Establishment of a complete alien tomato chromosome addition series in potato through the use of GISH and RFLP analyses

Selectie van een complete serie tomaatchromosoom additielijnen in aardappel door middel van GISH-en RFLP-analyse

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
Prof. Dr. Ir. L. Speelman
in het openbaar te verdedigen
op maandag 2 April 2001
des namiddags te 16:00 uur in de Aula

Propositions

1. The combined utilisation of RFLP and GISH analyses enables an easy and fast recovery of monosomic addition lines.
(This thesis)

2. Small chromosomes with non-uniform distribution of repetitive DNA are not suitable to study recombination by GISH or FISH.
(This thesis)

3. Utilisation of *Lycopersicon pennellii* as a bridging species between two sexually incompatible taxa is a reasonable way to circumvent crossing barriers.
(This thesis)

4. GISH on trigenomic triploids is not only colourful but also shows the way for utilising bridging species between *Solanum* and *Lycopersicon* for the first time.
(This thesis)

5. In future, if amylose-free potato starch could be modified through complementation with a monosomic addition containing the GBSS gene of tomato, the objections raised against transgenic plants could be avoided.
(This thesis)

6. The invention of protoplast fusion in plants has opened the prospects for wide hybridisation but the utilisation of such hybrids for introgression of characters requires strenuous efforts
(This thesis)

7. "The biggest issue for genomics today is no longer genes," "Whats interesting is what you do with those genes."
William A. Heseltine
Scientific American, July 2000

8. To be a human person means more than having a human genome, it means having a narrative identity of one's own. Likewise, membership in the human family involves a rich nexus of cultural links that cannot be reduced to taxonomy.
Alex Mauron – "Is the Genome the Secular Equivalent of the Soul"
Science, February 2001

9. Worship does not lie in copious prayer and fasting, but in the amount of contemplation in the works of God.

These propositions are a part of the thesis "*Establishment of a complete alien tomato chromosome addition series in potato through the use of GISH and RFLP analyses*" by S. N. Haider Ali, for the public defence at Wageningen University on Monday 2 April 2001.

To,

Next to God, my parents
My dear, my better half

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Glory to God, Most High, full of Grace and Mercy. He created all including Man. To man He gave a special place in His creation. He honoured man to be his agent, and endured him with intelligence and understanding, purified his affections and gave him spiritual insight, so that man should understand Nature, understand himself and know God through His wondrous Signs. For the fulfilment of this great trust, man was further given the Will and Knowledge so that his acts should reflect God's universal Will and Law to be able to experience the sublime joy of being in the harmony with the Infinite.

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I am writing this acknowledgement on gracefully completing my thesis under the able guidance of my teachers within the 4 years allocated period.

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Contents

1

General Introduction

Plant breeders have used sexual hybridisation in the past for the introduction of useful agronomic traits from alien taxa into crop varieties. Undoubtedly, there have been spectacular instances among crop plants where introgression of alien genes and chromosomes has made a great impact on crop improvement. One example is the introduction of rye chromosome segments into wheat cultivars that has nearly revolutionised wheat production in several countries (Wang, 1996). Less dramatic but equally important instances of introgression of characters can be mentioned in the case of certain major crops such as rice (Khush et al. 1994; Brar and Khush 1997; Khush et al. 1999), sugarcane (Price, 1957), tobacco (Bui et al. 1992) and potato (Ross 1986; Helgeson et al. 1993; Rokka et al. 2000) among others. In most of these cases, the conventional methods of sexual hybridisation were used. Nevertheless, the introduction of embryo and ovule rescue methods greatly enhanced the prospects of producing hybrids between distant taxa through overcoming some of the post-fertilisation barriers for hybridisation. Despite these developments, however, the scope for the utilisation of more distantly related taxa in breeding was highly restricted.

The restrictions to sexual methods of hybridisation were overcome by the introduction of the so-called para-sexual methods of hybridisation in higher plants during the late 1970's. The first somatic fusion hybrid was produced by fusing the protoplasts derived from leaf mesophyll cells of *Lycopersicon esculentum* and *Solanum tuberosum* (Melchers et al. 1978), between which sexual hybrids have never been reported. Following this landmark discovery, somatic fusion hybrids were produced in several taxa within Solanaceae (for review see Wolters, 1994). In addition, many intergeneric hybrids within the family of Cruciferae have also been produced (Keneko et al. 1999; Sigareva et al. 1999; Arumugam et al. 2000; Ren et al. 2000). One of the drawbacks of somatic hybridisation was, however, that a large majority of successful hybridisation's has been carried out in dicotyledonous species. However, in a few cases somatic hybrids have also been produced in monocotyledonous plants such as rice. Some of the more recent somatic hybrids that have been reported in Solanaceae together with one example from each of other families are summarised in Table 1.

The hurdles encountered in making somatic hybrids have been overcome by improving or modifying the tissue culture and/or selection techniques. Initially "symmetric hybrids' were made and as expected many of them were also fertile. With the increasing number of somatic hybrids produced it became obvious that there are a number of incompatibility barriers that occur at various steps after protoplast fusion. These barriers allow for example a few initial cell divisions or successful growth as undifferentiated callus, but no plant regeneration or only regeneration of highly abnormal or sterile plants. In some cases the fusion products could be regenerated to plants, but it resulted in unpredicted loss of chromosomes of one or both parents. This programmed loss of parts of one or both genomes from the fusion hybrids stimulated to look for a method for transferring only a part of a plant genome to allow the synthesis of fertile plants that could be more directly used in the breeding programmes. In this system the donor protoplasts were irradiated to break the genome in fragments followed by fusion with the recipient protoplast. These were called "asymmetric hybrids".

Production of somatic fusion hybrids is certainly the most important step to bypass the sexual incompatibility among distantly related species. Once they are produced, numerous obstacles impose limitations for their utilisation in further breeding work. Probably because of these limitations, very little, or no progress has been reported in many cases after the successful production of somatic fusion hybrids. In the first place, despite the fusion hybrids being allopolyploids, they can be highly sterile. An illustrative case is that of potato (+) tomato fusion hybrids produced for the first time by Melchers et al. (1978). They were never successfully backcrossed to either of the fusion parents. However, in the case of fusion hybrids involving more closely related taxa, fertility appeared to be less of a problem (Table 1).

Even after successful backcrossing of a fusion hybrid, the BC_1 or BC_2 progeny might be sterile either due to odd polyploidy or genomic imbalance. An example of the failure of the BC_2 progenies to produce BC_3 progenies was encountered in the case of potato (+) *Solanum nigrum* fusion hybrids (Horsman et al. 1999). Besides such

Table.1. A selection of somatic fusion hybrids made during the last decade in Solanaceae and some other families

Somatic hybrids between taxa used for fusion	Symmetric/ Asymmetric	Fertility	Reference	Follow up
Solanum brevidens (+) *S. tuberosum*	symmetric	yes	Rokka et al. 2000	BC_1 - resistance to potato leaf roll virus, tolerance to potato virus Y
Solanum commersonii (+) *S. tuberosum*	symmetric	yes	Seppanen et al. 2000	Selfed progeny from somatic hybrid- Cold-induced glutathione S-transferase activity studied
Brassica rapa (+) *B. oleracea*	symmetric	yes	Ren et al. 2000	BC_1 - resistance to *Erwinia* soft rot
Solanum nigrum (+) *S. tuberosum*	symmetric	yes	Horsman et al . 1999	BC_2 - resistance to *Phytophthora infestance*
Solanum etuberosum (+) diploid potato		yes	Dong et al. 1999	BC_2 - characterization of germplasm in various species
Oryza sativa (+) *Porteresia coarctata*	symmetric	yes	Jelodar et al. 1999	An attempt to use *P. coarctata* to transfer saline tolerance in rice
Solanum acaule (+) *S. tuberosum*	symmetric	yes	Rokka et al. 1998	Test underway for abiotic tolerance
Solanum bulbocastanum (+) *S. tuberosum*	symmetric	yes	Helgeson et al. 1998	BC_1 - resistance to late blight
Festuca (+) *Lolium*	asymmetric	yes	Sasaki et al. 1998	Not available
Solanum tuberosum (+) *S. pinnatisectum*	symmetric	yes	Thieme et al. 1997	BC_1 - resistance to *Phytophthora infestance*
Lycopersicon esculentum x *L peruvianum* (+) *Solanum lycopersicoides*	symmetric	yes	Matsumoto et al. 1997	Not available
Brassica oleracea (+) *Sinapis alba*	asymmetric	yes	Hansen and Earle 1997	Plants obtained via cuttings from somatic hybrids- resistance to *Alternaria brassicae*
Solanum tuberosum (+) *S. pinnatisectum*	both	-	Menke et al. 1996	Morphological and molecular analysis of somatic hybrids
Solanum tuberosum (+) *S. vernei*	symmetric	yes	Rasmussen et al. 1996	Restoration of fertility in fusion hybrids- resistance to *Globodera pallida* Pa2 and Pa3
Hibiscus rosa-sinensis (+) *Lavatera thuringiaca*	symmetric	no	Vazquez-Thello et al. 1996	Somatic hybrids showed increased chilling tolerance
Solanum brevidens (+) *S. tuberosum*	symmetric	-	Rokka et al. 1995	Haploids from another culture- Potato leaf roll lutevirus resistance transferred

Table.1. (continued)

Somatic hybrids between taxa used for fusion	Symmetric/ Asymmetric	Fertility	Reference	Follow up
Nicotiana tabacum (+) *N. megalosiphon*	both	yes	Donaldson et al. 1995	BC_1 - transferring disease resistance
Brassica oleraceae (+) *B. rapa*	asymmetric	yes	Heath and Earle, 1995	Improved agronomic characters in regenerated plants
S. tuberosum (+) *Lycopersicon pennellii*	symmetric	no	Sherraf et al. 1994	Hybrids showed 50% salt tolerance compared to *L. pennellii*
Lycopersicon esculentum (+) *S. tuberosum*	asymmetric	-	Schoenmakers et al. 1994	Producing asymmetric hybrids and effects of gamma irradiation studied
Brassica napus (+) *Thlaspi perfoliatum*	both	yes	Fahleson et al. 1994	Increase in Nervonic acid contents in fusion hybrids
Solanum tuberosum (+) *S brevidens*	symmetric	yes	Jacobsen et al . 1993	Meiotic recombination studied in megaspore mother cells
Solanum tuberosum (+) *S. bulbocastanum*	symmetric	yes	Austin et al. 1993	Fusion hybrids -resistance to nematodes
Citrus sinensis (+) *Atalantia ceylanica*	symmetric	-	Louzada et al. 1993	First report of fusion hybrids between these sexually incompatible genera

difficulties, although the fusion hybrids or their progenies can be grown in vitro, they do not produce vigorous plants in vivo in the greenhouse. Besides such difficulties, when the fusion parents belong to widely distant taxa, it might be difficult to obtain homoeologous pairing and intergenomic recombination in the fusion hybrids as well as in their backcross derivatives.

Besides producing fusion hybrids and their backcross derivatives, it is also equally important to monitor the backcross progenies for their chromosome composition and intergenomic recombination. Traditional cytogenetic methods, though useful, were highly ineffective and less dependable because the genomes and individual chromosomes could not be identified accurately. This situation changed dramatically during the last decade because of the introduction of molecular methods for the cytological identification of homoeologous chromosomes as well as mapping of the genomes through the use of DNA sequences, the so-called molecular probes. These techniques have enabled the identification of chromosomes regardless of their size. For example, the chromosomes of Solanaceae crops are extremely small and an accurate identification of individual chromosomes, with the exception of tomato, was nearly impossible. However, the molecular cloning of chromosome specific probes in tomato (Tanksley et al. 1992) and potato (Gebhardt et al. 1991) and the construction of molecular marker maps (Bonierbale et al. 1988) facilitates not only the identification of individual chromosomes but also recombination as a result of crossing over (Chetelat and Meglic, 2000). In addition, the procedures of direct DNA hybridisation in situ to chromosome preparations, genomic and fluorescent in situ hybridisation (GISH and FISH) enable the identification of genomes and chromosomes (D'Hont et al. 2000; Hou and Peffley, 2000). Especially, the combination of molecular markers (e.g., RFLP) and cytological in situ hybridisation procedures has provided a highly powerful tool for the identification of genomes and individual chromosomes (Jacobsen et al. 1995; Garriga-Calderé et al. 1997).

In spite of the difficulties associated with utilisation of the fusion hybrids as outlined above, success has been achieved in some cases in producing fertile backcross progenies. One example is the successful backcrossing of the potato (+) tomato fusion hybrid to potato (Jacobsen et al. 1994; Garriga-Calderé et al. 1998). In this case a hexaploid 2n=6x=72) potato (+) tomato fusion hybrid with four genomes of potato and two of tomato (PPPPTT) was backcrossed to potato (2n=4x=48). The resulting pentaploid (PPPPT) was again backcrossed to potato, the progeny of which consisted of alien tomato chromosomes in a potato background. This opened the prospects for the identification of a complete series of monosomic alien tomato chromosome additions in potato. In the past, alien addition lines have been produced in several crops and their usefulness has been clearly demonstrated for example in the case of wheat and rice. Some examples of alien chromosome addition series are summarised in Table 2.

Table.2. Summary of the monosomic alien addition lines in some crop species

Crop species	Alien donor	References	Monosomic Addition Series
Oryza sativa	*Oryza punctata*	Yasui and Iwata 1991	Partial
Beta vulgaris	Beta species	DeDong et al. 1994	Partial
Oryza sativa	*Oryza australiensis*	Multani et al. 1994	Partial
B. vulgaris	*Beta patellaris &B. procumbens*	Mesbah et al. 1996	Complete
Allium fistulosum	*Allium cepa*	Shigyo et al. 1996	Complete
Triticum aestivum	*Aegilops ovata*	Bijral et al. 1997	Partial
Oryza sativa	*Oryza* wild species	Brar and Khush 1997	Complete
Nicotiana sylvestris	*Nicotiana plumbaginifolia*	Suen et al. 1997	Partial
Raphanus sativus	Allied genera	Bang et al. 1997	Partial
Avena sativa	*Zea mays*	Ananiev et al. 1997	Partial
Oryza sativa	*Oryza eichingeri*	Qiang et al. 1998	Partial
Glycine max	*Glycine tomentella*	Singh et al. 1998	Partial
Lycopersicon esculentum	*Solanum lycopersicoides*	Chetelat et al. 1998	Complete
Brassica campestris	Allied species	Kaneko et al. 1999	Partial
Brassica napus	*Sinapis arvensis*	Snowdon et al. 2000	Partial
Triticum aestivum	*Aegilops speltoides*	Friebe et al. 2000	Complete

The practical breeding value of alien addition lines or the agronomic value for certain crops has been most clearly illustrated in the case of wheat (introgression of resistance to leaf rust, powdery mildew, *cephalsporium* stripe, seed storage proteins, genes for yellow pigmentation to flour) and rice (introgression of cytoplasmic male sterility, resistance to grassy stunt tenuivirus, bacterial blight, brown planthopper, rice blast from distantly related species into cultivated rice). To date there are several instances related to introgression of genes of interest in several crop species that have been achieved by producing fusion hybrids and alien addition lines. In *Brassica* species bacterial soft rot resistance (Ren et al. 2000), chlorosis correction (Arumugam et al. 2000), and resistance to *Alternaria brassicicola* (Sigareva et al. 1999) has been introduced. Likewise, resistance to tomato leaf curl virus (Kalloo and Banerjee, 1990), early blight (Kalloo and Banerjee, 1993), salt and drought stress (Eshed et al. 1992), chromosome segments to improve soluble solids (Eshed and Zamir, 1994) has been introduced into cultivated tomato from other wild tomato species. However, when complete series of alien chromosome additions are identified, they can be helpful for the accurate assignment of genetic markers to linkage maps (Dong et al. 2000); physical mapping of chromosomes (Zhong et al. 1996; Chen et al. 2000); construction of chromosome specific libraries (Ananiev et al. 1997); the assessment of the phenotypes of specific gene loci such as *Abg* gene on chromosome 10 of *S. lycopersicoides* (Rick et al. 1994) and positional cloning of genes. Considering these potential uses of alien chromosome addition series, an attempt was made to establish a complete series of alien tomato chromosome addition lines in the background of cultivated potato (Garriga-Calderé et al. 1997). In this endeavour only seven of the possible 12 monosomic alien tomato additions were identified. This study also indicated that the frequencies of transmission of different tomato chromosomes from BC_1 to BC_2 progeny was highly variable and there was little, if any, pairing and homoeologous recombination between the chromosomes of potato and tomato (Garriga-Calderé et al. 1999). Obviously, further work was needed in order to complete the series of tomato alien addition lines. Further more, an exploration of the prospects of obtaining recombination between potato and tomato chromosomes was needed. In order to achieve this objective, one approach was to use a "bridge species" whose chromosomes have the potential to pair with the chromosomes of both potato and tomato. One possible candidate to fill this role was *Lycopersicon pennellii* (formerly, *Solanum pennellii*) which occupies a phylogenetic position between the two concerned genera viz., *Lycopersicon* and *Solanum*. The concept of utilising the benefit of bridging species also mentioned as "stepping stone" procedure has regularly been used in other crop species where direct hybridisation was hampered. In potato, bridging by *Solanum acaule* helped to transfer genes from *S. bulbocastanum* into *S. tuberosum* (Dionne, 1963; Hermsen and Ramanna, 1973) and *S. sanctae-rosae* was used as a bridge between *S. tuberosum* and *S. tuquerrense* (Sams et al. 1977). Delibes et al. (1993), transferred resistance to cereal cyst nematode from *Aegilops ventricosa* to *Triticum aestivum* through *T. durum* that served as bridge species. Similarly, *Allium roylei* helped introgression of *A. fistulosum* into the genome of *A. cepa* (Khrustaleva and Kik, 2000). Following the same track of approach, we attempted to use *L. pennellii* (2n=2x=24) as a bridge species, which was successfully crossed with a tetraploid (2n=4x=48) potato (+) tomato fusion hybrid. The resulting trigenomic hybrid was used for an assessment of intergenomic recombination.

Aims of investigations:

- To complete the alien tomato chromosome addition series in the background of potato.
- To determine the usefulness of the monosomic alien addition lines in order to assess the phenotypic expression of a known genetic locus of tomato in the background of potato.
- To explore the potential of a "bridge species" in order to facilitate recombination between potato and tomato chromosomes.
- To determine whether or not it is possible to detect homoeologous recombination through GISH in the small chromosomes of these Solanaceous plants.

Outline of the thesis:

In chapter 2, the results on the completion of the alien tomato monosomic addition series are presented. The strategies for an efficient method of selecting the desired types of alien addition lines using RFLP and GISH analyses are outlined.

In chapter 3, using the amylose-free (*amf*) locus, which is localised on chromosome 8 in both tomato and potato, the phenotypic expression of the wildtype *Amf* locus in an alien monosomic addition line for chromosome 8 was assessed in simplex condition in different genotypes. Instead of the *Amf* locus of tomato, the variable expression of the wildtype *Amf* allele of potato was observed which produced different starch phenotypes in tubers after iodine staining are discussed.

Chapter 4 describes results on the use of *Lycopersicon pennellii* as a bridge species that was crossed successfully with the tetraploid (2n=4x=48) potato (+) tomato fusion hybrid. Based on GISH analyses of chromosome pairing behaviour during meiosis in the trigenomic hybrid, the prospects of introgression of tomato chromosome segments into potato are outlined.

In chapter 5, an assessment was made whether or not it is possible to cytologically detect homoeologous recombination in the case of small chromosomes with non-specific distribution of repetitive DNA on their chromosomes- such as in potato and tomato. Using the F_1 hybrid between *Lycopersicon esculentum* and *L. pennellii* and its backcross progeny, a GISH analysis of both meiotic and mitotic chromosomes was made and the limitations for monitoring introgression cytologically were discussed.

Finally, in chapter 6, the significance and implications of results obtained in these investigations are generally discussed and prospects are highlighted.

Introduction

Chromosomes from distantly related species and genera have often been added into a crop plant for breeding as well as for the purpose of fundamental studies. Among many examples, alien chromosome additions have been extensively exploited in crops such as wheat, sugarcane, rice and grasses, among others where interspecific and intergeneric hybridisation can be performed by sexual methods more easily as compared to dicotyledonous taxa (Hadley and Openshaw, 1980; Jiang et al. 1994; Multaini et al. 1994). Unlike in monocotyledonous plants, where hybridisation using protoplast fusion is difficult as yet, in several dicotyledonous species somatic hybridisation has been used for producing intergeneric hybrids (Sigareva et al. 1999; Arumugam, 2000; Ren et al. 2000). Among these, somatic hybridisation between distant species and genera of the family Solanaceae has received considerable attention (Shepard et al. 1983; Hassanpour-Estahbanati et al. 1986; Derks et al. 1992; Gavrilenko et al. 1992; Chetelat and Meglic, 2000). Despite the success achieved in producing numerous interspecific and intergeneric combinations of somatic hybrids within Solanaceae, there are only a few instances in which the fusion hybrids have been successfully backcrossed to any of the fusion parents. Some examples are however, the successful backcrossing of the potato (+) tomato fusion hybrids to potato (Jacobsen et al. 1994; Garriga-Calderé et al. 1997), *S. tuberosum* (+) *S. brevidens* (McGrath et al. 1994), *Solanum etuberosum* and *S. tuberosum* x *S. berthaultii* (Novy and Helgeson, 1994) and *S. tuberosum* (+) *S. bulbocastanum* (Naess et al. 2000). From the analysis of BC_2 and BC_3 progenies it has been demonstrated that the individual alien tomato chromosomes were transmitted through the female parent in variable frequencies (Garriga-Calderé et al. 1998). Based on RFLP and GISH analyses, single tomato chromosome addition lines of potato were identified (Garriga-Calderé et al.1998). Seven of the possible twelve monosomic alien tomato chromosome addition lines of potato were earlier identified in BC_2 populations. (Garriga-Calderé et al. 1998). Obviously, this opened the prospects for establishing a complete series of monosomic tomato alien chromosome addition lines of cultivated potato. Monosomic alien addition lines in different crops have already been created. The main reasons for making alien addition lines is the introgression of genes of interest from distant or wild relatives into the cultivated crops (Chetelat et al. 1998; Khrustaleva and Kik, 2000; Friebe et al. 2000) and construction of chromosome specific libraries (Ananiev, 1997; Schmidt et al. 1990) among others. In order to achieve this aim in potato, an attempt was made to complete the series through the identification of the remaining five different alien tomato chromosome additions. Using a combination of RFLP and GISH analyses in BC_3 populations, a complete series was established and is reported in this article.

Materials and Methods

Plant material

The three selected BC_2 plants containing at least one of the five missing alien tomato chromosomes were crossed with two different tetraploid potato pollinators (6704-3 and 6704-13). The berries were harvested on ripening to collect the seeds. These seeds were used for producing BC_3 populations. The BC_2 plants possessed different numbers of alien tomato chromosomes, which were as follows: 2103-5 (chr. 1 and 3), 6731-4 (chr. 3,6,8 and 9); 2303-5 (chr. 6,7 and 11). The four BC_3 populations (2808, 2520, 2701 and 2705) consisting of 105 plants, were used for RFLP and GISH analyses. The history of the plant material used for the identification of the five new monosomic alien addition lines is given in Table 1.

RFLP analysis

For RFLP analysis, DNA was isolated from young leaves according to Rogers and Bendich (1988). The procedures for DNA digestion and Southern hybridisation were according to Kreike et al. (1990). The DNA was digested with Eco RI or Eco RV enzymes in order to identify polymorphisms for tomato and potato genomes in the presence of alien tomato chromosome additions. For achieving clear polymorphisms, tomato-chromosome specific probes were used. Two criteria were taken into consideration to establish a polymorphism: a) a clear-cut difference between potato and tomato polymorphic bands that became evident after hybridisation with the probe DNA and b) the tomato polymorphic band was completely absent in potato. The tomato chromosome specific probes used were: TG53, TG366, TG23, TG118, TG438, TG160, TG8 and Ssp29 corresponding to chromosomes 1, 3, 5, 6, 7, 8, 9 and 11 respectively which were present in the BC_2 female parents. Prof. S.D. Tanksley, Cornell University, N.Y. U.S.A., kindly provided the TG probes.

Radioactive RFLP analysis was used and tomato probes were labelled with ^{32}P by random prime labelling according to the procedures described by the manufacturer monosomic additions, the disomic addition line was expected to be more useful for the high frequency of transmission for the alien chromosome in the sexual progeny. This was due to the fact that the presence of a pair of homologous alien tomato chromosomes in potato might facilitate their normal segregation during meiosis. In view of this, meiotic behaviour of the disomic chromosome addition genotype 2701-14 was investigated through GISH (Fig.2C,D).

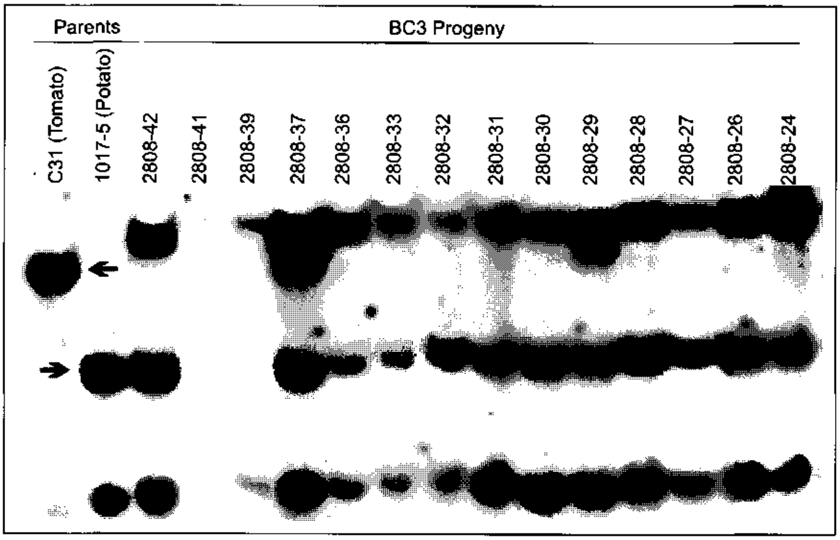


Fig.1. A representative autoradiogram of a Southern blot after EcoRI digestion, probed with TG366, specific for tomato chromosome 3. The polymorphism between tomato (C31) and potato (1017-5) is clearly visible (arrows). Both the bands are present in the BC_3 plants 2808-37 and 2808-29. Hence these plants carry tomato chromosome 3.

A notable feature was that this pair of chromosome 11 of tomato formed consistently two univalents at metaphase I stage (Fig.2E) instead of a bivalent. In order to monitor the pairing behaviour of this disomic addition, chromosomes at pachytene stage were analysed. Although pairing was observed in about half of the pollen mother cells, in other cases the homologous chromosomes were either unpaired or only partially connected. In 33 cells that were analysed 18 cells showed clear pairing whereas in 15 cells either there was no pairing or they were partially paired (Fig.2C,D).

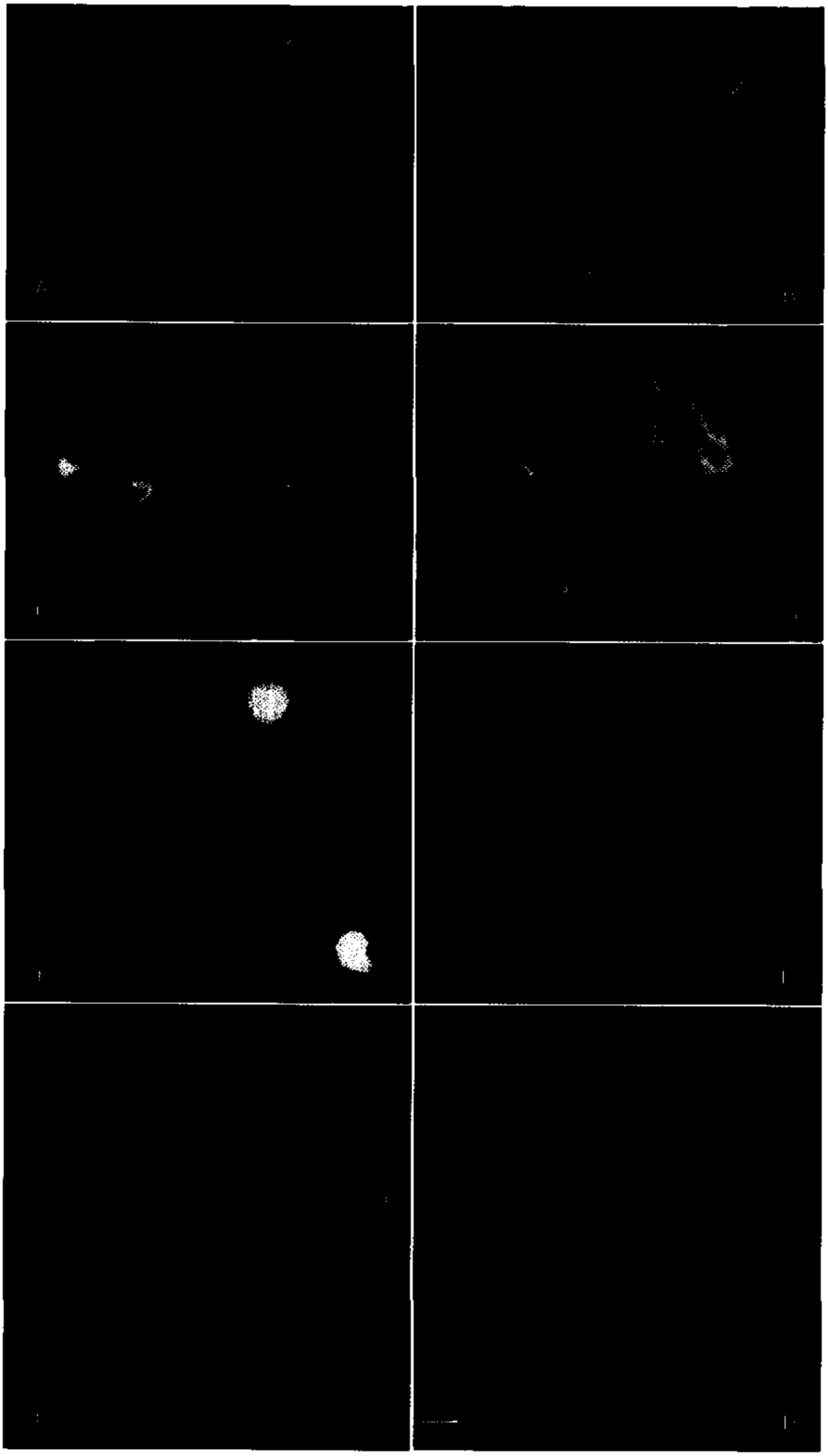
Table.1. Parentage of the BC_3 potato progenies from which the five new monosomic tomato alien chromosome addition lines were identified

Population		Pollinator		BC_1 plants		Pollinator		BC_2 plants		Pollinator		BC_3 population	Chromosome addition
C31-17-24	x	AM66.42	→	6739	x	AM66.42	→	2103-5	x	6704-3	→	2808	3
C31-17-1	x	AM66.42	→	6701	x	6020.22	→	6731-4	x	6704-3	→	2520	9
C31-17-5	x	AM66.42	→	2002	x	6706-2	→	2303-5	x	6704-13	→	2701	7 & 11
									x	AM66.42	→	2705	5

Table.2. Identification of alien tomato chromosomes (3,5,7,9 and 11) in different BC_3 potato populations and the frequencies (%) of their transmission based on RFLP's

Population	No. of plants analysed	No. of potato plants monosomic to alien tomato chromosome				
		3	5	7	9	11
2808	31	1 (3.2)				
2705	27		1 (3.7)			
2701	38			1 (2.6)		2 (5.2) *
2520	9				3 (33.3)	

* = One plant was a disomic addition for chromosome 11 after GISH analysis



At late prophase I and metaphase I stage only univalents of alien tomato chromosomes were observed. Obviously, the paired tomato chromosomes showed a precocious separation of the bivalent as compared to the potato chromosomes. This might result from the asynchrony of the chromosome behaviour of the alien genomes in general. For example, the centromere divisions and chromosome movements of the alien genomes were at odds also during later stages of meiosis (Fig.2E-H). Because of the formation of univalents the movement of the two univalents of tomato chromosomes was haphazard during anaphase I as well as anaphase II (Table 3). The ultimate result was that the anticipated (regular) meiotic behaviour of the disomic addition was lacking in this plant. Additional RFLP studies on 2701-14 did confirm the earlier observation of the presence of only alien tomato chromosome 11 in this plant (results not shown).

Frequency of transmission of alien tomato chromosomes from BC_2 plants to BC_3 populations

The frequencies of occurrence of the five alien monosomic additions were clearly variable and ranged between 2.6% (chromosome 7) and 33.3% (chromosome 9) in the different BC_3 populations (Table 2). Besides, the five tomato chromosomes (3,5,7,9,11) that were identified as monosomics, other tomato chromosomes (1,6 and 8) were also segregating in the same BC_3 populations. The frequency of transmission of these chromosomes from BC_2 plants to BC_3 populations was also calculated (Table 4) Each individual chromosome was transmitted with a different frequency in all combinations.

Fig.2. GISH on mitotic and meiotic cells. **A-B**. Representatives of a typical monosomic and disomic addition lines. **C-D**. Pachytene stages of a disomic (2701-14) for chromosome 11 showing two tomato chromosomes (yellowish green) not pairing at all and the same two chromosomes (yellow) pairing normally, the red ones are potato chromosomes. **E**. Metaphase I stage for disomic (2701-14), the two chromosomes (yellow) fall apart and are not oriented on the equatorial plate with potato chromosomes. **F**. Telophase II stage of disomic (2701-14) showing two tomato chromosomes (yellow) as laggards whereas other two chromosomes have moved to two poles. **G**. Disomic (2701-14), the tomato chromosomes (yellow) are still undivided indicating asynchrony of centromere division. **H**. In disomic (2701-14) at telophase II stage the chromosomes divide equally. In all cases the potato chromosomes are stained orange red because of the counter-staining of propidium iodide. The bar represents 10μm approximately.

There were two tomato chromosomes in three populations and three in one population that transmitted with variable frequencies. The transmission frequencies of chromosomes 1,6, and 8 were relatively high. This might have decreased or affected the transmission of the desired chromosomes 3, 5, 7, 9 and 11 as single copies in individual offspring plants.

However, all desired chromosomes were transmitted from BC_2 plants to BC_3 populations. All combinations of alien chromosomes were observed in four BC_3 offsprings except in population 2701, the combination of chromosome (6+7) and (6+7+11) was missing (Table 4).

In order to complete the whole series of alien tomato chromosome addition lines of potato, different genotypes of the fusion hybrids, BC_1 and BC_2 plants had to be used as female parents with different potato pollinators. This is enlisted in Table 5. Thus, the genetic background of this alien addition series is heterogeneous. However, the identification was completed by screening BC_2 populations for seven different monosomic additions and BC_3 populations for the additional five new monosomics. In this process, however, not just one genotype of a monosomic addition, but several genotypes for most of the chromosomes and for two chromosomes (10 and 11) disomic additions also became available (Table 5).

Characteristics of monosomic additions like morphology and fertility were also monitored. The potato addition lines for all the twelve tomato chromosomes resembled male potato parents in leaf shape, colour and morphology. The flower shape and colour was also similar to potato plants. A drawback of these addition lines is that due to the tetraploid background of the potato, the alien tomato chromosomes did not express any tomato specific phenotypes. The growth and fertility was not measured but in general all BC_3 plants showed vigorous growth and they were female fertile. The homoeologous pairing between potato and tomato chromosomes had already been monitored in the previous studies when the first seven monosomic addition lines were selected and therefore, this aspect was not further investigated here.

Table.3. Distribution (%) of alien tomato chromosomes in the potato line 2701-14 disomic for tomato chromosome 11

Meiotic stage	No. of cells analysed	Alien chromosome distribution to poles				
		1-1	**2-0**	**One lagging**	**Both lagging**	
Anaphase I/ Telophase I	56	10 (18.0)	15 (27.0)	24 (43.0)	7 (12.5)	
		1 each to 4 poles	**1 each to 2 poles + 2 lagging**	**2 each to 2 poles**	**1 each to 2 poles + 2 in 1 pole**	**1 each to 3 poles + 1 lagging**
Anaphase II/ Telophase II	68	16 (23.5)	19 (28.0)	19 (28.0)	6 (9.0)	9 (13.0)

Table.4. Summary of transmission frequencies (%) of tomato chromosomes from BC_2 plants to BC_3 populations

Population	No of plants	Mode of transmission of chromosomes							
		Together		Individual			Overall		
2802	31	**1 & 3**		**1**	**3**		**1**	**3**	
		3 (9.6)		13 (41.9)	1 (3.2)		16 (51.6)	4 (12.9)	
2705	27	**5 & 6**		**5**	**6**		**5**	**6**	
		2 (7.4)		1 (3.7)	6 (22.2)		3 (11.1)	8 (29.6)	
2701	38	**6 & 7**	**6 & 11**	**6**	**7**	**11**	**6**	**7**	**11**
		3 (7.8)	2 (5.2)	27 (71.0)	1 (2.6)	1 (2.6)	31 (81.5)	4 (10.5)	3 (7.8)
2520	9	**8 & 9**		**8**	**9**		**8**	**9**	
		1 (11.1)		1 (11.1)	3 (33.3)		2 (22.2)	4 (44.4)	

Discussion

This investigation demonstrates that through a combination of RFLP and GISH analyses the entire series of alien tomato chromosome addition lines in potato could be completed relatively easily. The combined effectiveness of these two techniques was already proven by Jacobsen et al. (1995) and Garriga-Calderé et al. (1998) for the establishment of tomato monosomic addition lines. If it were to be established through conventional cytogenetic techniques it would have been extremely laborious and frustrating. Laborious because the chromosomes of tomato are too small for chromosome identification in somatic cells and pachytene chromosomes are difficult to prepare for cytological analysis (Ramanna and Prakken 1976). It would have been frustrating because numerous genotypes would have been identified for chromosomes with higher frequencies of transmission. In other words, a pre-selection of a potentially useful genotype in the BC_1 and BC_2 generation would not be easy with the conventional approach. The advent of RFLP analysis has changed this situation drastically because the use of chromosome specific RFLP markers enabled us to make a pre-selection. During this process, it was possible to establish whether only one or more alien chromosomes were present in a given genotype. When only one alien chromosome was present, it was possible to identify such genotypes in more detail using more appropriate probes as well as through GISH. The combination of the two molecular techniques have been used earlier and proved successful both in sexual and somatic hybrids between *Lycopersicon esculentum* and *Solanum lycopersicoides* (Escalante et al. 1998) and in hybrids of *Gibasis* (Parokonny et al. 1992).

Although a combination of molecular techniques was crucial in establishing a complete series of monosomic alien addition lines, an appropriate strategy of using proper parents and populations was equally important. This is because of the following two aspects: 1) some of the alien chromosomes are transmitted through the female parent at a very low frequency or not at all (see Garriga-Calderé et al. 1998; chapter 2); 2) conversely, some of the chromosomes are transmitted at an extremely high frequency. So much so, that in some cases 100% of the progeny consisted of such an alien chromosome as in the case of chromosome 6 of tomato in a particular population (chapter 3). The differences in transmission rate of individual alien chromosomes in monosomic alien addition lines have been reported earlier by researchers in other crops. Such examples include rice (Jena and Khush, 1989), cotton (Rooney et al. 1991), tobacco (Suen et al. 1997) and tomato (Chetelat et al.

1998). The characteristic rate of transmission of individual chromosomes was also evident to some extent in this study. The chromosomes were transmitted in different frequencies in different combinations and this shed light on a peculiar genetic behaviour of a chromosome. For example chromosome 1 remains superior in transmission to chromosome 3 and together they have very low frequency of transmission and it seems as if the gametes that carry these two chromosomes together are less viable. Another example is chromosome 6, which in population 2705 had 29.6% of transmission, and in population 2701 it was transmitted at a much higher rate, 81.5% (Table 4). In an earlier study chromosome 8 could not be recovered at all in a BC_3 progeny (Chapter 3), whereas in this case there is a moderate transmission rate for chromosome 8 (22.2%). There is no clear explanation of the variable rate of transmission frequencies of tomato chromosomes in a potato background. The only valid explanation and that also seems convincing is that the different genetic background influences the transmission of alien chromosomes and each tomato chromosome has a unique genetic makeup that makes it exclusive in its behaviour. The GISH analysis of the disomic addition (Fig.2C-H) at pachytene and metaphase I stage also showed the irregular pairing behaviour of homologous chromosomes and in later stages their meiotic behaviour was highly disturbed.

Both of these aspects, viz., low transmission for certain chromosomes (3.2% for chromosome 3) as well as high transmission rate for other chromosomes (33.3% for chromosome 9; Table 2) can complicate the selection and identification of the desired alien addition line. However, the fertility of all alien addition lines was reasonably high and the alien chromosomes were transmitted to the next generation in most cases. Nevertheless, in view of the enormous amount of variation in the rate of transmission of individual chromosomes, the selection of proper backcross populations was important.

Previous cytological investigation on other monosomic alien addition lines has shown that there was very little or no homoeologous recombination between potato and tomato chromosomes (Garriga-Calderé et al. 1998). Therefore, no attempt was made during this investigation to establish whether there were any recombinant chromosomes among the newly selected alien tomato chromosome addition lines. It should be noted, however that it is extremely difficult, if not impossible, to detect cytogenetically the recombinant chromosomes involving a potato and tomato chromosome. This is because of the very small lengths of the euchromatic segments that are present in the somatic chromosomes of both species. Apart from the smallness of euchromatic segments, there might be another theoretical reason why homoeologous recombinants cannot be detected in potato and tomato. It has been well established that the distribution of repetitive DNA sequences on chromosomes is a crucial factor for differentiating genomes and chromosomes through GISH (Parokonny et al. 1992, D'Hont et al. 2000). Since there is very little or no repetitive DNA present in euchromatic regions, GISH cannot resolve easily recombinant segments in the species involved in the present material. There is no solid justification for the low homoeologous pairing between potato and tomato chromosomes and subsequently low transmission rate of alien chromosomes. However a convincing answer is that the presence of two genomes of tomato and four genomes of potato (for details see Garriga-Calderé et al. 1997) was not the best situation to stimulate homoeologous pairing of potato and tomato chromosomes. This has been observed frequently in interspecific hybrids and their backcross progenies of *Alstroemeria* (Kamstra et al. 1999) and *Lilium* (Lim et al. 2000) where the presence of only one genome of both species was influencing homoeologous pairing positively.

The completion of monosomic tomato chromosome addition lines of potato adds one more instance to the list of only a few crops in which such a series has been identified (see for reviews Khush, 1973; Garriga-Calderé et al. 1998). Such a series has been shown to be highly useful in assigning desirable genes to respective chromosomes and in breeding of rice (Brar and Khush, 1997). Apart from the use of alien addition lines in breeding (Janssen et al. 1997) they can also be useful for the study of chromosome organisation (Kamstra et al. 1997; Zhong et al. 1998). The monosomic or disomic alien tomato chromosome addition lines are also helpful in further cytological and molecular studies. Classification of respective polymorphic and non-polymorphic RFLP and AFLP markers on a particular chromosome will provide the possibility to know whether a chromosome is complete or not. Localisation of BAC's and YAC's on monosomic alien chromosomes will help and facilitate the characterisation and isolation of genes of interest.

Table.5. An overview of the establishment of a complete series of monosomic alien tomato chromosome additions from BC_2 and BC_3 generations to the cultivated potato.

Fusion Hybrid	Pollinator	BC_1	Pollinator	BC_2	Pollinator	BC_3	Tomato chromosomes	Number of monosomic *additions found*	Chr. transmission ! BC_1 to BC_2 in (%)	Remarks
C31-17-24	AM66.42	6739	AM66.42	TMA*	x	x	1	2103-1 2103-2 2103-4	8.0 - 41.4	Monosomics in (BC_2)
C31-17-5	6704-1	2003	6707-7	TMA	x	x	2	2102-5 2102-9 2403-6	6.9 - 92.0	Monosomics in (BC_2)
C31-17-24	AM66.42	6739	AM66.42	2103-5	6704-3	TMA	3	2808-37	3.4 - 28.0	Monosomics in (BC_3)
C31-17-24	AM66.42	6739	AM66.42	TMA	x	x	4	2101-7 2101-8 2103-10	6.9 - 28.0	Monosomic in (BC_2)
C31-17-5	AM66.42	2002	6706-2	2303-5	AM66.42	TMA	5	2705-4	10.3 - 11.6	Monosomic in (BC_3)
C31-17-5	AM66.42	2002	Desiree	TMA	x	x	6	2301-5 2301-6 2301-14 2301-18 2302-1 2303-3	13.8 - 88.4	Monosomics in (BC_2)
C31-17-5	AM66.42	2002	6706-2	2303-5	6704-13	TMA	7	2701-9	4.0 - 13.9	Monosomic in (BC_3)
C31-17-5	AM66.42	2002	Desiree	TMA	x	x	8	2301-2 2301-27	20.9 - 36.0	Monosomics in (BC_2)
C31-17-1	AM66.42	6701	6020.22	6731-4	6704-3	TMA	9	2520-1 2520-4 2521-2	0.0 - 32.0	Monosomics in (BC_3)
C31-17-24	AM66.42	6739	6706-1	TMA	x	x	10	2101-1	10.3 - 20.0	Disomic in (BC_2)
C31-17-5	AM66.42	2002	6706-2	2303-5	6704-13	TMA	11	2701-6 2701-14	8.0 - 13.8	Monosomic in (BC_3) Disomic in BC_3)
C31-17-24	AM66.42	6739	6704-6	TMA	x	x	12	2102-6 2303-9	13.8 - 18.6	Monosomics in (BC_2)

*** TMA = Tomato monosomic addition was found, ! = Data from Garriga-Calderé et al. 1998**

3

VARIATION IN STARCH PHENOTYPIC PATTERNS ARISING IN BACKCROSSING POTATO (+) TOMATO FUSION HYBRIDS TO AMYLOSE-FREE STARCH POTATO

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Submitted for publication

Abstract

In an attempt to introduce the wildtype Amf_t gene from tomato into nulliplex amylose-free potato ($amf_p amf_p amf_p amf_p$) (later indicated as $amf_p{}^4$), three BC_2 plants ($Amf_p amf_p{}^3/Amf_t$) possessing alien tomato chromosomes including chromosome 8, were backcrossed to nulliplex tetraploid (2n=4x=48) potato genotypes to produce BC_3 populations for RFLP and starch analyses. Since chromosome 8 of tomato was known to possess the wildtype Amf_t locus, coding for Granule Bound Starch Synthase (GBSS), the BC_2 populations were screened for the presence of this additional chromosome. The three BC_2 plants that were selected as female parents were simplex for Amf_p ($Amf_p amf_p/Amf_t$) and possessed besides chromosome 8, three to five other tomato chromosomes which were as follows: 2403-3 (1,2,3,6, and 8); 2403-10 (2,6, and 8) and 2403-11 (2,3,6 and 8). The phenotype of starch granules in the potato tubers of the BC_3 populations 98-2801, 98-2803 and 98-2804 was monitored through iodine staining. Besides the anticipated blue and red starch phenotypes in BC_3 populations, there were intermediate types of starch granules that were red, but possessed blue (amylose-containing) cores of varying sizes. This phenotype could be connected with the presence of Amf_t on tomato chromosome 8 in a nulliplex potato. Because of the very low transmission rate of the alien tomato chromosome 8, in none of the 60 BC_3 plants that were analysed through RFLP analysis this chromosome was present. On the other hand, in all 60 BC_3 plants tomato chromosome 6 was transmitted. The original potato parent (1017-5) used in the somatic fusion between potato and tomato was heterozygous for the Amf_p locus, therefore, one wildtype Amf_p allele was introduced into the hybrid material. From the segregation patterns observed in the three BC_3 populations, it appeared for the first time that the same Amf_p allele in simplex conditions might have different expression patterns depending on the genotypic background. There were clear indications that the deviating type of starch granules (intermediate type) did not arise because of the influence of any of the other alien tomato chromosomes.

Key Words: Amylose-free potato, Protoplast fusion, *Solanum tuberosum*, *Lycopersicon esculentum*, RFLP.

Introduction

It has been demonstrated that some of the somatic fusion hybrids of potato and tomato can be backcrossed to the cultivated potato (Jacobsen et al. 1992,1994). In the backcross progenies it was possible to select alien tomato chromosome additions in a tetraploid (2n=4x=48) potato background (Jacobsen et al. 1995, Garriga-Calderé et al. 1997,1998). In these alien tomato addition lines, however, it has not been possible to assess the phenotypic effects, if any, of the tomato chromosomes because of the tetraploid potato background of this material. In fact, all the monosomic alien addition lines selected so far by our group are similar to normal potato plants. One method of testing the phenotypic effect of alien chromosome additions is to genetically monitor the phenotypic expression of certain known genetic loci in the alien chromosome addition lines. For example, if a wildtype gene (A) of tomato is added into a potato genotype that is nulliplex for the same gene (aaaa), then it should be possible to monitor the expression of the wildtype alien gene (A).

In order to test the above hypothesis, we used the amylose-free (*amf*) mutant of potato, which behaves as a typically monogenic recessive in its inheritance (Jacobsen et al. 1989,1991). Besides this, the *amf* locus, which in wildtype form is coding for Granule Bound Starch Synthase (GBSS) was most appropriate for this purpose for the following reasons: 1) the phenotypic expression can be determined unambiguously by a simple test with potassium iodine staining starch granules in microspores, stomatal guard cells, columella cells of root tips and most importantly in tubers; 2) the phenotypic expression of this gene has been critically monitored through traditional genetic analysis (Bastiaanssen et al. 1996) as well as through molecular transformation methods such as transgenesis (Flipse et al. 1994) and antisense suppression (Kuipers et al. 1995) and 3) this gene for Granular Bound Starch Synthase (GBSS) has been molecularly cloned (Visser et al. 1989) and localised on chromosome 8 of potato (Gebhardt et al. 1991). The linkage maps of potato and tomato are nearly homosequential, except for a few translocations and inversions (Bonierbale et al. 1988 and Tanksley et al. 1992). Chromosome 8 of tomato also possessed the homoeologous locus coding for Granule Bound Starch Synthase in the distal part of the long arm of this chromosome (Gebhardt et al. 1991, Jacobs et al. 1995).

In view of the homoeologous positions of the amylose-free locus (*Amf*) of potato and tomato on the respective linkage maps, it was attempted to introduce the wildtype tomato Amf_t gene on chromosome 8 into a nulliplex (amf_p^4) genotype of potato through repeated backcrossing of the potato (+) tomato somatic hybrid. For this purpose, hexaploid potato (+) tomato fusion hybrids were repeatedly backcrossed to potato genotypes that were nulliplex for the amf_p locus and the progenies were analysed for the selection of the monosomic addition genotype amf_p^4/Amf_t. The direct fusion between diploid (amf_pamf_p) potato and wildtype (Amf_tAmf_t) tomato was not successful (unpublished results) but fusion hybrids between (Amf_pAmf_p) or (Amf_pamf_p) potato with tomato were obtained and some of them were successfully backcrossed with potato. Segregation analysis for starch phenotypes in the BC_3 progenies showed different types of tuber starch that could not be classified as either wildtype (blue) or mutant (red) after staining starch granules with potassium iodine. In order to elucidate the occurrence and segregation of blue and intermediate phenotypes of starch, a detailed investigation was carried out through RFLP analysis for the identification of the alien tomato chromosomes in the segregating populations.

Materials and Methods

Origin and history of the plant material

The fusion between potato and tomato was only successful out of the many protoplast fusion experiments carried out at this department. Initially a hexaploid (2n=6x=72) potato (+) tomato fusion hybrid C31-17-5 which was the result of a fusion between a tetraploidized potato ($Amf_p^2amf_p^2$) protoplast and tomato, was backcrossed to a tetraploid (2n=4x=48) potato (amf_p^4) (for details see Jacobsen et al. 1994). This fusion hybrid was used as a female parent in the backcrossing programme in view of its total male sterility. This hexaploid fusion hybrid possessed four genomes of potato and two of tomato (PPPPTT) (Garriga-Calderé et al. 1997). The expected genotype of this hybrid for *Amf* was ($Amf_p^2amf_p^2/Amf_t^2$).

The tetraploid pollen parents (6704-1, 6707-7 and 6704-13) were nulliplex for the amf_p- allele, resulting in amylose free starch (Table 1). Two approaches were used to confirm the presence of specific tomato chromosomes in the fusion hybrids and later for the BC_1 and BC_2 progenies. First, a genetic approach, in which the tomato chromosomes were identified through RFLP analysis. Second, cytological, using genomic in situ hybridisation (GISH), in which the chromosomes were visually identified (Garriga-Calderé et al. 1998).

Table.1. Pedigree chart for the BC$_3$ populations

Fusion hybrids	Pollinator	BC$_1$	Pollinator	BC$_2$	Pollinator	BC$_3$	Pop
C31-17-5	x 6704-1	→ 2003	x 6707-7	→ 2403-3	x 6704-13	→ 98-2801	33
				→ 2403-10	x 6704-13	→ 98-2803	40
				→ 2403-11	x 6704-13	→ 98-2804	35

The fusion hybrid (C31-17-5) was backcrossed to amf_p (amylose free) potato for BC$_1$ (2003). In the same way BC$_2$ and BC$_3$ progenies were obtained by crossing with amf_p tetraploid potato 6707-7 and 6704-13 respectively. These yielded the BC$_3$ populations. The RFLP and starch iodine analyses were done in BC$_3$ populations.

Plant material

The potato plants from three BC$_3$ progenies (98-2801, 98-2803 and 98-2804) were grown under greenhouse conditions. The tubers from these progenies were used for iodine staining and young leaves were used for DNA extraction.

Iodine staining of starch in different tissues

Iodine staining with Lugol's solution (I_2: KI) was used to visualise the presence of amylose in starch. A mixture of amylose and amylopectin stains blue, whereas only amylopectin stains red with iodine. Starch staining in potato was done in tubers with Lugol/H_2O (1:1). A single tuber was cut in the middle and a drop of staining solution was placed on the cut surface and left for two minutes. The cut and stained surface was visualised for any colour change and later it was rubbed against a microscopic slide to transfer the staining solution with starch granules on it to check the starch granule colour under the microscope. Staining of tuber starch was carried out as described by Hovenkamp-Hermelink et al. (1988) with slight modifications.

Microspores were directly used for starch staining with Lugol/H_2O (1:1). The microspores from the fresh flowers were directly placed on a glass slide and a drop of staining mixture was added to the microspores and left for a minute. On the drop containing the stained microspores a coverslip was placed and observed under a microscope. The microspores with blue, intermediate and red staining starch granules were also counted.

RFLP analysis

For RFLP analysis, DNA was isolated from young leaves as described by Rogers and Bendich (1988). The procedures for restriction digestion and Southern hybridisation were those of Kreike et al. (1990). The radioactive RFLP method was used for chromosome identification using chromosome specific probes. The DNA probe labelling and incorporation of radioactive isotope ^{32}P was done by random prime labelling according to the procedures described by the manufacturer (Amersham). The pre-hybridisation, hybridisation, washing of blot and exposure of blot to film were as follows. The blot after Southern hybridisation was left in 10-15ml of Church buffer (0.36M Na_2HPO_4, 0.14M $NaH_2PO_4.H_2O$, 1mM EDTA, 7%SDS (dodecilsulfate sodium salt) in a closed bottle in the oven at 65°C for 5-7 hours. For hybridisation, pre-warmed fresh Church buffer and labelled probe was added to the blot and it was left at 65°C overnight for hybridisation. The following day the blot was washed twice to a stringency of 0.2x SSC and 0.5% SDS at 65°C. The blot was exposed to a film by placing the cassette at -80°C.

For an unambiguous identification of the chromosomes, tomato chromosome specific probe like TG160 and an additional GBSS probe (Visser et al. 1991) for chromosome 8 were selected. The TG probes were kindly provided by S.D. Tanksley, Cornell University, N.Y., U.S.A and already earlier used in this kind of material (see Garriga-Calderé et al. 1997). RFLP analysis was done on plants that were selected from the three staining categories.

Results

Development of plant material

The somatic fusion hybrid C31-17-5 was crossed with *amf* potato (6704-1, 6707-7 and 6704-1) to make backcross progenies viz., (BC_1, BC_2). From the BC_2 population three simplex plants were selected that possessed besides chromosome 8, three to five, other tomato chromosomes: 2403-3 (1,2,3,6, and 8); 2403-10 (2,6, and 8) and 2403-11 (2,3,6 and 8) (Table 1). To dilute the presence of other alien tomato chromosomes the three selected plants from the BC_2 population were backcrossed again to an *amf* potato genotype to get BC_3 populations with the expectation to select a monosomic tomato chromosome 8 addition which would be nulliplex for amf_p and possess an

Amf_t locus. The plants from the three BC_3 progenies (98-2801, 98-2803 and 98-2804) were grown in the greenhouse for tuber production (Table 2).

Three starch phenotypes with iodine staining

The potato fusion parent, 1017-5, was heterozygous for the *Amf_p* locus (i.e., *Amf_p/amf_p*) and, as expected, the starch granules in the tuber were blue. This was also the case with the fusion hybrid, C31-17-5 that was used for backcrossing with the nulliplex (*$amf_p{}^4$*) potato parent (6704-1). C31-17-5 was a hexaploid consisting of four potato and two tomato genomes. The genetic situation with respect to the *Amf* gene was (*$Amf_p{}^2amf_p{}^2/Amf_t^2$*) because of the somatic doubling of the potato part before fusion. The BC_1 plant, 2003 was a pentaploid (2n=5x=60) with *Amf* alleles both from potato and tomato and the tuber possessed typically blue staining starch granules. The three BC_2 plants also gave the phenotype of blue staining starch granules. For the three BC_3 populations (98-2801, 98-2803 and 98-2804) a single tuber from all the plants in these three populations was stained with iodine (Table 2).The tubers after iodine staining showed three types of coloured starch granules, viz., blue, intermediate and red staining (Fig.2A-D). Within the blue (Fig.2A,B) or intermediate (Fig.2D) staining category, there were staining patterns showing different levels of amylose concentration spread over the entire granule. The segregation ratios were not amenable for a clear genetic explanation, however, if the two phenotypic classes of blue and intermediate types were pooled together, then in two cases (98-2801 and 98-2804) the ratio clearly fitted a simplex segregation ratio (1:1). In the remaining population, 98-2803, the (1:1) ratio did not hold true because of the high frequency of *amf* phenotypes.

Table.2. Segregation of three different BC_3 progenies for tuber starch granule phenotypes determined through potassium iodine staining

BC_3 population	No. of plants analysed	No. of plants with starch phenotype (%) Blue*	Intermediate*	Red*
98-2801	33	9 (27.3)	7(21.2)	17(51.5)
98-2803	40	3 (7.5)	10(25.0)	27(67.5)
98-2804	35	13 (37.1)	4(11.4)	18(51.4)

* blue = amylose containing starch granules

red = amylose free starch granules

intermediate = starch granules with blue and red sectors

The unusual appearance of intermediate starch phenotypes in this BC_3 material, the polymorphism obtained by using GBSS as probe (explained later in detail) and moreover the presence of several other alien tomato chromosomes in the three BC_2 parental plants made it necessary to do a detailed RFLP analysis using other tomato specific probes.

RFLP analysis with GBSS and tomato chromosome specific probes

The GBSS probe, which is specific for the Amf_t locus, was used to identify the presence of tomato chromosome 8 in the three BC_3 populations (98-2801, 98-2803 and 98-2804) that segregated for blue, red and intermediate starch phenotypes. There was a striking relationship between the tuber starch type and RFLP polymorphism obtained for the GBSS probe. In those plants that had a blue staining starch phenotype there were clearly two RFLP bands as against only one in plants with a red staining starch phenotype (Fig.1). Plants with intermediate starch also possessed two RFLP bands, as did the wildtype blue staining plants. In the case of the tomato parent there was only one band for the GBSS probe not polymorphic with the lower potato band. This means that, based on polymorphisms for the GBSS probe, the amf_p allele but not the wildtype *Amf* alleles of potato and tomato could be clearly discriminated in this material, which was not polymorphic with the lower potato band in the parent (Fig.1). The intermediate starch phenotype could be connected with Amf_t because of a lower activity in a potato background then the wildtype Amf_p allele.

The unusual starch phenotype (intermediate types), the distorted (1:1) segregation in population 98-2803 and the polymorphism obtained by GBSS probe made it mandatory to recheck the presence or absence of chromosome 8 of tomato in the BC_3 populations with another tomato specific probe. For this purpose, probe TG160 specific to chromosome 8 and closely linked to the Amf_t locus, was used to check the presence of chromosome 8. Unfortunately, there was no indication for the presence of chromosome 8 in the entire BC_3 population. This led to the conclusion that the lower band segregating in the BC_3 population (Fig.1) that is not polymorphic to potato and tomato is actually coming from potato. This lower band is actually responsible for the expression of two starch phenotypes (blue and intermediate).

The complementation of potato GBSS with a tomato homoeologue in a potato amf_p background was not achieved because of the very low transmission frequency of tomato chromosome 8 in this material. There was only one Amf_p allele inherited from the potato fusion parent 1017-5, indicating that this allele was solely responsible for the two starch phenotypic classes. This means that the intermediate starch phenotypic class was also due to the presence of one and the same Amf_p allele in a different genetic background. Because the three BC$_2$ parent plants possessed three to five, alien tomato chromosomes each, the BC$_3$ populations were expected to segregate for the alien chromosomes. In 60 plants from the three populations that were analysed (Table 2), chromosome specific tomato probes indeed identified tomato chromosomes, the number of which varied from one to three per plant.

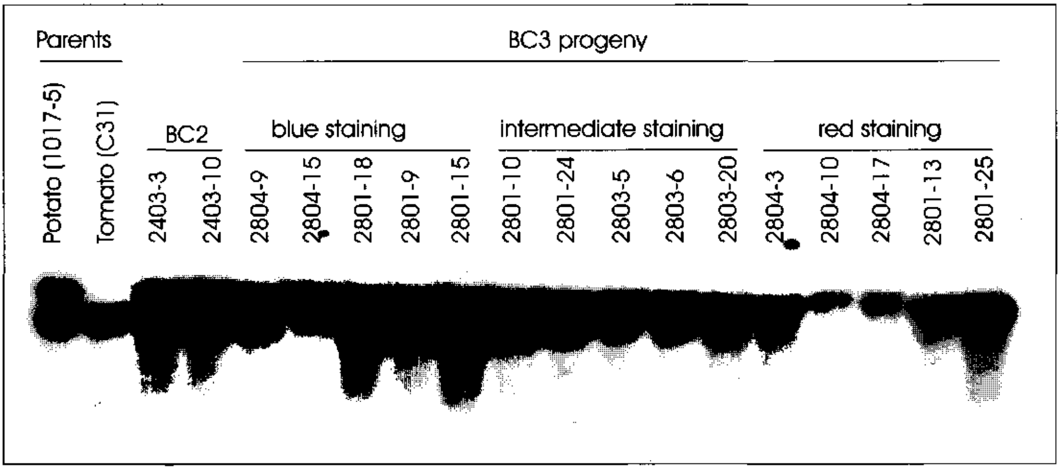

Fig. 1. A representative autoradiogram of Southern hybridisation showing two bands for the blue and intermediate staining plants in BC$_3$ populations, whereas red staining plants only have the single upper band. The enzyme used for digestion was Eco RV and probe was GBSS specific for chromosome 8 of tomato and potato.

Tomato chromosome 6 was found in all 60 BC$_3$ plants analysed. The presence or absence of other tomato chromosomes in BC$_3$ population was carefully assessed with respect to the phenotypes in all starch classes and it was concluded that they had no effect on the appearance of starch phenotypes.

Discussion

Our attempt to monitor the phenotypic effect of the wildtype *Amf*$_t$ allele on chromosome 8 of tomato in a nulliplex background of potato was not successful. This was mainly because of the low transmission rate of tomato chromosome 8 into the BC$_3$ material. We previously demonstrated that there are great differences between tomato chromosome transmission rates in progenies of alien addition lines which are sometimes connected with a disomic instead of monosomic situation for particular alien chromosomes (Garriga-Calderé et al. 1998). There are also clear indications that transmission rate of alien tomato chromosomes in a different genetic background of potato vary enormously (2.6-33.3%) in BC$_3$ populations (chapter 2). These differences arise due to the genetic content of the chromosomes as well as to the genotypic background of the material. For example, in three different BC$_2$ populations with alien tomato chromosome additions the rate of transmission of individual tomato chromosomes varied from 0 to 92% (Garriga-Calderé et al. 1998). Similarly, the rate of transmission of the same chromosome varied enormously in three different populations. As an illustrative example, these authors reported the transmission rate of chromosome 2 to be 8%, 17% and 92% in three different populations. This clearly indicates that the choice of BC$_3$ populations in this study was not appropriate for selecting individuals with alien tomato chromosome 8 additions. More trials preferably in BC$_2$ populations are obviously necessary in order to achieve this objective.

Based on the experience gained from this study, however, an approach with other material can be developed for the selection of a monosomic addition genotype for chromosome 8 of tomato in a nullisomic (*amf*$_p^4$) background of potato. Keeping in mind the failure to select an (*amf*$_p$*amf*$_p$) genotype as potato fusion parent to avoid the (*Amf*$_p$) locus in the first place, we can adopt some strategies. First, it is important to consider that while backcrossing BC$_1$ and BC$_2$ progenies to nulliplex (*amf*$_p^4$) potato genotypes, those alien additions that have tomato chromosomes 2 and 6 together with 8 of tomato should be avoided as parents to avoid the suppression of transmission of chromosome 8. Second, in the backcross progenies (BC$_3$) genotypes that have the single RFLP band for potato and tomato GBSS might be selected, but the intermediate starch phenotype is not a reliable criterion for the selection of the desired *Amf*$_t$ monosomic if the present wildtype *Amf*$_p$ allele is involved. Other tomato specific probes or preferably probe TG160 is the closest choice for an indirect detection of (*Amf*$_t$) or tomato chromosome 8.

Our additional interest of investigating the present material was because of the variation of tuber starch that showed an anomalous intermediate phenotype. Evidence shows that neither the alien chromosome 8 nor any other chromosomes of tomato were involved in causing the deviating starch phenotype. There was clear evidence for the presence of the wildtype (*Amf*$_p$) allele from potato in these populations. Then the question arises why the variation for starch phenotype was observed and what is the origin?

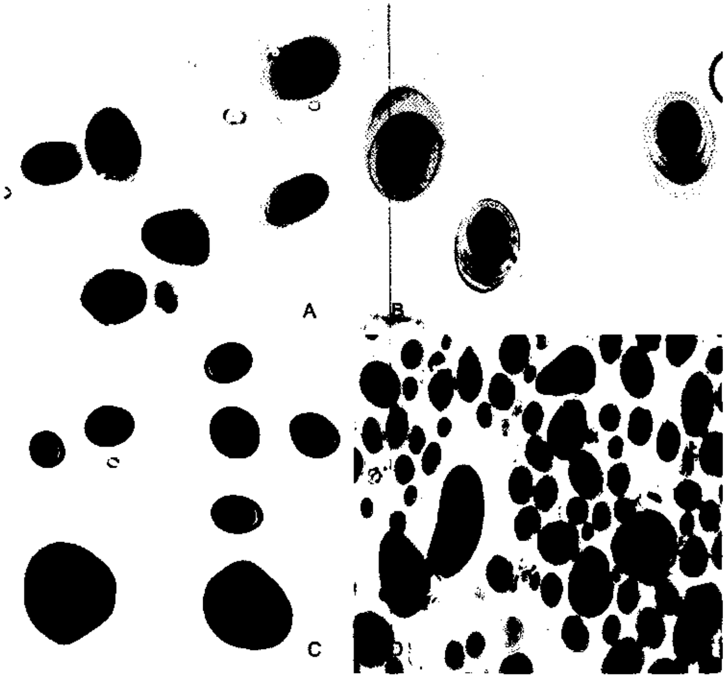

Fig.2. Iodine staining of tuber starch. **A**. Blue staining starch granules. **B**. Blue staining starch granules with concentric rings. **C**. Red staining starch granules. **D**. Intermediate staining starch granules

Previous studies by our group have shown indications for comparable degrees of variation for starch phenotypes for different reasons. When antisense GBSS constructs were introduced into wildtype potato (cultivars), complete inhibition of amylose production was observed in addition to cases of partial suppression of GBSS activity which resulted in starch granules with different sizes of blue cores in the hilum (Kuipers et al. 1991,1994). Similarly, using antisense and sense versions of parts of cDNA clones of the branching enzyme for transformation of amylose-free potato, Flipse et al. (1996) observed the formation of red starch granules with blue cores. When the heterogeneous cassava GBSS I was introduced into amylose-free potato, Salehuzzaman et al. (1999) observed many starch granules with a blue core of varying sizes indicating partial complementation besides red staining indicating no complementation at all. The same degree of complementation or higher is expected with the Amf_t allele on chromosome 8 of tomato.

Unlike the above instances, the presence of the Amf_p-allele, which leads to such a large variation of the starch phenotype, is a remarkable feature. Originally, only one dominant Amf_p-allele was introduced into the fusion hybrid through the heterozygous potato parent, 1017-5. The segregation patterns of the two populations (98-2801, 98-2804) justified the simplex (Amf_p) segregation pattern. In one genotype (98-2803), the segregation pattern was not 1:1 because of distorted segregation that has also been observed for several other loci in potato (Hermsen et al. 1974, Jacobs et al. 1995). It appeared in the BC_3 populations that there is a great possibility of a single Amf_p locus for a detectable diverse amylose expression in a different genetic background and in different genotypes of the heterozygous potato species. In a similar instance, in rice Sano et al. (1984) found that a difference in genetic background plays a determining role in GBSS expression levels. Flipse et al. (1996) for the first time showed in simplex potato genotypes different degrees of amylose synthesis, however it was not clear whether this phenomenon was related mainly to different wildtype Amf_p alleles or different genetic backgrounds

In this research contribution it is clearly shown that only a single wildtype (Amf_p) allele in a different genetic background showed different levels of GBSS expression and amylose activity resulting in two starch phenotypic patterns. The potato GBSS gene activity due to a different genetic background (interference) could have been reduced resulting in amylose formation in restricted zones of the starch granules of tubers with an intermediate starch phenotype.

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4

SUCCESSFUL HYBRIDIZATION OF A 4X POTATO (+) TOMATO FUSION HYBRID WITH *LYCOPERSICON PENNELLII* AND A GISH ANALYSIS OF THE TRIGENOMIC HYBRID

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Abstract

With the aim of utilising the 4x potato (+) tomato fusion hybrid (2n=4x=48) in a backcrossing programme, this hybrid was successfully crossed with diploid *Lycopersicon pennellii* (2n=2x=24) used as bridge species. All the resulting progenies were triploids (2n=2x=36) and possessed three different genomes, viz., potato, tomato and *L. pennellii*, therefore, they have been called trigenomic hybrids. Genome constitution of triploids was confirmed through genomic in situ hybridisation (GISH) in somatic cells as well as during meiosis. Total genomic probes of both *Lycopersicon* species were found to hybridise mutually whereas potato genome was clearly differentiated. During metaphase I stages of microsporogenesis trivalents, bivalents and univalents were observed. Bivalents were formed predominantly between tomato and *L. pennellii* chromosomes and the univalents of potato chromosomes were most common. Trivalents in all cases included homoeologous chromosomes of potato, tomato and *L. pennellii*. There was clear indication for chiasma formation between the chromosomes of potato and *L. pennellii*. In view of this, *L. pennellii* can serve as a bridge species to transfer genes from tomato to potato or vice versa. However, the triploids in the present experiment were totally sterile as determined from extensive crossing. On chromosome doubling of triploids by shoot regeneration from callus, hexaploids (2n=6x=72) were obtained. Despite having clear allohexaploid behaviour by forming 36 bivalents at meiosis, these were also completely sterile as their triploid counterparts. In spite of this drawback, the prospects of chromosome pairing between potato and *L. pennellii* genomes does open possibilities for introgressing genes between the two genera i.e. *Solanum* and *Lycopersicon*.

Key Words: Trigenomic triploids, GISH, bridge species, potato (+) tomato fusion hybrids.

Introduction

The first intergeneric hybrid between potato and tomato was made through protoplast fusion (Melchers et al. 1978), but this fusion hybrid could not be backcrossed to any of its parents. However, using a different set of potato (+) tomato fusion hybrids made by Jacobsen et al. (1992), a hexaploid (2n=6x=72) genotype of these was successfully back-crossed to *Solanum tuberosum* (2n=4x=48) and BC_1 and BC_2 progenies were obtained (Jacobsen et al. 1994). Following this breakthrough, it was possible to establish a series of monosomic tomato chromosome addition lines in the background of tetraploid potato (Jacobsen et al. 1995; Garriga- Calderé et al. 1997).

Although these monosomic tomato chromosome addition lines of potato are useful for basic research, they possess two main drawbacks. First, because of their polysomic background it is almost impossible to obtain the phenotypic expression of the recessive alien tomato genes in the present 4x material. Second, an extremely low frequency of homoeologous crossing-over has been found to occur between potato and tomato chromosomes (Garriga-Calderé et al. 1999). Obviously, this aspect is an impediment for introgressing segments of tomato chromosomes into potato. In view of paving the way for a more successful introgression of tomato chromosome segments into potato or vice versa, it is essential to generate more suitable plant material. This should preferably constitute alien chromosome additions at the diploid level and offer the prospects of higher levels of homoeologous crossing-over.

Solanum and *Lycopersicon*, both belonging to Solanaceae, had an independent and long evolution period (Hawkes and Smith, 1965). However, these genera still are highly conserved and according to Ramanna and Wagenvoort (1976), the pachytene chromosome morphology of potato and tomato is extremely similar. The high density molecular linkage maps made by Tanksley et al. in (1992) and RFLP maps made by Bonierbale et al. (1988) also prove that the genomes are homosequential except for some inversions and translocations. With all these similarities and the very low degree of homoeologous crossing-over, it was necessary to find a link between these two genera.

Fortunately, taxa within Solanaceae form a phylogenetic link between *Solanum* and *Lycopersicon*. These taxa include *S. lycopersicoides* and *L. pennellii* (formerly, *Solanum pennellii*) (Correll, 1962) which might be potentially useful as 'bridge species' for the purpose of crossing as well as introgression. Such bridge species has been previously used within the genus *Solanum* where *S. acaule* served as a bridge to transfer genes from *S. bulbocastanum* into *S. tuberosum* (Hermsen and Ramanna, 1973). Such an approach was successfully applied recently for onion by Khrustaleva and Kik (2000) using *Allium roylei* as a bridging species. However, there has never been an instance before where a bridge species was used between *Solanum* and *Lycopersicon*. In order to further explore the possibilities of bridge crosses, a tetraploid potato (+) tomato fusion hybrid was crossed with several Solanaceous species. In this article the outcome of these crosses and a cytological analysis of the hybrid using GISH (genomic in situ hybridisation) are reported and discussed.

Materials and Methods

The origin of the 4x- potato (+) tomato fusion hybrid (F8-10) was described by Jacobsen et al. (1992). The accessions of *Lycopersicon esculentum* and *L. pennellii* were used from the collection of the Laboratory of Plant Breeding, Wageningen University. Different accession numbers and the fusion hybrid used here are given in Table 1. The plants were grown in the greenhouse under standard growing conditions. Because of the crossability barriers and poor berry set, ovule rescue was necessary. Ovule rescue was performed on one to two week old berries according to the procedure described by Jacobsen et al. (1992).

Collection and Fixation of Anthers and Root Tips

To study the genomic constitution and behaviour of chromosomes, pollen mother cells from young flower buds were monitored for the right meiotic stage of development. One anther of each flower bud was squashed in a drop of aceto-carmine and examined under the light microscope. The remaining anthers were fixed in ethanol-acetic acid (3:1) for one to two hours. For analysis of chromosome constitution, the root tips were harvested in the morning, treated with 2mM 8-hydroxyquinoline overnight at 18°C in the dark and fixed in ethanol-acetic acid (3:1). The material was directly used for in situ studies or stored in 70% ethanol for 1 week at -20°C.

Table.1. Total number of crosses made to potato (+) tomato fusion hybrid F8-10 with different *Lycopersicon* species as male pollinators and embryos rescued leading to trigenomic plants

Male parents used	No. of pollinations made	No. of berries formed	No. of ovules cultured	No. of plants survived
		Lycopersicon species		
L. esculentum c.v. Money Maker	69	-	-	-
L. pennellii PV 960402	180	16	79	10*
L. cheesmannii PV 971321	40	-	-	-
L. hirsutum PV 971130	72	-	-	-
L. parviflorum PV 971061	38	-	-	-
L. esculentum PV 976451	46	-	-	-
Cherry Tomato PV 95199	54	-	-	-

* = Codes of the plants: 97-4705, 98-4804, 98-4805, 98-4806, HA99-2901, HA99-2902, HA99-2903, HA99-2904, HA99-2905, HA99-2906

Chromosome preparations

Chromosome preparations were made according to the method described by Zhong et al. (1996) with some modifications. The root tips and anthers were incubated in a pectolytic enzyme mixture (0.5% pectolyase Y23, 0.5% cellulase RS and 0.5% cytohelicase in 10mM citrate buffer, pH 4.5) for 2 hours at 37°C. The slides were made grease-free by leaving them in Chromosulfuric acid (Merck) for several hours followed by thoroughly rinsing with water. The slides were then left in absolute ethanol for 10 minutes. The macerated root tips or anthers were transferred to the slides in a droplet of water and crushed with a needle, removing the tissue material as much as possible. Then 20μl of 60% acetic acid was put for 2 minutes. A ring of freshly prepared ice cold ethanol-acetic acid (3:1) fixative was made on the slide around the drop containing the cells. A few drops of the same fixative were dropped on the slide from a small distance (10-20cm). The slides were shortly immersed in absolute ethanol and left for air-drying. Selected slides with good cells were used for in situ hybridisation.

Pre-treatments

For enzymatic digestions the chromosome preparations were pre-treated with 200μl/slide RNase A (100μg/ml) for 1 hour at 37°C and then washed with 2x SSC three times for 5 minutes. The slides were then incubated with 200μl/slide pepsin (5μg/ml) for 10 minutes at 37°C and washed with 2x SSC three times for 5 minutes. The slides were fixed in 4% (w/v) paraformaldehyde for 10 minutes at room temperature, washed in 2x SSC three times for 5 minutes, followed by dehydrating the slides in ice cold ethanol series (70%, 90% and 100%) for 3 minutes each. The procedure was carried out as described by Kuipers et al. (1997).

Genomic in situ hybridisation (GISH)

GISH was carried out according to Kuipers et al. (1997). The *L. pennellii* DNA used as probe was sonicated to a fragment size of 5-10 kb and labelled with digoxigenin following a standard nick translation protocol (Boehringer Mannheim). The potato DNA used, as blocking was autoclaved for 5 minutes to a fragment size of 100-500 bp. The hybridisation mixture (40μl/slide) included 50% demonised formamide, 10% (w/v) sodium dextran sulfate (sigma), 2x SSC, 0.25% SDS, 100ng/μl probe DNA and 2μg/μl blocking DNA. The hybridisation mixture was denatured at 70°C for 10 minutes and applied to the slides containing the chromosome spreads, covered with a cover slip. The slides were denatured at 80°C for 2 minutes and left for overnight hybridisation at 37°C. The slides were washed in 2x SSC buffer for 15 minutes at room temperature, 0.1x SSC for 30 minutes at 42°C and again in 2x SSC for 15 minutes at room temperature.

Digoxigenin-11-dUTP was detected with anti-dioxigenin-fluorescein (20μg/ml) (Boehringer Mannheim) and amplification was done by rabbit-anti-sheep fluorescein (20μg/ml) (Vector laboratories). Before each detection step the slides were incubated with 200μl/slide of blocking buffer (1% blocking reagent (Boehringer Mannheim) in buffer 1 (0.1M Tris HCl, 0.15M NaCl, pH 7.0) for 5 minutes. Incubation with the appropriate antibodies in 100μl blocking buffer was done at 37°C, proceeded with washing the slides in buffer 1 for 3 times for 5 minutes at 37°C. The preparations were counter-stained with 2μg/ml DAPI (4,6-diamidino-2-phenylindole) and 5μg/ml propidium iodide (in meiotic preparations only) and mounted in Vectashield (Vector Laboratories). Selected cells were photographed on Fuji 400 ISO colour negative film with an Axiophot microscope equipped with UV light and appropriate filters.

Negatives were scanned at 500dpi and images were optimised with routinely used image processing software.

Pollen tube growth

Growth of pollen tubes in the styles of trigenomic plants was examined after 24 and 48 hours of pollination with fresh pollen. Briefly, the technique is as follows: receptive styles were pollinated, later, they were fixed in freshly prepared FAA fixative for 24 hours or longer; left in 8N sodium hydroxide (NaOH) solution for at least 5-6 hours; rinsed with water; stained with 0.1% aniline blue dissolved in 0.1M potassium pyrophosphate; softened styles were mounted in glycerol and observed under the microscope. *Lycopersicon esculentum* (cv. Money Maker) and *L. pennellii* were used to pollinate the flowers (Fig. 1B). Crossing was done manually in the glass house. Pollen tube growth in styles was studied according to the modified technique of Martin (1959).

Results

Crossability of 4x- fusion hybrid with alien species

Because of the total male sterility of the fusion hybrid (F8-10), it could be used only as a female parent. Furthermore, the potato parent of the fusion hybrid was self-incompatible. This was also a consideration for the success of hybridising with a self-compatible species such as *L. esculentum*. In view of this, the male parents used for crossing consisted of both self-compatible and self-incompatible taxa. Among the large number of pollinations that were made, initial berry set was observed only in cross combinations involving *L. pennellii* as male parent (Table 1). The ovule culture using these berries did produce young plantlets and eventually vigorous plants. The frequency of the germination of ovules was relatively low. Once germinated from ovules, the plantlets grew normally and gave rise to vigorous plants that flowered profusely. Out of 79 cultured embryos, 10 plants were obtained in the last three years and their detail is mentioned in Table 1.

Confirmation of hybridity

Morphologically, the plants resulting from ovule culture showed a mosaic of parental, intermediate and novel characters. The plant colour was light green with growth habit resembling tomato. The flower colour was yellow, similar to tomato flowers but the shape-resembled potato flowers (Fig. 1A). Another clear indication for the hybrid nature of these plants was obtained from chromosome counting in the root tips (Fig. 1C and 1D). Because the plants originated from 4x-2x ($2n=4x=48$ and $2n=2x=24$ respectively) crosses, triploids ($2n=3x=36$) were expected in the progeny and this was indeed the case. These triploids are expected to possess three different genomes, i.e., one of *S. tuberosum*, *L. esculentum* and *L. pennellii* respectively. Although this is an allotriploid, it will be more appropriately referred to as trigenomic triploids in this article. The trigenomic nature of these plants was also evident from GISH analysis of chromosome pairing during microsporogenesis.

GISH analysis of chromosome pairing in trigenomic triploids

Being an intergeneric hybrid, it was not possible to spread meiotic chromosomes satisfactorily in order to analyse individual pollen mother cells completely. Nevertheless, it was possible to clearly distinguish univalents, bivalents and trivalents at metaphase I stage in some cells (Fig. 1E and 1F). In view of this, the estimates of chromosome pairing presented in table 2 are only approximate. For an estimate of chromosome pairing 50 cells were observed showing 25 trivalents between potato, tomato and *L. pennellii* chromosomes, 239 bivalents between tomato and *L. pennellii* chromosomes and all univalents were potato chromosomes. A notable feature was that when total genomic DNA of either *L. esculentum* or *L. pennellii* was used as a probe (FITC labelled), all bivalents fluoresced yellow whereas the univalents were red (propidium iodide) representing potato chromosomes (Fig. 1F). The frequency of bivalents varied from 2-10 and of trivalents from 0-3 per PMC (Table 2). Among trivalents, both chains of three as well as Y-shaped configurations were observed (Fig. 1F). Based on distinctly faint fluorescence of tomato chromosomes as compared to those of *L. pennellii*, it was possible to determine the position of the chromosomes of *L. pennellii* in the trivalent in several cases. In a chain trivalent, the chromosome of *L. pennellii* was always positioned in between those of potato and tomato chromosome. This was an indication that the chromosome of *L. pennellii* paired with potato as well as with those of tomato and formed chiasmata. There were also instances of allosyndetic pairing probably between the chromosomes of potato and probably *L. pennellii*, but there was no method of confirming this finding. Rarely, there were bivalent like structures involving only potato chromosomes like earlier observed in potato monohaploids (van Breukelen et al. 1975). It was not possible to confirm whether there were chiasmata connections between the non-homologous potato chromosomes.

Table.2. Chromosome pairing in 3x and 6x plants

Chromosome association/cell	Minimum number	Maximum number
3x (50 PMC's observed)		
Univalents	7	12
Bivalents	2	10
Trivalents	0	3
6x (45 PMC's observed)		
Univalents	0	4
Bivalents	32	36
Multivalents	0	0

Crossability of trigenomic triploids and hexaploids

All triploids proved to be completely male sterile and, therefore, they were used as female parents for crossing with several genotypes of *Lycopersicon* and *Solanum*. In spite of several hundreds of pollinations during the last three years, no success has been achieved so far in obtaining a berry with seeds. Nevertheless, some berries were obtained without seeds after crossing with *L. pennellii* (Table 3). This indicated that, probably because of their trigenomic constitution, the triploids are also highly female sterile. The trigenomic triploid plant (97-4705) was used for chromosome doubling. The plant material was treated with colchicine that yielded eight hexaploids plants, which were also highly sterile. The chromosome pairing was studied in 45 cells in hexaploids. Mostly there were bivalents and rarely univalents were present. There was not a single multivalent association between the three genomes of potato, tomato and *L. pennellii* (Table 2). Even then, these hexaploids were sterile.

Germination and Penetration of Pollen Tube

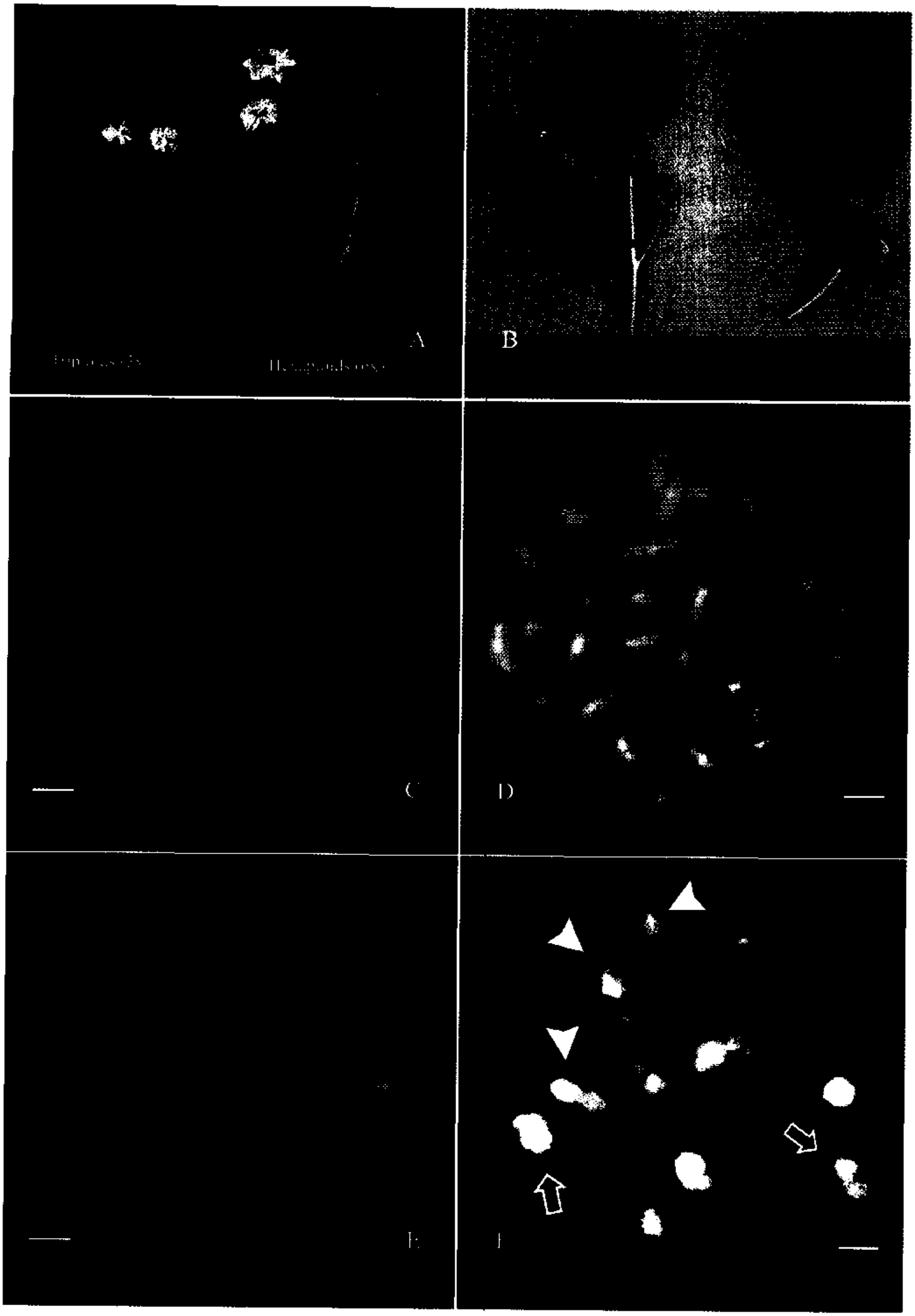
Because the phenomena of self-incompatibility (potato, *L. pennellii*) and self-compatibility (tomato) are involved in crossing of these taxa, it was essential to gain an insight into the processes of pollen germination, pollen tube growth in the styles and penetration into the ovules. The pollen grains of tomato cultivar, Money Maker (self-compatible) did germinate but the tube growth was totally inhibited just below the style. On the other hand, the pollen tube of *L. pennellii* (self-incompatible) did grow in the styles and reached up to the micropyle of the ovules.

Table.3. Total number of crosses made to trigenomic triploids (97-4705, 98-4804, 98-4805, 98-4806) with different male pollinators without seed set

Male parents used	No. of pollinations made
***Lycopersicon* species**	
L. esculentum c.v. Money Maker	701
L. pennellii - PV 960415	203
L. pennellii - PV 960416	118
L. pennellii - PV 960456	324
L pennellii - PV 960458	332
L. pennellii - PV 960402	457*
L. esculentum - PV 976451	80
L. cheesmannii - PV 971321	162
L. pimpinellifolium - PV 971025	75
L. hirsutum - PV 971130	131
Cherry Tomato - PV 95199	100
L. esculentum c.v. VFNT	20
L. esculentum x *peruvianum* hybrid PV 1127-443	35
L. esculentum x *peruvianum* hybrid PV 1124-437	43
L. esculentum x *peruvianum* hybrid PV 1125-440	46
Esculentum x *pennellii* hybrid	57
***Solanum* species**	
S. phureja - IVP 48	379
S. phureja - IVP 101	166
RH 89-031-35	148
RH 88-032-52	44
Katahdin	70
AM66-42	167
93-6706-2	189

* = Some berries were formed without seeds

However, there was clear indication of disintegration of pollen tube tips at or inside the ovules so that there was no fertilisation.


A
B
C
D
E
F

Discussion

The previous attempts to cross 4x-potato (+) tomato fusion hybrids to tomato parents, or related taxa, was not successful (Jacobsen et al. 1992). Trigenomic hybrids produced in the present investigation is indeed a step forward for using the fusion hybrids for introgression. One advantage of crossing the 4x- fusion hybrid to a diploid species such as *L. pennellii* is that it can open the way for creating diploid back-cross progenies through further back-crossing of the trigenomic triploids to diploid parents. Such crossings, when successful, should be able to give rise to monosomic or disomic alien addition lines at the diploid level.

Another important feature of the results of this investigation is the possibility of inter-genomic recombination by using *L. pennellii* as a bridge species. Previously it has been shown (Garriga- Calderé et al. 1999) that there is a very low frequency of recombination between potato and tomato chromosomes. In contrast, in the present trigenomic triploids there is clear evidence for the formation of trivalents resulting from the association between the chromosomes of potato, *L. pennellii* and tomato. In the past, bridging species have also been used and proved successful in overcoming the sexual hybridisation barriers. The work done by Khrustaleva and Kik (2000) on onions using *Allium roylei* as a bridging species made sexual hybridisation possible, including the possibilities of homoeologous recombination.

◄

Fig.1. Comparison of triploids and hexaploids and *L. pennellii* and *L. esculentum*. **A.** The triploids have short leaves and smaller flowers whereas the hexaploids have large rounded leaves and larger flowers. **B.** *L. pennellii* has round and waxy leaves whereas *L. esculentum* has cut leaf margins. Genomic constitution and meiotic behaviour of trigenomic triploids (F8-10xP) as revealed by GISH. **C.** Full metaphase complement of potato, tomato and *L. pennellii* chromosomes (2n=36) in somatic cells. Counterstained with 4,6-diamino-2-phenylindole (DAPI). **D.** Twenty-four tomato and *L. pennellii* chromosomes are green and potato chromosomes are blue. *L. pennellii* DNA used as probe labelled with FITC. **E.** Metaphase complement of potato, tomato and *L. pennellii* chromosomes (2n=36) in meiotic cells. Counterstained by DAPI. **F.** Total genomic DNA of *L. pennellii* used as probe and labelled with FITC shows twenty-four tomato and *L. pennellii* chromosomes fluoresce yellow. Twelve potato chromosomes are red due to propidium iodide counterstaining. (arrows) indicate bivalents, (arrowheads) indicate trivalents. One multivalent-like configuration (Fig E and F) occurs due to the association or clumping of nucleolar chromosomes. The scale bars in C-F represent 10μm.

In the past, double bridging species *S. acaule* and *S. phureja* used by Hermsen and Ramanna (1973) were within the *Solanum* species whereas in the present study we have utilised the benefits of bridging species after wide hybridisation between two different species viz., *Solanum* and *Lycopersicon* which are impossible to cross sexually. This clearly opens the possibility for transferring recombinant segments from potato to tomato or vice versa. The homoeologous pairing between chromosomes of potato, tomato and *L. pennellii* as revealed by GISH indicates that by using bridging species we can facilitate, indirectly, recombination between potato and tomato chromosomes. The chromosome pairing behaviour of *L. pennellii* with those of both potato and tomato, although intriguing, is not totally unexpected. *L. pennellii* was considered by taxonomists to be a *Solanum* species until recently on morphological grounds (Correll, 1962). Thus, in a phylogenetic sense, *L. pennellii* occupies an intermediate position between the genera of *Solanum* and *Lycopersicon*. To our knowledge, this is first instance in which the three genomes, viz., potato, *L. pennellii* and tomato are brought together in one complement and the pairing relationship is examined.

Out of the ten trigenomic plants, four plants (97-4705, 98-4804, 98-4805, 98-4806) were initially used for GISH analysis. Later when it was confirmed that they had the same behaviour and performance during the GISH experiments then only 97-4705 was selected for more critical observations on chromosomes pairing studies.

Despite strenuous efforts, we did not succeed in making backcrosses with trigenomic triploids. Looking to the chromosome pairing in the trigenomic triploids, bivalent formation between the chromosomes of *L. pennellii* and tomato one should expect the formation of near haploid gametes in some cases. If this is the case, it might be desirable to cross with various other accessions of *L. pennellii* or other genotypes. In the past, success has been achieved by extensive crossing with other new accessions as in the case of *L. esculentum* x *S. lycopersicoides* (Rick 1951). In this context, the results achieved by Rick et al. (1988) in a nearly comparable type of hybrid is of interest. By successfully crossing of the so-called sesquidiploid hybrids of *L. esculentum* x *Solanum lycopersicoides* to tomato, these authors did produce plant material with recombination between the alien genomes. Success of such work enabled them to establish a complete set of monosomic alien additions of *S. lycopersicoides* chromosome lines in tomato (Chetelat et al. 1998).

Another possible approach to make the backcrossing fruitful and improving the fertility of the plants is the use of re-fusion technique as applied by Karin Horsman in BC_1 backcross hybrids of *S. tuberosum* (+) *S. nigrum* with *S. tuberosum* (unpublished results). The pollen tube growth in the trigenomic plants revealed a selective growth pattern. As revealed by the investigation it was clear that pollen from *L. pennellii* was more effective in penetrating than *L. esculentum* cv. Money Maker. This result was also encouraging and provided the expectation that the best way to proceed further is to pollinate these plants with different pollinators and check the pollen tube growth with each pollinator. The one that gives the best results should be extensively used for further pollination. In addition, it might also be worthwhile to search for trigenomic hybrids that produce numerically unreduced (2n) gametes. It is well known that 2n-gametes do occur in some genotypes in numerous other plant taxa Kamstra et al. 1999). The possible hybridisation barriers in reciprocal crosses between *Solanum verrucosum* and *S. bulbocastanum* have been described by Hermsen and Ramanna (1976) and Hermsen (1994) has described in detail the barriers in interspecific hybridisation and possible ways to overcome them.

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Introduction

Non-isotopic in situ hybridisation techniques have greatly contributed to elucidating various phenomena in plant species, their hybrids and their backcross derivatives. For example, it has been possible to map repetitive DNA sequences and multigene families in the genomes to determine genome relationships and chromosome pairing behaviour much more critically than was possible with traditional cytological approaches and monitor alien chromatin in the derivatives of species hybrids (for review see Jiang and Gill, 1994; Schwarzacher and Heslop-Harrison, 2000). Although genomic in situ hybridisation (GISH) has been successfully used in a great variety of plant species hybrids and their derivatives, this technique is most informative in plants with fairly large chromosomes like *Alstroemeria*, Lily and wheat and when the genomes of the parental species are well differentiated. However, in the case of species with small chromosomes and when the genomes are less well differentiated, as for example in sibling species, GISH can be less informative especially with regard to the information on chiasma formation and crossing over between the homoeologous chromosomes.

Despite the above indicated difficulties, there are instances in which the chromosomes of closely related species have been clearly discriminated in species hybrids through GISH as in *Gibasis* (Kenton et al. 1991; Parokonny et al. 1992). As compared to *Gibasis*, *Solanum* and *Lycopersicon* species have much smaller chromosomes and even in these cases it has been claimed that the parental chromosomes can be clearly differentiated through GISH in hybrids between closely related species. For example, in a hybrid between *Lycopersicon esculentum* x *L. peruvianum*, the parental genomes have been clearly discriminated as well as homoeologous recombination has been detected (Parokonny et al. 1997). Among *Solanum* species the DNA of *S. phureja* has been shown to be present in the dihaploid of *S. tuberosum* as revealed by GISH (Wilkinson et al. 1995).

Besides being small, the chromosomes of both *Solanum* and *Lycopersicon* share an unusual characteristic of possessing the so-called centromeric, or pericentric, heterochromatin (Ramanna and Prakken, 1967; Ramanna and Wagenvoort, 1976). It has been estimated that 77% of the tomato DNA is located in the pericentric heterochromatin and 23% in the euchromatin parts (Peterson et al. 1996). This pattern of DNA distribution in the genome causes two important problems for the detection of homoeologous recombination through GISH: a) since recombination has been shown to be highly restricted to euchromatin parts in tomato (Khush and Rick, 1968; Stack, 1984), it is very difficult to demonstrate recombinant segments in the relatively small euchromatin segments and b) because most of the repetitive DNA is confined to pericentromeric heterochromatic segments, the euchromatic segments may possess little repetitive DNA that is so essential for differentiating the alien genomes in a species hybrid (Schwarzacher and Heslop-Harrison, 2000).

One of the important considerations for introgressing alien tomato chromosome segments into potato is to determine whether there is homoeologous recombination in the fusion hybrids/or their backcross derivatives. The RFLP maps developed in potato have been made by using tomato probes and it has been proven that potato and tomato genomes are highly homosequential (Bonierbale et al. 1988; Gebhardt et al. 1991). This question could not be resolved in our previous investigations in potato (+) tomato fusion hybrids and their backcross progenies (Garriga-Calderé et al. 1999; de Jong et al. 1993) because of two different types of approaches. Whereas electron microscopic analysis of synaptonemal complexes of potato (+) tomato fusion hybrids revealed homoeologous pairing, traditional staining methods and GISH of metaphase I chromosomes indicated a lack of pairing and crossing-over (Jacobsen et al. 1992; Garriga-Calderé et al. 1999). In both of these investigations on fusion hybrids the involved species were too distant from a phylogenetic point of view. With the main aim of elucidating whether or not homoeologous recombination can be demonstrated in plants with centromeric heterochromatin, the F_1 hybrids between *Lycopersicon esculentum* and *L. pennellii* and its backcross progeny were investigated through GISH. The main stimulus for this investigation was the report on the demonstration of homoeologous recombination between two closely related species such as *L. esculentum* and *L. peruvianum* (Parokonny et al. 1997). The results on the use of a stringent GISH method are described and discussed in relation to the difficulties associated with plants with centromeric heterochromatin as well as closeness of phylogenetic differentiation.

Materials and Methods

Plant material

The *Lycopersicon esculentum* and *Lycopersicon pennellii* accessions were obtained from the Laboratory of Plant Breeding, Wageningen University. The F_1 population (94166) was derived from a cross between *L. esculentum* cv. (Money Maker) and *L. pennellii* (LA 716). The BC_1 population (PV 95742) was a backcross of Money Maker used as female parent with the F_1 (94166-1) used as pollen parent. All the plant material was grown under greenhouse conditions. The F_1 hybrids and BC_1 population, both were highly fertile and backcrossing was successful in F_1 hybrids creating BC_1 progenies. A large progeny was obtained from seeds in the F_1 and BC_1 plants but only five genotypes from the F_1 (94166-1, 94166-2, 94166-3, 94166-4, 94166-5) and five genotypes from the BC_1 (PV 95742-1, 95742-2, 95742-3, 95742-4, 95742-5) were used in this investigation.

Chromosome preparations

For somatic chromosome preparations, roots were harvested from in vitro plants and meiotic preparations were made from fresh anthers, collected from plants in the greenhouse. Roots were collected in the morning and treated with 2mM 8-hydroxyquinoline overnight at 18°C in the dark. Chromosome preparations were made according to the method described by Zhong et al. (1996) with some modifications. The duration of treating the roots with 2mM 8-hydroxyquinoline instead of 6 hours was increased to over night.

Genomic in situ hybridisation (GISH)

The routine GISH procedures and pre-treatment of the selected slides was carried out according to Kuipers et al. (1997) with modifications. The treatment time of chromosome preparations with RNase was increased from one hour to two hours at 37°C in humid chamber. The pepsin treatment was extended to 30 minutes instead of 10 minutes at 37°C in humid chamber. With the standard protocol for in situ hybridisation it was not possible to differentiate between the two genomes of *L. esculentum* and *L. pennellii*, so modifications were made at hybridisation mixture and stringency washings steps. The high degree of cross hybridisation due to sequence homology between these two genomes made it very critical to standardise the procedure for differentiation.

The test experiments showed that using the DNA of *L. pennellii* as a labelled probe instead of *L. esculentum* gave good labelling and differentiation. The genomic DNA of *L. pennellii* was sonicated to a size of 5-10kb and labelled with digoxigenin following a standard nick translation protocol (Boehringer Mannheim). Genomic DNA of *L. esculentum* was used as blocking DNA after autoclaving to get a fragment size of 100-500bp. Test experiments showed that when blocking DNA exceeded the concentration of probe DNA by twenty folds a satisfactory level of differentiation could be achieved. This proved to be the critical factor and variations to this ratio did not give more optimal results. The stringency in the hybridisation mixture was achieved by increasing the formamide concentration from normal 50% to 60% (v/v).

Detection of digoxigenin-11-dUTP was done with anti-dioxigenin-fluorescein (20μg/ml) (Boehringer Mannheim) and amplification was done by rabbit-anti-sheep fluorescein (20μg/ml) (Vector laboratories). The stringency at post hybridisation washing was maintained at 73% [2x SSC at 42°C for 40 minutes]. Selected cells were photographed on Fuji 400 ISO colour negative film with an Axiophot Zeiss microscope equipped with UV light and appropriate filters. Scanned images were optimised with routinely used image processing software.

Results

The genomes of *Lycopersicon esculentum* and *L. pennellii* are highly homosequential sharing all the major families of the repeated DNA sequences. In order to determine whether or not the chromosomes of *L. esculentum* and *L. pennellii* could be clearly distinguished, and whether it was possible to detect any recombination or translocated segment between the two genomes, GISH analysis was done.

Chromosome differentiation in the F_1 hybrid as revealed by GISH

Mitotic and meiotic chromosome preparations of the five F_1 hybrids (94166/1-5) were used to verify the hybridity and to visualise any recombinant segment. Initially, the total genomic DNA of both Money Maker and *L. pennellii*, labelled with FITC were used as hybridisation probes to differentiate the respective chromosomes in the cells. Since the FITC labelled genomic probe of *L. pennellii* gave consistently better differentiation than the genomic probe of tomato, this total genomic probe was used in all cases for both somatic as well as meiotic chromosome preparations. Differentiation of the chromosomes of *L. pennellii* was clearly pronounced when the probe concentration was 100ng, with blocking DNA concentration of 2$\mu g/\mu l$ and the stringency in the hybridisation was 50% to 60% (Table 1). Any deviation from these conditions of hybridisation was not ideal for distinguishing the different genomes.

In the case of somatic metaphase cells from the F_1 hybrid (94166/1-5) a clear differentiation between the 12 chromosomes of *L. pennellii* and 12 chromosomes of Money Maker was not possible. After maintaining a precise level of hybridisation mixture and stringency washings (Table 1) considerable differentiation was achieved (Fig.1C,D). All the selected five genotypes of F_1 hybrids gave similar results so that one genotype (94166-1) was selected for further investigation. As genomic DNA of *L. pennellii* was used as a probe in this way bright green fluorescence of FITC represented *L. pennellii* chromosomes whereas the 12 chromosomes of Money Maker had faint signal because of cross hybridisation of the probe with the tomato genome (Fig.1E,F). A characteristic feature of all the FITC labelled chromosomes was that the distal parts of chromosomes (Fig.1E-arrow) were not as brightly fluorescent as the proximal parts. Such differences were also evident in Money Maker chromosomes but these were relatively less pronounced. This feature was also visible in DAPI stained chromosomes (Fig.1F-arrow). The satellite parts of both the nucleolar chromosomes were labelled because of the presence of highly conserved DNA sequences in these regions (Fig.1E,F).

Meiotic chromosome observations in F_1 hybrids were confined from late prophase I to telophase I stage. Because of the close taxonomic relationship between *L. esculentum* and *L. pennellii*, homoeologous chromosome pairing was expected to be high in F_1 hybrids. This was the case (Fig.1A) and on an average 9-10 ring bivalents were observed per cell which are expected with chiasma formation in both chromosome arms. A notable feature was that each bivalent consisted of a brightly yellow green fluorescent part and an orange-red part (Fig.1 B). This pattern obviously corresponded with FITC labelled *L. pennellii* and propidium iodide stained Money Maker chromosomes when they were paired during meiosis. This chromosome pairing behaviour obviously confirmed that there was normal pairing and crossing-over. But late prophase I or metaphase I stages were clearly not suitable to unravel the presence or absence of recombinant segments in the half-bivalents.

Table.1. Probe, blocking DNA concentrations and (%) stringency in hybridisation mixture and post hybridisation washings in the interspecific hybrid between *L. esculentum* and *L. pennellii*.

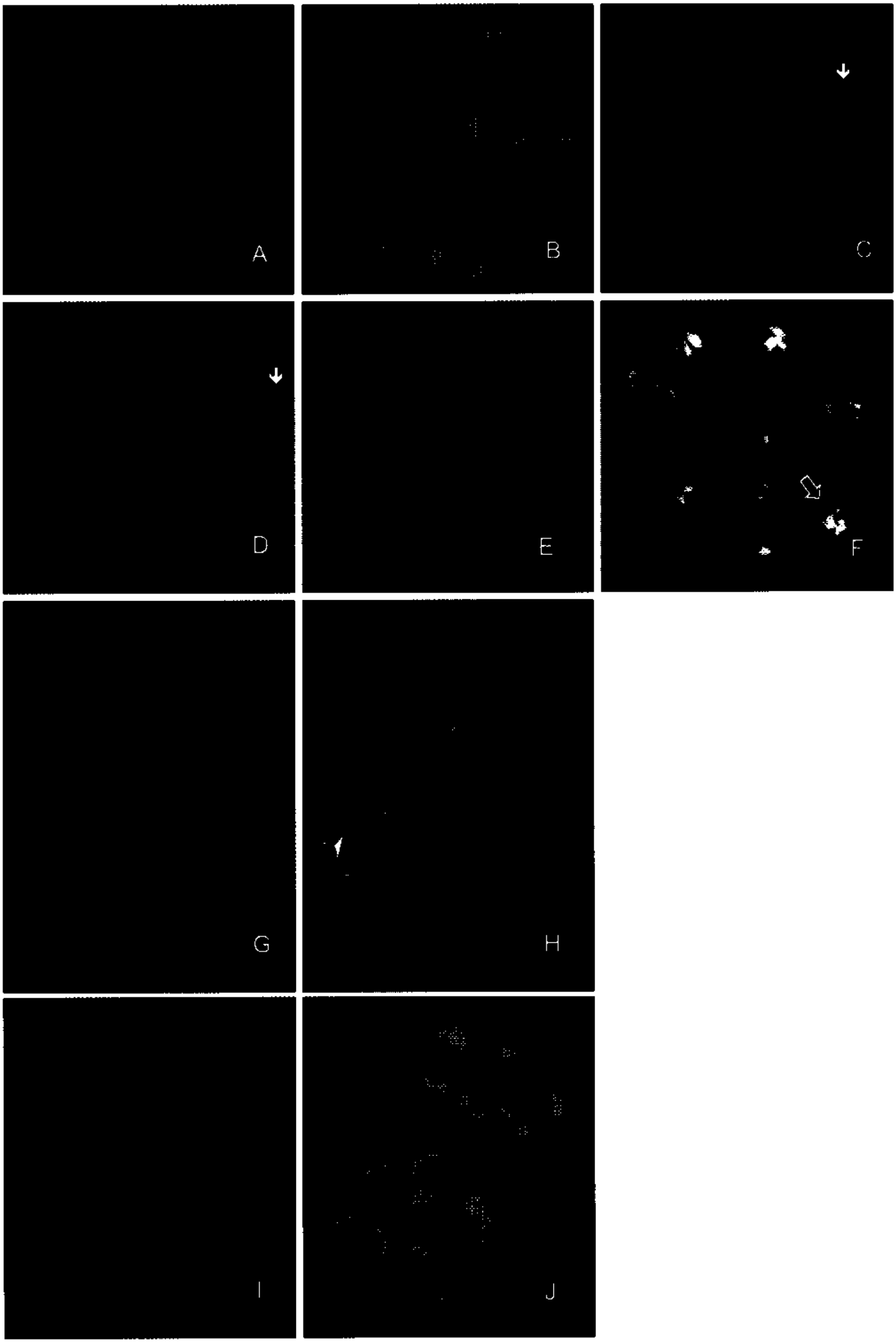
Sr.No.	Probe DNA concentration/µl	Blocking DNA concentration/µl	Stringency in hybridisation mixture (%)	Post hybridization stringency washing (2x SSC at 42°C)	Remarks
	L. pennellii				
1	50ng	2.5µg (50x higher)	50%	73%	No differentiation
2	100ng	5µg (50x higher)	50%	73%	No differentiation
3	50ng	1µg (20x higher)	50%	73%	No differentiation
4	100ng	2µg (20x higher)	50%	73%	Partial differentiation *
5	50ng	5µg (100x higher)	50%	73%	No differentiation
6	100ng	2µg (20x higher)	60%	73%	Partial differentiation*
7	50ng	5µg (100x higher)	70%	73%	No differentiation
	Tomato				
8	50ng	2.5µg	50%	73%	High cross hybridization
9	50ng	5µg	50%	73%	No differentiation

* = The chromosomes of *L. esculentum* and *L. pennellii* are not fully discriminated and there is some degree of cross hybridisation between the two genomes.

Fortunately, in *Lycopersicon* species the half-bivalent chromosomes during the late anaphase I and telophase I stage spread out very well so that the two chromatids of each half-bivalent could be clearly analysed cytologically (Fig1.H). The important advantage of analysing half-bivalents is that a non-recombinant and a recombinant chromatid are expected to be present together so that they might be detected readily. From an analysis of (20-25) pollen mother cells in the F_1 hybrid there was not a single instance in which a clear discrimination could be made between a recombinant and a non-recombinant chromatid in a half-bivalent. Almost invariably, the distal parts of the two chromatids fluoresced uniformly and were of identical size (arrowhead in Fig.1H). This led to the conclusion that the hybridisation signals of the distal parts resulted from the cross hybridisation of the probe to the highly conserved DNA sequences that are common to both of the parental species.

Chromosome differentiation in the BC_1 hybrid as revealed by GISH

By using Money Maker (LA 716) as female and F_1 hybrid (94166-1) as a pollen parent, a fertile BC_1 (PV 95742) backcross population was obtained. These BC_1 plants could easily be used for the development of BC_2 population. The observations on chromosome behaviour were restricted only to the BC_1 population. In the F_1 hybrids there were an equal number of chromosomes from each parent and pairing between the two genomes was normal with frequent bivalents. However, in the backcross progenies, there was an excess of Money Maker chromosomes as compared with *L. pennellii* chromosomes in both mitotic and meiotic cells (Fig.1I,J). The Money Maker genome is involved twice (once at fusion hybrid stage and another at backcrossing stage) consequently, the chromosomes of Money Maker are more in BC_1 progeny. In the five BC_1 genotypes the number of chromosomes with L. pennellii centromeric regions ranged from 8-10 chromosomes per cell and the rest were chromosomes with centromeric regions of Money Maker. The difference is visible with *L. pennellii* chromosomes labelled with FITC whereas Money Maker chromosomes show faint labelling (Fig.1J). Five different BC_1 genotypes and their karyotypes were investigated for this study. Morphologically all the plants looked alike with the same level of fertility. The chromosome behaviour of the five BC_1 genotypes (PV 95742/1-5) was also similar with no detectable difference in chromosome pairing between the two genomes in the sense that *L. pennellii* chromosomes paired normally with the homoeologous counterparts of Money Maker (Fig.1J). As in the F_1 hybrid, it was not


A
B
C
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J

possible even in the BC_1 to discriminate, non-recombinant chromatids from the recombinant chromatids in the half-bivalents.

Discussion

This investigation shows that the chromosomes of Money Maker and *L. pennellii* can be fairly well discriminated from each other through GISH in both mitotic and meiotic cells. The degree of differentiation is comparable to the results reported for the allodiploid involving the genomes of *L. esculentum* and *L. peruvianum* (Parokonny et al. 1997). The main difference is, however, that whereas the homoeologous recombinant parts could be detected in the case of the allodiploid of *L. esculentum* and *L. peruvianum*, we could not discriminate recombinant chromatids in the present study with *L. esculentum* and *L. pennellii*. The reason might be the differences in the stringency in the procedures used for GISH. It should be pointed out, however, that the differentiation of parental chromosomes observed by both Parokonny et al. (1997) and in the present case are much less discriminatory than the GISH results reported for potato (+) tomato fusion hybrids and their backcross derivatives (Jacobsen et al. 1995; Garriga-Calderé et al. 1997).

The level of differentiation achieved in this study was much dependent on the modifications made in the hybridisation mixture and stringency washings. The (1:20) ratio of probe with blocking DNA was very critical to achieve and maintain this discrimination between the two genomes

←

Fig.1. GISH on mitotic and meiotic chromosomes of *L. esculentum* and *L. pennellii* hybrids. **A.** Meiotic chromosomes of F_1 hybrids after DAPI. **B.** Meiotic chromosomes of F_1 hybrids with FITC signal. The distal parts of chromosomes do not hybridise completely (arrows). **C-D.** Meiotic chromosomes of BC_1 hybrids of *L. pennellii* fluoresce bright green (FITC) and Money Maker chromosomes have comparatively lighter signal. **E,F.** Somatic chromosomes of F_1 hybrids with DAPI and FITC signal. **G,H.** Half-bivalents with bright green signal at chromosome ends (arrowheads). **I,J.** The meiotic chromosomes (DAPI and FITC) of BC_1 hybrids show pairing between *L. pennellii* (bright chromosomes) and Money Maker (faint chromosomes).

In previous GISH experiments in backcross derivatives of *L. esculentum* (+) *L. peruvianum*, Parokonny et al. (1997) used a hundred fold higher concentration of blocking DNA and in *Saccharum* species D'Hont et al. (1995,1996) and in *Musa* species D'Hont et al. (2000) used equal amounts of probe and blocking DNA concentrations to achieve the differentiation. In this study it was not possible to get the differentiation unless the blocking DNA was used 20 times higher to probe DNA (Table 1). It is also important to mention that in somatic preparations the differentiation was possible to achieve at 50% stringency in a hybridisation mixture but in meiotic preparations the differentiation was achieved at 60% stringency in a hybridisation mixture (Table 1). The pre-treatments with RNase and pepsin had to be extended also to get a good labelling signal. When the temperature of stringency washings was increased beyond 73°C, it did not make any difference and often effected the morphology of the karyotypes.

In spite of clear differentiation of potato and tomato chromosomes at the centromeric heterochromatin regions it was not possible to establish conclusively whether or not homoeologous recombination can be cytologically detected through GISH. For instance Garriga-Calderé et al. (1999) concluded that there was a very low frequency, if ever, of crossing-over between the chromosomes of potato and tomato. However, abundant homoeologous pairing and exchange of pairing partners in the potato (+) tomato fusion hybrids as revealed from the analysis of the synaptonemal complexes have been reported (de Jong et al. 1993). It should be pointed, however, that the homoeologous pairing at prophase I do not guarantee chiasma formation and crossing over. In fact, analysis of synaptonemal complexes in polyploid *Gebasis* revealed homoeologous pairing but only autosyndetic bivalents were observed at metaphase I in this species (Davies et al. 1990). Similarly in potato (+) tomato fusion hybrids only autosyndetic bivalent formation was observed (Jacobsen et al. 1995).

In the context of the above situation, the following two possibilities can be assumed to occur: 1) there is very little or no crossover, between *Solanum* and *Lycopersicon* chromosomes and 2) there is homoeologous crossing over both in F_1 hybrid and BC_1 progeny but it cannot be detected through GISH. Only the heterochromatin region around centromeres is labelled and there is no crossing over in that region and the distal parts are not visibly labelled so detection of a cross over is difficult at this resolution. The latter of these is certainly the case in the present study because there is normal pairing and chiasma formation in the F_1 hybrid and BC_1 progeny but its difficult to impossible to detect any recombination through GISH analysis with the present resolution. The level of differentiation which is dependent on strength of signal from labelled probe DNA and where cross hybridisation between the two genomes sometimes makes it very critical to distinguish between the chromosomes of two genomes, detection of recombination and pairing is extremely difficult if not impossible which is even made worse because of the small chromosomes of tomato and *L. pennellii*.

Similarly, from the number of centromeric regions of *L. pennellii* estimated in the BC_1 progenies (Table 2), there appears to be an excess of alien chromosomes. Assuming a random assortment of the chromosomes of *L. pennellii*, we should expect approximately 5 to 7 alien chromosomes as the average class. However, the number of the BC_1 plants that were analysed was too small to make any definite conclusion for the presence of the number of *L. pennellii* chromosomes. Furthermore, it is questionable whether the identification of the two types of chromosomes was always accurate.

The similarities between the two genomes of tomato and *L. pennellii* sharing major repeated DNA sequences has been reported by Ganal et al. (1988) and Khush and Rick, (1963). However, in the present study we tried to discriminate between two genomes where the genome constitution and construction is very much similar. With standardising the protocol the parental genomes could be discriminated at the heterochromatin regions to a greater extent as it has been done earlier in other crops species.

Table.2. Number of chromosomes with centromeric regions of *L. pennellii* and Money Maker chromosomes in mitotic cells of BC_1 plants

BC_1 progeny	Centromeric regions counted	
	L. pennellii	Money Maker
1	8	16
2	10	14
3	8	16
4	8	16
5	10	14

Why is it difficult to detect homoeologous recombinants through GISH in *Lycopersicon* species?

There appear to be three different reasons why it might be difficult to demonstrate homoeologous recombination through GISH in *Lycopersicon* and related species. In the first place, the genome size of *Lycopersicon* is small (see Table 1, general discussion) which means that there is a relatively small amount of repetitive DNA. Secondly, about 77% of the repetitive DNA is confined to the proximal heterochromatin regions of the chromosomes (Peterson et al. 1996). This implies that relative lack of repetitive DNA sequences in the distal euchromatic regions is not favourable for discriminating homoeologous parts of chromosomes through GISH. Thirdly, close taxonomic relationship between *L. esculentum* and *L. pennellii* is a further obstacle for discriminating the chromosomes (or their parts) in the F_1 hybrid and the backcross progeny. The distal hybridisation sites observed in the half-bivalents (Fig. 1E) can be explained as due to the presence of highly conserved telomeric and telomere related DNA sequences that have been demonstrated to occur in tomato (Zhong et al. 1998). In view of this, it may be concluded that the procedure of GISH used in this study was unable to resolve homoeologous recombination between *L. esculentum* and *L. pennellii* even when we were able to discriminate the centromeric heterochromatin. In view of the absence of a cytological method for evaluating the genetic consequences of using *L. pennellii* as a bridge species (chapter 4) the use of molecular mapping methods such as RFLPs and AFLPs could be the alternatives.

6

General Discussion

The results presented in the four experimental chapters of this thesis mainly address the question of how to make use of the potato (+) tomato hybrids, and their backcross derivatives, and ultimately pave the way for introgression of tomato traits into potato. In this context, two main aspects including the establishment of a full series of monosomic alien tomato addition genotypes and the prospects of homoeologous recombination between alien chromosomes will be discussed in the following pages.

Despite belonging to two different genera within the Solanaceae, potato and tomato have been shown to possess nearly homosequential genomes. This has been established through the comparison of RFLP linkage maps (Bonierbale et al. 1988 and Tanksley et al. 1992), as well as through comparison of pachytene chromosome morphology (Ramanna and Wagenvoort, 1976). In view of this homoeology, it is attractive to transfer the wealth of desirable characters from *Lycopersicon* to *Solanum* species and vice versa. The establishment of the complete monosomic alien tomato chromosome addition series was, therefore, considered as useful. Based on pairing behaviour of homoeologous chromosomes during early meiotic prophase I stages there is clear evidence that the chromosomes of potato and tomato do pair (de Jong et al. 1993). However, there seems to be a very low frequency of cytological crossing over between the homoeologous chromosomes of the alien genomes (Garriga-Calderé et al. 1999). This obviously means that the alien additions are helpful to add entire tomato chromosomes into the potato genome rather than recombinant segments. However, monosomic addition series can be helpful to induce chromosome specific translocations between tomato and potato chromosomes if an efficient selection procedure for this purpose becomes available in the future.

Not unexpectedly, the frequencies of transmission of alien tomato chromosomes through gametes are highly variable (Table 4, chapter 2). These transmission rates were estimated only for female gametes because of the high degree of male sterility in the fusion hybrids as well as in the backcross derivatives. Generally in other plant species, the largest chromosomes of the genome were reported to have a lower transmission rate (Khush, 1973). This does not seem to be the case in the rates of transmission of alien tomato chromosomes into potato genotypes. The frequency of transmission of tomato chromosomes appears to be influenced by the genotype as well as chromosome morphology. For example the nucleolar chromosome (chromosome 2) was found to be transmitted at a very high frequency from BC_1 to BC_2 (Garriga-Calderé et al. 1998) but it was not so in the case of BC_2 to BC_3 transmission (chapter 3). This probably indicates the influence of the individual genotype. On the other extreme, chromosome 6 of tomato has been found to be transmitted at a high frequency consistently. In one situation it was transmitted in a frequency of 100% (chapter 3). The reason for this is not clear, but it should be emphasised that chromosome 6 of tomato has the lowest amount of heterochromatin when compared to others (Ramanna and Prakken, 1967). In any case, there appears to be very little or no relationship between the chromosome size and the rate of their transmission through the female gametes.

In order to facilitate more or less regular transmission of alien chromosomes from parent to progeny, disomic addition genotypes are useful in many other plant species. This is because, when a pair of homologous alien chromosomes are present, they are expected to pair as bivalents, disjoin regularly at anaphase I and thus behave normally during meiosis. The disomic additions found in the present investigations did not always fulfil these expectations. This was probably because of the precocious separation of the bivalent of the alien pair of chromosomes in relation to those of potato. Obviously, the univalents of tomato chromosomes are distributed haphazardly during meiosis and their gametic transmission is irregular. In view of the asynchrony associated with the disjunction and distribution of potato and tomato bivalents, the disomic addition line of tomato chromosome 11 appeared to be not useful in the genotype that was used in these investigations. One of the drawbacks of the potato genotypes possessing alien tomato chromosome additions is that they are tetraploid. Because of the polysomic condition, the phenotypic expression of any of the alien genes in these genotypes is not visible. It was tested whether or not a wild type tomato

Amf gene (Amf_t) can express its phenotype in a nulliplex genotype (amf_p^4) of potato. It was attempted to introduce the wildtype tomato Amf_t gene on chromosome 8 into a nulliplex (amf_p^4) genotype of potato through repeated backcrossing of the potato (+) tomato somatic hybrid. It should be kept in mind that potato and tomato genomes are homosequential and the amylose-free locus (*Amf*) shares the homoeologous position in potato and tomato on the respective linkage maps (Gebhardt et al. 1991, Jacobs et al. 1995). This attempt was not successful because the alien tomato chromosome 8 was not transmitted in a tetraploid nulliplex (amf_p^4) potato background. This research showed in addition that a single wildtype (Amf_p) allele in a different genetic background showed different levels of GBSS expression and amylose synthesis resulting in two starch phenotypic patterns i.e. after iodine staining, the starch granules were blue or red with a blue core (chapter 3). In another genetic background the same alien chromosome 8 was transmitted at a much higher frequency (22.2%, chapter 2) from BC_2 to BC_3 progeny.

It must be pointed out that the flower colour and the joint anthers of tomato are distinct characters that might be tested for the phenotypic expression in the backcross derivatives. The yellow colour is present only in *Lycopersicon* and not in *Solanum*. This character is neither visible in the fusion hybrid nor in backcross progenies except when *Lycopersicon* is crossed or fused with white flowering *Solanum*. For the expression of this trait, the tomato chromosomes possessing this trait should be present in the progeny. Expression of some characters of *Solanum lycopersicoides* has been achieved at the diploid level in tomato (Rick et al. 1951; Chetelat et al. 2000). In another example, the fusion hybrids of potato and tomato containing Cf-9 gene when induced with the avirulence gene product (*Avr*) showed a hypersensitive reaction in fusion hybrids and BC_1 progenies (unpublished results).

In order to achieve the above stated aim of introgressing specific tomato traits, the trigenomic hybrids obtained by crossing a 4x-potato (+) tomato fusion hybrid with diploid *L. pennellii* is a significant event. Such crosses offer the potential for establishing alien addition lines at the diploid level in addition of offering the prospects of "bridging" the chromosomes of potato and tomato. Considering the phylogenetic position of *L. pennellii*, which occupies a position between *Lycopersicon* and *Solanum*, there is reason to expect that the chromosomes of *L. pennellii* might have an affinity to pair with the chromosomes of both *Solanum* and *Lycopersicon*. Meiotic analysis of the trigenomic hybrid certainly vindicated this expectation. Considering meiotic pairing at metaphase I (chapter 4), there is a clear indication that recombination can be expected between the chromosomes of *Solanum* and *Lycopersicon*. Comparable results were achieved already in the case of *Solanum lycopersicoides* and tomato (Rick et al. 1988). This means as shown in the last analysis, that the introduction of recombinant segments, is still far from being successful.

For monitoring whether or not alien recombinant segments are present in the backcross derivatives, it would be most convenient if the alien segments can be cytologically identified through GISH. Such identification can indeed be most helpful to assign genetic loci or molecular markers to the introgressed alien recombinant segments. Cytological identification of recombinant segments has been very clearly demonstrated in sexual plant species hybrids with large chromosomes (for review see Jiang and Gill, 1994) through genomic in situ hybridisation. But most convincingly, through the analysis of meiosis in the interspecific hybrids and the karyotype analysis in the backcross progenies, the identification of recombinant segments has been reported in *Alstroemeria* (Kamstra et al. 1999), *Lilium* (Lim et al. 2000) and *Allium* (Khrustaleva and Kik, 2000). In these cases, obviously the introgression can be monitored by GISH very conveniently and accurately in the backcross progenies. The question remains whether the recombinant segments can be detected also in the case of plants with small chromosomes, in other words, in plants with very small genomes. One important feature of small genomes is that they possess relatively small amounts of repetitive DNA. This is less favourable for resolving differences between genomes of distantly related plant species with small chromosomes.

Besides the small genome size, another important feature might be the deciding factor for resolving recombinant segments in Solanaceous plants that possess rather small chromosomes. This is the pattern of distribution of repetitive DNA on the individual chromosomes. In numerous plants with small chromosomes, repetitive DNA is present mostly in the form of centromeric heterochromatin (also called pericentric heterochromatin) and tomato and potato are typical examples among these (Ramanna and Prakken, 1967; Ramanna and Wagenvoort, 1976). In such cases, the euchromatin regions are present in the distal parts of chromosomes and possess relatively much less repetitive DNA. It is doubtful whether homoeologous recombinant segments can be clearly detected through GISH in such cases. It is important to point out that the centromeric heterochromatin region in these small genomes could still be distinguished. The differentiation pattern between the two genomes makes it possible to identify *L. pennellii* chromosomes separate from chromosomes of Money Maker. Some examples of plants in which the genomes and chromosomes are very small and probably are less favourable for detecting alien recombinant segments through GISH are described in Table 1.

With the exception of the so-called allodiploid hybrid between *L. esculentum* and *L. perviunum* (Parokonny et al. 1997), in none of the plant species listed in Table 1, the recombinant segments have been described. Our attempt to identify recombinant segments in the F_1 hybrid between *L. esculentum* and *L. pennellii* (chapter 5) through GISH, was not successful although some degree of chromosome differentiation was observed between the parental genomes. Being closely related species with normal meiotic pairing, the half-bivalents were expected to show recombinant segments. In contrast to our results, Parokonny et al. (1997) did report on the clear differentiation of distal recombinant segments. They also assumed that chiasma formation occurred in the heterochromatic parts of chromosomes. Available evidence from the traditional cytological approach (Khush and Rick 1968) as well as from synaptonemal complex observations is that recombination is either absent or highly restricted in the proximal heterochromatic regions. In view of this, it should be concluded that, contrary to the observations of Parokonny et al. (1997), it is not possible to detect recombinant segments in the BC progenies and from our observations we do not agree with their findings on detecting recombination. In the absence of cytological methods for the detection of alien recombinant segments and phenotypically non-expressing alien traits, molecular mapping methods such as RFLP and AFLP analysis are the best alternatives. As far as the addition of whole chromosomes is concerned, their cytological detection through GISH is not a problem in the case of hybrids involving distant species such as potato and tomato or tomato and other *Lycopersicon* species. The molecular markers (e.g., RFLP, AFLP) are a good tool to see if introgression based on crossingover has taken place.

From the present investigations it is clear that fusion hybrids can be produced and utilised in backcrossing programmes. In case of backcrossing of the hexaploid fusion hybrid with four genomes of potato and two of tomato, once the first backcross was

Table.1. Some examples of plant species that possess a very small genome size, small chromosomes and unequal distribution of repetitive DNA sequences on chromosomes (i.e. differentiated into centromeric heterochromatin and distal euchromatin) and the outcome of GISH/FISH

Species/hybrids	Genomic size 2C DNA value in pg*	Chromosome differentiation	References**
Arabidopsis	0.3	Not clear	Fransz et al. 1996
Coffee arabica	2.4 - 2.6	Differentiated	Pinto-Maglio and de Cruz, 1998 Lashermes et al. 2000
Musa accuminata X *Musa balbisiana*	1.2 - 1.3 1.1	Differentiated Differentiated	D'Hont et al. 2000 Osuji et al. 1997
Beta vulgaris	1.5-1.6	Differentiated	Mesbah et al. 2000
Solanum tuberosum (2x)	1.8	Differentiated	Ramanna and Wagenvoort, 1976
Solanum tubersoum (+) *Lycopersicon esculentum*	1.8 1.5 - 2.0	Differentiated Differentiated	Ramanna and Wagenvoort, 1976 Ramanna and Prakken, 1967 Jacobsen et al. 1995
Lycopersicon esculentum X *Lycopersicon peruvianum*	1.5- 2.0 2.3	Differentiated	Parokonny et al. 1997
Oryza latifolia (4x)***	Not available	Not known	Fukui et al. 1997
Lycopersicon esculentum x *Lycopersicon pennellii*	1.5 - 2.0 2.5 - 2.8	Partly differentiated	This thesis (chapter 5)

* = values mentioned in Bennett and Leitch, 1995

** = include references on chromosome differentiation and GISH results

*** = the diploid species *O. sativa* has less then 1.0 pg of 2C DNA value obtained the subsequent backcrossing with potato was easy. This was probably due to the presence of the excess number of potato genomes in the 6x- fusion hybrid and the BC_1 plants. A similar trend was demonstrated when hexaploid somatic hybrids were obtained from *S. brevidens* (+) *S. tuberosum* fusion and pentaploid progeny was derived from the sexual cross with tetraploid potato (Helgeson et al. 1993). The situation was different in somatic fusion hybrids of potato (+) *S. nigrum* species (Horsman et al. 1999). In that case, the backcross progenies of potato (+) *S. nigrum* fusion hybrids were extremely sterile so that almost no further crossing was possible. A similar situation was found in the case of 4x- potato (+) tomato fusion hybrids with *L. pennellii*. The trigenomic hybrids were completely sterile and the somatic chromosome doubling did not restore the fertility either (chapter 4). Such high degree of sterility is indeed a formidable barrier for further crossing of either the trigenomic hybrid or its chromosome doubled form. It should be pointed out, however, that in the trigenomic hybrid we were able to test only a very limited number of genotypes. If this work has to be continued, it might be pertinent to use several genotypes of 4x- potato (+) tomato fusion hybrids in order to sexually hybridise with a multitude of accessions of the so-called *Neolycopersicon* species such as *Solanum lycopersicoides*, *L. pennellii* or the *Solanum* species of the *Etuberosa* section. Even a cursory survey of the literature suggests that a small fraction of the most sterile interspecific hybrids or triploids can produce fertile gametes. Utilisation of such gametes can be useful for introgression for two main reasons. It is known that in the case of 3x X 2x crosses, near diploid progeny can be obtained (reviewed by Brandham 1982). Such progenies can be potentially useful for producing alien addition or substitution lines at the diploid level as well as for introgression. Secondly, in the case of 3x X 4x crosses near tetraploid or even pentaploid progenies can be obtained in case 2n-gametes are produced by the 3x parent. Numerous instances of the occurrence of such progenies have been reported in plants (Brandham, 1982; Kuspira et al. 1986). However, these above-mentioned approaches might appear to be laborious and difficult, in order to utilise the somatic fusion hybrids in breeding. The investigations presented in the present thesis and a series of other studies reported from our research group (Jacobsen et al. 1993; 1994; 1995; Garriga-Calderé et al. 1997; 1998; 1999) indicate that potato (+) tomato fusion hybrids are directly useful for the creation of monosomic additions bearing important traits like resistances or *Amf*. The introgression of such traits by crossingover requires much more work. At least, these investigations have demonstrated that the fusion hybrids of potato and tomato are not as recalcitrant as were originally found by the pioneering work of Melchers et al. (1978).

The construction of monosomic alien addition lines though is a very cumbersome and lengthy process. But it opens many avenues of fundamental research and practical breeding possibilities. The alien chromosome in its own could be characterised and mapped for gene isolation. The classical cytological studies and molecular studies like BAC's and YAC's localisation on these alien chromosomes can help in chromosome walking for a target gene or unravelling the entire chromosomes constitution. The breeding programmes can benefit and already have been benefiting from the introduction of useful genes that furnish the new cultivars with various kinds of disease resistances and tolerances such as heat tolerance. The increase in yields and hybrid vigour of plants has already played a greater part in revolutionising agriculture.

Summary

The discovery of producing somatic fusion hybrids between distantly related non-crossable, taxa was a momentous event. Nevertheless, in most of the cases the fusion hybrids could not be utilised in introgression breeding simply because they could not be backcrossed to any of the fusion parents. One illustrative example was the somatic fusion hybrid between potato (*Solanum tuberosum*) and tomato (*Lycopersicon esculentum*) which was never successfully backcrossed to any of the parents. The first breakthrough was achieved at the Laboratory of Plant Breeding of Wageningen University when a hexaploid genotype (2n=6x=72) with four genomes of potato and two of tomato (PPPPTT) was successfully backcrossed to the cultivated potato (PPPP). The pentaploid BC_1 progeny (2n=5x=60; PPPPT) was again backcrossed to potato in order to produce BC_2 progenies. These achievements, obviously, opened the attractive possibilities of establishing potato genotypes with the addition of individual tomato chromosomes. Because the chromosomes of both potato and tomato are very small, the identification of potato genotypes with individual alien tomato chromosomes was a formidable task.

Fortunately, however, the recently developed molecular biological techniques have enabled the identification of genomes and individual chromosomes with great accuracy and efficiency. These techniques include, among others, genome analysis by molecular markers, amplified fragment length polymorphism (AFLP) restriction fragment length polymorphism (RFLP) and in situ hybridisation (ISH). In view of the availability of the unique plant material as well as efficient molecular techniques for chromosome identification, an attempt was made to elucidate certain basic aspects of introgression of chromosomes and genes from the genus *Lycopersicon* into *Solanum*. As a first step, an attempt was made to establish a complete series of alien monosomic addition lines of tomato chromosomes in a tetraploid potato background. By combining RFLP and GISH techniques, in earlier research on BC_2 populations, seven of the possible 12 alien tomato monosomic addition lines were identified. This investigation also revealed that there was only a very low frequency of intergenomic recombination between potato and tomato chromosomes.

The main aims of the present series of investigation, that constitute this thesis, were the following; a) to complete the series of monosomic alien tomato addition lines by identifying the remaining five; b) to gain more insight into the possible phenotype in a nullisomic potato background of a known genetic locus (amylose-free) present on one of the monosomic addition chromosome; c) to explore the possibilities of increasing the prospects of intergenomic recombination between potato and tomato chromosomes by crossing with a bridge species and d) to test whether homoeologous recombination segments can be cytologically detected, i.e., through GISH, so that the alien segments can be monitored in individual potato chromosomes for the purpose of introgression of tomato encoded traits in potato.

Using three different BC_3 populations consisting of 105 plants, all the remaining five alien tomato chromosome additions for chromosome 3,5,7,9,11 were identified. For this purpose, as in the previous studies, a combination of RFLP and GISH analyses proved to be most effective. One of the difficulties was that due to highly variable frequencies of transmission of alien chromosomes from BC_2 plants to BC_3 progenies, the selection and identification of all desired monosomic additions was not always easy. For example, specific alien chromosomes of tomato were found to be transmitted at an extremely high frequency so that it was a hindrance to select for a single monosomic addition for other chromosomes. Nevertheless, because of the availability of chromosome specific RFLP probes, a pre-selection of the BC_2 populations was made in order to maximise the chance of selecting the required monosomic alien addition lines. Because the RFLP analysis cannot discriminate between a monosomic and a disomic addition of the same chromosome, GISH analysis was used to determine whether a single or a pair of chromosomes was present in a particular genotype. This led to the discovery of a disomic addition for chromosome 11 of tomato. Meiotic analysis of this disomic addition had a tendency for precocious separation of the paired homologous alien chromosomes at metaphase I and anaphase I stages. Because of this, the alien chromosome was not always regularly included in all the nuclei at telophase II stages. During the course of this investigation, not just one but several genotypes were identified for some of the alien chromosome additions. Unlike the traditional cytogenetic approach, the present strategy of pre-selecting the BC_2 plants through RFLP was proven to be most efficient for the rapid completion of the monosomic series.

The present monosomic alien addition series has been established in the background of tetraploid potato. This high ploidy level is a drawback for the phenotypic expression of alien tomato genes. One approach of testing the phenotypic expression of an alien gene is to introduce a known dominant alien gene into a nulliplex potato background. To this end, an attempt was made to introduce the amylose-free wildtype locus of tomato (Amf_t), which is involved in the synthesis of amylose during starch formation, into a nulliplex (amf_p^4) genotype of potato. It is generally known that amylose-free starch is staining red and amylose-containing starch is blue staining with an iodine solution. By monitoring the starch phenotype in potato tubers of selected plants from BC_3 population through potassium iodide staining, considerable variation for iodine stained starch granules was expected and observed (chapter 3). The Amf_t is present on chromosome 8 of tomato that is homoeologous to chromosome 8 of potato. Unfortunately, the complementation for chromosome 8 of potato was not achieved through chromosome 8 (Amf_t) of tomato because of its too low transmission frequency. Despite this absence, two types of starch phenotypes were attributed to the simplex $Amf_p amf_p^3$ condition, clearly indicating variable expression of the wildtype Amf_p-allele in different genetic backgrounds. The two starch phenotypes observed consisted of blue or red starch granules with a blue core.

One of the important prerequisites for stable introgression of alien characters into a crop species is the introduction of recombinant alien chromosome segments rather than whole chromosomes. However, previous investigations by our group have shown that very little, if any, homoeologous recombination occurs between the chromosomes of potato and tomato. In order to overcome the lack of recombination between potato and tomato genomes, the use of a "bridge species", *Lycopersicon pennellii* was tested. For this purpose, the tetraploid potato (+) tomato fusion hybrid was successfully crossed with the diploid *L. pennellii* which gave rise to a trigenomic hybrid (chapter 4). The trigenomic hybrid consisting of one genome each of potato, tomato and *L. pennellii* indeed showed trivalent formation at metaphase I stage during microsporogenesis. Despite this encouraging result, this triploid hybrid could not be used for further crossing due to its sterility. After chromosome doubling through tissue culture of this triploid a typical hexaploid was produced. Despite the expected normal meiotic chromosome pairing, the hexaploid proved to be sterile as the triploid from which it was derived. In view of this difficulty, it is necessary to use a greater number of accessions of L. *pennellii* to cross with the tetraploid fusion hybrids and to test the trigenomic hybrids for fertility.

In spite of the evidence for homoeologous chromosome pairing in the trigenomic hybrids at metaphase I stage, it was not possible to demonstrate through GISH the presence of recombinant segments during meiosis. In order to test whether it is possible at all to cytologically identify recombinant segments, an investigation was carried out by using F_1 hybrids between L. *esculentum* and L. *pennellii* and its backcross progeny (chapter 5). Because these two species are relatively closely related the homoeologous chromosomes paired and crossing over as well as recombinant segments were certainly expected in the meiotic product of their F_1 hybrids. A detailed GISH analysis of both mitotic and meiotic chromosomes indicated that in the case of small chromosomes such as found in tomato and L. *pennellii* it is not possible to differentiate recombinant segments in a comparable way to those found in hybrids and their backcrosses in species with large chromosomes (e.g., *Alstroemeria* and Lily). However, in case of tomato x L. *pennellii* hybrids it was possible to visualise and differentiate the heterochromatic region around the centromeres of the chromosomes and we could differentiate between the chromosomes to these two genomes following the GISH hybridisation pattern. In the absence of cytological detection methods to prove introgression, an alternative has to be used such as RFLP and AFLP molecular mapping methods.

It might be concluded from the series of investigations of the potato (+) tomato fusion hybrids that have been conducted so far that: a) the fusion hybrids are not as recalcitrant for backcrossing as was originally conceived; b) potato genotypes do retain all alien tomato chromosomes to a reasonable extent (i.e., no preferential elimination) and that these alien chromosomes are transmitted to progenies; c) there is very little or no homoeologous recombination between potato and tomato chromosomes; d) the use of the bridge species, L. *pennellii* can facilitate exchange of genetic material between potato and tomato after sterility problems have been solved. In principle, potato (+) tomato fusion hybrids can be used for introgression, though the process to come to it is laborious.

The impact of monosomic addition lines is their enhanced agronomic value and their contribution in fundamental studies. The monosomic addition lines facilitate the transfer of interesting traits into cultivated species. With the advent of new techniques and the construction of addition lines the barriers to somatic hybridisation are decreasing. As mentioned in the general introduction (chapter 1: table 1 and 2) there are examples for the beneficial use of somatic hybrids and monosomic addition lines for transferring various kinds of disease resistances and abiotic tolerances. For fundamental studies monosomic addition or substitution lines provide opportunities for physical mapping, chromosome and gene characterisation, cytological and molecular studies and localisation of BAC's and YAC's on alien chromosomes for gene isolation.

Samenvatting

De ontdekking van de mogelijkheid om bij planten protoplasten van ververwijderde, niet kruisbare soorten, te kunnen fuseren en tot plant te regenereren is belangrijk geweest. Echter, in de meeste gevallen konden de verkregen somatische hybriden voor introgressie van eigenschappen via herhaalde terugkruising met één van de ouders niet of niet eenvoudig gebruikt worden. Eén van de meest illustratieve voorbeelden hiervan is de fusie tussen aardappel (*Solanum tuberosum*) en tomaat (*Lycopersicon esculentum*), die tot nu toe niet succesvol met één van de oudersoorten terug te kruisen was. De eerste doorbraak werd bij het Laboratorium voor Plantenverdeling bereikt toen een hexaploide aardappel-tomaathybride ($2n=6x=72$) met vier genomen aardappel en twee genomen tomaat (PPPPTT) succesvol teruggekruist werd met de normale autotetraploide (4x; PPPP) aardappel. De pentaploide BC_1 nakomeling ($2n=5x=60$; PPPPT) werd weer met aardappel teruggekruist om vervolgens BC_2 nakomelingschappen te produceren. Deze successen openden aantrekkelijke mogelijkheden om aardappelgenotypen te verkrijgen met een extra tomatenchromosoom. Op deze wijze kan er geselecteerd worden op aardappelgenotypen die chromosoom 1 t/m 12 van tomaat één voor één extra aanwezig hebben. Omdat de chromosomen van zowel aardappel als tomaat erg klein zijn is de identificatie van aardappelgenotypen met zo'n specifiek extra tomatenchromosoom niet eenvoudig.

Gelukkig hebben recente ontwikkelingen in de moleculaire biologie het herkennen van genomen en individuele chromosomen mogelijk gemaakt. Deze moleculaire technieken omvatten onder andere de genoomanalyse mbv de moleculaire merker analyse (Restriction Fragment Length Polymorphism; RFLP) bij de genetisch en de genomische in situ hybridisatie (GISH) techniek bij de moleculair cytogenetische analyse van planten. Met het oog op de aanwezigheid van het eerder aangegeven unieke plantmateriaal en de genoemde moleculair genetische technieken voor de identificatie van genomen en individuele chromosomen werd een begin gemaakt om tot introgressie van individuele chromosomen of delen ervan van het genus *Lycopersicon* in dat van *Solanum* te komen. Als eerste stap werd een begin gemaakt om tot een complete serie van monosome additielijnen van individuele tomatenchromosomen in een tetraploide aardappelachtergrond te komen. Door het moleculaire merkeronderzoek en de GISH technologie te combineren waren in een eerder stadium van het onderzoek zeven van de twaalf monosome addities van tomaat in een aardappelachtergrond van enkele BC_2 populaties geidentificeerd. Dit eerdere onderzoek gaf ook al duidelijk aan dat de frequentie van het optreden van recombinatie tussen aardappel en tomatenchromosomen (intergenoomrecombinatie) via overkruising tijdens de meiose erg laag was.

De hoofddoelen van het huidige onderzoek dat tot dit proefschrift geleid heeft waren: a) de serie monosome addities van chromosomen van tomaat in een aardappelachtergrond compleet te maken; b) meer inzicht te verkrijgen in het mogelijke fenotype van een bekende genetische eigenschap (amylose-vrij zetmeel) die afwezig is bij aardappel en aanwezig op één van de monosome additiechromosomen van tomaat; c) de mogelijkheden van het vooruitzicht op intergenomische recombinatie tussen aardappel en tomatenchromosomen te exploiteren door kruising met een brugsoort; d) de mogelijkheid te onderzoeken of, chromosoomsegmenten verkregen via homoeologe chromosoom recombinatie, cytogenetisch detecteerbaar gemaakt kunnen worden of niet. Op deze wijze zouden vreemde chromosoomsegmenten in aardappelchromosomen waargenomen kunnen worden met het doel tot introgressie van tomaatgecodeerde eigenschappen in aardappel te komen.

De ontbrekende vijf chromosoomaddities van tomaat coderend voor de chromosomen 3,5,7,9,11 werden in drie verschillende BC_3 populaties met 105 planten geidentificeerd (Hoofdstuk 2). Voor dit doel was, zoals eerder aangegeven, de combinatie van RFLP en GISH analyses het meest effectief. Eén van de problemen was de selectie en identificatie van alle gewenste monosome addities, die als gevolg van de erg variabele frequentie van transmissie van de soortsvreemde chromosomen in BC_2 of BC_3 nakomelingschappen niet altijd eenvoudig maakte. Er werd bijvoorbeeld gevonden dat de transmissie van specifieke chromosomen van tomaat zo hoog was dat zij een barriere vormden voor de selectie van monosome additties van andere chromosomen. Niettemin kon door de beschikbaarheid van chromosoomspecifieke RFLP probes een voorselectie in de BC_2 populaties gemaakt worden om de kans op de selectie van de gewenste monosome additielijnen te optimaliseren. Omdat de RFLP analyse geen verschil kan maken tussen een enkelvoudige (monosoom) en een dubbele (disoom) additie van het zelfde chromosoom werd de GISH analyse gebruikt om vast te stellen of er daadwerkelijk sprake was van de aanwezigheid van een monosome of disome additie in een specifiek genotype. Dit leidde tot de ontdekking van een disome additie voor chromosoom 11 van tomaat. Meiotische analyse van deze disome additie liet de tendens van een te vroege afstoting van de gepaarde homologe chromosomen van tomaat in de metafase I en anafase I zien. Door deze afwijking werd het soortsvreemde chromosoom niet altijd in alle kernen van het telofase II stadium opgenomen.

Gedurende de loop van het onderzoek werden niet altijd één maar vaak meerdere genotypen per monosome additie geidentificeerd. In tegenstelling tot de traditionele cytogenetische benaderingswijze was de huidige benaderingswijze gebaseerd op voorselectie met RFLPs van BC_3 planten het meest efficiënt om tot een snelle aanvulling van een volledige monosome serie te komen.

De huidige serie monosome addities is in een tetraploide achtergrond van aardappel gemaakt. Dit hoge ploidieniveau is echter nadelig voor de fenotypische expressie van de genen van tomaat. Een benaderingswijze om de fenotypische expressie van een tomatengen te testen is een bekend dominant gen van tomaat in een nulliplex aardappelachtergrond te plaatsen. Voor dit doel werden pogingen ondernomen om het amylose-vrije wildtype locus van tomaat (Amf_t), dat via het enzym korrelgebonden zetmeelsynthase voor de amylose vorming bij de zetmeelsynthese verantwoordelijk is, in een nulliplex ($amf_p{}^4$) aardappelgenotype te introduceren. Het Amf_t locus is evenals dat van aardappel aanwezig op chromosoom 8 van tomaat dat homoeoloog is met chromosoom 8 van aardappel. Het is algemeen bekend dat amylose-vrij zetmeel rood en amylose-bevattend zetmeel blauw met jodium kleurt. Door de zetmeelsamenstelling in de knollen van planten van geselecteerde BC_3 populaties met jodiumkleuring te onderzoeken werd zoals verwacht een aanzienlijke variatie in kleuring van zetmeelkorrels gevonden (Hoofdstuk 3). Helaas werd de verwachte "partiële" complementatie van het nulliplex amf_p allel niet verkregen door het uitblijven van een additielijn met chromosoom 8 van tomaat met daarop het wildtype Amf_t allel. Dit tomatenchromosoom had in deze genetische achtergrond een te lage transmissiefrequentie. Ondanks dat werden in aardappelplanten naast rood gekleurde zetmeelkorrels na jodiumkleuring nog twee typen gevonden, nml blauw en rood gekleurde zetmeelkorrels met een blauwe kern. Deze laatste categorie bleek alleen aan het simplex genotype ($Amf_pamf_pamf_pamf_p$) van de aardappel gelieerd te zijn, waarbij deze variabele expressie van het wildtype Amf_p allel aan de verschillende genetische achtergronden van de individuele aardappelplanten toegeschreven moest worden.

Eén van de belangrijste voorwaarden voor stabiele introgressie van soortsvreemde eigenschappen in een cultuurgewas is de mogelijkheid om tot de introductie en vestiging van recombinante chromosoomsegmenten van de wilde soort in één van de chromosomen van de cultuurplant te komen. Daarnaast is er de mogelijkheid van additie en substitutie van een volledig chromosoom of van een volledige chromosoomarm. Echter, eerder onderzoek in onze groep heeft aangegeven dat weinig of geen homoeologe recombinatie tussen chromsomen van de aardappel en die van tomaat optreedt. Om dit probleem het hoofd te kunnen bieden is het gebruik van *Lycopersicon pennelli* als brugsoort getest. Voor dit doel werd een tetraploide somatische aardappel (+) tomaat hybride succesvol met diploide *L. pennelli* gekruist die in een drieweghybride (trigenomic hybride) uitmonde (Hoofdstuk 4). De drieweghybride, bestaande uit een genoom van aardappel, tomaat en *L.pennelli*, liet zoals verwacht in de metafase I van de microsporogenese trivalent vorming zien. Ondanks dit bemoedigende resultaat kon deze triploide hybride niet in verdere kruisingen gebruikt worden vanwege haar hoge mate van steriliteit. Na mitotische chromosoomverdubbeling van deze triploid via weefselkweek werd een hexaploide plant verkregen. Echter, ondanks de verwachte normale chromosoom paring bleek deze hexaploid even sterk steriel te zijn als de triploide uitgangshybride. Met het oog op deze problemen, is het noodzakelijk een groter aantal accessies van *L. pennellii* te kruisen met de tetraploide fusiehybride en de verkregen drieweghybriden allereerst op fertiliteit te testen.

Ondanks het bewijs van homoeologe chromosoomparing in de metafase I bij de drieweghybriden was het niet mogelijk dmv GISH cytogenetisch de aanwezigheid van recombinante segmenten gedurende de meiose aan te tonen. Om te onderzoeken of het überhaupt mogelijk is cytologisch detecteerbare recombinatiesegmenten aan te tonen werd een onderzoek uitgevoerd met F_1 hybriden van tomaat en *L. pennellii* en hun terugkruisingsnakomelingen (Hoofdstuk 5). Omdat deze twee soorten relatief nauw verwant zijn paarden de homoeologe chromosomen normaal en werden zowel overkruising als het ontstaan van gerecombineerde segmenten zeker in de meiotische producten van de F_1 hybriden verwacht. Een gedetailleerde GISH analyse van zowel mitotische als meiotische chromosomen gaf aan dat in het geval van kleine chromosomen zoals hier bij tomaat en *L. pennelli* het niet mogelijk is gerecombineerde segmenten te identificeren die vergelijkbaar zijn met recombinaties die gevonden worden in hybriden en hun terugkruisingsnakomelingen van soorten met grote chromosomen zoals Lelie en Alstroemeria. Echter, in het geval van de kruising van tomaat met *L. pennellii* was het mogelijk in de F_1 hybride de heterochromatische regio rond het centromeer van de chromosomen van beiden soorten te onderscheiden mbv GISH. Door het afwezig blijven van een cytologische detectiemethode om introgressie aan te tonen moet een alternatief gezocht worden in de moleculaire merker technologie.

Uit het onderzoek aan de aardappel (+) tomaat hybriden kan geconcludeerd worden dat: a) de fusiehybriden niet zo erg recalcitrant voor terugkruising zijn als oorspronkelijk gedacht; b) aardappelgenotypen alle additionele tomatenchromosomen in voldoende mate stabiel aanwezig kunnen houden (geen preferentiële chromosoomeliminatie) en dat deze addities naar voldoende nakomelingen overgedragen worden; c) er geen of zeer sporadisch homoeologe recombinatie tussen chromosomen van aardappel en tomaat optreedt; d) het gebruik van de brugsoort *L. pennellii* de uitwisseling van genetisch materiaal tussen aardappel en tomaat kan helpen bevorderen nadat de steriliteitsproblemen opgelost zijn. In principe kan gezegd worden dat aardappel (+) tomaat hybriden voor introgressie gebruikt kunnen worden maar dat de weg ernaar toe lang is.

De betekenis van monosome additielijnen moet gezocht worden in hun toegevoegde agronomische waarde en in hun betekenis voor fundamenteel onderzoek. De monosome additielijnen maken de overdracht van interessante eigenschappen naar de cultuurplant mogelijk. Met de komst van nieuwe technieken en de constructie van additielijnen nemen de barrieres, die door de somatische hybridisatie opgeworpen zijn, af. Zoals in de algemene inleiding is aangegeven (Hoofdstuk 1: Tabel 1 en 2) zijn er voldoende voorbeelden van het nuttig toepassen van somatische hybriden en monosome additie of substituties voor het overbrengen van zieketeresistenties en abiotische toleranties beschreven. Voor fundamenteel onderzoek dragen de monosome addities de mogelijkheid in zich van betekenis te zijn voor fysische localisatie, chromosoom en genkarakterisatie, cytologische en moleculaire localisatie van BACs en YACs op het soortsvreemde chromosoom in het kader van genisolatie.

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Curriculum Vitae

Born on 26 May 1969, in Lahore, the heart of Pakistan, Syed Nurullah Haider Ali spent most of his life staying at different cities and towns, thanks to the nature of job of his father. Initial schooling was at different places and in 1984 from St. Bonaventure's High School at Hyderabad he completed his secondary school certificate with science subjects. Higher secondary school education was completed at F.G. Sir Syed College, Rawalpindi in 1987. Agriculture was the second most interesting subject for him so he entered Barani Agricultural College, Rawalpindi and completed his B.Sc. (Hons.) in Horticulture in 1991. He joined the University of Agriculture, Faisalabad in 1992 where he was interested and fascinated by Plant Tissue Culture studies. He completed his M.Sc. (Hons.) in 1994 after working on 'Micropropagation of Banana' and developing a protocol for virus-free in vitro production of banana plants. From November 1, 1994 to June 23, 1996 he worked at the Department of Horticulture at the same University as Research Associate in a project (Breeding for Seedless Kinnow) funded by Pakistan Science Foundation. He also joined the Ph.D. programme but in March 1997 he got the opportunity to join the Laboratory of Plant Breeding at Wageningen University, The Netherlands, to work for a Ph.D. degree in molecular cytogenetics. Presently, he has accepted a postdoctoral position at the Department of Genetics and Pathology, Rudbeck Laboratory, Uppsala University, Sweden to persue research on human cancer.

Publications

1. **Haider Ali**, MS Ramanna and E Jacobsen. 2001. Alien chromosome additions indicate taxonomic distance in Solanaceae. Proceedings fifth international Solanaceae conference. July 23-29, 2000. Botanical Garden of Nijmegen. Kotholieke University. Nijmegen. The Netherlands (in press)

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