986). *In vitro* NR activity with some (artificial) electron donors is shown in Table 4. For both A29 and C31 the low levels of NR activity with each of the individual

electron donors are in agreement with the hypothesis that these mutants are NAR mutants.

Table 4. In vitro NR activities of wild type and mutant tomato with NADH, methylviologen (MV), $FMNH_2$, or bromephenolblue (BPB) as electron donors.

Genotype	NADH	% of wt	MV	% of wt	$FMNH_2$	% of wt	BPB	% of wt
Wild type MM	60.2		25.3		18.5		29.5	
Mutant A29	1.0	2	0.9	4	0.3	2	1.1	4
Wild type GT	59.0		20.3		12.5		20.3	
Mutant C31	10.7	18	4.5	22	2.6	21	10.0	49

Activities are given as nmol NO_3^- reduced per minute per gram fresh leaf weight. Mutant activities are also given as a percentage of wild type (wt) activity.

NR can not only reduce nitrate but can also function as a diaphorase capable of carrying out NADH dependent reduction of a variety of artificial electron acceptors, such as cytochrome *c* (Hewitt et al. 1976). The *in vitro* CcR activity of the extracts and of the NR is shown in Table 5. MAb ZM 96(9)25 inhibits CcR activity while the control MAb 42(22) does not (data not shown). The NR-CcR activity of the mutants was not affected as much as were *in vitro* nitrate reduction and protein content, as determined by ELISA.

Table 5. In vitro cytochrome *c* reductase (CcR) activity in wild type (wt) and chlorate resistant genotypes of the tomato.

Genotype	CcR activity after preincubation in:		NR-linked CcR activity	% of wt NR-linked activity
	MAb 42(22) (a)	MAb 96(9)25 (b)	(a-b)	
Wild type MM	545	50	495	
Mutant A29	370	63	307	62
Wild type GT	393	48	345	
Mutant C31	333	59	274	79

The first column gives the total relative CcR activity of the extracts. 42(22) is a control MAb which does not bind tomato NR. The second column gives the NADH-CcR activity which is not linked to the NR. 96(9)25 is a MAb which reacts specifically with NR in the extracts. The third column gives the CcR activity linked to NR (a-b). (absorptions x 1000).

Gel electrophoresis of XDH

Polyacrylamide gel electrophoresis followed by enzyme assay showed that A29, A57, C3 and C42 lack XDH activity whereas MM, GT and C31 do show expression (Fig.5). Since XDH uses the same molybdenum cofactor (MoCo) as NR (Johnson 1980; Wray 1986) A29, A57, C3 and C42 are considered MoCo (*cnx*) mutants while C31 is classified as an apo-enzyme (*nia*) mutant. If A29 is a *cnx* mutant it is possible that XDH is expressed at low levels like NR; XDH activity might fall below the detection level of the assay system used.

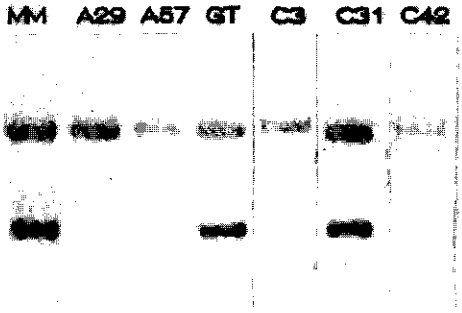

Fig.5. Xanthine dehydrogenase (XDH) activity detection after polyacrylamide gel electrophoresis (PAGE). The upper band is non-specific; the lower band is the XDH band.

RNA extraction and Northern blotting.

Northern blot analysis with the cloned tomato *nia* gene showed that mRNA for the NR apo-enzyme was present in both wild types and in the mutants. Expression in A29 was higher than in C31 and wild type (Fig.6). This is in agreement with previously observed over-accumulation of NR mRNA in tobacco when NR activity was inhibited by tungstate treatment (Deng et al. 1989) and in *Nicotiana plumbaginifolia* when NR activity was lost by mutation (Pouteau et al. 1989).

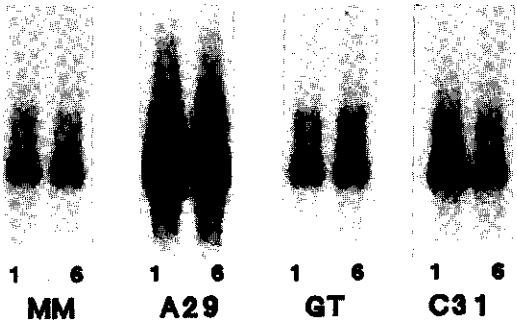

Fig.6. Northern blot analysis (autoradiogram) of MM, A29, GT and C31, harvested one (1) and six (6) hours after sunrise, probed with the cloned tomato *nia*-gene.

In tobacco NR expression is under the control of a circadian rhythm (Galangau et al. 1988; Deng et al. 1990). Such a rhythm, which is characterized by high NR mRNA levels at sunrise and low levels during the rest of the day, was not observed in our experiments with tomato leaf samples from greenhouse grown plants, frozen 1 and 6 h after sunrise.

Discussion

The present report shows that nitrate reductase deficient mutants of tomato can be selected efficiently at the seedling stage. Thus far five complementation groups have been identified represented by one *nia* and four different *cnx* types. In all plant species studied thus far at least one *nia* complementation group has been found. In *N. plumbaginifolia* six *cnx* complementation groups were identified among 70 mutants with reduced or absent activity of both XDH and NR (Gabard et al. 1988).

Mutants A29 and C31 were less resistant to chlorate than A57, C3 and C42 in dose response experiments in tissue culture (data not shown). Residual NR activity can account for this. In mutant A29, which has almost no NR activity, the residual activity is apparently sufficient to allow its survival in soil with nitrate as the only source of nitrogen.

Mutants of the *cnxA* type (Grafe and Müller 1983) are apparently not present among the *cnx* mutants described in this report because none of these mutants could be restored with molybdenum as tested in tissue culture on various media (results not shown).

Although in mutants A29 and C31 the *in vivo* level of NR was almost that of wild type tomato, the *in vitro* NR activity was much lower: immunochemical assays with anti-NR antibodies detected less protein and the mutant plants were resistant to chlorate. Therefore we consider the *in vivo* NR activity, as assayed according to Jaworsky (1971), not to be useful for the characterization of putative NAR mutants in tomato.

The reduction of nitrate by NR is a complex process, involving several steps of electron transfer. The measurement of partial catalytic activities allows the determination of which electron transfer step is affected in a specific mutant. The reduction of the electron acceptor cytochrome *c* involves the first two domains of the enzyme, an NADH cytochrome b_{557} reductase and cytochrome b_{557}. A mutation affecting one of these two domains will lead to the loss of CcR activity (Kleinhofs et al. 1985; Wray 1986; Campbell 1988; Kubo et al. 1988; Chérel et al. 1990). Mutants A29 and C31 do not appear to be affected in these two first domains since they express CcR activity levels close to that of

the wild type. MV, $FMNH_2$ and BPB can also be used as electron donors for the reduction of nitrate by NR. Chérel et al. (1990) have postulated that in *N. plumbaginifolia* nitrate reduction by $FMNH_2$ and MV requires a functional cytochrome b_{557} domain and MoCo binding domain, whereas the reduction of nitrate by BPB only requires the last domain. The comparison of terminal transferase activities with CcR activity suggests that A29 and C31 are defective in the last steps of electron transfer and are therefore probably affected in the MoCo-binding domain. Mutant A29 is also defective in XDH activity, which suggests that it lacks the MoCo and that its NR MoCo-binding domain is defective because of the absence of MoCo normally inserted into this domain. Since mutant C31 is not appreciably affected in XDH activity, we assume that the MoCo binding domain itself is affected in this mutant. In agreement with this is the observation that with different electron donors terminal transferase activities are differentially affected in the mutant. C31 is therefore classified as a nia mutant.

Interestingly enough A29 as well as C31 extracts contain NR protein which is detectable by ELISA with MAb 96(9)25. This MAb has recently been shown to recognize the heme group in the cytochrome b_{557} domain of the enzyme. As expected from CcR activity measurements this domain is detected in A29 and C31; however less antigen was detected in A29 than what would be expected on the basis of transcript analysis. It is possible that the cofactor has a stabilizing effect on the apo-enzyme, and that the apo-enzyme is degraded more rapidly in the absence of the cofactor.

The inability to grow in soil and the slow growth both as scions or *in vitro* on ammonium succinate containing medium limits the use of the extreme mutants A57, C3 and C42 in genetic experiments at both the level of the plant and of the cell. It is possible that MoCo deficiency results in loss of essential molybdoenzymes other than NR and XDH. For the "leaky" mutants A29 and C31 these negative pleiotropic effects are less pronounced but the chlorate resistance is also less. Thus, it is questionable whether the nitrate auxotrophy of A29 and C31 is sufficiently expressed at the level of the cell, to allow its use in somatic hybridization experiments. The use of the *nia* mutant for testing and characterizing genetic instability is facilitated by both the viability of the mutant and the availability of the cloned *nia* gene in tomato (Daniel-Vedele et al. 1989).

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CHAPTER 3

USE OF LEAKY NITRATE REDUCTASE-DEFICIENT MUTANTS OF TOMATO (*Lycopersicon esculentum* Mill.) FOR SELECTION OF SOMATIC HYBRID CELL LINES WITH WILD TYPE POTATO (*Solanum tuberosum* L.)

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Summary. Protoplasts of two "leaky" nitrate reductase deficient and thus nitrate auxotrophic (NAR) mutants of tomato and their wild types, were fused with protoplasts of monoploid potato. In all four combinations hybrid calli grew more vigorously than parental calli and this somatic hybrid vigour as such provided a useful enrichment for somatic hybrids. Selection against nitrate auxotrophy further increased the efficiency of the enrichment, particularly if a molybdenum cofactor mutation was used as the basis for the selection. It is concluded that the nitrate auxotrophy of these NAR mutants is sufficiently expressed at the level of the cell, to allow its use in somatic hybridization experiments with potato.

Introduction

In somatic hybridization experiments it is often necessary to select hybrid fusion products from a population, which also contains products of non-fused and fused protoplasts derived from one parent only. Selection can be performed at the level of the cell, the callus or the plant. Early selection of hybrid fusion products, shortly after fusion, is attractive because it increases the efficiency of the procedures.

Efficient selection at the level of the cell is possible on the basis of auxotrophies of one or both of the parents: Hybrid fusion products show genetic complementation for auxotrophy and can grow under normal conditions and under permanent selective pressure for hybridity. However, auxotrophic mutants are available only for a limited number of plant species (Gleba and Shlumukov, 1990). This limitation also holds for other mutations that can be used as a basis for hybrid selection such as dominant mutations causing antibiotic resistance and mutations causing resistance against amino-acid analogues and herbicides (Maliga et al. 1975; Bourgin 1978; Sato et al. 1988). Although the dominant antibiotic resistances obtained by transformation of plants with bacterial genes (Bevan et al. 1983; Herrera-Estrella et al. 1983; Waldron et al. 1985) are general applicable as cell selectable markers, their use is often limited by legal restraints to the use of transgenic plants.

The most generally used plant auxotrophes in somatic hybridization experiments are the nitrate reductase deficient (NAR) mutants of *Nicotiana spp.* (Glimelius et al. 1978; Grafe and Müller 1983; Lazar et al. 1983; Dirks et al. 1985; de Vries et al. 1987; Gabard et al. 1987; 1988; Koornneef et al. 1988;

Famelaer et al. 1989). Nitrate reductase (NR; EC 1.6.6.1 and EC 1.6.6.2), catalyses the reduction of nitrate to nitrite. NAR mutants cannot grow on a medium or soil containing nitrate as a sole nitrogen source and NAR mutations are expressed at the level of the cell, the callus and the plant (Wray, 1986). The difficulty in cultivating NR mutants *in vivo* limits their use in genetic experiments. Leaky mutations do not show these problems but their value for selection at the cellular level is uncertain.

Recently, we have described five NAR mutants in tomato (Schoenmakers et al. 1991), of which two (A29 and C31) are leaky. They display reduced NR activity and are capable of growth in soil and on normal tissue culture media. In this short communication we analyse whether the leaky NAR-mutations in tomato (*Lycopersicon esculentum*) are useful as a basis for selection at the cellular level in somatic hybridization experiments with potato (*Solanum tuberosum*).

Materials and methods.

Plant material.

For tomato two different NAR mutants, described by Schoenmakers et al. (1991), and their corresponding wildtypes were used in fusion experiments. The NR molybdenum cofactor (MoCo, *cnx*$^-$) mutant A29 was backcrossed once with its wild type cv. Moneymaker (MM) as a pistillate parent; the NR apo-enzyme (*nia*$^-$) mutant C31 was backcrossed once with its wild type cv. GT as a pistillate parent. F_3 lines, which were homozygous for chlorate resistance (Schoenmakers et al. 1991) and did not show any other visually detectable phenotypic abnormalities which were not associated with the NR mutations, were used for fusion. Potato 7322 monohaploid (2n=x=12) originated from prof. dr. G. Wenzel, Max Planck Institut, Köln, Germany. For detailed description see de Vries et al. (1987).

Isolation, fusion and culture of protoplasts.

Isolation, electrofusion and culture of protoplasts was carried out as described by Wolters et al. (1991). Protoplast media contained nitrate, vitamins and amino acids as a nitrogen source; ammonium was not present in these media. Minicalli were transferred to solidified TMcμZ greening medium (Wolters et al., 1991)

and subcultured every month. Larger calli were transferred to 1Z medium (modified 2Z medium (Thomas and Pratt, 1981) with 1 instead of 2 mg/l zeatin) for faster growth and regeneration.

Isoenzyme analysis.
For isoenzyme analysis 50-100 mg fresh callus tissue was ground in 30 μl 0.05 M TRIS-HCl, 0.1 g/l bromophenol blue, 20% (v/v) glycerol and 1% (v/v) ß-mercaptoethanol solution, pH 6.8. After centrifugation in an Eppendorf centrifuge for five minutes at maximum speed (16,000 r.p.m.) the supernatants were subjected to polyacrylamide gel electrophoresis. Glutamate-Oxaloacetate Transaminase (GOT; EC 2.6.1.1) and 6-PhosphoGluconate DeHydrogenase (6-PGdH; EC 1.1.1.44) activities were analysed using 30 μl samples on a 5 % stacking and 7 % running gel (Desaphor VA equipment, Desaga, V=400 V, I=125 mA). The electrophoresis buffer consisted of 20 mM Tris-HCl and 50 mM glycine, pH 8.8. After electrophoresis the gels were incubated in staining solutions prepared according to Vallejos (1983).

Results and discussion

Electrofusion resulted in 2-5 % fusion products, estimated directly after fusion. Protoplasts divided and many microcalli developed from all fusion combinations. Cultures of parental protoplasts with and without fusion treatment and mixed cultures without fusion treatment hardly gave microcalli. This suggests that somatic hybrid calli grew more vigorously and that this hybrid vigour as such provides a useful enrichment for somatic hybrids. Earlier publications, describing somatic hybridization of tomato and potato (Melchers et al. 1978; Shepard et al. 1983), where selection was based on the phenotype of regenerated plants, did not report this hybrid vigour at the cellular level.

The hybridity of the calli was determined by GOT and 6-PGdH isozyme analysis of randomly selected calli, two to five months after protoplast fusion (Fig. 1). GOT and 6-PGdH zymograms of callus of A29, C31, MM and GT were identical (data not shown).

The somatic hybrid vigour was a more efficient selection criterion in combinations with GT than in combinations with MM since MM itself displayed a better callus growth capacity from protoplasts than GT. The use of the "leaky"

NAR mutants further increased the efficiency of the enrichment for somatic hybrid calli (Table 1). Also here, the *cnx*$^-$ mutant of MM displayed a better callusing response than the *nia*$^-$ mutant of GT.

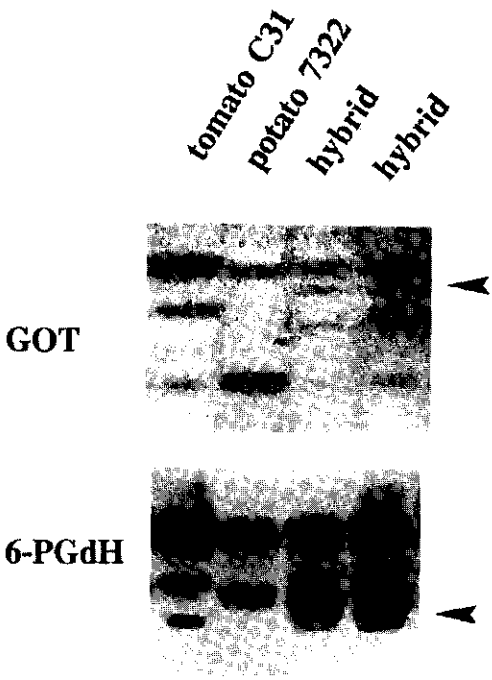

Fig.1. GOT and 6-PGdH isoenzyme analysis of tomato NAR mutant C31; potato 7322 and two hybrid fusion products. GOT and 6—PGdH are dimeric enzymes. Zymograms of fusion products show an extra hetero-dimeric band (◄) for both isoenzymes.

Table 1. GOT isoenzyme analysis of randomly selected calli, two to five months after protoplast fusion of tomato NAR mutants C31 and A29 and their corresponding wild types GT and MM with potato 7322.

Fusion combination	Number of calli			Total	% hybrid calli
	Hybrid	Tomato	Potato		
MM (+) 7322	4	28	0	32	13
A29 (+) 7322	23	7	0	30	77
GT (+) 7322	47	6	0	53	89
C31 (+) 7322	68	0	0	68	100

If the *nia*$^-$ mutant C31, which is exclusively impaired in nitrate reductase activity, was one of the fusion parents, the enrichment of hybrid calli by selection for nitrate-dependence was hardly more efficient than if the wild type GT was one of the parents. The *cnx*$^-$ (MoCo) mutant A29 is not only impaired in nitrate reductase activity but also in the expression of other MoCo containing enzymes like Xanthine Dehydrogenase and Sulfite Oxidase. If this *cnx*$^-$ mutant was one of the fusion parents, the enrichment of hybrid calli by selection for nitrate-dependence was improved sixfold. However, this does not necessarily imply that *cnx*$^-$ mutations are more useful as the basis for selection than *nia*$^-$ mutations, because the genetic background of A29 (MM) differs from that of C31 (GT) and the *cnx*$^-$ mutant A29 displays a lower nitrate reductase activity than the *nia*$^-$ mutant C31 (Schoenmakers et al. 1991). Experiments without selection for nitrate auxotrophy were not carried out.

In addition to the somatic hybrid vigour at the level of callus growth, regeneration capacity on 1Z medium was much better for hybrid than for tomato calli. Hybrid and tomato shoots showed morphological differences (data not shown). The somatic hybrid plants will be analysed in the near future.

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CHAPTER 4

ALLOTRIPLOID SOMATIC HYBRIDS OF DIPLOID TOMATO (*Lycopersicon esculentum* Mill.) AND MONOPLOID POTATO (*Solanum tuberosum* L.).

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Summary. Allotriploid somatic hybrids were obtained from fusions between protoplasts of diploid tomato and monohaploid potato. The selection of fusion products was carried out in two different ways: 1) Fusion of nitrate reductase deficient tomato with potato gave rise only to hybrid calli if selection was performed on media without ammonium. Parental microcalli were rarely obtained and did not regenerate. 2) Fusion of cytoplasmic albino tomato with potato gave rise to albino and green hybrid calli and plants. Allotriploids were identified from the two somatic hybrid populations by counting chloroplast numbers in leaf guard cells and by flowcytometry of leaf tissue. Although some pollen fertility of allotriploids and pollen tube growth of tomato, potato and *Lycopersicon pennellii* into the allotriploid style were observed, no progeny could be obtained thus far. The relevance of allotriploid somatic hybrids in facilitating limited gene transfer from potato to tomato is discussed.

Introduction

Asymmetric somatic hybrid plants, that combine the total genome of a recipient species with part of the genome of a donor species, can be constructed in various ways. Many investigators used ionizing irradiation of donor cells or protoplasts prior to fusion with recipient protoplasts. The irradiation causes changes or loss of bases, different types of crosslinking, DNA fragmentation and thus the elimination of damaged nuclear DNA of the donor in fusion products. However, the extent of elimination of donor DNA is often unsatisfactory and the irradiation treatment also reduces the efficiency of recovering somatic hybrids. Furthermore, endoreduplication during cell culture increases the ploidy level of asymmetric somatic hybrids, the genomic balance is often disturbed and, in general, fertility of the hybrids is low or absent, which prevents further elimination of donor DNA during meiosis by possible recombination between recipient and donor genomes.

Techniques such as the fusion of donor microprotoplasts, which contain micronuclei with one or a few chromosomes (Verhoeven et al. 1991), with recipient protoplasts and micro-injection of isolated chromosomes (de Laat et al. 1989) or micronuclei theoretically result in highly asymmetric hybrids. However, these procedures are technically complicated and require cell cultures of the donor species of which the cell cycle can be synchronized efficiently.

A more established approach to reduce the contribution of the donor genome to that of the hybrid, is to use haploid unirradiated genotypes instead of the normally used diploid irradiated donor protoplasts for fusion. Haploid protoplasts can be either of gametophytic or of sporophytic origin. In theory, one complete set of chromosomes (one genome) is transferred by such a fusion. For phylogenetically related combinations this means that the genomic balance of the somatic hybrids is less disturbed than in asymmetric somatic hybrids that are constructed with the aid of ionizing radiation.

Experimental data suggest that both the efficiency of recovering triploid somatic hybrids of related solanaceous species by this procedure and the fertility of these allotriploid somatic hybrids are higher than those of asymmetric somatic hybrids constructed with ionizing radiation (Lee and Power 1988; Pental et al. 1988; Pirrie and Power 1986).

Partial fertility has also been described for sexual allotriploid hybrids between solanaceous species. Soost (1958) described the construction of fertile allotriploid (sesquidiploid, 2n=3x=36) hybrids derived from the interspecific cross *Lycopersicon esculentum* (tomato) (2n=4x=48) x *L. peruvianum* (2n=2x=24). Progenies from open pollination, assumed to be allotriploid x *L. peruvianum* (2n=2x=24) crosses, showed reduced chromosome numbers, ranging from 26 to 34. Rick et al.(1986; 1988) performed intergeneric crosses between *L. esculentum* (2n=2x=24) and *Solanum lycopersicoides* (2n=2x=24), doubled the ploidy level of the F_1 hybrid and subsequently could make the backcross with *L. esculentum* (2n=2x=24). The resulting allotriploids (2n=3x=36) could be crossed with *L. pennellii* (2n=2x=24). Progenies from these crosses showed introgression of *S. lycopersicoides* traits into diploid lines.

In this report we describe the isolation and analysis of allotriploid somatic hybrids between diploid tomato and monohaploid potato (*Solanum tuberosum*). Tomato and potato are related, solanaceous species with the same basic chromosome number (x=12); they cannot be hybridized sexually. Somatic hybrid plants between tomato and potato have been described by Melchers et al. (1978) (hyper-allotetraploids), Shepard et al. (1983) (allohexaploids) and Jacobsen et al. (1992) (allotetraploids and allohexaploids).

Materials and methods.

Plant material.

Two different tomato (*Lycopersicon esculentum*) genotypes were used in fusion experiments. The nitrate reductase deficient apo-enzyme (*nia*) mutant C31, described by Schoenmakers et al. (1991), was backcrossed with its wildtype cv. GT. GT is a tomato mosaic virus resistant line, kindly provided by DeRuiterseeds, Bleiswijk, the Netherlands. The F_3 line C31-244 (derived from GT x C31 crosses), that was homozygous for chlorate resistance and did not show any other visually detectable phenotypic abnormalities, that were not associated with the *nia* mutation, was used for fusion. The tomato with a cytoplasmic inherited albino mutation was obtained as follows: *In vitro* grown shoots of a cytoplasmic albino mutant of tomato cv. Large Red Cherry (ALRC; originating from dr. M.R. Hanson, Section of Genetics and Development, Cornell University, Ithaca, USA - for description see Hosticka and Hanson (1984)) were grafted *in vitro* onto tomato c.v. Moneymaker root-stock. Grafted albino shoots were transferred to the greenhouse and crossed with tomato genotype MsK8, which exhibits good regeneration capacity (Koornneef et al. 1987), as a staminate parent. From the totally albino F_1 population, genotype ALRCxM8-7 was selected because of its good regeneration capacity from root and hypocotyl explants (Koornneef et al. 1993). The potato (*Solanum tuberosum*) 7322 monoploid originated from dr. G. Wenzel, Germany. For detailed description see de Vries et al. (1987). Plants were grown *in vitro* in 380 ml plastic containers on 50 ml Murashige and Skoog (1962) medium without hormones, with 58.4 mM (for C31-244 and 7322) or 175 mM (for ALRCxM8-7) sucrose, pH 5.8 before autoclaving and solidified with 8 g/l agar (these media are designated MS20 and MS60 respectively), at a light intensity of 10 W/m^2 under a 16h-8h light-dark regime at 25 °C.

For pollinations various species were used. Homozygous recessive morphological markerlines of tomato were used as pistillate parents. Various diploid tomato lines, including cv. Moneymaker and MsK9, the F1 hybrid (*L. esculentum* cv Solentos x *L. peruvianum* LA 2157) and *L. pennellii* LA 716 were used as staminate parents, as was the dihaploid (2n=2x=24) potato RH87-343-25, kindly provided by R. Eijlander, Department of Plant Breeding, Wageningen Agricultural University, the Netherlands.

Isolation, fusion and culture of protoplasts; regeneration and culture of plants. Isolation, fusion and culture of protoplasts was carried out as described by Wolters et al. (1991). Minicalli were subcultured monthly on solidified TMcμZ greening medium (Wolters et al. 1991). Calli with a diameter of approximately 5 mm were transferred to 1Z medium (modified 2Z medium (Thomas and Pratt, 1981) with 1 instead of 2 mg/l zeatin) to induce shoot regeneration. Regenerated green shoots were rooted on MS20 medium; albino shoots were rooted on MS60 medium. Vegetative propagation of somatic hybrid plants was carried out *in vitro*. Aseptic shoots were rooted in Jiffy-7 peat pellets (Jiffy Products Ltd. Norway) and transferred to the greenhouse. Cuttings of somatic hybrid plants were grafted onto tomato root-stock for better growth and flowering.

Flowcytometric analysis.
For flowcytometric analysis of leaf tissue approximately 0.1 g of tissue was placed in a 6 cm petridish and 0.5 ml of a nuclei buffer, consisting of 10 mM sperminetetrahydrochloride, 200 mM hexylene glycol, 10 mM NaCl, 10 mM TRIS-HCl, 0.025 % (v/v) triton-X100 and 2.5 μg/ml 4,6-*D*-diamidino-2-phenylindole (DAPI), pH 7.0, was added. The tissue was chopped into small pieces with a very sharp razor blade and the suspension was filtered through a 85 μm pore nylon filter. The filtrate was used directly for flowcytometry. For flowcytometric analysis of protoplasts, the protoplasts were suspended in nuclei buffer (10^4 protoplasts/ml) and disrupted mechanically by passing them twice through a 26 gauge needle prior to the flowcytometric assay.

Cytological analyses.
The number of chloroplasts per guard cell pair was determined in the lower epidermis from leaves of *in vitro* grown plants or in lower epidermis strips from leaves of greenhouse-grown plants as described by Koornneef et al. (1989). Chromosome counts and karyotype analyses were performed on unbanded chromosome preparations of spread root tip meristems prepared as follows. Root tips were collected from *in vitro* grown hybrids and from the parental species grown in the greenhouse. They were incubated in 2 ml 2 mM 8-hydroxyquinoline for 4 h at 17°C and afterwards fixed in cold Carnoy's solution (ethanol - glacial acetic acid, 3:1) for 18 h or more at 4°C. The fixed root tips were rinsed in demineralized water (demiwater) and subsequently incubated in 1-2 ml 10 mM citric acid - sodium citrate buffer pH 4.2-4.5 containing 0.1% each of cellulase RS, pectolyase Y23 and cytohelicase enzymes for 1 h at 37°C.

Then the enzyme solution was carefully removed and demiwater was added. With a Pasteur pipette root meristems were transferred to a clean slide (cleaned in 70% ethanol). Excess water was removed and the cells were loosened with the aid of fine needles under a binocular. Quickly a small drop of 60% acetic acid was added with a Pasteur pipette and the cells were suspended in this drop. Then, with a pipet tip the suspension was surrounded with freshly prepared, ice-cold Carnoy's solution, after which one drop of Carnoy's solution was put on top of the suspension. The slide was air dried for $\pm$ 1 h. For staining the slides were first incubated for 15 min in 2 x SSC at 60°C and subsequently rinsed for 1-2 min in demiwater. Then the chromosomes were stained in 2% Giemsa in 10 mM Sörensen's phosphate buffer pH 6.8 for 30 min at room temperature, rinsed in Sörensen's buffer and demiwater, air dried and mounted in Entellan with a large cover slip.

Isoenzyme analysis.
Polyacrylamide gel electrophoresis (PAGE) of Glutamate-Oxaloacetate Transaminase (GOT; EC.2.6.1.1) and 6-PhosphoGluconate deHydrogenase (6-PGdH; EC.1.1.1.49) was performed according to Schoenmakers et al. (1992).

Morphological analysis and fertility.
In vitro and greenhouse grown plants and scions were analyzed for their leaf and flower morphology. Pollen viability was determined by **a.** staining with a solution consisting of 6 mM fluorescein diacetate (FDA) and 0.2 M sucrose in a 1:1 (v/v) mix of dimethylsulfoxide (DMSO) and water (viable pollen stained yellow under UV, non viable pollen did not stain) and **b.** pollen germination on a solidified medium consisting of 0.3 M sucrose, 2.2 mM $Ca(NO_3)_2.4H_2O$, 1.6 mM H_3BO_3, 2.0 mM citric acid, 0.6% agar, pH 5.9. Reciprocal crosses were made between allotriploid somatic hybrids and diploid tomato, *Lycopersicon pennellii*, *L. peruvianum* and potato. Pollen tube growth in the pistils was assessed 24h or 48h after pollination according to Ramanna and Mutsaerts (1971) with aniline blue. Observations were made with a Nikon microscope equiped with high quality fluor objectives and filter set DM400.

Results

Fusion and culture of protoplasts; regeneration of hybrid plants.

From the fusion combination C31-244 (+) 7322, further designated C7, 170 hybrid calli (each from a single fusion event) yielded somatic hybrid plants. Parental microcalli were rarely obtained in control and fusion plates; they turned brown at a very early stage. From the combination ALRCxM8-7 (+) 7322, further designated A7, few viable, albino tomato calli could be obtained in control and fusion plates. Also here, tomato nor potato regenerants were found. From the A7 combination 275 hybrid calli gave green somatic hybrid plants while five hybrid calli gave albino plants. Hybridity of calli and plants was determined with GOT and 6-PGdH isoenzyme analysis (zymogrammes not shown). The somatic hybrid vigour of fusion products of tomato and potato was also observed in earlier experiments (Schoenmakers et al. 1992). Up to four independent, adventitious shoots (designated A, B, C and D) were regenerated per fusion callus. These shoots were rooted on MS20 (green plants) or MS60 medium (albino plants).

Chloroplast number in leaf guard cell pairs.

For tomato (Koornneef et al. 1989) and potato (Frandsen 1968, Karp et al. 1984) the number of chloroplasts per guard cell pair is positively correlated with the ploidy level. This correlation was also found for the somatic hybrids of tomato and potato (Fig.1.). However, because *in vitro* rooted shoots formed significantly more chloroplasts than non-rooted shoots and because of the small differences between allotriploid and allotetraploid somatic hybrids, it was difficult to identify allotriploids from the somatic hybrid populations on the basis of the average number of chloroplasts per guard cell pair only. Within well rooted allotriploid, allotetraploid and allohexaploid populations no significant differences were found between the average numbers of chloroplasts per guard cell pair.

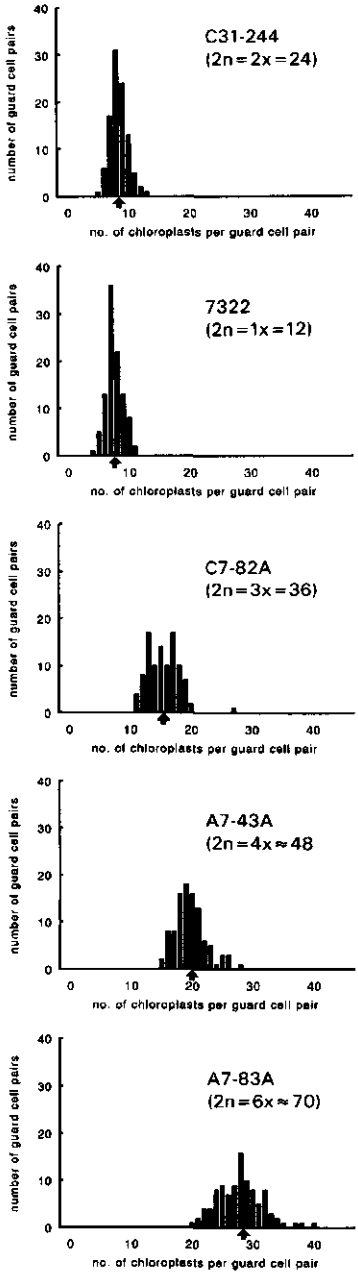

Fig.1. Number of chloroplasts per guard cell pair of in vitro grown tomato C31-244, potato 7322, allotriploid C7-82A, allotetraploid A7-43A and allohexaploid A7-83A. The average number of chloroplasts per guard cell pair is indicated with an arrow. (n=number of guard cell pairs counted=100).

Flowcytometric analysis.

Parental protoplast populations were analyzed for their relative DNA content, expressed as C-values, by means of flowcytometry (Table 1). The C-values of diploid tomato (2C) and diploid potato (2C) are almost equal. The presence of higher C-values (especially 4C in diploid tomato and 2C and 4C in monoploid potato) can be explained either by the presence of cells in G2-phase or because of polyploidization, as is described by Nootebos et al. (1989) for tomato and by Uijtewaal (1987) for potato.

With this procedure, it was possible to detect one triploid leaf sample in a mixture of 20 leaf samples of equal weight, each derived from a different plant. For each flowcytometric analysis 10 mg leaf pieces of 10 somatic hybrids were pooled. Subsequently, plants from pools containing triploids were analyzed individually (Table 1, Fig.2). From the somatic hybrid population C7 one albino and nine green allotriploids were selected. From population A7 12 green allotriploids were selected. The discrepancy between the numbers of isolated allotripoids and the numbers as estimated from summed flowcytometric histograms from 10 plants each (Table 1) is probably related to differences in **a)** the degree of polyploidization per plant, **b)** the fraction of cells in G2, **c)** the cell weight, and to equipment imprecision.

Table 1. Relative C-value distribution (in percentages) of parental protoplast populations (ppp) and somatic hybrid populations (shp) C7 and A7. For shp the distribution was estimated from summed flowcytometric histograms from pools of 10 plants each. n = number of analyzed protoplasts or somatic hybrids.

Population	Number of C equivalents									n
	1C	2C	3C	4C	5C	6C	7C	8C	>8C	
ppp C31-244		86		14				0		841
ppp ALRCxM8-7		82		18				0		704
ppp 7322	66	30		4						989
shp C7			5	60	11	12	0	12	0	170
shp A7			8	56	12	12	0	11	1	280

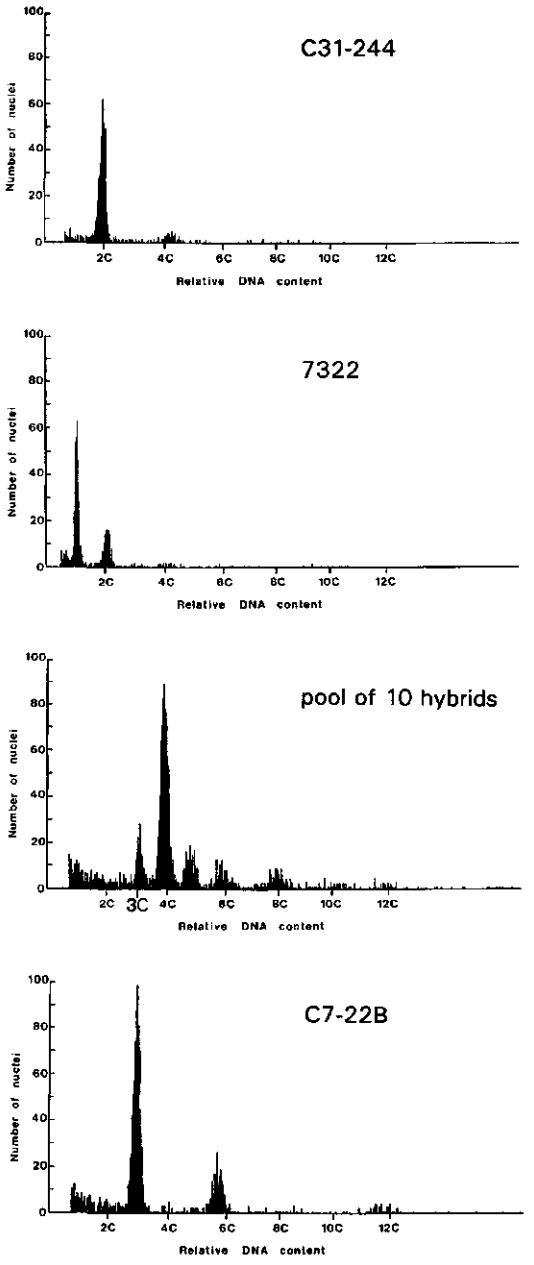

Fig.2. Flowcytometric histograms of protoplasts of fusion parents **a.** tomato C31-244 (2n=x=24) and **b.** potato 7322 (2n=1x=12), **c.** leaf samples from a pool of 10 somatic hybrid plants and **d.** allotriploid fusion product C7-22B (2n=3x=36).

Chromosome numbers.

In addition to flowcytometric selection of allotriploids, the triploid character was confirmed by counting chromosomes in root tip cells as is shown in Table 2 and Fig.3. Although karyotypes of the tomato and potato genotypes used are very similar, the satellite chromosomes (the numbers 2 in both parental karyotypes) can easily be distinguished in mitotic metaphase plates by the strikingly bigger size of the tomato satellites, compared with the potato satellites. In most allotriploids both tomato satellite chromosomes were present, but in three hybrids containing 35 chromosomes, one chromosome 2 of tomato was missing. It appeared that less than half of the hybrids contained the euploid triploid number (36) of chromosomes.

Table 2. Number of allotriploid somatic hybrids of diploid tomato and monoploid potato containing 34, 35, 36 or 37 chromosomes. The number of hybrids with 35 chromosomes possessing 1 or 2 tomato satellite chromosomes ("sat") are indicated. All other hybrids possessed both satellite chromosomes from tomato.

Fusion combination	Number of triploid hybrids with a chromosome number of					
	34	35 (1 sat)	35 (2 sat)	36	37	total
C31-244 (+) 7322	1	2	3	5	-	11
ALRCxM8-7 (+) 7322	2	1	4	5*	2	14

*One hybrid formed roots with cells containing 36 chromosomes and roots with cells containing 37 chromosomes.

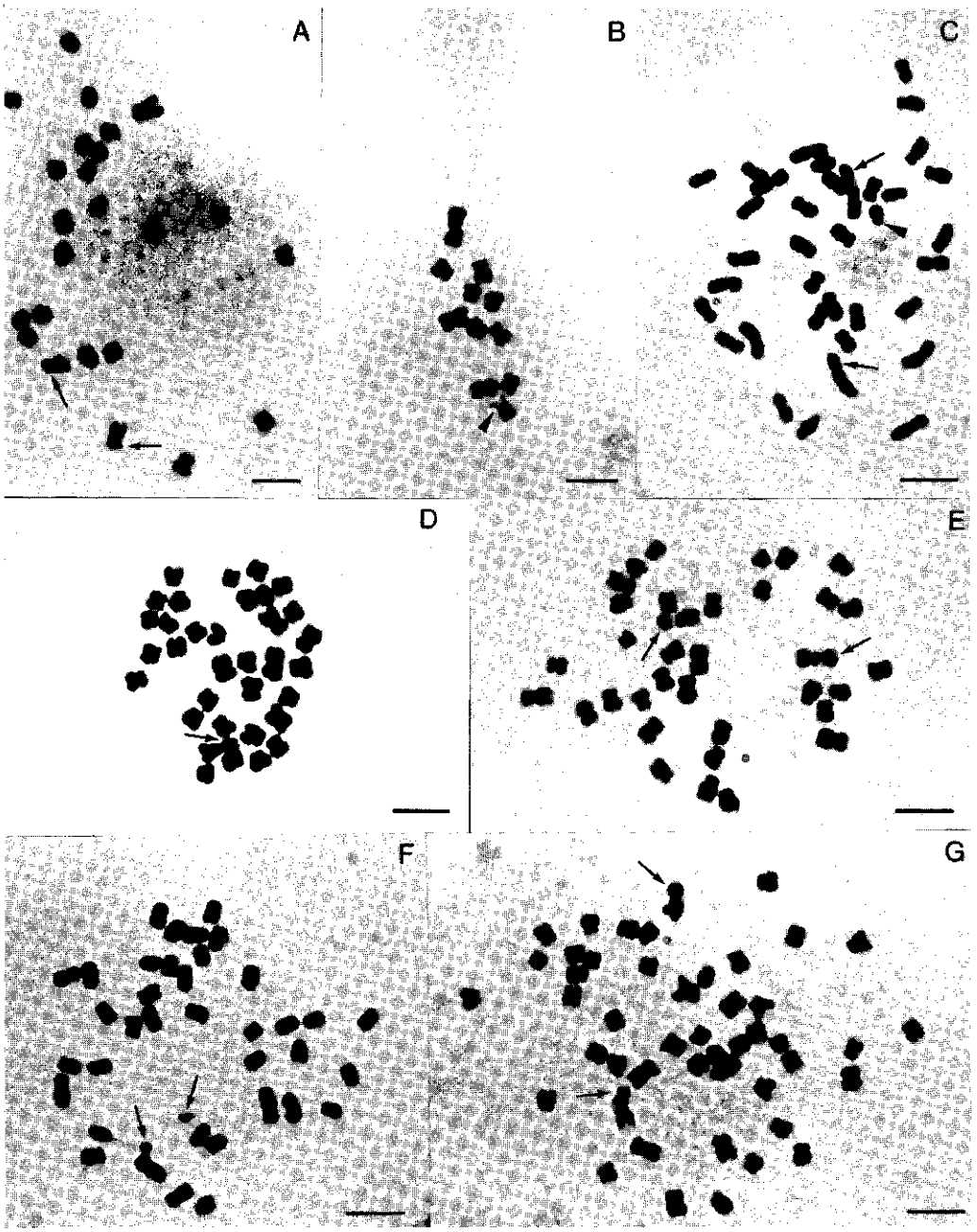

Fig.3. Metaphase plates of root tip cells from **A.** tomato C31-244 (2n=2x=24), **B.** potato 7322 (2n=x=12), **C.** allotriploid somatic hybrid C7-133A (2n=3x=36), **D.** allotriploid C7-149A (2n=3x=35, with 1 tomato satellite chromosome), **E.** allotriploid C7-22A (2n=3x=35, with 2 tomato satellite chromosomes), **F.** allotriploid A7-74B (2n=3x=37) and **G.** allotetraploid C7-23A (2n=4x=48). Arrows indicate tomato satellites, arrowheads indicate potato satellites. Horizontal bars represent 5 μm.

Morphology

Although growth of the allotriploids *in vitro* was vigorous, greenhouse grown plants displayed a retarded growth and developed local necrosis. Upon grafting both growth and flowering improved, but poorly growing plants still produced no flowers. The leaf morphology of both allotriploids and allotetraploids was intermediate between the two fusion parents and was highly variable between allotriploids (Fig 4). Allotriploids were more tomato-like than allotetraploids. The colour of young flowers of allotriploids was intermediate (light-yellow) between tomato (yellow) and potato (white). The light-yellow colour faded to white in older flowers. The flower morphology was variable between allotriploids (Fig.5a,b). Allotetraploid flowers (Fig.5c) were white. The anther morphology of allotriploids was tomato-like (Fig.5d).

Surprisingly, there was no obvious link between the plant and flower morphology and the number of chromosomes or the absence of satellite chromosomes of these plants. Vigorously growing plants, with normal appearing leaves and which produced many flowers, were found in both euploid and aneuploid populations. On the other hand, several euploid plants remained stunted in their development.

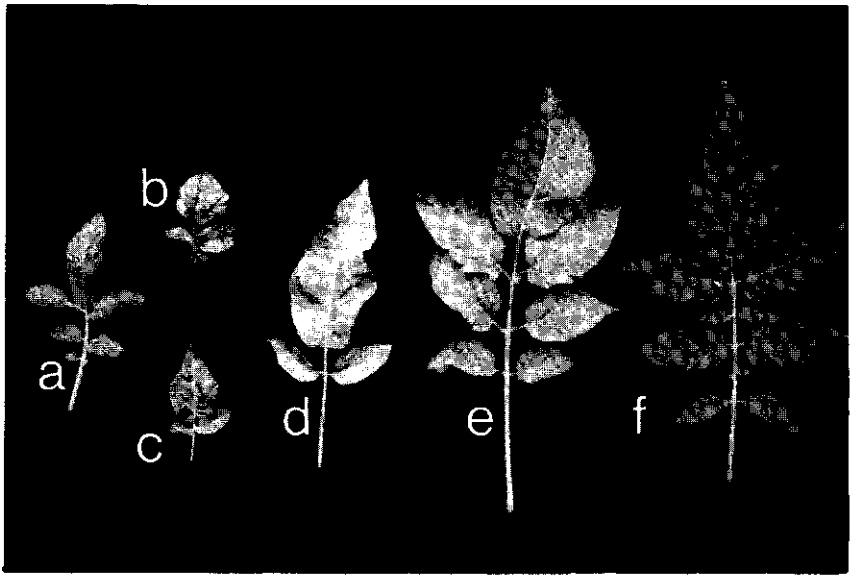

Fig.4. Leaves of **a.** monoploid potato 7322, **b.** allotriploid somatic hybrid C7-22A, **c.** allotriploid A7-58A, **d.** allotriploid A7-74B, **e.** allotriploid A7-82A and **f.** diploid tomato C31-244.

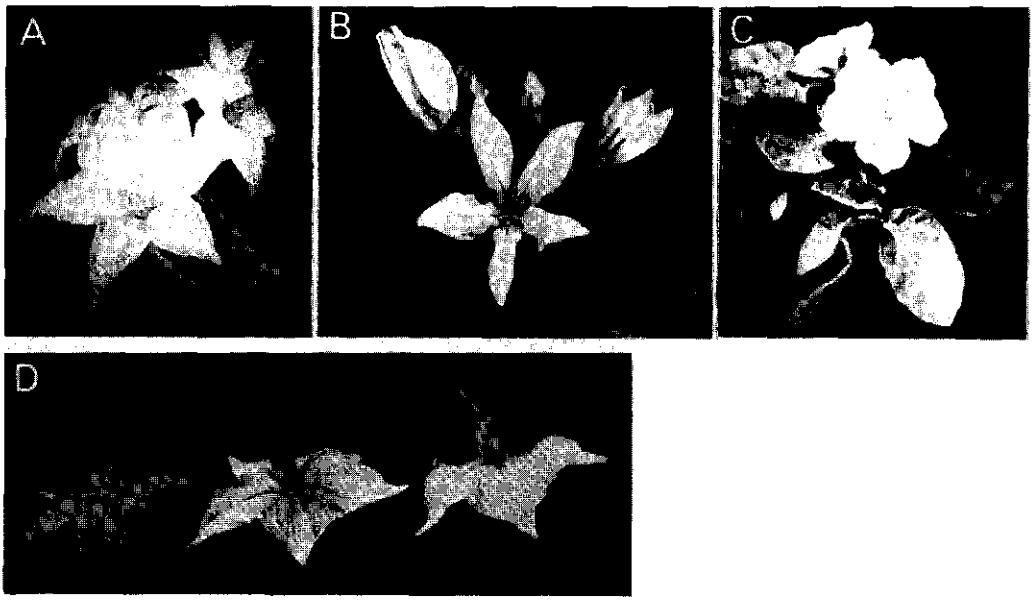

Fig.5. Flowers of **a.** allotriploid somatic hybrid G7-82A, **b.** allotriploid somatic hybrid A7-146D, **c.** allotetraploid somatic hybrid G7-101B. **d.** from left to right, flower morphology of tomato, allotriploid G7-82A and diploid potato: tomato and allotriploid anthers have sterile tips.

Fertility

Only six of the 22 allotriploid somatic hybrids (C7-22A, C7-167A, A7-58A, A7-74B, A7-82A,B,C,D, A7-146A,B,C,D) produced full-grown flowers with calyx (sepals), corona (petals), pistils and stamen. Pollen viability of these hybrids, as tested with FDA staining, was low (approximately 5%) for A7-82A (2n=36), A7-82B (2n=35) and A7-146D (2n=34), and less than 1% for C7-22A (2n=35), C7-167A (2n=35), A7-58A (2n=36) and A7-74B (2n=36/37). Pollen germination on artificial medium was rarely observed (less than 0.1%; control tomato 45%) as was pollen tube growth in the styles of tomato and allotriploid somatic hybrids. Therefore, allotriploids were mainly used as staminate parent in crosses with tomato, *Lycopersicon pennellii* and potato. These crosses did not yield progeny. Tomato and potato pollen tube growth was inhibited in the style (Fig.6a). *L. pennellii* pollen tubes grew further into the styles of the hybrids. However, tube growth was arrested near the ovules (Fig.6b,c). Allotriploids A7-74B and A7-82A spontaneously produced berries with some immature seeds. Embryos from these seeds, cultured on HLH medium (Neal and Topolenski 1983), did not develop further.

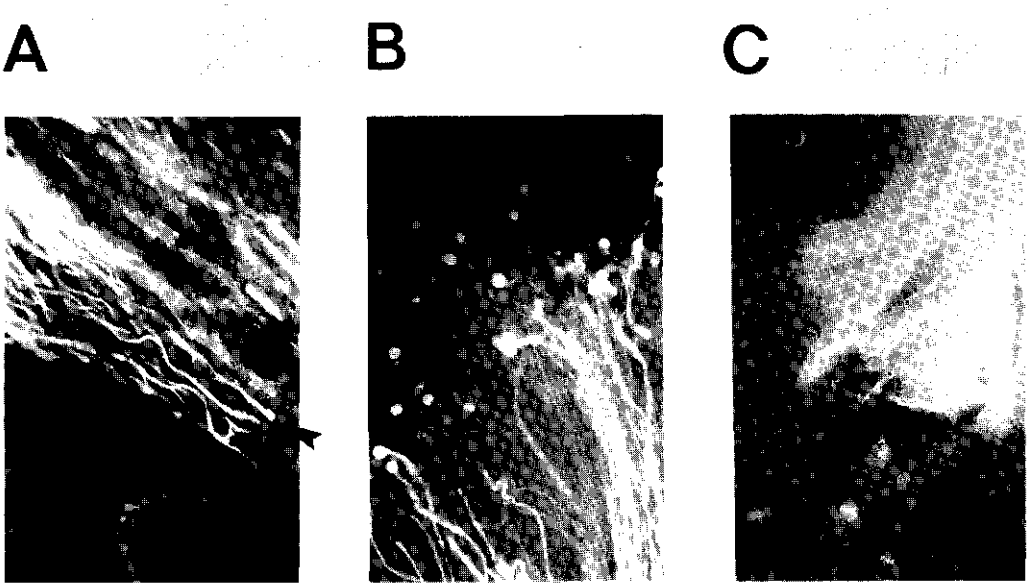

Fig.6a. Tomato pollen tube growth in the pistil of A7-82A (2n=36). Tube growth was inhibited in the style, where callose was formed at the top (◄). **b.** and **c.** *Lycopersicon pennellii* pollen tube growth in the pistil of A7-82A. Tube growth was inhibited near the ovules, where callose was formed (◄) and where tubes sometimes spirulated.

Discussion

Altogether, 25 allotriploids were selected from 450 somatic hybrids. From flowcytometric histograms of parental protoplast populations it was expected that a large fraction of both somatic hybrid populations would be allotriploid. However, allotriploids were rarely found while allotetraploids were present at a high frequency. Several explanations are possible for this: **a.** monoploid potato protoplasts did not take part in the fusion as much as diploid potato protoplasts did, **b.** the fusion treatment yielded many multiple fusions (this also explains the presence of allopentaploids), **c.** polyploidization of allotriploid cells upon fusion resulted in hexaploid somatic hybrids (these were also found at a high frequency) and **d.** allotriploid fusion products displayed a low fitness during cell and tissue culture as compared with e.g. tetraploid hybrids. This lower fitness might have been caused by, for example, the difficult combination of potato chloroplasts with a prevailing tomato genome (Wolters et al. 1991)

As compared to asymmetric somatic hybrids of tomato and potato that have been produced with the aid of gamma irradiation of potato protoplasts prior to fusion (Schoenmakers et al. submitted), these fusion experiments demonstrated a far better and quicker regeneration of phenotypically normal appearing somatic hybrid plants.

Only six of the 25 allotriploids produced full-grown flowers but vital pollen was seldom found. Apart from aneuploidy of some of the allotriploids, the arrest of floral development and the male sterility of flower producing allotriploids can partly be explained by assuming recombination between mitochondrial DNAs of tomato and potato in the somatic hybrids. Recombination between parental mitochondrial DNAs in somatic hybrids, which has been described for several related combinations (Derks et al. 1991; Kofer et al. 1991), is suggested as the cause of cytoplasmic male sterility. Kofer et al. (1991) further postulated that nuclear-mitochondrial interaction occurs at several stages in tobacco floral development and that expression of several mitochondrial genes is essential for normal stamen and corolla development.

Male (and also female) sterility of allotriploid somatic hybrids might further be influenced by homoeologue pairing between tomato and potato chromosomes and the formation of trivalents during meiosis. Lee and Power (1988) reported complete sterility of, presumably trivalents forming, autotriploid and complete fertility of allotriploid somatic hybrids in *Petunia*. Rick et al. (1986) described a very low fertility of tomato autotriploids and a higher fertility of allotriploids, containing two tomato genomes and one *S. lycopersicoides* genome. In these latter hybrids the tomato chromosomes exhibited strong preferential pairing, whereas the *S. lycopersicoides* chromosomes remained unpaired and got lost during meiosis. Since *S. tuberosum* is less related to tomato than *S. lycopersicoides* is, we expect that homoeologue paring is very limited and that, as a consequence, a fair number of gametes with a balanced tomato genome are formed. The observed homoeologue paring in some hypotetraploid somatic hybrids of *Lycopersicon esculentum* (+) *Solanum tuberosum*, described by de Jong et al. (1993) must also be considered as incidental. Homeologue crossing over would offer further possibilities for introgression of potato traits in tomato. We assume that only gametes without or with a small number of potato chromosomes are genetically sufficiently balanced to be able to take part in crosses.

Apart from the observed male and possible female sterility, hybridization might also be hampered by a genetic incongruity between the allotriploids and its crossing partners. (Unilateral) incongruity in *Lycopersicon* and *Solanum* species, expressed at the level of pollen tube growth and zygotic abortion, has been described by Chetelat and Deverna (1991), Chetelat et al. (1989), Deverna et al. (1987), Gadish and Zamir (1986), Jacobsen et al. (1992), Nettancourt et al. (1974), Rick (1963) and Smith and Desborough (1986). The suppressed pollen germination, the arrested pollen tube growth and the observed embryo abortion indicate incongruity between the allotriploids and tomato and potato. Possibly, crosses with *L. pennellii*, followed by embryo rescue, will ultimately give progeny.

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CHAPTER 5

ASYMMETRIC SOMATIC HYBRIDIZATION BETWEEN TOMATO (*Lycopersicon esculentum* Mill) AND GAMMA IRRADIATED POTATO (*Solanum tuberosum* L.): A QUANTITATIVE ANALYSIS

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Summary. We analyzed 110 asymmetric fusion products, obtained by fusion of hygromycin resistant tomato protoplasts and gamma irradiated kanamycin resistant potato protoplasts that expressed β-glucuronidase (GUS). The fusion products were selected for antibiotics resistance, and were subsequently analyzed for their shoot regeneration potential, GUS activity, expression of two potato isoenzymes, chloroplast type, total genomic DNA content and relative genomic composition. No viable plants could be obtained and calli were highly polyploid. All hybrids expressed GUS activity, whereas they displayed a large variation with respect to the other traits.

Introduction

Asymmetric somatic hybridization is often described as a new plant breeding technique, that allows the introduction of genes from a donor species into a recipient crop species, and that can be useful if these species cannot be hybridized sexually. Asymmetric hybridization can be performed by fusion of protoplasts of a recipient species with protoplasts of a donor species of which the nuclear DNA has been damaged e.g. by ionizing irradiation. Ideally, the resulting asymmetric somatic hybrids combine the complete recipient genome with a small fraction of the donor genome. Further elimination of unfavourable and preservation of favourable donor traits can be achieved by means of recurrent sexual hybridization with the recipient species and selection for the desired traits. In some cases, asymmetric somatic hybrids could successfully be used as parents in subsequent sexual hybridization (Bates 1990; Dudits et al. 1980, 1987; Gleba et al. 1988; Sjödin and Glimelius 1989; Somers et al. 1986). Most of these partially fertile hybrids are highly asymmetric: they contain only a minor part of the donor genome. However, many reports describe poor growth and regeneration of asymmetric fusion products, limited elimination of donor DNA, high aneuploidy, strong polyploidization and, in case plants were obtained, sterility of the asymmetric somatic hybrids (e.g. Hinnisdaels et al. 1991; Imamura et al. 1987; Itoh and Futsuhara 1983; Sacristan et al. 1989; Wolters et al. 1991; Wijbrandi et al. 1990a,b; Yamashita et al. 1989).

Little information is available about the factors that influence regeneration, chromosome elimination and polyploidization and the relations between these various biological processes after asymmetric hybridization. For example, one can envisage that there is a selective advantage of asymmetric somatic hybrids with higher ploidy levels, and that this advantage subsequently affects shoot regeneration in a negative way. In addition, the role of organelle DNA should be considered because cybrids with potato chloroplast DNA but without potato nuclear DNA were not obtained after fusion of tomato protoplasts with gamma irradiated potato protoplasts (Wolters et al. 1991). The incongruity between a recipient nucleus and donor chloroplasts, associated with large phylogenetic distances between parental plants, was also described by Derks et al. (1992) and Wolters et al. (1993) for combinations of other solanaceous species.

The present study describes the genetic characterization of a large number of asymmetric fusion products that resulted from fusion of protoplasts from two hygromycin resistant tomato genotypes with gamma irradiated protoplasts from a kanamycin resistant, β-glucuronidase (GUS) expressing potato genotype. Fusion products were obtained by selection for resistance against both antibiotics and then analyzed with respect to several other unselected parental traits. In addition, the chromosomal complement of these fusion products was analyzed.

Materials and Methods

Plant material

The tomato recipient genotype was obtained by crossing genotype *Xa-2/xa-2* (Persson 1960) with MsK9 (Koornneef et al. 1987a). From the F_1 progeny an *Xa-2/xa-2* genotype, designated Xa11, with good regeneration capacity from roots and hypocotyl, was selected according to Koornneef et al. (1993). Transformation of Xa11 with *Agrobacterium tumefaciens* strain Ach5, containing the plasmid pJW6 which transfers hygromycin-B resistance, was carried out as described by Koornneef et al. (1987b). Two diploid, hygromycin resistant transformants, which did not show any other visually disturbed phenotype, designated Xa11-H4 and Xa11-H8, were used in protoplast fusion experiments. For potato the monoploid (2n=x=12) genotype 7322, which originates from Prof. Dr. G. Wenzel, Germany (for

detailed description see de Vries et al. 1987), was transformed in the same way with *A. tumefaciens* strain C58, containing the plasmid pZ707C which transfers kanamycin resistance and the ß-glucuronidase (GUS) reporter gene (Jefferson et al. 1987). A somatically doubled diploid (2n=2x=24) kanamycin resistant GUS expressing transformant, designated 7322-K5, was used as 'donor' genotype in protoplast fusion experiments.

Protoplast isolation, gamma irradiation, electrofusion and culture, selection of fusion products and regeneration

Protoplast isolation, gamma irradiation, electrofusion and culture were carried out as described by Wolters et al. (1991). Fusion products were selected on culture media containing 50 mg/l hygromycin-B and 150 mg/l kanamycin, from one week until half a year after fusion. Minicalli were transferred to solidified TMCμZ greening medium (Wolters et al. 1991) one month after fusion and subcultured every month. Larger calli were transferred to 1Z medium (Wijbrandi et al. 1990b), approximately 3 to 6 months after fusion, for shoot regerenation. Calli were classified as regenerable when small shootlike structures were observed. Regenerated shoots were grown on solidified medium according to Murashige and Skoog (1962) without hormones; 20 g/l sucrose and 8 g/l agar were added. The pH was adjusted to 5.8 before autoclaving. This medium was designated MS20.

Fluorometric ß-glucuronidase assay

ß-Glucuronidase (GUS) activity was analysed according to Jefferson et al. (1987). Approximately 20 μg of callus tissue was ground in 20 μl of extraction and staining buffer containing 1 mM 4-methyl umbelliferyl glucuronide (MUG; Sigma M-9130) as a fluorogenic substrate. After 15 min incubation at 37°C the reaction was stopped with 0.2 ml 1 M Na_2CO_3 and fluorescence was registered under UV light.

Flowcytometric analysis.

For flowcytometric analysis of callus tissue approximately 0.5 g tissue was placed in a 6 cm petridish and 1 ml of a nuclei buffer, consisting of 10 mM sperminetetrahydrochloride, 200 mM hexylene glycol, 10 mM NaCl, 10 mM TRIS-HCl, 0.025 % (v/v) triton-X100 and 2.5 μg/ml 4',6-diamidino-2-phenylindole (DAPI), pH 7.0, was added. The tissue was cut up fine with a

sharp razor blade and the mixture was filtered through a 85 μm pore nylon filter. The filtrate was directly used for analysis in a flow cytometer (IPC22, Ortho Diagnostic Systems, Beerse, Belgium). Calf thymocytes and chicken erythrocytes were used as internal, absolute standards; tomato haploids, diploids and tetraploids were used as references. The nuclear DNA content was expressed as relative C-value.

In our flowcytometric analysis the diploid nuclear weights and relative C-values of tomato and potato were almost identical. Similar weights were also reported by Arumuganathan and Earle (1991).

DNA isolation, DNA probes, Southern blot and dot blot analysis

Total DNA from leaves of tomato MsK9 and potato 7322 and from fusion calli was isolated as described by Wolters et al. (1991). Probes were radioactively labeled by means of the Boehringer Mannheim Random Primed DNA Labeling Kit. The chloroplast DNA composition of fusion products was analyzed by hybridization of Southern blots of HinfI digested total DNA to the *Petunia hybrida* chloroplast DNA clone pPCY64 (Derks et al. 1991).

Determination of the ratio of tomato:potato genomic DNA was carried out by means of dot blot analysis according to Wolters et al. (1991). Several concentrations of parental DNA were applied to two identical dot blots to make a calibration plot of the radioactivity per dot in relation to the amount of DNA from one species. Total DNA from the fusion products was applied to the same dot blots. Two identical filters were prepared for every analysis. One was probed with pTHG2, a tomato-specific repetitive DNA probe (Zabel et al. 1985). The insert of pTHG2 represents a moderately repetitive DNA fragment that is evenly dispersed on all tomato chromosomes, as was shown by *in situ* hybridization (Zabel et al. 1985). The other filter was probed with P5L, a potato-specific repetitive DNA probe (Visser et al. 1988). On all potato chromosomes hybridization with this fragment occurs predominantly in the telomeric and centromeric regions. With the calibration plots for both species-specific probes the amount of tomato and potato DNA per dot could be estimated, and the percentage of nuclear DNA of the fusion products that originated from potato could be determined.

The diploid nuclear weights of tomato and potato were considered identical. The flowcytometric data together with the dot blot data were used to estimate the number of genome equivalents of tomato respectively potato that were present in hybrid calli.

Isoenzyme analysis

Glutamate oxaloacetate transaminase (GOT; EC 2.6.1.1) and malate dehydrogenase (MDH; EC 1.1.1.37) are dimeric enzymes. GOT and MDH zymograms of symmetric fusion products of tomato and potato do not only display parental, homodimeric bands but also intermediate, heterodimeric hybrid bands. The elimination of all potato alleles that encode for a specific isoenzyme-subunit results in the disappearance of the correspondig heterodimeric, hybrid band in the zymogram.

Isoenzyme analysis of GOT and MDH was performed as follows. Approximately 0.25 g of fresh callus tissue was ground in 0.25 ml of 0.05 M TRIS-HCl, 0.1 g/l bromophenol blue, 20% (v/v) glycerol and 1% (v/v) β-mercaptoethanol solution, pH 6.8. After centrifugation in an Eppendorf centrifuge for 5 min at maximum speed the supernatants were subjected to polyacrylamide gel electrophoresis (PAGE) according to Schoenmakers et al. (1992). Staining reactions were performed according to Vallejos (1983).

Results

Selection of fusion products and regeneration.

Tomato protoplasts were able to divide and regenerate plants in culture media without kanamycin. Without irradiation, potato microcalli could rarely be obtained in hygromycin-free media. These microcalli turned brown and died at a very early stage. After gamma irradiation with a dose of 150 or 500 Gy, potato protoplasts never yielded microcalli. Only fusion products were able to grow on culture media that contained both hygromycin and kanamycin. Altogether 47 "150-Gy" and 63 "500-Gy" hybrid fusion calli were recovered from fusion experiments Xa11-H8 (+) 7322-K5, 150 Gy irradiated, and Xa11-H4 (+) 7322-K5, 500 Gy irradiated, respectively. Regeneration of somatic hybrids was inhibited by gamma irradiation of the potato protoplasts prior to fusion: 38% of the 150-Gy calli and 23% of the 500 Gy calli formed small shootlike structures within 18 months after fusion. Symmetric "0-Gy" fusion products, selected in he same way, showed an earlier regeneration at a significantly higher frequency (approximately 95% within 18 months after fusion). Most asymmetric somatic hybrid shoots showed gross morphological

abnormalities and grew poorly as compared to symmetric hybrids. None of the shoots from asymmetric fusion experiments could be rooted in vitro on MS20 medium and transferred to the greenhouse. Nearly all shoots from symmetric fusion experiments could be rooted on MS20 medium and grown in the greenhouse.

Fluorometric GUS assay

All calli, selected on media that contained both hygromycin and kanamycin, displayed GUS activity (a trait derived from potato and absolutely linked to the kanamycin resistance), which confirmed the hybridity of the calli. Half a year after fusion, the selected calli were removed from the selection medium and were subsequently subcultured every two months on medium without antibiotics for half a year. During this time all calli retained their GUS activity (GUS activity was determined every three months), which suggests that limited further elimination of potato DNA took place under these non-selective conditions.

Flowcytometric and nuclear DNA analysis

Flowcytometric analysis of approximately one year old hybrid calli showed that the genomic C-value, which corresponds to the ploidy level of both tomato and potato, was highly variable between calli. Nearly all calli were hyper-tetraploid. For the 150-Gy calli the average ploidy level was 10.0 (ranging from 6.0 to 18.6); for the 500-Gy calli this was 7.6 (ranging from 3.9 to 18.2). Some preference could be observed for ploidy levels that were multiples of 4 (Fig.1, Fig.4). The genomic DNA constitution, calculated from dot blots, was also highly variable between fusion calli. For the 150-Gy calli the average estimated percentage of potato nuclear DNA was 12.6 (ranging from 0.9 to 25.8). For the 500-Gy calli this was 10.3 (ranging from 0.3 to 39.9).

Estimated numbers of tomato and potato genome equivalents of individual calli are presented in Fig.1. The fact that both the average ploidy level and the average percentage of potato nuclear DNA of the 500-Gy calli were lower than that of the 150-Gy calli demonstrates a stronger elimination of potato nuclear DNA following 500 Gy irradiation.

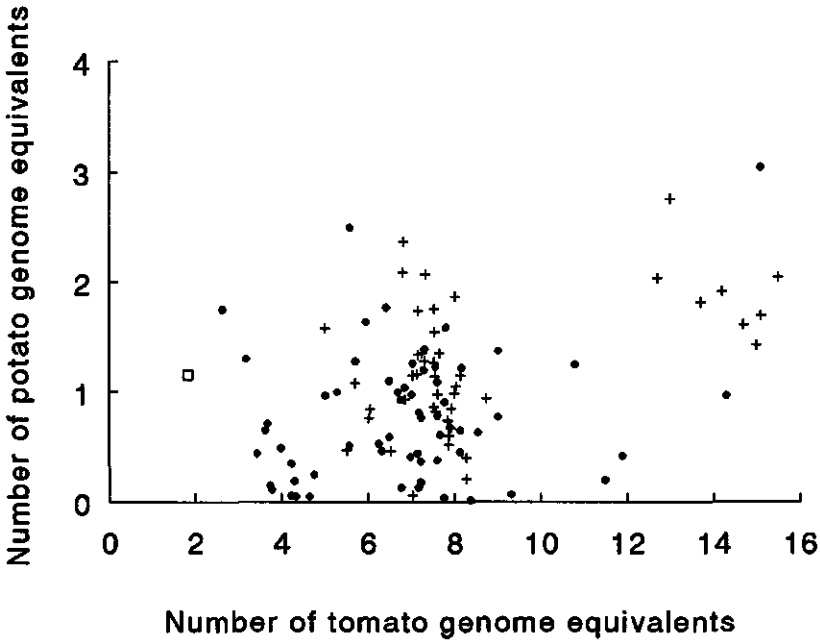

Fig.1. Estimated numbers of potato and tomato genome equivalents of individual 150-Gy (+) and 500-Gy (•) calli, calculated from C-values and dot blot hybridization signals. A C-value of 1 corresponds with one complement of 12 tomato or potato chromosomes. □ = reference: an allotriploid somatic hybrid (A7-146D, described by Schoenmakers et al. (1993) of a diploid tomato (with a C-value of 2) and a monohaploid potato (with a C-value of 1).

Isoenzyme analysis

The absence of heterodimeric, hybrid GOT and/or MDH bands is an indication of elimination of potato nuclear DNA (Fig.2). Of the 150-Gy calli, 7 % did not express a potato GOT isoenzyme while 31 % did not express a potato MDH isoenzyme. For the 500-Gy calli these figures were 53% and 56% respectively. Tomato specific bands were always present.

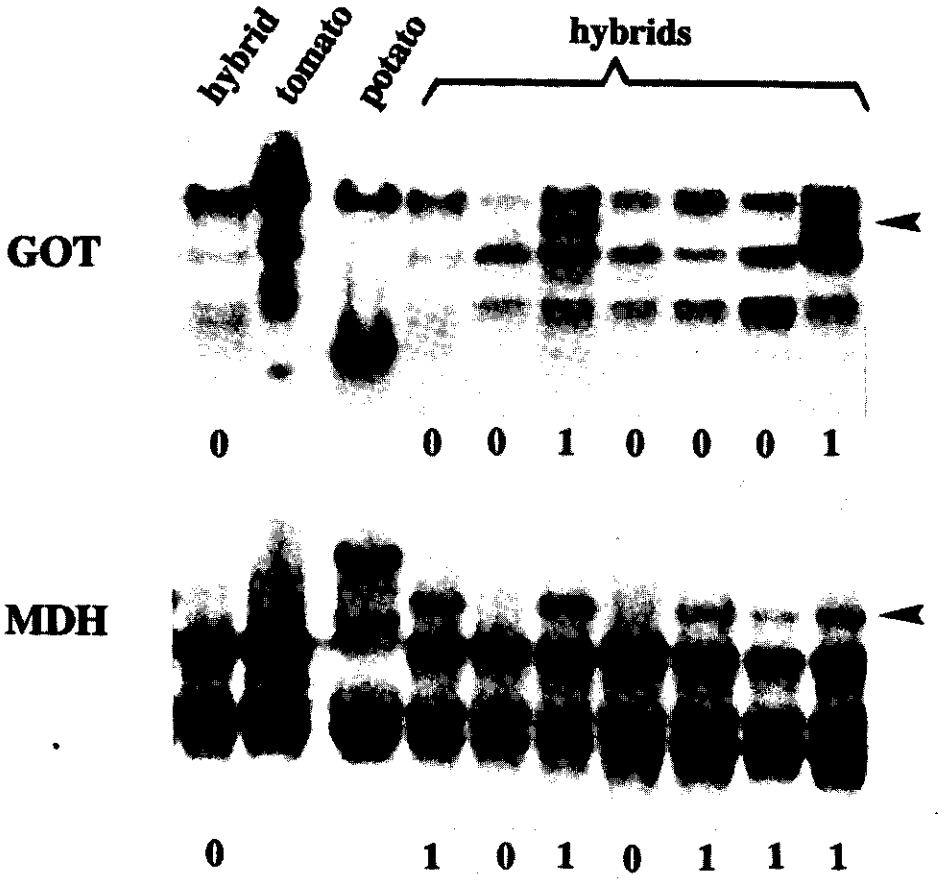

Fig.2. GOT and MDH zymograms of tomato, potato and some asymmetric fusion products. The heterodimeric band (◄) is present (=1) or absent (=0).

Chloroplast DNA analysis

Restriction Fragment Length Polymorphism (RFLP) analysis of total DNA of hybrid calli with the *Petunia hybrida* chloroplast clone pPCY64 suggests that calli contain either tomato or potato chloroplasts (Fig.3). No novel restriction patterns were found. Of the 150-Gy calli, 96 % possessed tomato chloroplasts. For the 500-Gy calli this preference was not found: 51 % of the hybrids contained tomato chloroplasts.

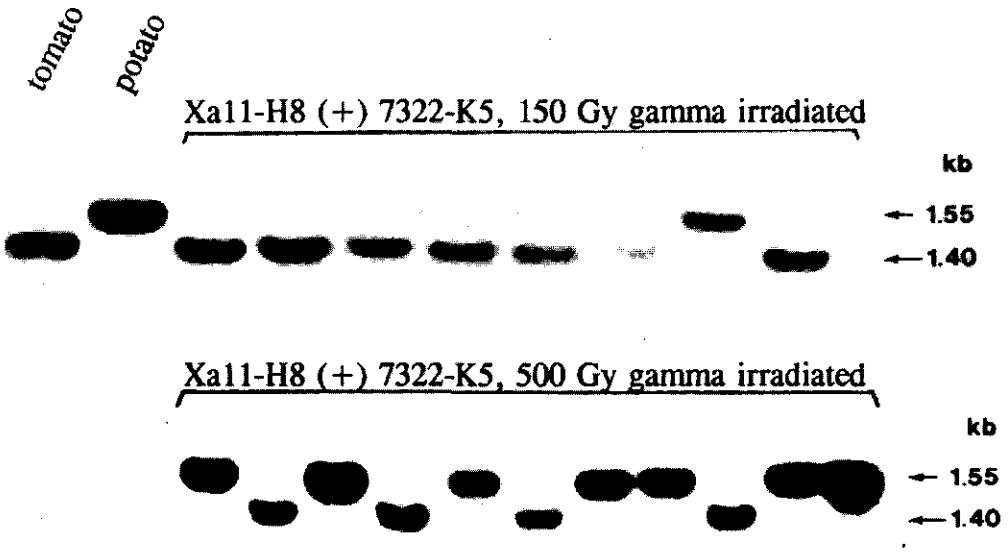

Fig.3. Identification of chloroplast types in fusion products. RFLP analysis of total DNA of parental and hybrid tissue with the chloroplast clone pPCY64 of *Petunia hybrida* used as a probe. All lanes contain 4 μg HinfI-digested total DNA.

Relations between various genetic parameters

Relations between shoot regeneration, the flowcytometric C-value, the nuclear DNA constitution, the presence or absence of the heterodimeric isoenzymes for GOT and MDH and the chloroplast type are presented in Fig.4 and Table 1 for both doses. Remarkably, many of the genetic parameters behave independently. Only for the 150-Gy calli the retainment of the potato MDH isoenzyme was significantly stronger at higher ploidy levels. Furthermore, 500-Gy calli with a lower percentage of potato nuclear DNA displayed a significantly better shoot regeneration. The presence or absence of potato GOT and potato MDH were correlated for the 500-Gy calli.

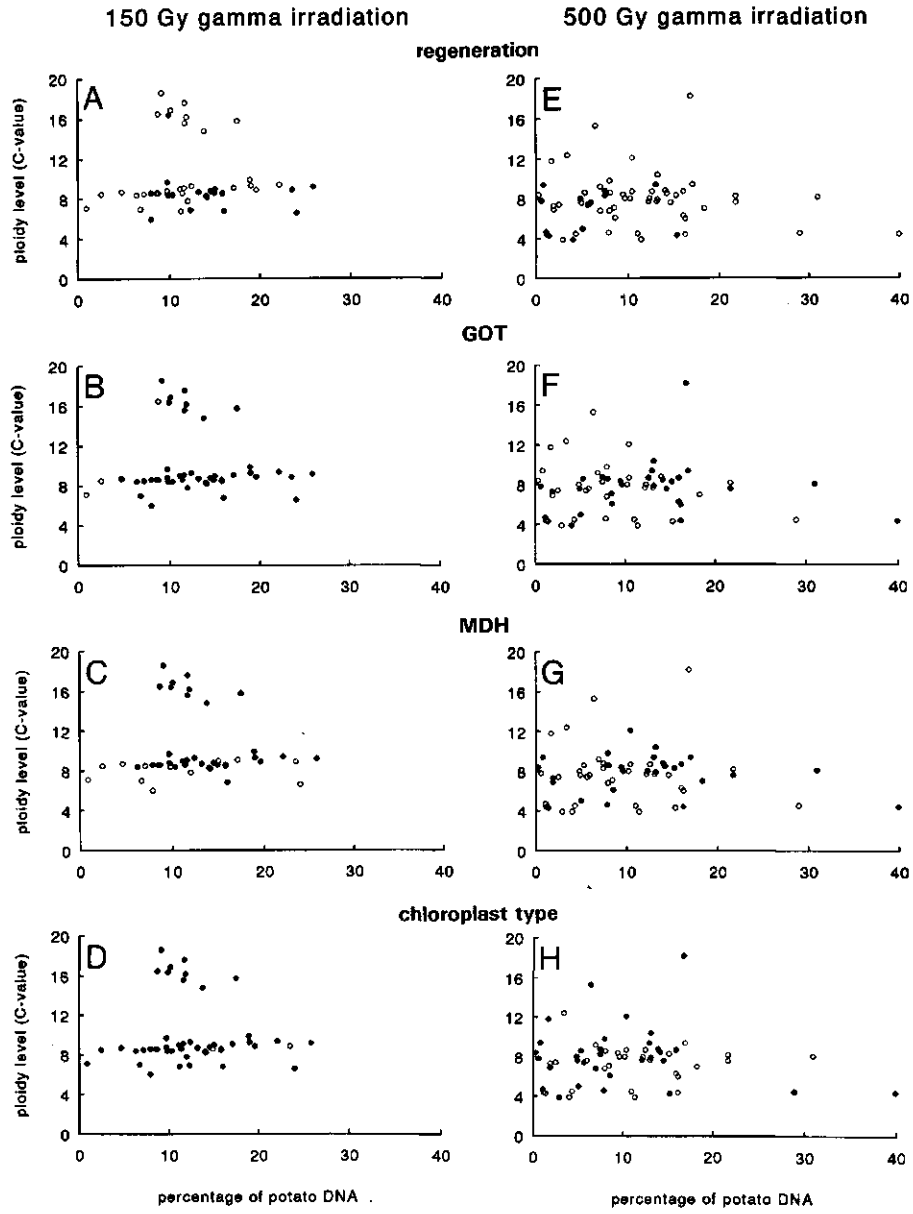

Fig.4. Relations between the percentage of potato nuclear DNA in individual 150-Gy hybrid calli, the ploidy level and **a.** the regeneration (● = regeneration; o = no regeneration), **b.** the presence (●) or absence (o) of potato GOT, **c.** the presence (●) or absence (o) of potato MDH and **d.** the presence of tomato (●) or potato (o) chloroplasts. For 500-Gy calli these relations are shown in **e.**, **f.**, **g.** and **h.** respectively.

Table 1. Chi square values for a test of independence between the various genetic parameters: the regeneration (REG), the ploidy level (PLOI) (higher or lower than 12 for 150-Gy calli and higher or lower than average (=7.6) for 500-Gy calli), the nuclear DNA constitution (NDC) (higher or lower than the average percentage of potato DNA), the presence or absence of the heterodimeric isoenzymes for GOT and MDH and the chloroplast type (CP) (tomato or potato) for the fusion products Xa11-H8 (+) 7322-K5, 150 Gy irradiated and Xa11-H4 (+) 7322-K5, 500 Gy irradiated $\{P(\underline{X}_1^2 > 3.84) < 0.05;\ P(\underline{X}_1^2 > 6.64) < 0.01\}$. Significant correlations are indicated with * ($P < 0.05$) or ** ($P < 0.01$).

	150-Gy calli					500-Gy calli				
	REG	PLOI	NDC	GOT	MDH	REG	PLOI	NDC	GOT	MDH
PLOI	3.03					0.89				
NDC	3.74	1.53				10.14**	1.42			
GOT	1.77	0.36	2.35			0.11	0.00	1.54		
MDH	1.85	5.08*	0.00	1.90		1.65	1.27	1.48	5.03*	
CP	3.69	0.49	3.08	0.15	0.35	3.07	0.77	0.77	0.06	0.59

Discussion

Asymmetric somatic hybrids between tomato and potato were selected only on the basis of their ability to grow at least as callus. The analysis of seven genetic parameters of these hybrids shows that fertile asymmetric hybrid plants, with a relative strong elimination of donor DNA are very difficult to obtain under the experimental conditions that we used. The irradiation of the donor prior to protoplast fusion caused elimination of donor traits and donor nuclear DNA. We found that with 500 Gy gamma irradiation the elimination of potato isoenzymes for GOT and MDH and the elimination of potato nuclear DNA were stronger than with 150 Gy irradiation. This radiation dose dependent elimination of donor traits has also been described by Bonnema et al. (1992); Melzer and O'Connell (1992) and Menczel et al. (1982) for other solanaceous species.

In the 150-Gy calli the potato MDH was more frequently eliminated than the potato GOT. Possibly, the potato GOT gene is more closely linked to a potato trait which plays an important role in the development of the fusion

product or the kanamycin resistance gene. Chances for elimination might also depend on the location on the chromosome. Although centromeres are necessary for retainment of the donor chromosomes and/or chromosome fragments, extensive rearrangements, due to the irradiation treatment, may result in more distal chromosome regions with nearby centromere fragments. Furthermore, damaged DNA fragments can be 'rescued' by somatic recombination with recipient chromosomes (Parokonny et al. 1992; Piastuch and Bates 1990). The fact that in the 500-Gy calli the elimination of the potato GOT was as strong as that of the potato MDH while in the 150-Gy calli the potato GOT was more frequently retained than the potato MDH, suggests that, apart from an increased donor DNA elimination following 500 Gy irradiation, more donor chromosome breakage and/or rearrangements have taken place after 500 Gy gamma irradiation. Indications for extensive donor chromosome rearrangements after irradiation were already reported by Wijbrandi et al. (1990a) and Parokonny et al. (1992).

In all hybrid calli the recipient DNA amount has increased considerably, irrespective of the percentage of potato DNA. This polyploidization of the recipient genome after asymmetric fusion was also observed by Gleba et al. (1988) and Wijbrandi et al. (1990a). Possibly, polyploidization of the recipient tomato genome is necessary to buffer the negative effect of the additional genetic material of the donor and aneuploidy.

The observation that the percentage of potato DNA was not correlated with the regeneration nor with the ploidy level suggests that it should be possible to select highly asymmetric somatic hybrids of tomato and potato when large numbers of somatic hybrids are available. However, the polyploidization of the tomato genome, which was observed in all hybrid calli, might be inevitable and poses an important restriction to the use of asymmetric somatic hybridization for plant breeding in this species combination. Sexual hybridization between solanaceous genotypes differing in ploidy level is extremely difficult.

Recent studies of asymmetric somatic hybridization between *Lycopersicon esculentum* as a recipient and *Solanum* species as a donor suggest that a certain amount of *Solanum* DNA is required to establish functional *Solanum* chloroplasts in the fusion product (Derks et al. 1992; Wolters et al. 1991). However, we did not observe a stronger retention of potato DNA in calli with potato chloroplasts in our experiments. We can only conclude that the equivalent of approximately one potato chromosome is sufficient to establish

potato chloroplasts. Because rearrangements occur frequently in these asymmetric hybrids (see above) it is possible that this chromosome equivalent represents parts from different chromosomes.

From earlier studies we know that chloroplasts in symmetric and asymmetric fusion products of *Lycopersicon* and *Solanum* species sort out to homogeneity for either parent during cell divisions (Levi et al. 1988; O'Connell and Hanson 1987; Schiller et al. 1982). The results obtained for the 500-Gy calli indicate that sorting out of tomato and potato chloroplasts was at random. This observation matches the data of Derks et al. (1991), who showed that irradiation did not lead to a significant reduction of the irradiated chloroplast genome in somatic hybrids of *L. esculentum* and *L. peruvianum* which was irradiated with 50, 300 or 1000 Gy, or left unirradiated. However, it contradicts the observation of Bonnema et al. (1992) that sorting out was radiation-dose dependent in somatic hybrids of *L. esculentum* and *L. pennellii* which was irradiated with 50, 100, 150, 250, 500 or 1000 Gy, or left unirradiated. Bonnema et al. (1992) also reported that the chloroplast genotype of the asymmetric fusion products reflected the predominant nuclear genotype. We did not observe this correlation in either hybrid series. No explanation can be given for the almost complete absence of potato chloroplast DNA in the 150-Gy hybrids.

Apart from a possible irradiation-dependent and nuclear composition-dependent sorting out, the symmetric fusion experiments of Pehu et al. (1989) and Li and Sink (1992) and the asymmetric fusion experiments of Bonnema et al. (1992) demonstrated that the direction in which sorting out of chloroplasts takes place is strongly dependent on undefined differences in experimental conditions. Because our 150-Gy and 500-Gy fusions were carried out at different times with different transformants of one tomato genotype, the two callus population cannot be compared with respect to their chloroplast constitution.

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CHAPTER 6

ASYMMETRIC FUSION BETWEEN PROTOPLASTS OF TOMATO (*Lycopersicon esculentum* Mill.) AND GAMMA IRRADIATED PROTOPLASTS OF POTATO (*Solanum tuberosum* L.): THE EFFECTS OF GAMMA IRRADIATION.

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Summary. This chapter describes (1) the aggregation of nuclei in heterokaryons of tomato and unirradiated or irradiated potato protoplasts and (2) the effect of gamma irradiation of potato and tomato protoplasts on single and double strand DNA fragmentation, DNA repair and DNA synthesis with the aid of alkaline and pulsed field gel electrophoresis and an immunocytochemical technique. Prospects, to obtain highly asymmetric somatic hybrids of tomato and gamma irradiated potato, are discussed.

Introduction

Somatic hybridization or protoplast fusion is a technique by which the genomes of two plant species are brought together. It provides the possibility to circumvent sexual crossing barriers and to introduce desirable traits of a wild, donor species into a recipient, e.g. crop species. However, the symmetric combination of the two genomes also introduces many undesirable traits from the wild parent. In combinations of phylogenetically related species the removal of these traits is sometimes possible through repeated backcrossing of the symmetric somatic hybrid to the recipient. In symmetric combinations of less related species this is not possible because regeneration and fertility of the hybrids are strongly reduced or absent.

However, in some fusion combinations that involve species of different genera, subfamilies or families, it was found that the elimination of a large part of the donor genome enabled the regeneration of asymmetric somatic hybrid plants that could be used in subsequent sexual hybridizations (Dudits et al. 1980, 1987; Gleba et al. 1988; Somers et al. 1986). These investigators isolated highly asymmetric hybrids by means of selection at the cellular level for a nuclear trait of the donor. The strong elimination was induced by gamma or X irradiation of the donor prior to protoplast fusion. These rays produce various kinds of damage in the DNA, like single and double strand DNA breaks, crosslinking within the helix, crosslinking to other DNA molecules and to proteins and changes or loss of bases (Casarett 1968; George and Cramp 1987). After protoplast fusion, during the early stages of development, the damaged donor DNA is expected to be more susceptible to elimination than the recipient DNA.

Unlike the unique highly asymmetric and fertile hybrids, many more problematical asymmetric fusion combinations have been described (Famelaer et al. 1989; Imamura et al. 1987; Müller-Gensert and Schieder 1987; Wijbrandi

et al. 1990; Wolters et al. 1991; Yamashita et al. 1989). In general, these fusion experiments displayed a limited elimination of donor DNA, a poor regeneration potential, an aberrant morphology and sterility of regenerated plants. Furthermore, many investigators found that the irradiation of the donor lowered the frequency at which hybrid calli could be obtained. It is not clear why only in a very few cases and at a low frequency highly asymmetric fertile somatic hybrids were obtained.

Irradiation of plant protoplasts has been used extensively to induce elimination of genetic material of the irradiated parent after fusion with unirradiated recipient protoplasts. However, hardly any studies have been published on the effects of irradiation on parameters thought to be important in obtaining the desired effects. (1) A delayed karyogamy, as a side effect of irradiation, can possibly explain the low frequencies at which asymmetric hybrid calli are normally obtained. Possible reasons for the limited effect of ionizing irradiation are (2) an insensitivity of donor cells to irradiation, (3) repair of the donor DNA after irradiation and (4) *de novo* synthesis of donor DNA after irradiation.

Ad (1). To test if the low frequency, at which asymmetric hybrids calli are obtained, is caused by an inhibited or a delayed karyogamy, the aggregation of nuclei in heterokaryons should be studied microscopically (Constabel et al. 1975). For this analysis fusions between distinguishable protoplasts have to be performed. Only in this way it is possible to identify fusion products from unfused protoplasts and fusion products derived from one parent only, that are also present in the fusion mixture.

Ad (2). In order to determine whether a limited elimination of donor DNA is the result of a limited irradiation damage in the DNA, the effect of the ionizing irradiation on DNA fragmentation should be analyzed. DNA damage has been monitored by means of non-unwinding neutral filter elution, neutral or alkaline sucrose gradient centrifugation or (pulsed field) gel electrophoresis of isolated DNA. With pulsed field gel electrophoresis (PFGE) of double strand (ds)DNA it is possible to separate fragments up to approximately 12 Mbp. This system can be used to detect dsDNA breaks in irradiated cells (Ager and Dewey 1990; Ahn et al. 1991; Blöcher and Kunhi 1990; Grimsley et al. 1991; Iliakis et al. 1991). With alkaline gel electrophoresis of single strand (ss) DNA it is possible to separate fragments up to approximately 100 Kb (Sambrook et al. 1989) and to detect ssDNA breaks after irradiation.

Ad (3). Whether DNA repair of ds- and ssDNA breaks in irradiated donor protoplasts limits the irradiation damage in the donor genome, can also be determined with these two systems: ligation of fragmented DNA molecules results in a lower migration capacity with gel electrophoresis. In animal cells short dsDNA breaks (dsb) repair times of only a few hours were described: Ahn et al. (1991) found in mouse cells a constant repair rate of 44 dsb per minute during three hours after 50 Gy X-irradiation. Metzger and Iliakis (1991) reported biphasic repair after 50 Gy X-irradiation, with half or more of the total dsb repaired in 10-15 minutes and the rest very slowly (repair half-times between 60-90 minutes) for Chinese hamster ovary cells. For plants, Howland et al. (1975) described extensive ssDNA repair in *Daucus carota* protoplasts after 200 Gy gamma irradiation: Within 5 min, 50% of the ssDNA breaks were repaired and 1 h upon irradiation no more breaks could be detected. Hall et al. (1992a,b) could not detect extensive DNA breakage after irradiation with PGFE analysis, and suggested that plant DNA is either relatively insensitive to irradiation or that repair takes place rapidly, thus escaping detection. These data suggest that irradiation of plant cells is relatively inefficient to promote the elimination of irradiated DNA, especially when genetic damage such as point mutations are complemented by the recipient genome. However, it remains possible that the irradiation damage does not allow a proper DNA replication in most cells and that therefore the elimination of irradiated nuclei as a whole occurs frequently.

Ad (4). To test if *de novo* synthesis of donor DNA after irradiation opposes the DNA damage and elimination, irradiated protoplasts should be cultured in the presence of either tritiated thymidine or the thymidine analogue bromodeoxyuridine (BrdU). These molecules are incorporated in the newly synthesized DNA strand. Tritiated thymidine can be detected radiochemically while BrdU can be detected immunocytochemically (Wang et al. 1989).

In this report we analyzed the effects of gamma irradiation on (1) karyogamy in fusion products of protoplasts of tomato (*Lycopersicon esculentum*) and potato (*Solanum tuberosum*), (2) ds and ssDNA fragmentation in tomato and potato, (3) dsDNA and ssDNA repair in tomato and potato and (4) *de novo* DNA synthesis in potato.

Materials and methods

Plant material

The tetraploid potato (*Solanum tuberosum*) genotype 7322-K1, described by Wolters et al. (1991), was used as a donor in protoplast fusion experiments. The diploid potato genotype 1029-31 was kindly provided by Dr. E. Jacobsen, department of Plant Breeding, Wageningen Agricultural University, the Netherlands. Shoots of these genotypes were subcultured aseptically every four weeks in 500 ml translucent plastic containers on 80 ml Murashige and Skoog medium (1962) without hormones, with 20 g/l sucrose, pH 5.8 before autoclaving and solidified with 8 g/l agar, and grown at a light intensity of 10 W/m^2 (Philips TLD 36W) under a 16h-8h light-dark regime at 25°C. A cell suspension of the maternally inherited albino mutant of tomato (*Lycopersicon esculentum*) cv. Large Red Cherry (ALRC), described by Hosticka & Hanson (1984), was used as a recipient in fusion experiments and for irradiation studies. Cell suspensions of ALRC were cultured according to Wolters et al (1991).

Isolation, gamma irradiation, fusion and culture of protoplasts

Isolation, gamma irradiation, fusion and culture of protoplasts of tomato ALRC and potato 7322-K1 was carried out as described by Wolters et al. (1991). Fusion frequencies were 1 - 5%. Isolation of protoplasts of potato 1029-31 was carried out according to Wolters et al. (1991) with some modifications. After floatation in the AS medium, protoplasts were rinsed and pelleted twice in W5 medium (Menczel et al. 1981) (5 min, 70 x g) after which the protoplast concentration was determined with a haemocytometer. After another centrifugation (5 min, 70 x g) protoplasts were resuspended in TM2G medium (Wolters et al. 1991) to a final concentration of $5*10^5$ protoplasts/ml (pp/ml) for culture according to Wolters et al. (1991). For gamma irradiation, protoplasts of 1029-31 were resuspended in W5 medium to a final concentration of $3*10^6$ pp/ml. The irradiation was carried out on ice using a ^{60}Co irradiation source with a dose rate of 0.41 Gy/sec. After irradiation, protoplasts were centrifuged (pelleted) (5 min, 70 x g) at 0°C and resuspended in ice-cold W5 medium at a concentration of $6*10^7$ pp/ml for direct agarose embedding and subsequent DNA isolation. Protoplasts that were not immediately used for DNA isolation were resuspended in a 9:1 (v/v) mix of W5 : AS medium to a final concentration of $3*10^6$ pp/ml and put in darkness at 25°C. After culture, irradiated protoplasts were pelleted (5 min, 70 x g) and subsequently floatated

on AS medium (10 min, 125 x g) to separate protoplasts from disintegrated structures. (Gamma irradiation inhibited cell wall formation.) The protoplasts were pelleted in W5 medium (5 min, 70 x g), resuspended in W5 medium at a density of $6*10^7$ pp/ml and embedded. Cultured unirradiated protoplasts were directly pelleted in W5 medium (5 min, 70 x g), resuspended in W5 medium at a density of $6*10^7$ pp/ml and embedded. (Cell wall formation did not allow another floatation step.)

Cytological studies on heterokaryons
At various times after protoplast fusion, cultures were fixed by the addition of glutaraldehyde to a final concentration of 2.5 % (v/v) and subsequent incubation at 4°C for at least 8h. Fixed cultures were centrifuged for 10 min at 70 x g to pellet the fusion mixture. The pellet was stained in citrate phosphate buffer that contained 1 mg/l of the DNA binding fluorochrome Hoechst '33258' (bisbenzimide) (Laloue et al. 1980) and microscopic preparations of small samples were made on uncoated slides. We could distinguish heteroplasmic fusion products through a fluorescence microscope with the Zeiss dichroic filter set UG09. Heteroplasmic fusion products revealed a red autofluorescence of the potato chloroplasts together with the yellow autofluorescence of the typical thread-like structures of the albino tomato cytoplasm. The Hoechst '33258' stained nuclei could be distinguished subsequently in these fusion products, with the Zeiss filter combination UG01.

Preparation of high molecular weight DNA in agarose plugs.
225 µl of the $6*10^7$ pp/ml protoplast suspension in W5 medium, warmed briefly to 37°C, was mixed with an equal volume of a 1% (w/v) DNA grade agarose (Bio-Rad Laboratories, Richmond, USA) solution in W5 medium at 37°C. The mixture was pipetted directly in an ice-cold perspex mould and kept on ice for 30 min to allow the agarose to solidify. Solidified plugs were removed from the mould, cut in four equal pieces, collected in 7 ml lysis mix, that consisted of 0.5 M Na_2EDTA, 1 % (v/v) N-lauryl-sarcosinate and 2 g/l proteinase-K (Merck, Darmstadt, Germany), pH 8.0, and incubated for 48 hours at 50°C. Subsequently, the plugs were stored in 0.5 M Na_2EDTA, pH 8.0 at 4°C for several weeks. Agarose plugs that contained intact chromosomal DNA from *Aspergillus niger* were kindly provided by dr F. Debets of our department; agarose plugs that contained intact chromosomal DNA from

Schizosaccharomyces pombe were purchased from Bio-Rad. These DNA molecules were used as length markers in pulsed field gel electrophoresis.

Pulsed field gel electrophoresis.
A CHEF (Contour clamped Homogeneous Electric Field) DR II system (Bio-Rad) was used for pulsed field gel electrophoresis (PFGE). DNA-agarose plugs were loaded according to Debets et al. (1990) on 0.5 % (w/v) DNA grade agarose gels. Gels were electrophoresed at 9°C in circulating 0.5xTAE buffer (Maniatis et al. 1982) at 45 V (approximately 1.5 V/cm) with 60 pulse intervals of 50 min followed by 60 pulse intervals of 33 min. After electrophoresis gels were stained in 0.5x TBE buffer (Maniatis et al. 1982) with 0.5 mg/l ethidium bromide for one hour, destained in distilled water for 2 hours and photographed under UV illumination at 302 nm.

Tritiated thymidine labelling
[Methyl-^{3}H]thymidine (TRK758, Amersham, Buckinghamshire, UK), with a specific activity of 2.96 TBq (80 Ci)/mmol, was supplied to freshly transferred, fast growing ALRC cell suspensions (multiplication rate five in four days) at a concentration of 2.5 nM (2 μCi/ml). Incubation was carried out for four days during culture. The incorporated label amounted approximately 10.8 counts/min/isolated protoplast.

Preparation of DNA for alkaline gel electrophoresis; ^3H detection
$2.8*10^6$ Protoplasts were frozen at -80°C in 1 ml W5 medium, thawed and lysed in 7.5 ml of a solution that contained 0.05 M Na_2EDTA, 0.1 M TRIS, 1% (v/v) sodium dodecyl sulphate and 200 mg/l proteinase-K during 3 h at 37°C. Per 8.5 ml sample, 0.25 ml was transferred to an Eppendorf tube, treated with 7.5% (v/v) trichloroacetate (TCA) during 30 min on ice to precipitate DNA and peptides and centrifuged (15 min, maximum speed Eppendorf centrifuge). The pellet was washed with 5% TCA (v/v) and with 70% ethanol and incubated in 0.25 ml 0.2 M NaOH during 1h, 65°C. 3 ml of the water soluble liquid scintillation cocktail Ultima Gold (Packard, USA) was added and the radioactivity was measured twice in a Tricarb Liquid Scintillation Analyzer (Packard), 20 min per sample.

The rest of the sample (8.25 ml) was centrifuged (10 min, 500 x g), supernatants were carefully transferred to a new tube and one volume of Sevag (Maniatis et al. 1982) was added. After gentle turning the tube for 5 min,

followed by centrifugation (5 min, 500 x g), the aqueous phase was transferred to a new tube. Precipitation of DNA was carried out overnight at -20°C after adjustment of the solution to 60% (v/v) isopropanol. After centrifugation (30 min, 500 x g) the DNA pellet was carefully washed with 70% ethanol, air dried, dissolved in water at a concentration of 40 ng/μl and subjected to alkaline gel electrophoresis (AGE).

Alkaline gel electrophoresis

Tritiated thymidine labelled DNA was separated on a 0.5 % DNA grade agarose gel that contained 20 mM NaOH and 5 mM $(Na)_2EDTA$. The electrophoresis buffer also consisted of 20 mM NaOH and 5 mM $(Na)_2EDTA$. Electrophoresis was carried out at 0.7 V/cm during 40 h. After electrophoresis, less than 2% of the total radioactivity in the gel was present in the electrophoresis buffer. The gel was stabilized in a solution that contained 1 M Tris-HCl and 1.5 M NaCl, pH 7.5 for 45 min, stained in 1x TAE buffer (Sambrook et al. 1989) with 1 mg/l ethidium bromide for 45 min and destained in distilled water for 2 h.

Southern blot hybridization.

After electrophoresis, staining and destaining, the DNA in the PFGE and AGE gels was fragmented by two applications of 0.5 M HCl, each for 15 min at room temperature. The DNA was transferred to Hybond-N+ (Amersham, UK) by means of an alkaline blotting procedure with 0.4 M NaOH (Reed and Mann, 1985). Blots were shortly rinsed in SSPE 2x (Sambrook et al. 1989), wrapped in Saran-Wrap plastic foil and kept at 4°C until use.

Total DNA of potato was isolated according to Rogers and Bendich (1988). The DNA was purified on a cesium chloride gradient and radioactively labelled by means of the Boehringer Mannheim (Germany) Random Primed DNA Labelling Kit. Hybridization was performed in glass bottles in a Hybaid hybridization oven (Hybaid, UK) at 65°C for 16 hours. The blots were rinsed twice in 2x SSC, 1% SDS, 0.1% Na-PPi (tetra-sodium pyrophosphate) (Sambrook et al. 1989) at 65°C for 30 min and twice in 0.1x SSC, 1% SDS, 0.1 % Na-PPi at 65°C for 30 min. Autoradiography was performed on Konica X-ray film. Sections were cut from the filters, put in scintillation vials, 5 ml of the liquid scintillation cocktail Ultima Gold (Packard) was added and radioactivity per region was measured in a Tricarb Liquid Scintillation Analyzer (Packard), 10 min per section.

For the blots of AGE gels that contained tritiated thymidine labelled DNA, sections were directly cut from the filters. 3 ml of liquid scintillation cocktail was added and after one day in the dark, radioactivity per section was measured twice, 10 min per section.

Immunocytochemical detection of DNA synthesis

Bromodeoxyuridine incorporation, fixation of cultured protoplasts and immunocytochemical detection of DNA synthesis was carried out according to a modified procedure of Wang et al. (1989) with a cell proliferation kit and fluorescein-conjugated rabbit anti-mouse IgG antibody (Amersham, UK). Two antibody pre-incubation steps with 10% (v/v) goat serum in PBS buffer (Sambrook et al. 1989) were added to the protocol to inhibit non-specific binding. Nuclei were counterstained with Hoechst '33258'.

Results

Aggregation of nuclei in heterokaryons

Heteroplasmic fusion products of cell suspension protoplasts of albino tomato and leaf mesophyll protoplasts of potato could be distinguished through a fluorescence microscope. Subsequently, Hoechst '33258' stained interphase nuclei in these fusion products were counted. Potato nuclei could not be distinguished from tomato nuclei. The aggregation of nuclei was observed from two days after fusion but in most fusion products it took place later, especia[redacted] from four days after fusion (Fig.1). Constabel et al. (1975) described th[redacted] nuclear aggregation in heterokaryons of soybean and pea took place before the first mitosis and within four days after protoplast fusion. In our experiments the aggregation always occurred before cell division. Mitotic stages were rarely found in the fusion products. It could not be observed whether the aggregation of nuclei was pursued directly by karyogamy or whether karyogamy occurred after mitosis of the aggregated nuclei. In aggregated nuclei no sub-structures could be distinguished. Cell division of fusion products was observed from five days after fusion when the potato protoplasts were not irradiated. No cell division was observed within 7 days after fusion when potato protoplasts were 50 or 500 Gy gamma irradiated.

Irradiation did prevent nor retard the nuclear aggregation significantly (Fig.1c,d). The presence of more than one nucleus in the isolated protoplasts (Fig.1a,b) might be caused by spontaneous fusion during the protoplast isolation steps (Miller et al. 1971) and by acytokinesis, as was described by Huang and Chen (1988). The relative large number of fusion products with more than two nuclei probably resulted from multiple fusions and the involvement of multinucleate protoplasts in the fusion process. Presumably, the reduction of the percentage of hybrid cells with more than two nuclei, within two days after fusion, was mainly caused by the aggregation of tomato nuclei. This aggregation was also observed in isolated tomato protoplasts two days after isolation.

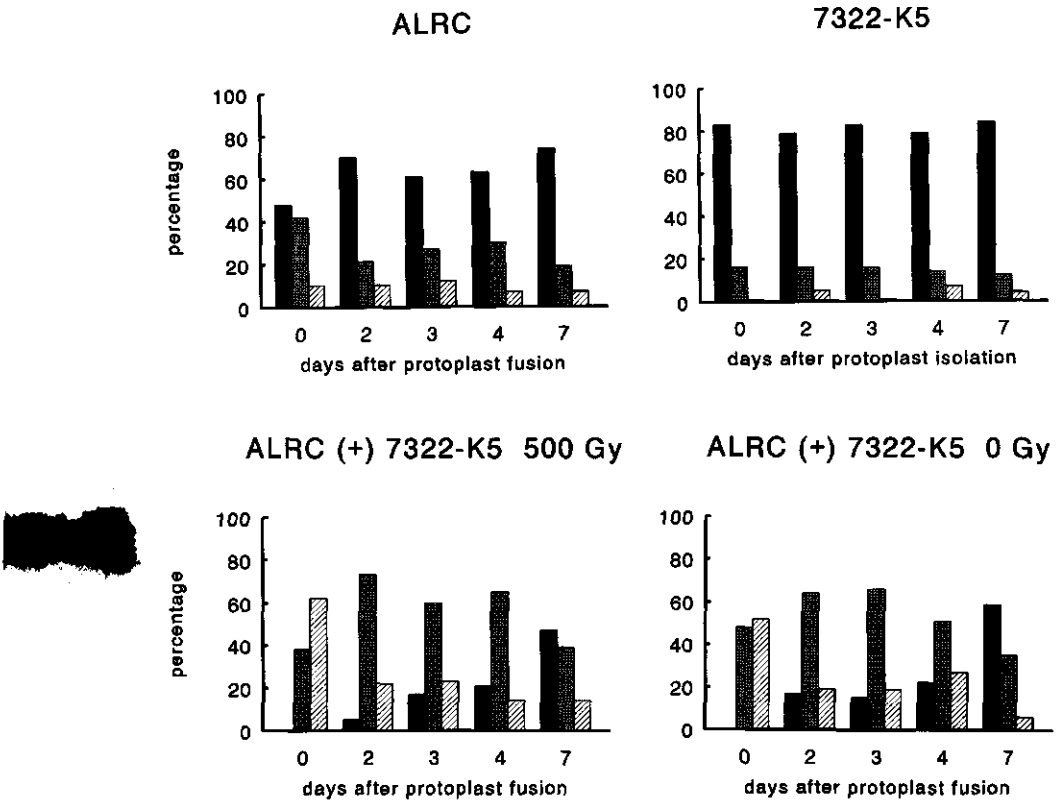

Fig.1. The numbers of protoplasts or heteroplasmic fusion products (expressed as percentages) with 1 nucleus (■), with 2 nuclei (▒) and with more than 2 nuclei (▨), present in **a.** tomato ALRC protoplasts, **b.** potato 7322-K1 protoplasts, **c.** heteroplasmic fusion products of ALRC and unirradiated 7322-K1 and **d.** heteroplasmic fusion products of ALRC and 500 Gy gamma irradiated 7322-K1. Observations were made directly after protoplast isolation and/or fusion, after 2, 3, 4 and 7 days. n=100 for each day and each population.

The effect of gamma irradiation on dsDNA fragmentation and dsDNA repair

The potato 1029-31 was used for irradiation studies because vegetative propagation of this genotype was efficient and many protoplasts could be obtained routinely. With PFGE it was shown that gamma irradiation produced dsDNA breaks in the DNA of potato protoplasts. With an increase of the irradiation dose a stronger fragmentation (more smaller and less larger fragments) of the dsDNA was observed (Fig.2). The fragmentation was quantified by means of Southern blotting of the DNA from the Pulsed Field Gel, followed by hybridization with labelled total DNA of potato (Fig.3). During PFGE a significant percentage of dsDNA was not released from the plugs. This was most evident for higher irradiation doses. Several causes have been proposed (Blöcher and Kunhi 1990; Grimsley et al. 1991; Metzger and Iliakis 1991) for the retainment of DNA in the plugs: (1) incomplete lysis of embedded cells or protoplasts, (2) reduced migration of replication forks, (3) disentanglement of large DNA molecules, (4) a prolonged pathway of extended DNA molecules through the chain of pores in the agarose plug and through a part of the plug, where immobilized DNA is left behind, and (5) association of large DNA fragments with branched and looped structures (crosslinking). This non-elutible DNA could be detached from the gel by nicking with HCl treatment before Southern blotting. The employed PFGE conditions resulted in a compression zone at the bottom of the lanes. This zone, which was most evident for non-irradiated protoplasts (Fig.2 and 3), most likely contained degraded and organellar DNA

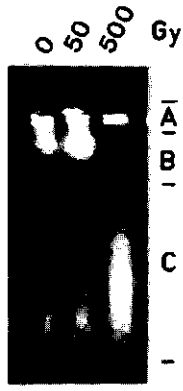

Fig.2. Ethidium bromide stained Pulsed Field Gel Electrophoresis (PFGE) gel of dsDNA of 0, 50 and 500 Gy gamma irradiated potato 1029-31 protoplasts. Section A contains dsDNA fragments larger than approximately 10 Mbp; section B contains fragments smaller than approx. 10 Mbp and larger than approx. 4 Mbp; section C contains fragments smaller than approx. 4 Mbp.

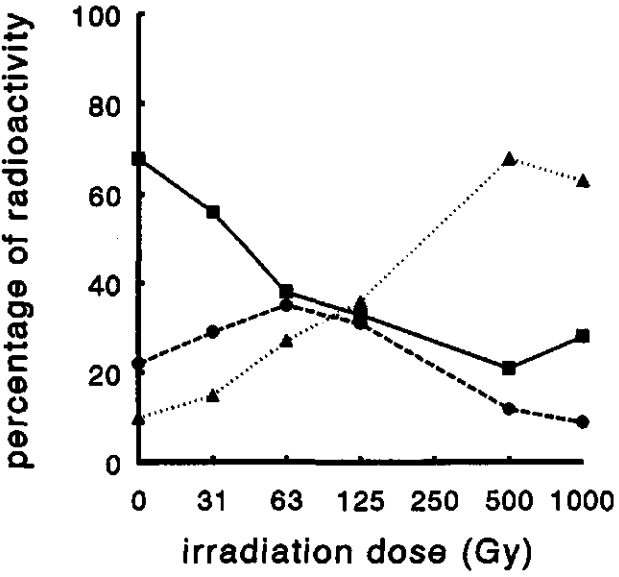

Fig.3. Relative dsDNA fragment distribution (in percentages of the total amount of DNA in the lane) of 0, 31, 63, 125, 500 and 1000 Gy gamma irradiated potato 1029-31 protoplasts. Protoplasts were lysed directly after irradiation, dsDNA was isolated and subjected to PGFE, a Southern blot was made and probed with total DNA of potato, radioactive sections were cut from the filters and liquid scintillation analysis was performed. Sections A (—■—), B (--●--) and C (···▲···) correspond to those in Fig.2.

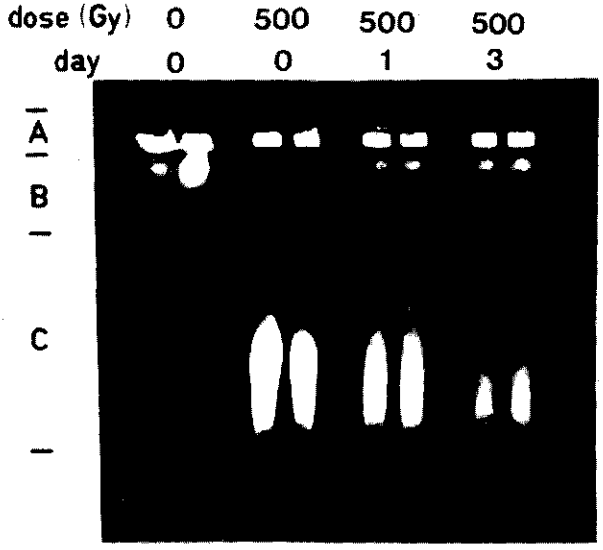

Fig.4. Ethidium bromide stained PFGE gel of dsDNA of unirradiated (0 Gy) and 500 Gy irradiated protoplasts of potato 1029-31, directly embedded (day 0), embedded after 1 and after 3 days of culture. The contiguous lanes contain protoplast samples that were treated identically.

When 500 Gy irradiated potato protoplasts were cultured for one or three days prior to embedding and lysis, the PFGE gels and liquid scintillation analysis of labelled blot sections A, B and C showed relatively fewer small and more large dsDNA fragments in intact protoplasts (Fig.4). This fragment shift was also observed after 125, 250 and 1000 Gy gamma irradiation of potato protoplasts (data not shown).

The effect of gamma irradiation on ssDNA fragmentation and ssDNA repair

By means of alkaline gel electrophoresis (AGE) it was shown that gamma irradiation produced ssDNA breaks in the DNA of tomato ALRC and potato 1029-31 protoplasts. With an increase of the radiation dose a stronger fragmentation of the DNA was observed. Tomato and potato responded similarly to gamma irradiation (data not shown). ^{3}H radioactively labelled tomato cell suspension protoplasts were used to quantify the degree of fragmentation and the degree of repair of the ssDNA breaks. Labelled protoplasts that were 500 Gy gamma irradiated or left unirradiated were either directly lysed or lysed after 10 h of culture. The DNA was isolated and subjected to AGE. After 10 h of culture, post-irradiation repair of ssDNA breaks was deduced from the appearance of larger and the disappearance of smaller ssDNA labelled fragments (Fig. 5). The divergence between the AGE patterns of unirradiated and irradiated protoplasts in Fig. 5b suggested an incomplete DNA repair, 10 h after irradiation.

The effect of gamma irradiation on the synthesis of DNA

To find out whether DNA synthesis occurred after 500 Gy gamma irradiation, isolated cell suspension protoplasts of tomato ALRC were irradiated or left unirradiated and cultured for two days in the presence of 5-bromodeoxyuridine (BrdU). Approximately 30% of the cultured protoplasts died because of the BrdU treatment. Lower BrdU concentrations hindered a unambiguous screening. In unirradiated cultures, 45% of the examined cells (n=200) could be stained with the fluorescein-conjugated rabbit anti-mouse IgG antibody against BrdU (Fig.6a,b). Cultures without BrdU were not stained. At this stage no division had occurred. Surprisingly, also 45% of the 500 Gy gamma irradiated

protoplasts (n=200) could be stained, thus indicating that post-irradiation DNA synthesis takes place. We presume that the amount of BrdU that is incorporated during excision DNA repair is much smaller than that during DNA replication. The amount of incorporated BrdU that results from excision DNA repair probably falls below the detection level.

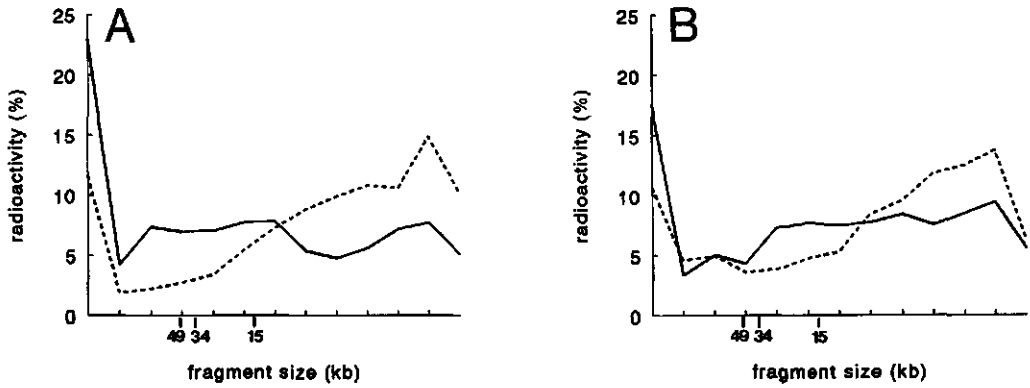

Fig.5. Relative ssDNA fragment distribution (in percentages of the total amount of DNA in the lane) of unirradiated (———) and 500 Gy gamma irradiated (--------) tomato ALRC cell suspension protoplasts **a.** directly after irradiation and **b.** after 10 h of culture. Cells were labelled with ^{3}H-thymidine, protoplasts were isolated and irradiated, DNA was isolated and subjected to Alkaline Gel Electrophoresis, a Southern blot was made, radioactive sections were cut from the filters and liquid scintillation analysis was performed. Phage Lambda DNA and XhoI digested lambda DNA were used as length markers

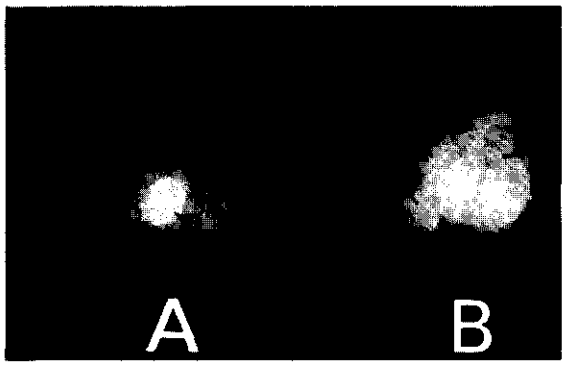

Fig.6. Immunocytochemical detection of DNA synthesis in tomato ALRC protoplasts. A.shows the BrdU labelled nucleus (←—), which was visualized by a fluorescein-conjugated rabbit anti-mouse IgG antibody against BrdU. B.shows the same protoplasts counterstained with Hoechst '33258'.

Discussion

Gamma irradiation of potato protoplasts before fusion with tomato protoplasts did not inhibit nuclear aggregation in fusion products. However, it was demonstrated that the irradiation retarded the cell division of fusion products. This retardation and a possible complete obstruction of the initial cell division process might explain the low frequencies of proliferating fusion calli that have been observed by many investigators.

From various experiments with animal cells it has been concluded that the number of dsDNA breaks increases linearly with the dose of gamma or X rays and ranges between $2.4*10^{-3}$ and $22*10^{-3}$ breaks/Mbp/Gy gamma or X rays in the presence of oxygen (Ager and Dewey 1990; Ahn et al. 1991; Blöcher and Kunhi 1990; Charlton et al. 1989; Menke et al. 1991; Ward 1990). Ahn et al. (1991) also showed that the dsDNA fragmentation occurred randomly throughout the genome in mouse cells. With this relationship, 50 Gy gamma irradiation produces dsDNA fragments with an average size between 0.9 and 8.4 Mbp, whereas 500 Gy produces fragments with an average size of 0.1 to 0.8 Mbp. These calculations are in coordinance with our PFGE-data on the fragmentation of the DNA from irradiated potato protoplasts. These data suggest that the physical properties of gamma irradiation are similar for plant and animal DNA.

Investigators also found that the number of ssDNA breaks increases linearly with the dose of gamma or X rays: Howland et al. (1975) determined that for *Daucus carota* 0.040 ssDNA breaks/Mb/Gy gamma irradiation were produced. Mullenders et al. (1983) observed a value of 0.083 breaks/Mb/Gy X irradiation for human HeLa cells whereas Menke et al. (1991) found a similar value of 0.11 for *E. coli*. 500 Gy gamma irradiation would thus produce ssDNA fragments with an average size of approximately 18 to 50 kb. The stronger fragmentation, that we observed after AGE, was probably caused by breakage of long DNA molecules by DNA shearing forces during the isolation and electrophoresis steps. Furthermore, it is known that gamma irradiation produces alkali-labile DNA bonds that can break during alkaline blotting and alkaline gel electrophoresis (George and Cramp (1987). Possibly, also shearing damage is stronger in irradiated DNA.

We studied ssDNA repair after gamma irradiation by means of AGE of prelabelled DNA. With the labelled DNA it was possible to discriminate between DNA repair and *de novo* DNA synthesis. The long recovery time (10

h) after irradiation was chosen to obtain information on the presence of breaks in the donor DNA at the time of protoplast fusion but prior to karyogamy and mitosis. Like Howland (1975) with *Daucus carota* and Tano and Yamaguchi (1977) with *Hordeum distchum*, we observed a strong fragmentation directly after gamma irradiation and a subsequent ligation of ssDNA fragments. The observed divergence between the AGE patterns of unirradiated and irradiated protoplasts that were cultured for 10 h, was probably caused by a renewed breakdown of the quickly and thoroughly repaired DNA in the 10 hours after gamma irradiation. This renewed breakdown was also reported by Tano and Yamaguchi (1977) for *Hordeum distchum* within 5 hours after irradiation.

The repair of dsDNA breaks was studied with PFGE. The observed disappearance of small (section C) and the appearance of large (sections A and B) dsDNA fragments and a fairly constant amount of hybridizing dsDNA in time suggested a slow dsDNA repair rate during three days. However, several investigators reported very fast and highly efficient dsDNA repair within several hours after irradiation. Therefore we assume that the observed small dsDNA fragments that were present one day after irradiation, resulted from the renewed breakdown of initially repaired DNA. This renewed breakdown also suggested a declining DNA polymerase and ligase activity and cell death due to the irradiation treatment. The disappearance of small and the appearance of large DNA molecules at day three after irradiation was probably caused by a selective degradation of small DNA fragments in combination with *de novo* DNA synthesis of large fragments. Post-irradiation DNA synthesis was also described by George and Cramp (1987) for various mammalian cells, whereas Hall et al. (1992a) even found cell divisions in heavily irradiated (450 Gy) cells of *Beta vulgaris*. Probably, Hall et al. 1992a,b did not find a strong initial DNA fragmentation in 300, 450 or 1000 Gy gamma irradiated cells and protoplasts of *Beta vulgaris* because irradiations were carried out at room temperature and cells or protoplasts were not directly lysed but from one until 18 h after irradiation. Between irradiation and lysis, cells and protoplasts were kept at room temperature. This allowed extensive DNA repair.

It was not possible to investigate DNA repair and synthesis in heterokaryons after fusion because the number of heterokaryons, necessary for PFGE, AGE and for the immunocytochemical analysis could not be isolated. However, it is conceivable that quantitative differences in the renewed DNA breakdown and replication within irradiated donor protoplasts, related or not to a possibly increased donor DNA preservation and synthesis upon fusion with 'helper'

unirradiated recipient protoplasts, result in a large variation of elimination between fusion products. It is questionable whether this variation already expresses itself as a selective proliferation of fusion products that harbour relatively much donor DNA.

From these experiments we cannot explain why highly asymmetric, animal somatic hybrids can be constructed efficiently with low irradiation doses (Pontecorvo 1971) or even without irradiation (Davis 1991) whereas these hybrids are extremely difficult to obtain with plant cells. Possibly, differences in the length of the cell cycle are responsible for this striking difference. In newly isolated plant protoplasts and fusion products it takes several days before the first nuclear and cell divisions take place; in animal cells and fusion products the first divisions usually occur within several hours. This long period in fusion products of plant cells thus might allow the irradiation-damaged nucleus either to recover or to break down its damaged DNA. The subsequent maintenance of only a minor part of the donor DNA, which should contain the gene where selection is based on, might be more difficult to establish than the entire breakdown of the donor DNA. This then can explain why cybridization experiments, where selection is based on a cytoplasmic trait of the irradiated parent and not on a nuclear encoded trait, are less difficult to perform than asymmetric somatic hybridization experiments.

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CHAPTER 7

GENERAL DISCUSSION AND SUMMARY

7.1 Recapitulation

Somatic hybridization has the potential to combine genetic information of species that cannot be hybridized by sexual crosses. The technique to obtain useful asymmetric hybrids that contain the complete genome of a recipient and part of the genome of a donor, requires **(1)** the availability of efficient cell and tissue culture systems, **(2)** the availability of selection systems for somatic hybrids, **(3)** an efficient and directed elimination of a large part of the donor genome, **(4)** insight in the effects of somatic incongruity and ways to circumvent it, and ultimately **(5)** introgression of donor traits into the recipient genome by means of mitotic or meiotic recombination between the donor and recipient genomes.

In this thesis, tomato (*Lycopersicon esculentum* Mill.), potato (*Solanum tuberosum* L.) and their fusion products were used to investigate the possibilities and limitations of somatic hybridization as a plant breeding technique. Tomato provides efficient cell and tissue culture systems, especially those genotypes bred for favourable cell and tissue culture traits (Koornneef et al. (1987). Together with potato, the species is also an excellent genetic model plant (Hille et al. 1989), for which extensive genetic maps (Tanksley et al. 1992) and species specific, respectively locus specific DNA probes are available. Thus, a detailed genetic analysis of the somatic hybrids could be performed.

This thesis describes specifically **(1)** the isolation and characterization of nitrate reductase-deficient mutants in tomato; **(2)** the use of two of these mutants in somatic hybridization experiments with potato; **(3)** the use of one of these mutants and of an albino tomato to produce triploid somatic hybrids with monoploid potato; **(4)** the isolation and characterization of asymmetric somatic hybrids between hygromycin resistant tomato and gamma irradiated, kanamycin resistant potato and **(5)** a study on the effects of gamma irradiation on karyogamy in heterokaryons of tomato and potato and on DNA fragmentation, DNA repair and DNA synthesis in irradiated tomato and potato protoplasts.

7.2 Selectable marker genes and the use of the nitrate reductase-deficiency marker in tomato for the selection of fusion products with potato.

To obtain large numbers of somatic hybrids it is useful to select for hybrids during the early stages of cell culture. This selection can be carried out efficiently with dominant cell-selectable markers that are present in one but not in the other parent. Antibiotic resistances such as the kanamycin and hygromycin resistances that are introduced in the plants by transformation, are excellent examples of such dominant cell-selectable markers (Chapter 5). Recessive auxotrophs that can be complemented by the wild type allele from the other parent suit the same purpose. The genetic transformation of tomato with genes that encode for antibiotic resistances, is more easy than the isolation and characterization of nitrate reductase deficient mutants. Furthermore, the vegetative propagation of transgenics is not as complicated as that of auxotrophs. Nevertheless, we decided to isolate auxotroph, nitrate reductase-deficient (NAR) mutants of tomato (Chapter 2) because of legal restraints to the use of transgenic plants. These NAR mutants were successfully used in somatic hybridization experiments with potato (Chapters 3 and 4).

Most somatic hybrids are not immediately suited for agricultural use. They often require subsequent sexual breeding steps. When (transgenic) marker genes have been used for the selection of fusion products, these steps may be utilized to remove the marker genes together with other unwanted traits from the donor.

7.3 Symmetric somatic hybridization as a plant breeding technique

Whereas in crosses haploid meiotic cells are fused, somatic hybridizations are mainly carried out with diploid somatic protoplasts. Instead of the diploid F_1 hybrids derived from crosses, protoplast fusions generate mainly tetraploid hybrids. The sterility that is exhibited in several interspecific allodiploid F_1 hybrids and that sometimes can be abolished by doubling the ploidy level, thus can be circumvented with the aid of protoplast fusion. However, further breeding steps, i.e. crosses between allotetraploid hybrids and autodiploid species, are often extremely difficult to perform and require embryo rescue,

especially in solanaceous species. In general, allotriploid hybrids offer better possibilities in crosses with autodiploid species. These crosses show less degeneration of the endosperm than crosses between allotetraploids and autodiploids. Furthermore, the chromosomes of the allotriploid that remain unpaired during meiosis, are divided among the gametes. This distribution allows the elimination of chromosomes that are present in gametes that do not take part in sexual hybridizations. For these reasons, allotriploid somatic hybrids between diploid tomato and monoploid potato were produced (Chapter 4). Triploid somatic hybrids can be considered asymmetric because the genome contribution of both parents is unequal. Unfortunately, our triploid somatic hybrids could not yet be hybridized with tomato or potato. Apart from the extremely low pollen viability, genetic incongruity between the triploids and its crossing partners tomato and potato apparently restricted further breeding steps.

The limitations of symmetric somatic hybridization as a plant breeding technique are increasingly better understood. Only slightly larger genetic distances can be overcome by somatic hybridization than by interspecific sexual crosses in combination with embryo rescue. The genetic incongruity in somatic tissues imposes a strong limitation and almost prohibits to bridge gaps beyond a certain phylogenetic distance. E.g. nucleo-cytoplasmic incongruity is already expressed in somatic hybrids between tomato and potato (Wolters et al. 1991). This incongruity is even more profound in combinations between potato and *Nicotiana plumbaginifolia* (Wolters et al. 1993).

The spontaneous and induced chromosome elimination that was described for several intergeneric symmetric hybrids ((Babiychuk et al. 1992; Gilissen et al 1992a,b; Hoffmann and Adachi 1981; Tempelaar et al. 1991; de Vries et al. 1987; Wolters et al. 1993) can theoretically result in useful asymmetric hybrids. However, this elimination is often biparental and it occurs in combination with unwanted polyploidization. In addition, the elimination is rather unpredictable and it can sometimes require a long period of tissue culture. We expected that the artificial induction of chromosome elimination by means of ionizing irradiation would offer better possibilities for a quick and directed chromosome elimination.

7.4 Asymmetric somatic hybridization as a plant breeding technique

Asymmetric somatic hybrids contain the complete genome of a recipient species and part of the genome of a donor species. The elimination of donor DNA can be induced by gamma or X-irradiation of donor protoplasts prior to fusion with recipient protoplasts. The removal of part of the donor genome conceivably limits the genetic incongruity in the somatic hybrid tissue. However, aneuploidy, mutations and chromosome rearrangements (Wijbrandi 1989) that are induced by elimination treatments, may result in reduced somatic performance and fertility. Although many researchers tried to obtain fertile asymmetric hybrids, only few have succeeded. The fertile hybrids between species of different genera, families or subfamilies, that have been described, are highly asymmetric whereas those between more related species can contain more donor DNA. Presumably, the genomic imbalance is better tolerated in phylogenetically close combinations because less genes contribute to somatic incongruity.

We did not obtain fertile asymmetric somatic hybrids between tomato and gamma irradiated potato. Although shoot and sometimes root regeneration was observed, no viable plants were recovered. The hybrid calli were highly polyploid and showed an increase in the amount of tomato DNA. Some calli demonstrated an extensive elimination of potato DNA but this elimination did not affect regeneration (Chapter 5).

In addition to gamma irradiation, we used UV irradiation of donor protoplasts to eliminate donor DNA (see Hall et al. 1992). We observed some dsDNA breakage with pulsed field gel electrophoresis but the UV treatment had extremely toxic effects on protoplast cultures. Tomato protoplasts even did not divide when they were mixed 10:1 with UV irradiated potato protoplasts; fusion products could not be obtained.

Apart from the elimination of donor DNA with irradiation, loss of donor traits was described when isolated nuclei of donor protoplasts were fused with recipient protoplasts (Saxena et al. 1986). This technique also circumvents possible nucleo-cytoplasmic incongruity (Wolters et al. 1991). We performed fusion experiments between hygromycin resistant tomato protoplasts and nuclei of kanamycin resistant potato and tobacco protoplasts that expressed β-glucuronidase (GUS). Although fusion was observed frequently through a microscope and significant GUS activity was found in the fusion mixture, up to two weeks after fusions, fusion products did not

proliferate.

The damaging and elimination of donor DNA is normally induced prior to protoplast fusion. However, we also tried to specifically damage the donor DNA after protoplast fusion: Stetten et al. (1976) demonstrated that it was possible to trigger DNA damage: Bromodeoxyuridine (BrdU) was incorporated in the DNA of isolated animal cells. Cell death could be induced by the addition and uptake of the DNA fluorochrome Hoechst '33258' and subsequent illumination. It was presumed that the combined BrdU-Hoechst-illumination treatment caused unrepairable DNA damage. In theory, it is possible to incorporate BrdU in cell suspension cells of the donor and to fuse BrdU-labelled protoplasts with recipient protoplasts. The donor DNA can be damaged subsequently by the addition of Hoechst '32258' and the illumination. We labelled cell suspension cultures of ALRC tomato with BrdU. Protoplasts were isolated and cultured for two days in the dark. Subsequently, Hoechts '32258' was added to the cultures and the mixtures were illuminated. Although we observed that Hoechst '32258' entered living cells, the BrdU-Hoechst '32258'-illumination treatment did not cause cell death. Therefore, this 'time-bomb' method could not be applied in protoplast fusion experiments.

The preservation of genetic information of a donor in a recipient species is assured optimally when this information is physically integrated in the recipient genome. In a few cases the direct introgression of donor DNA in the recipient genome has been reported. Piastuch and Bates (1990) and Parokonny et al. (1992) described the formation of translocation chromosomes that were made up from DNA of the two fusion parents. It is not clear whether these mitotic crossing over events are restricted to homoeologuous (parts of) chromosomes.

Homoeologuous recombination rarely takes place during meiosis because pairing between the chromosomes of different species is usually absent. Furthermore, the two combined genomes might be spatially separated, as was observed by Gleba et al. (1987) in *Nicotiana plumbaginifolia* (+) *Atropa belladonna* fusion products. Also genes have been described that suppress homoeologuous recombination during meiosis, e.g. the *Ph* gene in wheat (Riley and Chapman 1958; Gillies 1989; Jenkins 1989). Further elimination of donor chromosomes and selection for recombinogenic genotypes may

ultimately lead to meiotic recombination. The existence of genes that suppress meiotic recombination in somatic hybrids of tomato and potato is suggested by the observed early meiotic pairing and recombination nodules in hypotetraploid hybrids (de Jong et al. 1993) and the observed absence of paring in presumed eutetraploids (Jacobsen et al. 1992).

Recombination between chromosomes can also occur during the mitotic cell cycle. The phenomenon is observed commonly in fungi (Orr-Weaver and Szostak 1985; Whittaker et al. 1989) and occasionally in plants (Evans and Paddock 1980; Harrison and Carpenter 1977; Sibi et al. 1984). The stimulation of mitotic recombination might be useful to obtain asymmetric hybrids with already recombined chromosomes. We used genotypes that are heterozygous for nitrate reductase-deficiency and semidominant for *xa-2* (a partially dominant chlorophyll mutant that was described by Persson (1960)) to study mitotic recombination in tomato at the level of the cell and that of the plant respectively. Homoeologuous mitotic crossing over in NR^+/NR^- heterozygotes can result in homozygous nitrate reductase deficient cells, that can be selected on media that contain chlorate. In *Xa-2/xa-2* heterozygotes mitotic recombination can express itself by the appearance of two adjacent homozygous *Xa-2/Xa-2* and *xa-2/xa-2* 'twin-spot' regions. Chemicals, that were suggested to increase mitotic recombination, were tested for their effectiveness. However, these chemicals were either ineffective or too toxic for tomato. Therefore, they were not used in protoplast fusion experiments.

7.5 A stronger elimination of donor DNA in somatic hybrids

Many investigators used gamma or X-irradiation of donor protoplasts prior to fusion with recipient protoplasts to eliminate the donor DNA in asymmetric hybrids. However, often a limited elimination was observed whereas other investigators described extensive DNA repair after irradiation. This, together with other side-effects of the irradiation, lead to our investigations of the early effects of gamma irradiation. Gamma irradiation damages the nuclear DNA and this damage can be increased by raising the irradiation dose. Our results (Chapters 5 and 6) suggest that more donor DNA is eliminated after a higher irradiation dose. However, the dose cannot be raised indefinitely because of toxic effects on cell metabolism. Doses over 50 Gy already completely suppress cell division in tomato and potato although only limited

DNA fragmentation was observed with pulsed field gel electrophoresis at this dose. We performed fusion experiments between tomato protoplasts and 150 or 500 Gy gamma irradiated potato protoplasts. These doses were sufficient to eliminate a large part of the donor DNA in several hybrids.

In theory, the initial damage in protoplasts can be maintained by an inhibition of the DNA repair. However, the enzymes that are primarily involved in the repair process (polymerases and ligase), are also essential during DNA replication and cell division. Therefore, the blockade of repair has to be short and easy to elevate. The chemicals 1-ß-D-arabinofuranosylcytosine that is known for its repair inhibiting action, and cytidine, that is known for its elevating action (Snyder and Lachmann 1989) were used in protoplast culture and asymmetric fusion experiments. However, no highly asymmetric calli were obtained.

The renewed DNA breakdown after the presumed quick and efficient DNA repair is probably opposited by the fusion with 'helper' recipient protoplast. An increase of the time between irradiation and fusion might allow more unrepairable DNA damage and a stronger elimination of the damaged DNA to occur.

Apart from the sometimes observed spontaneous chromosome elimination in fusion products, especially in combinations of unrelated species, the elimination of donor chromosomes can be induced chemically (Griesbach et al. 1983; Roth and Lark 1984). Again, we used NR^+/NR^- and *Xa-2/xa-2* genotypes of tomato to study spontaneous and induced chromosome elimination. Although some interesting results were obtained these were not translated to our fusion experiments.

7.6 Concluding remarks

Our results indicate that it is extremely difficult to obtain fertile asymmetric somatic hybrids between tomato and potato that can be used in subsequent breeding programs. True asymmetric hybrids need the screening of very large populations of fusion products and a stringent selection for regeneration. This might imply selection for hybrids with limited incongruity problems. Alternative ways to eliminate the donor DNA were not successful in our preliminary experiments, but demand thorough investigation.

The allotriploid somatic hybrids between diploid tomato and monoploid potato might ultimately yield progeny when a large variation of staminate parents, e.g. species that may act as bridges between the hybrids and tomato, is used and when embryo rescue will be applied on a large scale.

It remains questionable whether the transfer of traits between sexually remote species needs the procedure of asymmetric somatic hybridization. Somatic incongruity strongly limits wide species combinations whereas many undesired genes of the donor are transferred with the procedure. The techniques to clone specific genes and the methods to transfer these genes to the plant still develop rapidly. The transfer of monogenic traits alleviates the need of less precise fusion and transplantation techniques that seem more complicated than earlier results suggested. The transfer of quantitative traits that are encoded by various genes remains to be resolved and for this somatic hybridization may be used.

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SAMENVATTING

Bij de veredeling van planten wordt dikwijls gebruik gemaakt van kruisingen met wilde soorten omdat deze vaak nuttige eigenschappen zoals bijvoorbeeld ziekte-resistenties, speciale inhoudsstoffen en nieuwe bloemkleuren in zich bergen. De bruikbare genetische variatie wordt echter sterk beperkt door kruisings-barrières die tussen veel soorten bestaan. Het samensmelten van protoplasten van somatische cellen geeft de mogelijkheid om van soorten, die niet kruisbaar zijn, hybriden te verkrijgen. In principe kan met deze techniek de genetische informatie van twee planten, hoe onverwant ook, gecombineerd worden. Bovendien kunnen met behulp van protoplastenfusie nieuwe cytoplasma-kerncombinaties (cybriden) verkregen worden.

Protoplastenfusie heeft echter een aantal beperkingen. Soorten die te onverwant zijn leveren na een fusie geen normale planten op. De fusieprodukten zijn vaak zelfs niet tot plant te regenereren. Daarnaast is het verkrijgen van nieuwe allotetraploiden meestal ongewenst. Dit omdat de overdracht van eigenschappen vanuit allotetraploiden naar een diploid cultuurgewas via kruisingen vaak zeer moeilijk is.

Een procedure die bovengenoemde problemen mogelijk kan omzeilen is het gamma of Röntgen bestralen van de protoplasten van één soort (doorgaans de wilde soort of donor) vóór fusie met protoplasten van de andere soort (het cultuurgewas of recipiënt). De bestraling veroorzaakt schade aan het donor DNA. Na fusie ontstaan asymmetrische hybriden waarin slechts een deel van het donor-genoom gecombineerd is met het intakte genoom van de recipiënt. Deze situatie benadert in principe datgene wat meestal in de plantenveredeling gewenst is, namelijk het toevoegen van één of enkele eigenschappen uit een wilde soort aan een verder onveranderd cultuurgewas. Het genetische donor-materiaal dat via asymmetrische somatische hybridisatie wordt overgedragen, kan hele chromosomen of fragmenten daarvan, welke al dan niet ingebouwd zijn in het recipiënte genoom, omvatten.

In dit proefschrift wordt nagegaan wat de mogelijkheden en beperkingen zijn van asymmetrische somatische hybridisatie voor de plantenveredeling. De volgende aspecten zijn hierbij in detail onderzocht:

1) Het gebruik van genetische merkers voor de selektie van fusieprodukten op het niveau van de cel.
2) Het isoleren van asymmetrische somatische hybriden, waarbij de donor bestraald wordt vóór fusie met de recipiënt.
3) Het isoleren van triploïde somatische hybriden, waarbij in plaats van een bestraalde diploïde een onbestraalde haploide donor gefuseerd wordt met de diploïde recipiënt.
4) De genetische karakteristieken van asymmetrische hybriden. Er is bepaald wat de bijdrage is van de donor aan het genoom van de hybride.
5) De karakteristieken van de effecten van gamma bestraling op karyogamie en celdeling bij fusieprodukten en op DNA fragmentatie, het herstellen van de aangebrachte breukschade en op *de novo* DNA synthese bij de donor.

In de beschreven experimenten werd de tomaat (*Lycopersicon esculentum* Mill.) als recipiënt gebruikt. Als donor werd de aardappel (*Solanum tuberosum* L.) gebruikt. De tomaat en de aardappel zijn onderling niet kruisbaar. Genetische kaarten laten evenwel zien dat de soorten sterk verwant zijn.

Hoofdstuk 2 beschrijft de isolatie en karakterisering van vijf nitraat reductase-deficiente (NAR) mutanten van tomaat. Deze werden geisoleerd uit een M_2 populatie van ethylmethaansulfonaat behandelde zaden. De selektie van NAR mutanten werd uitgevoerd op chloraatresistentie. Alle vijf mutaties waren monogeen, recessief en niet allel. Met biochemische en moleculair biologische technieken werd bepaald dat van vier NAR mutanten het co-enzym veranderd of afwezig was en dat van één mutant het apo-enzym veranderd was.

Hoofdstuk 3 beschrijft het gebruik van de NAR apo-enzym mutant en een NAR co-enzym mutant van tomaat (beide met een verminderde nitraatreductase aktiviteit) en van hun bijbehorende wildtypen in fusie-experimenten met een monoploide aardappel. Fusieprodukten groeiden sneller dan protoplasten van de fusie-ouders. Deze hybride groeikracht was bruikbaar als selektie-kenmerk. Omdat protoplasten van de beide NAR mutanten

slechter groeiden op medium zonder ammonium dan die van de wildtypen, verliep de selektie van hybriden in fusie-experimenten met NAR mutanten efficiënter dan wanneer wildtype tomaat gebruikt werd.

Allotriploide somatische hybriden van diploide tomaat en monoploide aardappel (Hoofdstuk 4) werden verkregen uit fusie-experimenten met de NAR apo-enzym mutant van tomaat. Selektie van fusieprodukten vond plaats op media zonder ammonium. Oudercalli werden nauwelijks verkregen en vertoonden geen regeneratie. Ook fusies met een cytoplasmatische albino tomaat leverden voornamelijk fusieprodukten op. Triploiden werden uit de beide populaties van somatische hybriden geselekteerd door het bepalen van chloroplast-aantallen in de sluitcellen van huidmondjes en met behulp van flowcytometrische en cytologische analyse. Hoewel enkele triploiden vitaal pollen produceerden en hoewel pollenbuisgroei van tomaat, aardappel en *Lycopersicon pennellii* in stijlen van een triploid waargenomen werd, mislukten alle kruisingen met de allotriploiden. Enkele spontaan gevormde vruchtjes bevatten onvolgroeide embryo's. Het lukte evenwel niet hieruit planten te verkrijgen in de weefselkweek.

Hoofdstuk 5 beschrijft de fusie tussen hygromycine-resistente protoplasten van tomaat en gamma bestraalde, kanamycine-resistente aardappelprotoplasten met β-glucuronidase-aktiviteit. Na selektie op antibioticum-resistenties werden 110 asymmetrische fusieprodukten verkregen. Deze werden vervolgens geanalyseerd voor hun regeneratievermogen, GUS aktiviteit, de expressie van twee isoenzymen van aardappel, het chloroplast type en de totale genomische DNA hoeveelheid en de relatieve bijdrage van beide fusie-ouders hierin. Er konden geen levensvatbare planten verkregen worden. De calli waren sterk polyploid en vertoonden allen GUS aktiviteit. Voor de overige parameters werd een grote variatie gevonden zonder duidelijke correlatie.

Hoofdstuk 6 beschrijft het effect van gamma bestraling van aardappel-protoplasten op de karyogamie en celdeling in heterokaryons van gefuseerde tomaat- en aardappelprotoplasten. Terwijl karyogamie nauwelijks vertraagd werd door bestraling gebeurde dit wel met het celdelingsproces. Daarnaast vond na bestraling uitgebreide DNA reparatie en *de novo* synthese van DNA plaats. Dit werd onder andere zichtbaar gemaakt met Pulsed Field Gel Electrophorese. De reparatie en synthese van DNA leiden tot een verminderde eliminatie van donor DNA in fusieprodukten en waarschijnlijk ook tot chromosomen en andere mutaties die de vitaliteit van het fusieprodukt negatief beinvloeden.

Uit de resultaten blijkt dat het verkrijgen van geschikte asymmetrische somatische hybriden van tomaat en aardappel, dat wil zeggen goed groeiende en fertiele planten, zeer moeilijk is. Bestraling van de aardappel vóór fusie leidt tot een sterk verminderde opbrengst aan fusieprodukten, beperking van de regeneratie, groei en bloei. Somatische triploiden bieden weliswaar betere perspectieven maar laten vooralsnog ook steriliteit zien.

CURRICULUM VITAE

Hermann Christianus Hendrikus Schoenmakers werd op 7 maart 1962 geboren te Oss (Noord Brabant). Na het behalen van het Atheneum-B diploma aan het Maasland College te Oss, begon hij in 1980 aan zijn studie Plantenveredeling aan de Landbouwhogeschool te Wageningen. De doctoraalfase omvatte het verzwaarde hoofdvak Plantenveredeling en het hoofdvak Erfelijkheidsleer. De studie werd in juni 1987 afgerond. Per augustus 1987 werd hij aangesteld als celbioloog bij het kweekbedrijf Ropta Z.P.C. te Metslawier (Friesland). Per maart 1988 werd hij aangesteld als Assistent In Opleiding bij de vakgroep Erfelijkheidsleer van de Landbouwuniversiteit te Wageningen op een vierjarig onderzoeksproject gesteund door de Programma commissie Landbouw Biotechnologie (PcLB). Het onderzoek werd uitgevoerd onder leiding van prof. dr ir Maarten Koornneef en prof. dr Christa Heyting. Reeds tijdens het promotie-onderzoek werd een begin gemaakt met wat later zou uitgroeien tot een eigen bedrijf: Het Subsidie Adviesbureau Plantenbiotechnologie.