Somatic hybrids of *Solanum tuberosum* and species of the *Solanum nigrum*-complex and their backcross progeny

Somatische hybriden van *Solanum tuberosum* en soorten uit het *Solanum nigrum*-complex en hun terugkruisingsnakomelingen

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STELLINGEN

1. Een goede groei van fusieprodukten *in vitro* zegt niets over hun groeikracht *in vivo.*
(dit proefschrift)

2. De verhouding van het aantal genomen dat de betrokken soorten bijdragen aan een fusieprodukt is van invloed op de resultaten van terugkruisingsexperimenten met dit fusieprodukt.
(dit proefschrift)

3. Het is onterecht dat fusieprodukten tot de genetisch gemodificeerde organismen moeten worden gerekend.

4. Evenals somatische verdubbeling biedt somatische hybridisatie van ver-verwante soorten in veel gevallen slechts een kleine kans op introgressie.
(Lim et al., Chromosome Research 8: 119-125)

5. Het plan van het College van Bestuur van de Universiteit van Amsterdam om het promotietraject van een vierjarige in een driejarige opleiding te veranderen door de begeleiding te intensiveren is vergelijkbaar met de verwachting dat een sporter een wereldrecord zal realiseren wanneer zijn coach daartoe gemaand wordt door de sportbond.

6. De biologische landbouw zou een extra impuls kunnen krijgen wanneer zij genetische modificatie zou accepteren.

7. Het barbecue-gen ligt op het y-chromosoom.

8. De term "kenniseenheid" is verzonnen door een optimist.

9. De wetenschappen bedrijven en niet van mensen houden dat is als een fakkel ontsteken en de ogen sluiten.
(Chinees gezegde)

Stellingen behorende bij het proefschrift "Somatic hybrids of *Solanum tuberosum* and species of the *Solanum nigrum*-complex and their backcross progeny" door Karin Horsman, in het openbaar te verdedigen op 14 mei 2001, te Wageningen.

ABSTRACT

This thesis describes the production of somatic hybrids of potato and species of the *S. nigrum*-complex, wild relatives of the cultivated potato which are potentially interesting sources of genetic variation. The resulting fusion products could be used in backcross experiments with potato which, however, had a very low success rate and stopped after the production of BC2-progeny. To improve the crossability of the backcross derivatives, the genomic compositions of a BC1 and a BC2-genotype were successfully altered by adding two haploid potato genomes by somatic hybridisation. Unfortunately crossability did not improve, although the resulting BC1-somatic hybrids could be backcrossed again with potato. Through the application of genomic in situ hybridisation (GISH) it was shown that genomic constitutions of somatic hybrids and backcross derivatives were close to or exactly the expected constitutions and that homoeologous pairing occurred in the BC1 as well as in the BC2 generation.

CONTENTS

1

General introduction

TRADITIONAL POTATO BREEDING

In plant breeding wild relatives of a cultivated species are interesting sources of genetic variation. In many instances these wild species have important characteristics that can not be found within the gene pool of the cultivated species. The traditional method of introducing a specific trait from a related species is sexual hybridisation followed by recurrent backcrossing. In potato breeding several species have been used for the transfer of desirable traits into the potato gene pool. Many of the potato cultivars bred in Europe and North America carry genes from *S. demissum*, *S. andigena* or *S. vernii* (Hermsen, 1994), mainly coding for resistance to late blight, cyst nematodes and viruses.

Often one or more reproductive barriers have to be overcome in the process of introgression of a specific trait. Depending on the type of barrier, application of, for instance, rescue pollination or the use of bridging species could be a solution to the problem. A successful example of the latter was the introgression of late blight resistance from diploid *S. bulbocastanum* (B) into tetraploid potato (T), by using two late-blight-susceptible bridging species, tetraploid *S. acaule* (A) and 2*x S. phureja* (P). The introgression scheme, which also involved doubling of triploid hybrids, resulted in the ABPT clones among which some highly resistant segregants were discovered which have been used as breeding parents (Ramanna and Hermsen, 1971; Hermsen and Ramanna, 1973).

Despite the possibilities that were created by these classical approaches, many non-tuberous *Solanum* species still cannot be sexually hybridised with potato and used for introgression breeding.

SOMATIC HYBRIDISATION

The discovery of the fusion of protoplasts opened new prospects for further broadening of the genetic base of potato because this technique offered the possibility to use distantly related species (Shepard *et al.*, 1983). Within the family of the Solanaceae somatic hybridisation has been applied in many instances, mostly for the introduction of a single interesting trait. However, the consequence of the application of somatic hybridisation is that the complete genome of the donor species is introduced into the recipient species, or a large part in case of an asymmetric fusion. In either case intensive backcrossing is inevitably but surprisingly, only a few successful somatic hybridisation experiments followed by recurrent backcrossing were described in literature. Within the Solanaceae the combinations *S. bulbocastanum* (+) potato, *S. brevidens* (+) potato and potato (+) tomato have been studied most extensively.

Somatic hybrids of S. bulbocastanum *(+) potato*

S. bulbocastanum is a source for resistance to late blight (Niederhausen and Mills, 1953) and *Meliodogyne chitwoodi* races 1 and 2 (Brown *et al.*, 1989) and to *M. hapla* (Janssen *et al.*, 1996). *S. bulbocastanum* is extremely difficult to cross with potato but, as described previously, by using bridging species it could be made accessible for potato breeding (Hermsen and Ramanna, 1973). Others choose to bypass the sexual incompatibility of these species by applying somatic hybridisation like Helgeson *et al.* (1998) who produced somatic hybrids between diploid *S. bulbocastanum* and tetraploid potato with the aim to transfer late

blight resistance. First and second generation backcrosses could easily be obtained after crossing with tetraploid potato. Late blight resistance was transferred to both generations.

Brown *et al.* (1995) analysed *S. bulbocastanum* (+) potato somatic hybrids (Austin *et al.*, 1993) and progeny for their resistance to *Meliodogyne chitwoodi* races 1 and 2 and *M. hapla*. They reported male sterility of the somatic hybrids and BC1 genotypes but crossing experiments with tetraploid potato resulted in seeds. The BC2 population of 62 plants was used for mapping the resistance to *M. chitwoodii* with RFLP's (Brown *et al.*, 1996).

Somatic hybrids of S. brevidens *(+) potato*

S. brevidens is an example of a non-tuberous Solanaceae species which has been of interest for potato breeding because of its resistance to potato leaf roll virus (PLRV), potato virus Y (PVY), potato virus X (Ehlenfeldt and Helgeson, 1987; Gibson *et al.*, 1988; Pehu *et al.*, 1990), and several bacterial strains causing *Erwinia* soft rot (Austin *et al.*, 1988) but it was not easily accessible. Numerous attempts have been undertaken to sexually hybridise *S. tuberosum* and *S. brevidens* (Ramanna and Hermsen, 1981) but no hybrids resulted from these experiments. The alternative approach of somatic fusion seemed to be much more successful, as was shown by a number of research groups (Fish *et al.*, 1988, Preiszner *et al.*, 1991; Jacobsen *et al.*, 1993, Valkonen *et al.*, 1994). Although in most instances the somatic hybrids were found to be at least partially fertile, backcross hybrids of the *S. brevidens* (+) *S. tuberosum* fusion products are not frequently reported. Some research was mainly focused on the molecular, cytogenetic and morphological analysis of the somatic hybrids (Pehu *et al.*, 1989), analysis of their resistance against PVY, PVX and PLRV (Gibson *et al.*, 1988; Pehu at al., 1990; Valkonen *et al.*, 1994), determination of nuclear DNA content, chromosome number and flowering capacity (Valkonen *et al.*, 1994) or characterisation of morphological variation and cold resistance (Preiszner *et al.*, 1991). However, in some instances the somatic hybrids were successfully used in backcross experiments. Ehlenfeldt and Helgeson (1987) as well as Jacobsen *et al.* (1993) produced progeny from *S. brevidens* (+) *S. tuberosum* somatic hybrids after crossing with the latter species. They both used tetraploid as well as hexaploid hybrids with respectively two and four genomes of potato. Crossability of the tetraploid somatic hybrids was poor with both 2*x* and 4*x* potato genotypes, whereas the hexaploid somatic hybrids crossed very well with tetraploid potato. Rokka *et al.* (1994) were also successful in producing backcross progeny from *S. brevidens* (+) potato somatic hybrids. In their experiments the hexaploid somatic hybrids, although being aneuploids, could be used as male parents, in contrast to Ehlenfeldt and Helgeson (1987) and Jacobsen *et al.* (1993) who were only successful when the somatic hybrids were used as the female parent.

Rokka *et al.* (1994) and Jacobsen *et al.* (1993) only described the production of the first generation backcrosses whereas Williams *et al.* (1993) and McGrath *et al.* (1994) analysed first and second generation BC-genotypes with molecular markers. Their results suggested the occurrence of recombination by crossing-over between potato and *S. brevidens* which is a prerequisite for the introgression of useful traits of *S. brevidens* into the potato gene pool.

In 1995 Watanabe *et al.* described the first successful attempt of a direct cross between *S. brevidens* and *S. tuberosum* by using a rescue pollinator, IVP35. Besides parental characteristics the F1 hybrids also expressed *S. phureja* traits. A major advantage of the production of these sexual hybrids is the fact that, as a result of this, the somatic hybrids of this combination of species no longer are considered to be genetically modified organisms.

Somatic hybrids of potato (+) tomato

One of the best studied combinations in somatic hybridisation is that of potato and tomato (*Lycopersicon esculentum*). Melchers *et al.* (1978) were the first to describe the production of potato-tomato somatic hybrids but progeny was never reported by them. Wolters *et al.* (1991) analysed both symmetric and asymmetric somatic hybrids and Schoenmakers *et al.* (1993) tried unsuccessfully to obtain progeny from triploid fusion hybrids with two genomes of tomato and one of potato. Fifteen years after Melchers *et al.* (1978) reported the first potato-tomato somatic hybrids, Jacobsen *et al.* (1994) were the first to succeed in making backcrosses of a hexaploid somatic hybrid, containing two genomes of tomato and four of potato, with tetraploid potato. Over 3400 immature seeds were cultivated to obtain the first BC1 genotype which was male sterile but demonstrated female fertility. The production of another six BC1 plants was described by Garriga-Calderé *et al.* (1997) who used hexaploid somatic hybrids of the same series as Jacobsen *et al.* (1994) as female parents. The effort they had to put in was much lower with only 406 cultured immature seeds resulting in six BC1 plants. The first step in the backcross programme appeared to be by far the most laborious and difficult one since it took only a few pollinations for the production of second backcross progeny (Jacobsen *et al.*, 1994). A combined total of 97 BC2 plants from three populations were screened for the presence of potato genotypes with alien tomato chromosome additions. Out of the 12 possible monosomic addition types, seven different ones (1, 2, 4, 6, 8, 10 and 12) were detected by Garriga-Calderé (1998). At present all 12 monosomic addition lines are available (Haider Ali *et al.*, in press).

SOMATIC HYBRIDS WITHIN THE BRASSICACEAE

Another plant family from which several species have been used in somatic fusion experiments are the Brassicaceae. The most important crop species within the Brassicacea are the species of the genus *Brassica*: *B. campestris* (e.g. chinese cabbage, genome AA), *B. nigra* (genome BB), *B. oleraceae* (e.g. cabbage, genome CC), *B. juncea* (genome AABB), *B. napus* (rapeseed, genome AACC), *B. carinata* (genome BBCC). Most of the previously mentioned species have been used in interspecific somatic hybridisation experiments whereas a majority of the intergeneric and intertribal fusion experiments involved *B. napus* as one of the parents.

Interspecific somatic hybrids

Interspecific somatic hybridisation within the *Brassicacea* has been performed between several combinations of diploid and allopolyploid species. For increasing the

genetic diversity of rapeseed, *Brassica napus* (AACC) was resynthesised by a fusion of *B. campestris* (syn. *B. rapa*, AA) and *B. oleraceae* (CC) (Sundberg *et al.*, 1987). Somatic hybridisation in this combination of species was not applied in order to circumvent crossing barriers since sexual hybridisation in combination with chromosome doubling is another possibility to resynthesise *B. napus* (Ozminkowski Jr. and Jourdan, 1994a and 1994b).

Important breeding goals for rapeseed are resistances to blackleg (*Phoma lingam*, anamorph *Leptosphaeria maculans*) and to clubroot (*Plasmodiophora brassicae*) (Sjödin and Glimelius, 1989a, b). Resistance to *Phoma lingam* was found in *B. nigra* , *B. juncea* and *B. carinata* and after production of symmetric as well as asymmetric somatic hybrids between these gene-donors and rapeseed, resistant hybrids were obtained. Stable inheritance and possible introgression of the gene(s) for resistance to *Phoma lingam* have been recorded in lines derived from a backcross programme of the original hybrid with rapeseed (Waara and Glimelius, 1995).

Intergeneric somatic hybrids

Several intergeneric hybrids have also been produced via protoplast fusions between species from the genera *Brassica*, *Eruca*, *Sinapis*, *Raphanus*, *Moricandia*, *Diplotaxis* and *Trachystoma*. In general all intergeneric combinations resulted in hybrid plants but, compared to the interspecific somatic hybrids, they showed a larger variation in chromosome number, with more aneuploid hybrids as well as polyploid hybrids. Attempts to produce backcross progeny were not successful in all combinations. One of the goals was to introduce resistance to beet cyst nematod (BCN) from *Raphanus sativa* into rapeseed but the hybrids showed reduced fertility and could not be backcrossed to *B. napus* (Lelivelt and Krens, 1992). Experiments with *Sinapis alba* (+) *B. napus* hybrids also failed in producing progeny because the somatic hybrids were mitotically unstable and sterile (Lelivelt, 1993). Somatic hybrids of *S. alba* (+) *B. juncea* however, were fertile and BC1 progeny was obtained after crossing with *S. alba*, just like somatic hybrids between *B. napus* and *Eruca sativa* which enabled backcrossing to rapeseed and introduction into breeding programmes (Fahleson *et al*, 1988). The correlation between chromosome elimination in somatic hybrids and genetic distance between the fusion parents was investigated by Sundberg and Glimelius (1991) by comparing interspecific and intergeneric somatic hybrids. They concluded that a larger genetic distance resulted in a higher degree of chromosome elimination.

Intertribal somatic hybrids

Intertribal somatic hybrids within the Brassicacea can only be produced via somatic hybridisation. The first combination was *Arabidopsis thaliana* (+) *B. campestris* (Gleba and Hoffman, 1979, 1980), followed by *A. thaliana* (+) *B. napus* (Forsberg *et al.*, 1994); *Thlasi perfoliatum* (Fahleson *et al.*, 1994b) or *Lesquerella fendleri* (+) *B. napus* (Skarzhinskaya *et al.*, 1996); *Barbarea vulgaris* (+) *B. napus* (Fahleson *et al.*, 1994b) or *B. campestris*. More spontaneous asymmetric hybrids were found among the intertribal hybrids than among the intrageneric and intergeneric hybrids. Interestingly, though,

when comparing the intertribal hybrids with those obtained from hybridisation experiments between more closely related species, no differences in the frequency of hybrid fusions or hybrid shoot regeneration were recorded. However, the intertribal combinations were, in general, more difficult to culture to mature plants in the greenhouse. This was especially pronounced in the fusion *Barbarea vulgaris* (+) *B. napus* which never resulted in plants that could grow under greenhouse conditions (Fahleson *et al.*, 1994a).

Nevertheless, intertribal somatic hybrids have been produced that differentiated and developed into normal hybrid plants that could even be backcrossed, like some of the *Arabido-Brassica* hybrids (Forsberg *et al.*, 1994) or *B. napus* (+) *L. fendleri* asymmetric hybrids (Skarzhinskaya *et al*, 1996).

APPLICATION OF GISH AND GENETIC MARKERS FOR THE MONITORING OF THE PROCESS OF RECURRENT BACKCROSSING

There are quite a few bottlenecks for introgression of alien chromosomes, like

- Preferential elimination of alien chromosomes from the fusion hybrids and backcross progenies;
- extreme degree of sterility in the backcross progenies;
- lack of genetic recombination between the species involved;
- non-transmission of alien chromosomes through the gametes.

Besides these drawbacks the monitoring and evaluation of backcross progeny is not simple and can be highly laborious. Fortunately, a number of recent molecular techniques like genomic *in situ* hybridisation (GISH), restriction fragment length polymorphisms (RFLP) and amplified fragment length polymorphisms (AFLP) have opened immense possibilities to monitor the progress of introgression. These techniques enable the determination of: 1) whether alien genomes or chromosomes are intact; 2) intergenomic recombination; 3) transmission of the alien chromosomes to the progeny.

GISH and the RFLP-technique were both applied to the progeny of the first and second backcross progeny of the potato-tomato somatic hybrids in order to improve the efficiency of introgression of genetic material into cultivated potato. (Jacobsen *et al*, 1995; Garriga-Calderé *et al.*, 1997; Garriga-Calderé *et al.*, 1998). The combination of both techniques resulted in the elucidation of the genomic constitution of all hybrids involved. With the application of GISH it was possible to establish the number of alien tomato chromosomes whereas RFLPs were used for the identification of individual chromosomes. The first BC1 genotype was shown by using the GISH-technique to contain nine tomato chromosomes besides the 24 potato chromosomes. The RFLP-analysis indicated the presence of only six different chromosomes which led to the conclusion that three chromosomes were in disomic condition. So with these techniques it was clearly demonstrated that chromosomes from alien genera within the Solanaceae can be successfully transferred and that regularly plants are obtained with alien chromosomes in disomic condition. This phenomenon is the result of lack of separation of the chromatids or homologs during meiosis.

A very powerful demonstration of the application of GISH and FISH (fluorescence *in situ* hybridisation) was performed by Kamstra

during his analysis of *Alstroemeria aurea* and *A. inodora* hybrids. Chromosomes of both species could be distinguished with GISH whereas FISH with repetitive probes (two species specific and two ribosomal rDNA repeats) was used to identify all individual chromosomes (Kamstra *et al.*, 1997). The same techniques were used to study pairing of chromosomes and establish whether or not recombination had occurred between the two species involved. It was shown that recombination had occurred frequently in the original hybrid since chromosomes with 1-4 recombination points per chromosome were discovered in all BC1 genotypes (Kamstra *et al.*, 1999). In the recurrent backcross generations, on the other hand, it was recorded that recombination was almost absent. Based on these results Kamstra concluded that breeders that are aiming at introgression should emphasise more on the production of sufficient BC1 genotypes.

S. nigrum AND SPECIES OF THE *S. nigrum* COMPLEX

In view of the previously mentioned possibilities it appears attractive to utilise more distantly related species in somatic hybridisation experiments, for example the species of the *S. nigrum* complex which belong to the section *Solanum,* more usually referred to as the section Morella (Seithe, 1962). Species of this section are distributed in all major continents and are being used as leafy herbs and vegetables, as a source of fruits and for medicinal purposes in Africa and Southeast Asia (Edmonds and Chweya, 1997). However, in many cases they are considered to be weeds which means they are highly adaptable, resistant to many biotic and abiotic factors and probably possess immense physiological and genetic diversity. The greatest diversity and concentration is found in the New World Tropics, particularly in South America. The generic type *S. nigrum,* commonly known as the black, garden or common nightshade is predominantly an Eurasian species which does not occur naturally in South America (Edmonds, 1979). The *S. nigrum* complex comprises of species with several levels of ploidy, ranging from diploid (*S. americanum* Miller; *S. chenopodioides* Lam.) to tetraploid (*S. villosum* Miller) and hexaploid (*S. nigrum* L.) species.

Some of the previously mentioned species have been used in experiments with the aim to explore their genetic diversity. A few sexual hybrids of *S. nigrum* or *S. villosum* with respectively *S. tuberosum* and *S. demissum*, were produced by Eijlander and Stiekema (1994) who circumvented the strong barriers to sexual hybridisation through embryo rescue. These flowering sexual hybrids were found to be highly sterile and until now the production of backcross progeny has not been reported.

One of the interesting traits was the resistance of the *S. nigrum* species to *Phytophthora infestans*, which is based on a strong hypersensitive reaction pattern (Colon *et al.*, 1993; Vleeshouwers et al., 2000). *S. nigrum* as well as the sexual hybrids of *S. nigrum* related species and potato (Eijlander and Stiekema, 1994) were shown to be highly resistant to *P. infestans*,.

In the past, Binding *et al.* (1982) have performed protoplast fusions between *S. nigrum* and *S. tuberosum* in order to transfer the atrazine resistance from black nightshade to potato (Gressel *et al.*, 1984), but successful backcross experiments have not been reported so far. In this thesis somatic hybridisation

experiments with four species of the *S. nigrum* complex are described:

- *S. americanum* Miller: $2n = 2x = 24$. Most widespread, highly variable species, one of the ancestors of *S. nigrum*. It is found throughout the world.
- *S. chenopodioides* Lam.: $2n = 2x = 24$. One of the most easy identifiable species, native to the eastern parts of South America, might be an ancestor of *S. villosum*.
- *S. villosum* Miller: $2n = 4x = 48$. It is thought to be native to Eurasia and has been sparingly introduced to Australia, New Zealand and North America. It is one of the ancestors of *S. nigrum*, but its own ancestry is unknown.
- *S. nigrum* L. ssp. *schultesii*: $2n = 6x = 72$. It is considered to be native to Eurasia, has not been found yet in South or Central America. The derivation of this species from *S. villosum* and *S. americanum* is now very well established (Edmonds, 1979).

AIMS AND OUTLINE OF THIS THESIS

The principle aims of the work presented in this thesis were the following: 1) To hybridise the non-tuberous *S. nigrum*-species with cultivated potato; 2) to backcross the fusion products to potato; 3) to evaluate the BC progenies with GISH and AFLPs and for resistance to *P. infestans*; 4) to gain an insight into the aspects of introgression of alien chromosomes into potato.

In **chapter 2** the fusion experiments between diploid or tetraploid potato genotypes and four species belonging to the *S. nigrum* complex, namely *Solanum nigrum*, *S. villosum*, *S. chenopodioides* or *S. americanum* are described. The results of the sixteen fusion combinations showed that somatic combining abilities were influenced by the ploidy level as well as the genotype of the parental species. The main aim was to select flowering somatic hybrids that could be used for backcross experiments.

Chapter 3 describes successful backcrosses of *Solanum nigrum* (+) $2x$ potato somatic hybrids with both *S. nigrum* and potato. A considerable difference in success rate was detected between both backcross programmes. While first and second backcross progeny with *S. nigrum* could be obtained easily, it required thousands of pollinations, resulting in 505 cultured ovules, to obtain two potato-BC1 genotypes. The production of BC2 progeny was equally laborious and so far no BC3 plants could be obtained. The vigour and resistance to *Phytophthora infestans* of twelve BC2 genotypes has been described.

Since no progeny was obtained from the BC2 genotypes, alternative approaches were sought to overcome the sexual crossing barrier. In **chapter 4** is described how by somatic hybridisation it was attempted to add two additional potato genomes to the BC1-6738 and BC2-9017 to improve crossability of the backcross derivatives. An approach for the selection of vigorous somatic hybrids, that can be used for making recurrent backcrosses, involving flow cytometry to estimate the DNA content of the regenerants, crossing experiments and genomic *in situ* hybridisation results are discussed. Plants with a DNA content higher than that of the BC1 or BC2 genotypes were considered to be potential somatic hybrids. The application of genomic *in situ* hybridisation (GISH) made it possible to

distinguish clearly between *S. nigrum* and potato chromosomes in mitotic and meiotic chromosome spreads which enabled us to determine the genomic composition of hybrids.

In **chapter 5** the chromosome constitution and meiotic behaviour of a somatic hybrid of *Solanum nigrum* ($2n=6x=72$) and diploid potato ($2n=2x=24$) and its BC1 and BC2 progeny was described. The somatic hybrid F21-26 was found to be an octaploid with six genomes of *S. nigrum* and two of potato. A cross between F21-26 and a 4*x* potato genotype produced BC1-6738, a hexaploid with 36 chromosomes each of both species. With GISH it was impossible to determine whether the 36 chromosomes of *S. nigrum* represented three complete genomes. However, AFLP-data showed that none of the AFLP-specific markers that was amplified in the *S. nigrum* fusion parent and the somatic hybrid was missing in BC1-6738, which is an indication that no major chromosome elimination had taken place. A second backcross with 4*x* potato resulted in eleven BC2 genotypes with a near-pentaploid genomic constitution. Molecular cytological investigations on nuclei of BC2 tetrad cells showed that transmission of alien *S. nigrum* chromosomes to BC3 progeny is likely. Meiotic analysis of metaphase I in BC1-6738 and in BC2-9019 indicated clearly that allosyndetic pairing occurs in these genotypes. Association of homoeologous chromosomes leading to both bivalent and trivalent formation was observed but recombination based on crossingover was not detected.

In **chapter 6** the results of the earlier chapters are discussed including the perspectives of this approach and material for future research.

2

Somatic hybridisation between *Solanum tuberosum* and species of the *S. nigrum* complex: selection of vigorously growing and flowering plants

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ABSTRACT

Fusion experiments were performed between diploid (2*n*=2*x*=24) or tetraploid (2*n*=4*x*=48) potato genotypes and four species of the *Solanum nigrum* complex, namely *S. nigrum* (2*n*=6*x*=72), *S. villosum* (2*n*=4*x*=48), *S. chenopodioides* (2*n*=2*x*=24) or *S. americanum* (2*n*=2*x*=24 and 2*n*=6*x*=72). All five accessions of the *S. nigrum*-species were successfully hybridised with at least one of the potato genotypes. Somatic combining abilities were influenced by the ploidy level as well as by the genotype of the parental species. The use of kanamycin or hygromycin resistance as cell-selectable markersystem had no influence on somatic combining ability, but such markers can be useful to improve efficient selection of somatic hybrids in sufficient numbers. At least 20% of the hybrids of each successful combination performed well *in vitro*. However, only 60 genotypes out of 761 somatic hybrids were vigorous as well as flowering in the greenhouse. Analysis of the DNA content of somatic hybrids could be used as a criterion for the indirect selection *in vitro* of hybrids that were vigorous in the greenhouse. Flowering somatic hybrids of *S. nigrum* (+) 2*x* potato and *S. americanum* (+) 4*x* potato were selected with the aim of introgression of resistance traits after recurrent backcrossing with cultivated potato.

INTRODUCTION

In potato breeding several related species have been used on a wide scale for the introduction of useful traits into the gene pool of the cultivated potato. Although most tuber-bearing wild *Solanum* species can be crossed easily with the cultivated potato, some valuable source species are recalcitrant in this respect (Hermsen, 1994). Several approaches have been used to overcome this barrier. Via bridging species genes from distantly related species of the series Bulbocastana, Pinnatisecta and Etuberosa have been successfully introgressed into the cultivated potato (Hermsen, 1966; 1983; Hermsen and Ramanna, 1973; Hermsen and Taylor, 1979). The application of embryo rescue has been another successful approach in producing sexual hybrids of potato and recalcitrant species, such as three species of the series Etuberosa (Watanabe *et al.*, 1995) or *Solanum nigrum* and *S. villosum* (Eijlander and Stiekema, 1994).

A third method, to circumvent strong crossability barriers, is somatic hybridisation. However, when somatic hybrids are obtained, new barriers may block subsequent backcrosses that are essential for introgression of desired traits into the potato genome and for elimination of the undesired traits. *S. brevidens* is an example of a species that has been used successfully as a fusion parent in combination with potato, resulting in fertile somatic hybrids that could be crossed with potato (Ehlenfeldt and Helgeson, 1987; Jacobsen *et al.*, 1993). In this way backcross progenies with resistances to *Erwinia* soft rot (Austin *et al.*, 1988), potato leaf roll virus, potato virus X and potato virus Y (Pehu *et al.*, 1990) were obtained. The fusion combination of potato and tomato (*Lycopersicon esculentum* Mill.) is more complex. It has been studied elaborately for a long time, starting in the seventies (Melchers *et al.*, 1978; Jacobsen *et al.*, 1992; Schoenmakers *et al.*, 1993). Backcross progeny from the potato-tomato hybrids were less readily obtained than from *S. brevidens*-potato somatic hybrids. Jacobsen *et al.* (1994) were the first to succeed in backcrossing potato (+) tomato hybrids with potato. At present the transfer of a number of valuable traits, partly related to non-host resistance, is being investigated in several BC-populations (unpublished data).

Black nightshade (*S. nigrum*) is another interesting non-tuberous species with useful traits. Colon *et al.* (1993) described a high level of late blight resistance in *S. nigrum* ($2n=6x$) and the closely related species *S. villosum* ($2n=4x$). A few sexual hybrids of *S. nigrum* or *S. villosum* with *S. tuberosum* or *S. demissum* respectively, were produced by Eijlander and Stiekema (1994). These sexual hybrids were found to be highly sterile and until now, the production of backcross progeny has not been reported. In the past, Binding *et al.* (1982) have performed protoplast fusions between *S. nigrum* and *S. tuberosum* in order to transfer the atrazine resistance from black nightshade to potato (Gressel *et al.*, 1984), but successful backcross experiments have not been reported so far.

This paper describes the production and selection of vigorously growing and flowering somatic hybrids of *S. nigrum*-related species and potato that are suitable for backcross experiments. For increasing the success of somatic hybridisation two factors were tested. Firstly, selective growth markers were applied with the objective to isolate somatic hybrids on a large scale. Secondly, the influence of the ploidy levels of the fusion parents on the production of somatic hybrids was evaluated.

MATERIALS AND METHODS

Plant material

Four species of the *S. nigrum* complex and three genotypes of *S. tuberosum* ssp. *tuberosum* were used in our fusion experiments (Table 1). This table includes the accession number, the ploidy level, the abbreviation(s) of the species and the serial number(s) of the plants that were propagated *in vitro* and used in the fusion experiments. The abbreviations and serial numbers are used throughout this paper.

The *S. nigrum* ssp. *schultesii* accession (*ngr*, SN14-0, $2n=6x=72$) was provided by the Gradina Botanica in Rumania. The accession of the diploid species *S. chenopodioides* (*che*, SN13-1, $2n=2x=24$) was collected at Miseglia, Apuane in Italy. In one fusion combination, a hygromycin resistant genotype of *S. chenopodioides* (che^H, SN13-1^H) was involved, which was obtained by *Agrobacterium* transformation (strain LBA 4404 with plasmid PVU 1011). The accession of the species *S. villosum* (*vll*, SNF1-1, $2n=4x=48$) was collected at Montpellier (France). Both *S. americanum* accessions (ame_{2x}, Sam90-3, $2n=2x=24$ and ame_{6x}, Sam88-1, $2n=6x=72$) were supplied by the Botanical Garden of the University of Nijmegen (the Netherlands).

The *S. tuberosum* fusion parents were an amylose-free (*amf*) mutant (tbr_{2x}, 1029/31, $2n=2x=24$) described by Jacobsen *et al.* (1989), a kanamycin resistant transformant of 1029/31 (tbr_{2x}, $AM10^K$) and the Dutch potato cultivar Désirée ($2n=4x=48$).

Protoplast fusion procedure

The protoplast fusion procedure and media used were according to Jacobsen *et al.* (1992). Solid-callus growth medium (MS11), shoot induction medium (MS12) and shoot elongation medium (MS13) were prepared according to Mattheij *et al.* (1992). When resistance genes in parental clones allowed the use of selective agents, kanamycin was used at concentrations of 50 mg/l in TMD, 75 mg/l in MS11, 100 mg/l in MS12, MS13, MS20 and MS30. Hygromycin was used at concentrations of 10 mg/l in TMD and 20 mg/l in MS11, MS12, MS13, MS20 and MS30.

Identification and analysis of somatic hybrids

The first screening of the putative hybrids was based on the type and density of hairs on the stem of the regenerated shoots. The potato genotypes had few long, non-glandular hairs on their stems, whereas the stems of species of the *S. nigrum* complex were covered with short, glandular hairs. Hybrid plants had both types of hairs and the density was intermediate.

Hybridity of the plants was confirmed with the isozyme marker 6-phosphogluconate dehydrogenase (6-PGDH) with the phast system (Jacobs *et al.*, 1995).

Flow cytometric analysis of the DNA content of the parental plants and hybrids was carried out according to De Laat *et al.* (1987).

Propagation and culture of hybrids

In vitro plants were propagated on MS30 in glass tubes or low glass jars. Vigour and morphology of the hybrids were assessed in a glasshouse that complied with Government biosafety rules.

Table 1. Indication and details of the plant material used in the fusion experiments of four species of the *S. nigrum* complex with one tetraploid and two diploid genotypes of *S. tuberosum*.

Species	Accession/variety	Ploidy	Abbreviation
S. nigrum ssp. *schultesii*	88BG 278 02	6*x*	*ngr*
S. chenopodioides	88BG 186 09	2*x*	*chp*
		2*x*	*chp*^{H}
S. americanum	Sam 904750023	2*x*	*ame*$_{2x}$
	Sam 884750061	6*x*	*ame*$_{6x}$
S. villosum	-	4*x*	*vll*
S. tuberosum	87.1029/31	2*x*	1029/31
		2*x*	AM10^{K}
	Désirée	4*x*	-

^{H} = Hygromycin resistance, ^{K} = kanamycin resistance

Results

Regeneration of fusion parents

The regeneration capacity of all parental genotypes, *S. nigrum*-related species as well as potato genotypes, was evaluated in several control experiments with the aim to use this characteristic as a selection criterion in our fusion experiments. Based on these results, it was possible to assess whether selection markers like kanamycin and hygromycin resistance would be necessary or mainly be useful in our fusion experiments. Regenerants of the *S. nigrum*-related species were observed within one month, whereas the potato genotypes required on average two months to start regeneration. The regeneration of the potato genotype 1029/31 was studied in more detail. After three months 47% of the calli had produced regenerants, increasing to 62% after four months and 67% after seven months. Désirée and AM10^{K} behaved similarly. Regeneration experiments with mixtures of *S. nigrum* and potato protoplasts resulted in shoots of both species but, as expected, the first shoots were always of *S. nigrum* origin.

These observations indicated that the use of a cell-selectable marker, such as kanamycin resistance, could improve the efficiency of regeneration of somatic hybrids considerably. Application of two cell-selectable markers, one in each fusion parent, could theoretically increase the efficiency to 100% of somatic hybrids. All three approaches using zero, one or two cell-selectable markers have been applied in our experiments.

Identification of somatic hybrids

Somatic hybrids were identified both morphologically (type and density of the hairs on stems) and biochemically (isozyme patterns). The reliability of the morphological identification

of somatic hybrids was evaluated on the basis of the pooled results of two fusion experiments, involving the combinations *vll* (+) 1029/31 and *ngr* (+) 1029/31. All 147 regenerants obtained from these two experiments were tentatively classified either as "somatic hybrid phenotype", "parental phenotype" or "inconclusive" on the basis of characteristics of their hairs. Subsequently, this morphological classification was compared with results from the isozyme analysis. Out of 112 somatic hybrids, 110 (98%) had been successfully identified on the basis of their hairs only two being inconclusive. In the category of 32 parental phenotypes only 20 (63%) were identified as such by their hairs and 7 (22%) genotypes had erroneously been classified as somatic hybrids; five (16%) were classified as "inconclusive". Not a single somatic hybrid that was detected by the isozyme test, was classified as "parental phenotype" by stem hair type.

From these results we concluded that the structure and density of stem hairs were useful morphological markers for initial screening of somatic hybrids among the regenerants from the two fusion combinations investigated. Nevertheless, isozyme tests were necessary for the confirmation of hybrid nature.

Analysis of the fusion experiments

Sixteen different fusion combinations were performed in which one of the indicated *S. nigrum*-related species was combined with one of the three genotypes of cultivated potato. All combinations are listed in Table 2 along with the results obtained.

a. Fusion experiment with two cell-selectable markers. In this experiment both fusion parents contained a cell-selectable marker; the potato parent ($AM10^K$) the kanamycin and *S. chenopodioides* the hygromycin resistance gene. The experiment yielded 1025 calli that could grow on media with both antibiotics. Regenerants were obtained from only 48 calli, of which 42 (88%) were hybrids. Only 26% of the hybrid plants grew vigorously *in vitro*. Unfortunately none of these hybrids, including the ones that grew well *in vitro*, were capable of growing in the greenhouse.

b. Fusion experiments with one cell-selectable marker. In five fusion combinations only the potato fusion parent ($AM10^K$) carried a cell-selectable marker, the kanamycin resistance gene, whereas the four *S. nigrum*-related species were wild type genotypes. The number of kanamycin resistant calli ranged from 250 to 1450 across the five experiments. Regeneration efficiency of the calli varied from less then 1% with diploid *S. americanum* to 56% with the hexaploid *S. americanum* accession as fusion parent. The single somatic hybrid that resulted from the combination ame_{2x} (+) $AM10^K$ grew vigorously *in vitro* but did not grow well in the greenhouse. The fraction of hybrids among the other four combinations was high, ranging from 83% to 97%. Such high frequencies were expected from fusion combinations in which the faster regenerating parent lacked the antibiotic resistance. The proportion of plants with good growth capacity *in vitro* ranged from 20% to 69%, depending on the combination. In the greenhouse only 15 (16%) of the $ngr\text{-}tbr_{2x}$ hybrids and 2 (2%) of the $ame_{6x}\text{-}tbr_{2x}$ hybrids grew vigorously and flowered well, whereas the remaining 365 hybrids performed poorly or died within one month.

In the combination *S. chenopodioides* (+) $AM10^K$ no essential differences were found between the results of the experiments with one or two cell-selectable markers.

c. Fusion experiments without cell-selectable markers. Ten fusion combinations were performed with genotypes lacking a cell-selectable marker. The experiments involved all the *S. nigrum*-related species on the one hand and the potato genotypes 1029/31 (2*x*) and cv. Désirée (4*x*) on the other. From the five experiments with the diploid potato fusion parent the combination with *S. americanum* (2*x*) was not successful. Only two *S. americanum* (2*x*) plants and not a single hybrid regenerated from the 150 calli obtained. In the remaining four combinations, calli (450-1800) and regenerants (29-86%) could easily be obtained. As a consequence of the absence of cell-selectable markers, the percentages of hybrids among those regenerants were low. The highest frequencies, 43 and 30%, resulted from the combinations with the two hexaploid species, *S. americanum* (6*x*) and *S. nigrum* respectively, whereas only 2% of the 1549 regenerants of *S. villosum* (+) 1029/31 were somatic hybrids.

The performance *in vitro* and in the greenhouse of the hybrids of these four fusion combinations was to a large extent comparable to the performance of somatic hybrids from fusion experiments with AM10^{K}. Although between 36 and 69% of the hybrids of all four combinations performed well *in vitro*, only the combination with *S. nigrum* resulted in a reasonable number of hybrids that was vigorous in the greenhouse. Only one of the *chp*-tbr_{2x} hybrids and four out of 138 ame_{6x}-tbr_{2x} hybrids grew vigorously and flowered.

The results of fusion experiments with the tetraploid cv. Désirée deviated from those obtained with the diploid potato fusion parents in several respects. Regenerants were obtained from all five combinations, even with *S. americanum* (2*x*), but no hybrids resulted from the fusion combinations with *S. villosum* and *S. nigrum*, possibly due to the high ploidy level of the expected hybrids. Nevertheless, out of the 355 regenerants from ame_{6x} (+) Désirée, eight hybrids with an expected ploidy level of 10*x* were obtained, though none of these hybrids was viable in the greenhouse. The fusion experiments with the two diploid species *S. chenopodioides* and *S. americanum* yielded both 21 hybrids which is respectively 8 and 20% of the regenerants that were obtained. The majority of these hybrids performed rather well *in vitro*, but only plants of the combination of ame_{2x} (+) Désirée showed vigorous growth in the greenhouse and flowered profusely.

The absence of a cell-selectable marker influenced the fraction of regenerants that was shown to be somatic hybrids. Among the ten combinations investigated this fraction varied between 0 and 0.43, which is much lower than the fractions in experiments with one or two cell-selectable markers (0.82-1.00).

An example of the time frame of regeneration of somatic hybrids and parental plants is shown in Table 3. It indicates the number of regenerants within a period of 12 months that resulted from the four fusion combinations with *S. chenopodioides*. In both combinations in which a selection marker was involved, somatic hybrids were found among the first regenerants. When the experiments were conducted without a cell-selectable marker, the regeneration of somatic hybrids appeared to be delayed. From the combination *chp* (+) 1029/31, not a single potato regenerant was obtained, whereas in the combination of *chp* (+) Désirée potato regenerants appeared, although regeneration was delayed. Time frames of regeneration in the fusion combinations with the other *S. nigrum*-species were comparable.

Table 2. Regeneration and number of somatic hybrids as percentage of the total number of calli per fusion combination. Number of vigorous plants *in vitro* and *in vivo* as percentage of the total number of somatic hybrids.

Fusion combination		Cell selectable marker[1]	Total number of calli	Regeneration efficiency[2] (number of regenerants)	Percentage (number) of somatic hybrids	Fraction of hybrids among the regenerants	Percentage (number) of vigorous hybrids *in vitro*	Percentage (number) of vigorous hybrids *in vivo*
chp^{H}	(+) AM10^{K}	++	1025	5% (48)	4% (42)	0.88	26% (11)	0%
ame$_{2x}$	(+) AM10^{K}	+	550	<1% (1)	<1% (1)	1.00	100% (1)	0%
chp	(+) AM10^{K}	+	1400	7% (95)	6% (79)	0.83	34% (27)	0%
vll	(+) AM10^{K}	+	1450	7% (100)	7% (97)	0.97	20% (19)	0%
ngr	(+) AM10^{K}	+	1091	9% (101)	8% (92)	0.91	58% (53)	16% (15)
ame$_{6x}$	(+) AM10^{K}	+	250	56% (139)	46% (114)	0.82	69% (79)	2% (2)
ame$_{2x}$	(+) 1029/31	-	150	1% (2)	0%	-	-	-
chp	(+) 1029/31	-	665	29% (191)	4% (25)	0.13	36% (9)	4% (1)
vll	(+) 1029/31	-	1800	86% (1549)	1% (24)	0.02	46% (11)	0%
ngr	(+) 1029/31	-	650	50% (328)	15% (99)	0.30	69% (68)	23% (23)
ame$_{6x}$	(+) 1029/31	-	450	72% (322)	31% (138)	0.43	41% (57)	3% (4)
ame$_{2x}$	(+) Désirée	-	400	27% (107)	5% (21)	0.20	90% (19)	71% (15)
chp	(+) Désirée	-	725	36% (262)	3% (21)	0.08	71% (15)	0%
vll	(+) Désirée	-	250	72% (179)	0%	-	-	-
ngr	(+) Désirée	-	100	38% (38)	0%	-	-	-
ame$_{6x}$	(+) Désirée	-	450	79% (355)	2% (8)	0.02	50% (4)	0%

[1] ++ = hygromycin and kanamycin resistance gene; + = kanamycin resistance gene; - = no cell-selectable marker; [2] Only those regenerants were counted that were large enough for isozyme testing.

Table 3. Time course of regeneration of somatic hybrids and parental genotypes from the fusion combination *chp*^{H} (+) AM10^{K} with two cell-selectable markers, *chp* (+) AM10^{K} with one cell-selectable marker, *chp* (+) 1029/31 and *chp* (+) Désirée both without a cell-selectable marker (sh = somatic hybrid)

Number of months	Two cell-selectable Markers			One cell-selectable marker			Without cell-selectable markers					
	AM10^{K}	sh	*chp*^{H}	AM10^{K}	sh	*chp*	1029/31	sh	*chp*	Desirée	sh	*chp*
2	0	9	1	-	-	-	0	0	98	0	0	59
3	0	4	1	1	2	0	0	1	35	0	0	87
4	0	9	0	3	2	0	0	0	7	0	0	8
5	0	12	2	2	14	0	0	0	6	21	1	5
6	-	-	-	2	8	0	-	-	-	9	3	6
7	-	-	-	5	13	0	0	3	2	1	0	7
8	0	8	0	-	-	-	0	3	5	5	4	3
9	-	-	-	3	20	0	-	-	-	9	8	1
10	-	-	-	0	5	0	0	5	3	-	-	-
11	-	-	-	-	-	-	-	-	-	3	0	2
12	-	-	-	0	15	0	0	13	10	6	5	9
Total	0	42	4	16	79	0	0	25	166	54	21	187

The results shown in Tables 2 and 3 clearly indicate that in the combinations tested a cell-selectable marker is not essential for the selection of somatic hybrids if a quick and reliable morphological method to detect putative hybrids is available. However, a cell-selectable marker is useful in those combinations from which large numbers of regenerants have to be screened, for instance, when the fraction of hybrids among the regenerants is small, or when only a small proportion of the somatic hybrids grows vigorously in the greenhouse. In the combinations from which not a single vigorous hybrid was obtained, it might be effective to use different genotypes of the species involved. Indications of such genotypic effects were found after comparison of potato cv. Aminca with cv. Désirée in fusion experiments (data not shown). In five fusion combinations with cv. Aminca, only one somatic hybrid was obtained.

Table 4. Somatic combining ability of the three potato fusion parents with each of the five species of the *S. nigrum*- complex resulting in somatic hybrids.

	ploidy	*ame* 2*x*	*chp* 2*x*	*vll* 4*x*	*ngr* 6*x*	*ame* 6*x*
1029/31	2*x*	-	+	+	+	++
AM10^K	2*x*	+-	+*	+	++	++
Désirée	4*x*	+	+	-	-	+

* with and without hygromycin resistance in *S. chenopodioides*.

- = no somatic hybrids
+ - = <1% of the calli resulted in a somatic hybrid
+ = >1% but <10% of the calli resulted in a somatic hybrid
++ = >10% of the calli resulted in a somatic hybrid

The somatic combining abilities of the individual *S. nigrum*-related species with the three different genotypes of potato are summarised in Table 4. It is apparent from the data that each of the wild species was able to form somatic hybrids with the cultivated potato. Furthermore, the fraction of somatic hybrids among the regenerants was highly variable and probably influenced by the genotype as well as the ploidy level of the fusion parents.

Ploidy level of somatic hybrids

The ploidy level of all fusion parents involved was known. A total of 245 somatic hybrids were subjected to flow cytometric analysis to determine their DNA content, which gave an indication of the ploidy level of the hybrids. The DNA content of the desired hybrids should equal the sum of the parental DNA contents. Deviating DNA contents may originate from asymmetric fusion, loss of chromosomes after fusion, or multiple fusion events. Assignment of ploidy levels to plants with a deviating DNA content was complicated because of different C-values between the *S. nigrum*-related species and potato.

No selection of somatic hybrids *in vitro* or *in vivo* was applied before the flow cytometric analysis, except among plants of the combination *S. nigrum* (+) AM10^K, from which most of the poorly performing hybrids were removed. Within each fusion combination somatic hybrids with the expected or deviating ploidy levels were distinguished (Table 5). Clearly different results were obtained from comparable combinations like *chp* (+) 1029/31 and *chp* (+) AM10^K, which only differ in the presence or absence of the kanamycin resistance gene. The fusions with AM10^K resulted in a higher proportion of plants

with the expected DNA content than the comparable combination with 1029/31. Exceptions were the two fusion combinations with *ame*$_{6x}$, which showed equally high proportions of hybrids with the expected DNA content, indicating that the use of a cell-selectable marker does not result in a higher fraction of expected hybrids in this combination.

In case a deviating DNA content was observed, the hybrids with an expected ploidy level of 4*x* or 6*x* mostly contained a surplus of DNA, but a deficit when 8*x* was the expected ploidy. In the combination *ngr* (+) 2*x* potato it was shown that vigorous plants had, as a rule, the expected DNA content, whereas non-vigorous plants had mostly a deviating DNA content.

Table 5. Analysis of the DNA content of somatic hybrids from different fusion combinations

Fusion combination		Expected ploidy	Observed number of somatic hybrids		
			with expected DNA content[1]	with deviating DNA content	total
chp	(+) 1029/31	4*x*	1	16	17
chp	(+) AM10^{K}	4*x*	6	13	19
chp^{H}	(+) AM10^{K}	4*x*	6	6	12
vll	(+) 1029/31	6*x*	4	17	21
vll	(+) AM10^{K}	6*x*	23	20	43
ame$_{6x}$	(+) 1029/31	8*x*	25	11	36
ame$_{6x}$	(+) AM10^{K}	8*x*	13	7	20
ngr	(+) 1029/31	8*x*	12	34	46
ngr[2]	(+) AM10^{K}	8*x*	15	3	18
ame$_{2x}$	(+) Désirée	6*x*	3	0	3
chp	(+) Désirée	6*x*	6	4	10

1 Hybrids with the sum of the parental DNA contents ±10%.
2 Most of the poorly growing genotypes from this combination had been removed.

Description of somatic hybrids

All successful fusion combinations resulted in hybrids with *in vitro* growth levels ranging from vigorous to poor (Table 2). Somatic hybrids of different fusion combinations were morphologically very similar. They differed mainly in the size of their leaves. The somatic hybrids with *ngr* as parental genotype had the largest leaves *in vitro* (approximately 2 cm long), whereas the smallest leaves (0.5 cm) were observed on *in vitro chp*-hybrids. Hybrids with

ame$_{6x}$ as one of the parents showed more anthocyanin pigmentation than hybrids from other fusion combinations.

The *in vivo* growth capacity of 682 somatic hybrids was analysed in the greenhouse. Ninety-seven somatic hybrid plants died *in vitro* before they could be transferred to the greenhouse which was partly due to infection. Only sixty if the 682 plants grew vigorously and flowered profusely. Fifty-three of those hybrids resulted from three fusion combinations, i.e. *ngr* (+) 1029/31, *ngr* (+) AM10^{K} and *ame*$_{2x}$ (+) Désirée. Plants of the first two combinations resembled each other. They were very vigorous and could reach a height of approximately two meters. Leaves were simple, dentate and of light green colour and in some cases leaves were slightly irregular. All plants showed an early aging of leaves. The size of the flowers was intermediate between those of potato and *S. nigrum*. Petals were white and folded back for three quarters of the length. Some tuber-like structures were obtained from three somatic hybrids. Plants of these fusions resembled *ngr* more than potato.

The *ame*$_{2x}$ (+) Désirée hybrids had a completely different morphology. Plants reached a height of one meter at most. Leaves were compound, irregular, and dark green. The plants produced many flower buds, which rarely opened. Petals had a tinge of purple and opened half, if they did at all. Some hybrids produced tuber-like structures. In contrast to the *ngr* (+) 1029/31 and *ngr* (+) AM10^{K} hybrids these plants resembled potato.

Among the plants of three other fusion combinations, *ame*$_{6x}$ (+) 1029/31, *ame*$_{6x}$ (+) AM10^{K} and *chp* (+) 1029/31, some well growing and flowering hybrids were found. Plants of all other combinations died or grew very poorly *in vivo*. Some genotypes flowered shortly before dying. Somatic hybrids with *ame*$_{6x}$ as a parent showed both *in vitro* as in the greenhouse intense anthocyanin pigmentation.

Discussion

Production of somatic hybrids

The four parental species of the *S. nigrum* complex that were used in our fusion experiments are closely related. *S. villosum* (2*n*=4*x*) and *S. americanum* (2*n*=2*x*) are assumed to be progenitors of the hexaploid *S. nigrum* (Edmonds, 1979). Our results show that all five accessions of the four species were able to form somatic hybrids with potato.

The sixteen investigated fusion combinations showed many differences in somatic combining ability. The ploidy level of the species is a factor that may have influenced the results. Some combinations between parental plants with a relatively high ploidy level did not yield viable hybrids. Although potato performs poorly at higher ploidy levels (Pijnacker and Sree Ramulu, 1990), high ploidy levels need not be troublesome since *S. nigrum* is still fertile as dodecaploid genotype (Singh and Roy, 1985). Our results imply that, besides ploidy level, also the species as such is a determining factor, as is shown by the differences in somatic combining ability of the diploid species *S. chenopodioides* and *S. americanum*$_{2x}$ with *S. tuberosum*.

The different results of fusion experiments with cv. Aminca and with cv. Désirée clearly indicated a genotypic effect on somatic combining ability. In interspecific somatic hybridisations, the effect of the genotype within a species on successful hybridisation has not been described in detail in literature, but interspecific sexual hybridisation has clearly shown the

importance of this factor in many combinations. Hermsen (1966), for instance, presented the differences in berry set among 538 different cross combinations between 20 accessions of *S. acaule* and 28 of *S. bulbocastanum*. Genotypes of both species differed in their intercrossability from (very) good to bad. Recently Watanabe *et al.* (1995) found indications for genotypic effects in the results of their crosses between diploid potato and non-tuberbearing *Solanum* species. Since sexual and somatic interspecific hybridisation both result in the combination of genetic material of two different species in one plant, it is assumed that also in somatic hybridisation the combination of different parental genotypes is of importance.

Selection and detection of somatic hybrids

Comparison of the fractions of hybrids resulting from experiments with zero, one or two cell-selectable markers led to the conclusion that the selection for these markers did not influence somatic combining ability. However, in some combinations, a cell-selectable marker is useful to minimise the effort necessary to obtain and identify a sufficient number of somatic hybrids. This is especially true when no easy detection method is available like the type and density of hairs on the stem. The use of two cell-selectable markers did not have an advantage over the use of only one such marker. Contrary to our expectation, the fusion combination in which both fusion parents contained a cell-selectable marker did not result in the isolation of 100% somatic hybrids. The same combination of cell-selectable markers, used in fusion experiments involving *S. tuberosum* and *S. brevidens*, resulted in 100% somatic hybrids (Jacobsen *et al.*, 1993). In our experiment, some *S. chenopodioides* genotypes occurred among the regenerants as well. Either these regenerants were asymmetric hybrids in which the *tbr*-allele encoding for 6-PGDH was lost, or the selection pressure was not strong enough.

Vigour of somatic hybrids in vitro *and* in vivo

Our results clearly show that somatic hybrids that are vigorous *in vitro* are easily obtained, since at least 20% of the hybrids of each successful combination performed well *in vitro*. Nevertheless it was observed that only from the combinations *ngr* (+) 1029/31, *ngr* (+) AM10^{K} and *ame*$_{2x}$ (+) Désirée a substantial part of the hybrids was also vigorous in the greenhouse. Three other combinations resulted in a low percentage of relatively well growing hybrids: *chp* (+) 1029/31, *ame*$_{6x}$ (+) 1029/31 and *ame*$_{6x}$ (+) AM10^{K}. The latter two combinations are comparable in ploidy to *ngr* (+) *tbr*$_{2x}$. Among the hybrids of seven other combinations, not a single plant was able to grow in the greenhouse.

The DNA measurements showed that the percentage of hybrid plants with the expected DNA content varied between the different combinations but that most well-performing hybrids in the greenhouse had the expected DNA content. Thus, DNA measurements can be used as an additional means of indirect selection of a higher fraction of hybrids that are vigorous in the greenhouse.

The striking contrast between *in vitro* and *in vivo* vigour was observed earlier in potato-tomato hybrids. Several genotypic combinations resulted in somatic hybrids that performed very well in the greenhouse, whereas plants of other genotypic combinations all died *in vivo* (Garriga-Calderé *et al.*, 1997). Poor performance of potato (+) tomato somatic hybrids *in vivo* was also reported by

Schoenmakers *et al.* (1993), but in their case also a low frequency of vigorous plants was obtained.

A possible cause of poor vigour of somatic hybrids in the greenhouse might be organelle-nucleus incongruity. However, in our hybrids incongruity between chloroplasts and nucleus is unlikely, since Gressel *et al.* (1984) showed that the *ngr* (+) *tbr* hybrids, that resulted from their fusion experiments, grew well *in vivo* whether they contained *S. nigrum* chloroplasts or potato chloroplasts. Wolters (1996) stated that somatic incongruity is stronger when the species are less related. However, the factor of relatedness of the species does not explain the striking differences in vigour between hybrids of the different fusion combinations we performed since the phylogenetic distances between each of the four *S. nigrum*-related species and potato are expected to be similar.

It can be concluded that three out of sixteen fusion combinations provided flowering hybrids that can be used in crossing experiments with potato as backcross parent.

3

Successful first and second backcrosses of *S. nigrum* (+) *S. tuberosum* somatic hybrids with both *Solanum* parents

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ABSTRACT

Somatic hybrids of *Solanum nigrum* (+) 2*x* potato were successfully crossed with *S. nigrum* and with potato. First and second generation backcross progeny with *S. nigrum* could easily be obtained. One of the BC1 genotypes was already self-fertile. Backcrosses with potato had a much lower success rate. Only pollinations with tetraploid potato resulted in seed containing berries. Two BC1 genotypes were obtained after 5000 pollinations from which 505 ovules were cultured. The first BC1 genotype was vigorously growing *in vitro* and in the greenhouse and flowered abundantly. The second BC1 showed many abnormalities and dropped its flowers before anthesis. The first BC1 was again crossed with tetraploid potato and also in this generation the success rate was low. Over 5000 pollinations resulted in 1750 berries from which over 3000 ovules were obtained. Twelve plants germinated from these ovules, which were not as vigorous *in vitro* and *in vivo* as the BC1 parent. Some of the BC2 genotypes were used for further backcrosses but so far no BC3 plants could be obtained. BC1 and BC2 genotypes that resulted from the backcross programme with potato were tested for their resistance to *Phytophthora infestans*. The BC1 genotype was as resistant as the *S. nigrum* fusion parent but among the eight BC2 genotypes scored six were resistant whereas two genotypes showing lesions were concluded to be susceptible.

INTRODUCTION

Somatic hybridisation followed by recurrent backcrossing is a method for the introduction of useful traits from recalcitrant species into a recipient genome. In potato breeding this approach has been used for several species like *S. brevidens* (Austin *et al.*, 1988; Ehlenfeldt & Helgeson 1987; Jacobsen *et al.*, 1993), *S. bulbocastanum* (Austin *et al.*, 1993) *S. circaeifolium* (Mattheij *et al.*, 1992) and tomato (*Lycopersicon esculentum*) (Jacobsen *et al.*, 1994). Successful backcrossing of somatic hybrids depends on various factors, like the phylogenetic distance of the parental species and the genotypes of the fusion parents. The ratio of the parental genomes was shown to be a major factor in the backcrossing programmes of *S. brevidens* (+) potato and of potato (+) tomato fusion hybrids.

Jacobsen *et al.* (1994) reported the first successful backcross of a potato (+) tomato somatic hybrid with potato, 16 years after the first somatic hybrids of this intergeneric combination were described (Melchers *et al.*, 1978). Garriga-Calderé *et al.* (1997) described the production of more BC1 plants and of several BC2 populations. All BC1 genotypes had resulted from crosses of hexaploid somatic hybrids containing four genomes of potato and two genomes of tomato with tetraploid potato genotypes as the male parents. Attempts to produce backcross progeny from tetraploid fusion products had not been successful.

Backcrosses of the hexaploid *S. brevidens* (+) potato somatic hybrids with tetraploid potato were also more successful than backcrosses of the tetraploid fusion products. Backcrosses with diploid potato gave poor results on both hexaploid and tetraploid somatic hybrids. A total of over 140 plants was obtained from more than 3000 ovules that were cultured *in vitro*. The majority of 114 BC1 genotypes that was obtained, germinated from 865 ovules from crosses between the hexaploid somatic hybrids and tetraploid potato (Jacobsen *et al.*, 1993). Ehlenfeldt and Helgeson (1987) reported similar results from their crossing experiments with fusion hybrids of *S. brevidens* (+) potato.

The production of somatic hybrids between species of the *S. nigrum* complex and potato has been described by Horsman *et al.* (1997). Diploid, tetraploid and hexaploid *S. nigrum* related species were hybridised with 2*x* and 4*x* potato genotypes in order to produce somatic hybrids with a range of ploidy levels from 4*x* to 10*x*. Only the fusion combinations 6*x* *S. nigrum* (+) 2*x* potato and 2*x* *S. americanum* (+) Désirée resulted in plants that were vigorous in the greenhouse. *S. nigrum* (+) 2*x* potato somatic hybrids have also been described by Binding *et al.* (1982) but no backcrosses were reported by them. In the present report the successful first and second generation backcrosses of the *S. nigrum* (+) 2*x* potato fusion products with both potato and *S. nigrum* have been described. The influence is shown of the ratio of the genomes in these somatic hybrids on the success rate of backcrosses with potato compared to backcrosses with *S. nigrum*.

The aim of the backcrossing programme with potato was the transfer of the resistance of *S. nigrum* to *Phytophthora infestans* into cultivated potato. *S. nigrum* as well as the sexual hybrids of *S. nigrum* related species and potato (Eijlander and Stiekema 1994) were shown to be resistant to *P. infestans*, based on a strong hypersensitive reaction (Colon *et al.*, 1993). The genotypes that resulted from the backcrosses of *S. nigrum* (+) 2*x* potato fusion products with potato were analysed for their resistance to *P. infestans*.

MATERIALS AND METHODS

Plant material and culture

Somatic hybrids of three fusion combinations were used as female parents in crossing experiments: 1) 2*x* *S. chenopodioides* (+) 2*x* *S. tuberosum* clone 87.1029/31; 2) 2*x* *S. americanum* (+) *S. tuberosum* cv. Désirée; 3) 6*x* *S. nigrum* (+) 2*x* *S. tuberosum* clone AM10. *S. chenopodioides* (2*n* = 2*x* = 24), *S. americanum* (2*n* = 2*x* = 24) and *S. nigrum* (2*n* = 6*x* = 72) all belong to the *S. nigrum* complex of the section *Solanum*. The diploid *S. tuberosum* genotype 87.1029/31 was an amylose-free mutant described by Jacobsen *et al.* (1989). AM10 was a transformant of clone 87.1029/31, containing the GUS-gene and the kanamycin resistance gene (Horsman *et al.*, 1997).

The *S. chenopodioides* (+) 1029/31 and the *S. americanum* (+) Désirée somatic hybrids were pollinated with the cultivars Désirée, Frieslander, Gloria and Katahdin and with the tetraploid breeding clone AM66-42. The potato backcross parents for the *S. nigrum* (+) AM10 somatic hybrids were: 1) the tetraploid breeding clones AM66-42, HB93-7133-1, HB93-7133-2, HB93-7133-3, HB93-7133-4 and HB93-7133-5; 2) the cultivars Désirée, Escort, Hertha, Monalisa, Mansour and Vital. The *S. nigrum* backcross parent was the same genotype as used in the initial fusion experiments between diploid potato and *S. nigrum*.

Backcrosses of the BC1 and BC2 genotypes were performed with the cultivars Adora, Désirée, Frieslander, Fresco, Gloria, Katahdin, Mansour, Sebago, Turbo and the breeding clone AM66-42.

Somatic hybrids and backcross genotypes were maintained *in vitro*. Plants were grown on basal MS medium with 30 g/l sucrose. All plants were grown in a greenhouse from February to October. The somatic hybrids and the backcross genotypes were grown according to government safety rules for genetically modified organisms.

Ovule culture

Berries of the somatic hybrids were harvested between 10 and 15 days after pollination (DAP) for ovule rescue. Berries of the BC1 DJ93-6738 were harvested 16-52 DAP, but most frequently between 19 and 25 DAP. Ovule culture was performed using HLH-medium according to Neal and Topoleski (1983). Ovules were transferred to fresh medium every fortnight.

STS-treatment

BC2 genotype KH94-9017 was treated with silverthiosulphate (STS) to prevent early flower drop. STS was prepared by adding a 0.4 mM silvernitrate solution to an equal volume of a 3.2 mM sodiumthiosulphate solution containing 0.1% Tween 20. The entire plant in the greenhouse was sprayed once a week.

GUS assay

The method used for screening of the GUS-activity was based on Jefferson *et al.* (1987). Young leaves of *in vitro* plants were incubated in 50 mM sodium phosphate buffer pH 7.2 for 1 h. The leaves were transferred to GAB-buffer containing 50 mM sodium phosphate, 10 mM EDTA (ethylene diamine tetra acetic acid), 0.1% (v/v) Triton X-100, 0.1% ß-mercapto-ethanol and 1 mM X-Gluc (5-bromo 4-chloro 3-

indol ß-glucuronide). Incubation took place overnight at 37°C. Leaves were screened for the occurrence of blue coloured regions.

Determination of kanamycin resistance

Backcross genotypes were tested for the presence of the NPTII-gene. Shoots of all genotypes were put on MS-medium containing 30 g/l sucrose and 100 mg/l kanamycin. After one, two and four weeks the formation of roots was determined. Absence of roots indicated susceptibility to kanamycin.

Determination of resistance to P. infestans

In vitro plants were first transferred to the greenhouse and four weeks later to a screencage in order to adapt the plants to the outdoor environment. At an age of eight to ten weeks the plants were transferred to a climate chamber with a temperature of 15°C and a daylength of 16h. Three days after their transfer the plants were put in a plastic tent within the climate chamber with a moisturiser. Before inoculation the moisturiser was switched on for four hours.

P. infestans isolate 90128, race 1,3,4,6,7, 8,10,11, which was provided by the Department of Phytopathology of Wageningen University, was used to make the inoculum. Ten ml of sterilised water was added to two rye-agar plates with mycelium of *P. infestans*. The plates were put at a temperature of 4°C for two hours to induce the release of zoospores. Five leaves starting with the first full-grown leaf were inoculated on the lower epidermis with a 10 µl drop of a zoospore suspension with a concentration of 4.10^4 zoospores per ml. Inoculations were performed in the afternoon. After inoculation the lights were switched off and the moisturiser was switched on overnight. The occurrence of lesions was observed 3, 4, 5, 6 and 7 days after inoculation.

Results

Crosses of somatic hybrids of 2x species of the S. nigrum *complex (+) 4x or 2x* S. tuberosum *with potato varieties*

From six hexaploid hybrids of the fusion combination 2*x S. americanum* (+) Désirée two plants of each hybrid were put in the greenhouse at three time points during the crossing season. As expected these hybrids morphologically resembled potato more than *S. americanum* since they contained two genomes of the latter species and four of potato. Leaves showed some abnormalities but flowers had a normal morphology. Early in the season all six hybrids dropped most of their flower buds before anthesis but later in the season it was possible to carry out pollinations. Vigour and flowering capacity varied between the different *S. americanum* (+) Désirée hybrids. Two of them, F54-3 and F54-16, reached a height no more than 0.25 m, and produced only two and five flowers, respectively, that could be pollinated. F54-1, F54-9 and F54-11 were moderately vigorous, had a height of approximately 0.4 m. and flowered better. A total of 24, 16 and 33 pollinations respectively could be made. The most vigorous genotype was F54-10 of which 94 flowers could be pollinated. In all these cases no berries were obtained.

The fusion combination *S. chenopodioides* (+) 2*x* potato resulted in one flowering,

tetraploid somatic hybrid. Most flowers dropped before anthesis. The remaining flowers of six plants of this hybrid were pollinated with pollen of tetraploid potato varieties. A total of 31 pollinations did not result in any berry set.

Crossing experiments with somatic hybrids of 6x S. nigrum (+) 2x potato

Backcrosses with S. tuberosum: BC1, BC2 and BC3: The most vigorous hybrids resulted from fusion experiments between a hexaploid *S. nigrum* accession and a diploid potato genotype. Eighteen somatic hybrids were pollinated with diploid and tetraploid potato genotypes. Pollinations with diploid potato mainly resulted in empty berries (data not shown). The results of pollinations with tetraploid potato genotypes are summarised in Table 1.

Between 24 and 469 pollinations per genotype were made with twelve tetraploid potato genotypes. HB93-7133-2 was the only potato genotype that did not induce berry formation. All other genotypes produced berries and ovules. The somatic hybrids F21-5, -7, -16 and –45 were removed from the crossing programme because of their stunted growth, parthenocarpic berry set and low number of ovules per berry. Among the remaining 14 genotypes berry set varied from 11 to 64%.

Ovule rescue was performed between 9 and 19 days after pollination (DAP). Most berries were harvested before 15 DAP because berry abscission increased exponentially after this point in time. Among the 14 selected somatic hybrids the number of ovules per berry varied from 0.11 to 0.90. Ten somatic hybrids produced between 0.33 and 0.57 ovules per berry. The eight genotypes with the highest berry set were the genotypes with the lowest production of ovules per berry. The six genotypes with the lowest berry set had the highest production of ovules per berry. A total number of 505 ovules was cultured from 1750 berries.

Two of the cultured ovules developed into BC1 plants. The first BC1 genotype, DJ93-6738, resulted from the cross F21-26 x AM66-42. Rescue was applied at 15 DAP. After 14 weeks the first sign of germination was noticed. The seed coat cracked and two weeks later a green callus had emerged. After another two weeks during which shoot meristems had developed the callus was transferred to shoot induction medium for twelve days. Subsequently the callus was cultured on MS13 + 30 g/l sucrose. Twelve shoots were harvested which were subcultured as individual genotypes.

No morphological differences were found between the twelve BC1-shoots and therefore they were considered to be one and the same genotype. Isozyme analysis using SKDH demonstrated the hybrid nature of the BC1, being a descendant of F21-26 and AM66-42. BC1 DJ93-6738 was a vigorously growing genotype that was able to reach a height of several meters. As expected from the genomic constitution, leaf and flower morphology were intermediate between *S. nigrum* and *S. tuberosum*. Some pollen could be collected from the flowers but their stainability with lactophenol acid fuchsin was just 10-15%. Formation of tuber-like structures was observed, whereas the somatic hybrids showed only some stolons with a few thickened parts.

The second BC1, KH93-9100, germinated directly from an ovule that originated from the cross F21-59 x Désirée and was rescued at 13 DAP. Under *in vitro* conditions this genotype

Table 1. Results of backcrosses of *S. nigrum* (+) 2*x* potato somatic hybrids with tetraploid potato genotypes.

Somatic hybrid	Number of pollinations	Number of berries	Berry set	Number of ovules	Ovules/ berry	Number of BC1 plants
F21-4	366	117	32%	39	0.33	0
F21-5	110	66	60%	5	0.08	0
F21-6	395	238	60%	33	0.14	0
F21-7	24	5	21%	0	0	-
F21-8	469	227	48%	85	0.37	0
F21-9	465	189	41%	73	0.39	0
F21-15	275	106	39%	18	0.17	0
F21-16	29	1	3%	0	0	-
F21-24	66	14	21%	6	0.43	0
F21-26	133	14	11%	8	0.57	1
F21-30	457	274	60%	29	0.11	0
F21-35	296	190	64%	67	0.35	0
F21-37	273	42	15%	38	0.90	0
F21-44	164	24	15%	10	0.42	0
F21-45	45	42	93%	4	0.10	0
F21-49	301	45	15%	25	0.56	0
F21-58	299	96	32%	36	0.38	0
F21-59	194	60	31%	29	0.48	1
Total	4362	1750	36%	505	0.40	2

was not as vigorous as the BC1 described in the previous paragraph. In the greenhouse it reached a height of approximately 1 meter, showed deformation of leaves and dropped its flowers before opening.

During three successive seasons crosses were made with the vigorously growing BC1 genotype DJ93-6738. It was mainly used as the female parent. Diploid and tetraploid potato genotypes, *S. demissum* and *S. nigrum* were used as pollinators. Pollinations with *S. demissum*, *S. nigrum* and diploid potato genotypes did not result in berry set. Only after pollination with pollen of tetraploid potato genotypes seed containing berries were obtained (Table 2). Berry set depended among other factors on the pollinator. Cv. Frieslander showed the highest number of berries per pollination in all three seasons with an average of 39%, whereas cv. Gloria had a berry set of only 14%. Besides the pollinator, also plant age and temperature influenced the crossing results considerably. For instance, berry set varied from 15% in 1995 to 28% in 1996. The low percentage in 1995 was largely due to the high temperatures in the greenhouse in July and August.

A total of 5474 pollinations with tetraploid potato genotypes resulted in 1251 berries (Table 2). Ovules were rescued from these berries between 9 and 52 DAP with an average of 22.7 DAP. Seventy-six percent was rescued between 17 and 24 DAP. A total of 3065 ovules was rescued from which less then 1% (27

ovules) germinated. Fifteen embryos died shortly after germination or showed severe abnormalities, whereas twelve embryos developed into complete plants. Ten of these BC2 genotypes showed a good growth capacity *in vitro* (Table 3) which was comparable to the vigour of the BC1 parent. Two genotypes were obviously less vigorous.

All BC2 genotypes were analysed for their kanamycin resistance and GUS-activity, since the potato fusion parent contained T-DNA with both genes (Table 3). Both BC1 genotypes and ten BC2 genotypes were still able to form roots on kanamycin containing medium and showed GUS-activity. Only KH96-9017 and KH96-9304 were kanamycin susceptible and had no GUS-activity. Since the results of the tests for kanamycin resistance and GUS-activity were in agreement, the latter two genotypes were considered not transgenic.

Reciprocal crosses were also performed but pistil preparations showed that BC1 pollen was not able to produce pollen tubes.

All twelve BC2 genotypes were propagated and transferred to the greenhouse. Compared to the BC1 parent all BC2's were less vigorous (Table 3). KH94-9019 was the most vigorously growing genotype that reached a height of approximately 1.5 meter. Because of the second backcross to potato, leaf morphology was as expected to be more potato-like than leaf morphology of the BC1 DJ93-6738. KH94-9019 flowered abundantly with normal looking white flowers. Stylar preparations showed that pollen of several tetraploid potato genotypes, for instance Gloria and Désirée, were able to form pollen tubes, but 1155 pollinations did not result in any berry set (Table 2). The tubers that were produced by this BC2 genotype and also by the other BC2 genotypes were more potato-like than tubers of the BC1 parent, but still showed abnormalities because of the presence of *S. nigrum* chromosomes.

KH94-9017 and KH96-9320 were less vigorous than the previously described genotype but could still reach a height of approximately 1 meter. KH94-9017 showed early senescence of leaves and dropped its flower buds prematurely. STS treatment prevented this early flower drop but also induced the formation of some spontaneous berries which were all empty. Most of the BC2 genotypes produced spontaneous berries during a particular part of the growing season, mostly at the end. KH96-9320 showed spontaneous berry set at the beginning of the season. Later on 20 pollinations could be carried out which resulted in five berries (Table 2). Four ovules were rescued and cultured on HLH medium. After four weeks one of those ovules germinated but the resulting seedling stopped *in vitro* growth at a height of 2 cm.
Some pollinations could be made on the BC2's KH94-9072, KH94-9084, KH96-9379 and KH96-9380 (Table 2). These genotypes were stunted, showed abnormalities and produced spontaneous berries. Some of these pollinations resulted in a few empty berries. KH94-9026 and KH96-9304 did not produce any flowers. Grafts onto tomato or *S. nigrum* were made of all genotypes but no improvement of flowering and/or vigour was observed. Three BC2 genotypes, KH94-9053, KH96-9345 and KH96-9365, were not able to grow *in vivo* at all. All plants of these genotypes died a few weeks after they were transferred to the greenhouse.

Backcrosses with S. nigrum*: BC1 and BC2*

The S. *nigrum* (+) 2*x* potato hybrids were backcrossed with *S. nigrum* as well.

Table 2. Results of crossing experiments of BC1 and BC2 genotypes with tetraploid potato.

Genotype	Generation	Number of pollinations	Number of berries (berry set)	Number of ovules	Ovules per berry	Number of germinating ovules	Number of vigorous plants *in vitro*	Number of viable plants *in vivo*
DJ93-6738	BC1	5474	1251 (23%)	3065	2.5	27	12	9
KH94-9017	BC2	136	0	-	-	-	-	-
KH94-9019	BC2	1155	0	-	-	-	-	-
KH94-9072	BC2	163	0	-	-	-	-	-
KH94-9084	BC2	14	0	-	-	-	-	-
KH96-9320	BC2	22	5	4	1.3	1	-	-
KH96-9379	BC2	20	6	0	-	-	-	-
KH96-9380	BC2	6	2	0	-	-	-	-

Table 3. Characteristics of two BC1 and twelve BC2 genotypes that resulted from backcrosses of *S. nigrum* (+) 2*x* potato somatic hybrids with tetraploid potato genotypes (DAP=days after pollination; n.d.=not determined; R=resistant; S=susceptible).

Code	Cross	DAP	Growth *in vitro*	Growth *in vivo*	Flowering	Kanamycin resistance	Gus-activity	Resistance to *P. infestans*
DJ93-6738	F21-26 x AM66-42	15	++	+++	++	+	+	R
KH93-9100	F21-59 x Désirée	15	+/-	+/-	+/-	+	+	n.d.
KH94-9017	DJ93-6738 x Katahdin	21	++	+	+/-	-	-	R
KH94-9019	DJ93-6738 x Katahdin	23	++	++	++	+	+	S
KH94-9026	DJ93-6738 x Gloria	41	+	+/-	-	+	+	R
KH94-9053	DJ93-6738 x Katahdin	23	+/-	-	-	+	+	n.d.
KH94-9072	DJ93-6738 x Désirée	24	+	+/-	+/-	+	+	R
KH94-9084	DJ93-6738 x Gloria	23	+	+/-	+/-	+	+	R
KH96-9304	DJ93-6738 x Frieslander	16	++	+/-	-	-	-	n.d.
KH96-9320	DJ93-6738 x Frieslander	38	++	+	+	+	+	S
KH96-9345	DJ93-6738 x Turbo	28	++	-	-	+	+	n.d.
KH96-9365	DJ93-6738 x Mansour	21	+/-	-	-	+	+	n.d.
KH96-9379	DJ93-6738 x Désirée	20	+	+/-	+/-	+	+	R
KH96-9380	DJ93-6738 x Désirée	20	+	+/-	+/-	+	+	R

Pollinations were made during the winter, which is not the optimal season for performing crossing experiments. The *S. nigrum* fusion parent that was maintained *in vitro*, was used as pollinator. A total of 31 pollinations on somatic hybrid F21-6 resulted in 18 berries that contained 31 ovules. Although the number of ovules per berry resulting from backcrosses with *S. nigrum* was higher than from backcrosses with potato, it was still significantly lower than the number of ovules normally observed in berries of *S. nigrum* or potato.

Thirteen BC1 plants were derived from 31 cultured ovules. Four of these BC1 plants were transferred to the greenhouse for further analysis and crossing experiments. One genotype, DJ93-6732-1 was stunted and did not flower. The remaining three genotypes, DJ93-6732-2, -3 and –5, were vigorous plants and did flower. Pollen stainability of BC1 plants 6732-2 and –3 was low, 1 and 10% respectively, but genotype 6732-5 was self-fertile with 75% pollen stainability.

The three selected BC1 genotypes were backcrossed with *S. nigrum* again. Berry set was high, for instance 75% for 6732-3. Ovules could be rescued from all berries, which easily resulted in three BC2 populations of 12, 27 and 11 plants respectively. Pollinations with pollen of 6732-5 on each of the two other BC1 genotypes, 6732-2 and 6732-3, also resulted in two populations of 13 and 11 plants. Fifteen BC2 genotypes were obtained from selfing of 6732-5.

Compared to the backcross programme with potato, the success rate of backcrosses of the *S. nigrum* (+) 2*x* potato somatic hybrids with *S. nigrum* was much higher, and more comparable to results from backcrosses of *S. brevidens* (+) potato somatic hybrids with potato.

Resistance to P. infestans

The resistance to *P. infestans* of the somatic hybrid F21-26, the BC1 genotype DJ93-6738 and eight BC2 genotypes was determined and compared to the resistance of *S. nigrum*. During two successive seasons drop inoculation experiments were conducted with all previously mentioned genotypes, except for the BC2 genotypes KH96-9320, KH96-9379 and KH96-9380. Plants to test these genotypes were naturally infected in the screencage in 1997. The occurrence of lesions on these plants was determined in the screenhouse.

Results of the drop inoculation experiments and the spontaneous infection are summarised in Table 3. The somatic hybrid F21-26 and BC1 genotype DJ93-6738 were as resistant as the *S. nigrum* fusion parent. Some necrotic spots could be observed on the inoculation site as they could also be found on the resistant *S. nigrum* control plants.
The BC2 genotypes KH94-9017, KH94-9019, KH94-9026, KH94-9072 and KH94-9084 were also tested with the drop inoculation method. From these genotypes KH94-9019 developed lesions and was concluded to be susceptible. The remaining four BC2 genotypes formed necrotic spots like *S. nigrum* or in some instances slightly larger necrotic regions and were considered resistant.

After the natural infection with *P. infestans* in the screenhouse lesions were observed on leaves of KH96-9320. This genotype was therefore concluded to be susceptible to *P. infestans*. The BC2 genotypes KH96-9379 and KH96-9380 did not show any lesions. Most likely these two BC2 genotypes were resistant. So from eight BC2 genotypes six were resistant and two were susceptible to potato late blight.

Discussion

Crosses with somatic hybrids

This report described the first backcrosses that were made with *S. nigrum* (+) potato somatic hybrids. In 1982 Binding *et al.* reported about the first fusion hybrids of this combination, but crossing experiments were not mentioned. Eijlander and Stiekema (1994) succeeded in obtaining sexual hybrids from the cross *S. nigrum* x Désirée and *S. nigrum* x a diploid breeding clone. Until now successful backcrosses of these sexual hybrids have not been reported.

Backcrosses of the *S. nigrum* (+) potato somatic hybrids with both *S. nigrum* and with potato were described in this report. Considerable differences between both crossing programmes were observed. Backcrosses with *S. nigrum* had a high success rate. Only a few pollinations were necessary to obtain twelve BC1 plants from which one was already self-fertile. BC2 progeny was easily obtained.

Backcrosses of the *S. nigrum* (+) potato fusion hybrids with potato had a much lower success rate. Over 4300 pollinations resulted in 1750 berries from which 505 ovules were obtained. Only two BC1 plants resulted from *in vitro* culture of these ovules. Jacobsen *et al.* (1994) and Garriga-Calderé (1997) showed an equally low success rate in their crossing experiments with potato (+) tomato somatic hybrids. Both tetraploid somatic hybrids (2*x* potato (+) 2*x* tomato) and hexaploid ones (4*x* potato (+) 2*x* tomato were crossed with 4*x* potato genotypes. Crosses with the hexaploid somatic hybrids resulted in a berry set of 25%, which was comparable to the berry set in our experiments. On the other hand, the number of ovules per berry was much higher. On average 35 ovules per berry were cultured in the potato (+) tomato crossing experiments, whereas from the *S. nigrum* (+) potato crosses only 0.4 ovules per berry could be rescued. Nevertheless only one BC1 plant germinated from the almost 3500 ovules that resulted from the potato (+) tomato backcrosses with potato. Crosses with the 4*x* potato (+) tomato hybrids did not result in any progeny.

Differences in crossability were also observed between *S. brevidens* (+) potato somatic hybrids with different genomic constitutions. Ehlenfeldt and Helgeson (1987) were the first to produce backcross progeny from this combination. They used tetraploid hybrids with two genomes of both species and hexaploid hybrids that resulted from fusion experiments between *S. brevidens* and a tetraploid potato genotype. Tetraploid somatic hybrids crossed poorly with both 2*x* and 4*x* potato genotypes, whereas the hexaploid *S. brevidens* (+) potato somatic hybrids crossed very well with tetraploid potato. Jacobsen *et al.* (1993) showed similar results after backcrossing *S. brevidens* (+) potato somatic hybrids. In their experiments the hexaploid somatic hybrids with four genomes of potato crossed better than the tetraploid fusion hybrids.

Rokka *et al.* (1994) were also successful in producing backcross progeny from *S. brevidens* (+) potato somatic hybrids. In their experimentsthe hexaploid somatic hybrids, although being aneuploids, could be used as male parents, in contrast to Ehlenfeldt and Helgeson (1987) and Jacobsen *et al.* (1993) who were only successful when the somatic hybrids were used as the female parent.

According to Ehlenfeld and Helgeson (1987) their results with *S. brevidens* were in

accordance with the EBN hypothesis. Genetic studies of EBN have shown that a slight EBN excess on the female side can be overcome. This might explain the success of the 6*x* (5EBN) *S. brevidens* (+) *S. tuberosum* hybrids in comparison to the 4*x* (3EBN) hybrids in crossing experiments with potato. The EBN hypothesis could not be applied to our crossing experiments since no sufficient crossing data of *S. nigrum* data with standard species are available. Another aspect was that EBNs are based on normal seed set in ripe berries. Our results related to crossing experiments in which ovule culture was applied 2-3 weeks after pollination.

Development of BC2 and BC3 progeny

The main difference between the *S. nigrum* (+) potato and the potato (+) tomato backcross experiments with potato, was the production of BC2 and BC3 progeny. The *S. nigrum* (+) potato BC1 was again crossed with tetraploid potato genotypes, resulting in an average berry set of 23% and 2.5 ovules per berry, the latter being an improvement compared to the number of ovules per berry from the somatic hybrids. Germination rate of the ovules was again low, less than 1%. Only 12 plants with good *in vitro* vigour germinated from over 3000 cultured ovules. In the greenhouse all BC2 genotypes were less vigorous than the BC1 parent. Further backcrosses with potato have not resulted in BC3 progeny.

In the potato (+) tomato backcrossing programme BC2 progeny from different BC1 genotypes (Garriga-Calderé *et al.*, 1997) could easily be obtained. Ovule culture was still found to be necessary, but mainly to improve the low germination rate of the ovules.

So in the crossing experiments of the potato (+) tomato somatic hybrids with potato the first backcross was the main obstruction. Crosses of the *S. nigrum* (+) potato somatic hybrids with potato faced a new obstruction in every generation. This might be caused by the genomic constitution of the initial somatic hybrid, which contained two genomes of potato and six of *S. nigrum.* The BC1 that resulted from a cross with 4*x* potato was expected to be a hexaploid genotype, that contained three genomes of both species. The plants that resulted from a second backcross with 4*x* potato were most likely to be pentaploid, but aneuploids for both species. During the backcross programme of the *S. nigrum* (+) potato hybrids the genomic ratio had to be reversed.

In the potato (+) tomato backcross programme the somatic hybrids as well as the BC1 and BC2 genotypes contained four genomes of potato and an additional number of tomato chromosomes that depended on the generation. All hybrids had a surplus of potato genomes. In this report we described crossing experiments with somatic hybrids of a diploid *S. nigrum*-related species and cv. Désirée which had a genomic ratio that was comparable to the hexaploid potato (+) tomato somatic hybrids. Flowers of the *S. americanum* (+) Désirée fusion hybrids were pollinated with pollen of several potato genotypes, but no berry set was obtained although pollen tube growth was observed. Additional fusion experiments of *S. americanum* with potato cultivars Kanjer, Elkana and Désirée resulted in new somatic hybrids. The *S. americanum* (+) Kanjer hybrids showed good *in vitro* vigour but were not able to survive in the greenhouse. Among the *S. americanum* (+) Elkana and *S. americanum* (+) Désirée hybrids some vigorously growing and

flowering genotypes were selected, but again pollinations did not yield berries. The fusion experiments should be repeated with other *S. americanum* genotypes from different accessions in order to select for combinations with a higher cross compatibility.

Another possibility might be to change the genomic ratio of the BC genotypes that have resulted from the *S. nigrum* (+) potato backcross experiments by somatic hybridisation. Fusion experiments between the BC1 and diploid potato genotypes are expected to result in hybrids with three genomes of *S. nigrum* and five of potato. The results of these kind of experiments will be described elsewhere.

Another option might be to use the microprotoplast technique (Ramulu *et al.*, 1995), but the possibility to use this technique depends on the genetics of the trait that has to be transferred. Our aim was to transfer the resistance to *P. infestans*. So far nothing is known about the genetics of this resistance. Should it turn out to be a polygenic trait with loci on different chromosomes, the microprotoplast technique might not be the optimal approach.

Resistance to P. infestans

The somatic hybrid *S. nigrum* (+) potato, the first backcross and eight second backcross genotypes to potato that resulted from the crossing experiments, were tested for their resistance to *P. infestans*. The somatic hybrid and the BC1 showed an equally high level of resistance as the initial *S. nigrum* fusion parent with a strong hypersensitive reaction. Colon *et al.* (1993) also observed this hypersensitive reaction in *S. nigrum* and the sexual hybrid of *S. nigrum* x diploid potato.

Among the eight BC2 genotypes two showed the formation of lesions and were concluded to be susceptible. The results of the *P. infestans* tests might indicate that one or more chromosomes were missing in the susceptible BC2 genotypes. It might also be possible that not all genomes of *S. nigrum* contribute to the resistance since *S. nigrum* is expected to be an allohexaploid (Edmonds, 1979).

4

Alteration of the genomic composition of *Solanum nigrum* (+) potato backcross derivatives by somatic hybridisation: selection of fusion hybrids by DNA measurements and GISH

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ABSTRACT

Fusion experiments were performed with a first (BC1-6738) and a second (BC2-9017) generation backcross hybrid which resulted from a cross between 6*x* *S. nigrum* (+) 2*x* potato somatic hybrids and potato. Because no progeny was obtained from the BC2 genotypes, alternative approaches were sought to overcome this sexual crossing barrier. Five potato genotypes, of which one contained the hygromycin resistance gene, were used in the fusion experiments. All vigorous regenerants were used for the estimation of nuclear DNA content through flow cytometry. Plants with a DNA content higher than that of the fusion parent BC1-6738 or BC2-9017 were considered potential somatic hybrids. Forty-nine potential somatic hybrids resulted from fusion experiments with BC1-6738, from which 20 grew vigorously in the greenhouse and flowered. after pollination with several 4*x* potato cultivars eight genotypes produced seeded berries and five genotypes gave seedless berries. In addition, eleven of these thirteen somatic hybrids were selected for GISH-analysis to determine their genomic composition. Nine of them had exactly or approximately the expected number of 36 *S. nigrum* and 60 potato chromosomes. In one genotype only 22 instead of 36 *S. nigrum* chromosomes were found and one potato chromosome was possibly missing as well. Only five potential somatic hybrids were detected among the 79 flow-cytometrically analysed regenerants from BC2-9017 (+) 2*x* potato fusion experiments. Two of these hybrids were rather vigorous and did flower, but pollinations with potato have not as yet, set any berries.

INTRODUCTION

Several research groups have attempted to use *Solanum nigrum* as a donor species in potato breeding, for instance for resistance to *Phytophthora infestans* (Colon *et al.*, 1993). Binding *et al.* (1982) produced somatic hybrids of *S. nigrum* and potato but did not report successful backcross experiments. Eijlander and Stiekema (1994) produced a few sexual hybrids but so far further crossing experiments have not been described.

Successful backcross programmes of interspecific somatic hybrids of several combinations of Solanaceae species have shown the influence of the genomic composition of the fusion hybrid on the results of backcross experiments. Somatic hybrids with an excess of genomes of the species that was used as the backcross parent frequently gave better results in backcross experiments. For example, in the potato (+) tomato backcross programme offspring could be obtained from crosses between hexaploid somatic hybrids with four potato genomes and two tomato genomes and tetraploid potato (Jacobsen *et al.*, 1994; Garriga-Calderé *et al.*, 1997). First generation backcross hybrids were not obtained easily but the production of BC2 and BC3 genotypes was less laborious. On the other hand, backcrosses with tetraploid somatic hybrids possessing two potato and two tomato genomes were not successful. Somatic hybrids of *S. brevidens* (+) potato showed similar results (Ehlenfeldt and Helgeson, 1987; Jacobsen *et al.*, 1993; Rokka *et al.*, 1994). The hexaploid genotypes with four genomes of potato were more fertile than the tetraploid genotypes. After pollination with tetraploid potato, BC1 plants were obtained in low frequencies but in each following generation fertility improved rapidly. Somatic hybrids of *S. nigrum*-related species [*S. americanum* (2*x*), *S. chenopodioides* (2*x*) and *S. villosum* (4*x*)] and potato with a more favourable genomic composition were produced as well (Horsman *et al.*, 1997). Most fusion combinations resulted in plants that were not sufficiently vigorous in the greenhouse. Some vigorous plants of the fusion of 2*x* *S. americanum* (+) 4*x* potato were selected but pollinations on these hybrids did not result in berries containing seed.

In this contribution the alteration of the genomic composition of a first (BC1-6738) and a second (BC2-9017) generation backcross hybrid that have resulted from the *S. nigrum* (+) potato backcross programme with potato is described. The BC1 genotype is a vigorous plant with three genomes each of *S. nigrum* and potato. In comparison to the BC1, the selected BC2 genotype was not as vigorous but was still one of the best-performing genotypes among the BC2's. Two genomes of potato were added to these backcross hybrids by somatic hybridisation. The successful approach for the selection of the fusion hybrids is discussed. Putative somatic hybrids were selected after measurements of the DNA content of the regenerants. The hybrid nature of the selected fusion hybrids was confirmed by chromosome counting after genomic *in situ* hybridisation. Finally, the entire selection pathway of somatic hybrids that grew vigorously in the greenhouse and flowered is described.

MATERIALS AND METHODS

Plant materials

The two backcross genotypes that were used in the fusion experiments, a BC1 and a BC2 plant,

were both hybrid derivatives of *Solanum nigrum* and *S. tuberosum* (Horsman *et al.*, 1999). The BC1-6738 was the result of successful backcrossing of a somatic hybrid of 6*x S. nigrum* and a 2*x* potato genotype, AM10, with a tetraploid potato genotype, AM66-42. Over 4000 pollinations and ovule rescue were needed to obtain two hexaploid BC1 genotypes (Horsman *et al.*, 1999) of which one genotype, BC1-6738, was vigorous, flowered profusely and could in its turn be crossed with tetraploid potato. Over 5000 pollinations with several potato cultivars resulted in 12 BC2 genotypes. The BC2 genotype BC2-9017 resulted from a cross of the BC1-6738 and the cv. Katahdin. None of the BC2 genotypes was as vigorous as the BC1 parent and most of the BC2's suffered from severe growth abnormalities. Despite many attempts, no BC3 progeny was obtained.

The BC1-6738 contained the kanamycin resistance gene from the potato fusion parent of the initial hybrid, AM10. The kanamycin resistance gene appeared to be absent in BC2-9017.

Five 2*x* potato genotypes were used as fusion parents in our experiments. PVU1 was a hygromycin-resistant genotype which was obtained after *Agrobacterium* transformation (strain LBA 4404 with plasmid PVU1011) of the amylose-free (*amf*) mutant 87.1031/29 (Jacobsen *et al.*, 1989). RH90-052-1 was a diploid BC1 clone of a *S. tuberosum* x *S. microdontum* hybrid, provided by Dr. Ronald Hutten of the Laboratory of Plant Breeding of Wageningen University. The remaining three genotypes 6486-4, 6486-19 and 6487-9 all resulted from crosses between 1024-2, an *Amfamf* genotype, and dihaploids of the cv. 'Gineke'. They were selected because of their favourable cell culture traits. Somatic hybrids and backcross genotypes were maintained *in vitro*. Plants were grown on basal MS medium with 30 g/l sucrose.

Somatic hybridisation

The protoplast fusion procedure and media used were according to Jacobsen *et al.* (1992). Solid-callus growth medium (MS11), shoot induction medium (MS12) and shoot elongation medium (MS13) were according to Mattheij *et al.* (1992). When resistance genes in parental clones allowed the use of selective agents, kanamycin was used at concentrations of 50 mg/l in TMD, 75 mg/l in MS11, 100 mg/l in MS12, MS13, MS20 and MS30. Hygromycin was used at concentrations of 10 mg/l in TMD and 20 mg/l in MS11, MS12, MS13, MS20 and MS30.

Selection of potential somatic hybrids

Regenerants from experiments in which one or both fusion parents contained a cell-selectable marker were put on medium containing kanamycin and/or hygromycin during callus growth, regeneration and plant growth. BC1-6738 still contained the kanamycin resistance gene, which allowed the use of kanamycin for selection. One fusion combination in which BC1-6738 was involved, BC1-6738 (+) 052-1, was carried out without kanamycin selection. Double selection with both kanamycin and hygromycin was applied in the fusion experiments of BC1-6738 (+) PVU1 because the potato fusion parent PVU1 was hygromycin resistant.

All regenerants that were able to grow *in vitro*, were analysed flow cytometrically for their DNA content. Regenerants with a DNA

Table 1. Results of fusion experiments of BC1-6738 (+) 2*x* potato and BC2-9017 (+) 2*x* potato and analysis of the vigour of the somatic hybrids.

	Fusion combination	Selection marker(s)	Results of fusion experiments: number of			Results of flow cytometry: number of			Analysis of growth of potential somatic hybrids: number of		
			calli	regene-rants	flow cyto-metrically analysed regenerants	BC1 or BC2 regene-rants	potato regene-rants	potential somatic hybrids	vigorous potential somatic hybrids *in vitro*	vigorous potential somatic hybrids *in vivo*	flowering potential somatic hybrids
A.	BC1-6738 (+) 052-1	-	950	238	84	58	23	3	2	1	1
	BC1-6738 (+) 6486-4	kana[1]	600	171	43	41	0	2	2	1	1
	BC1-6738 (+) 6486-19	kana	750	197	69	64	0	5	4	2	2
	BC1-6738 (+) 6487-9	kana	1000	243	71	68	0	3	3	1	1
	BC1-6738 (+) PVU1	kana,hygro[2]	1150	337	40	1	3	36	28	15	18
	Total		4450	1186	307	232	26	49	39	20	23
B.	BC2-9017 (+) 6487-9	-	800	138	75	71	0	4	4	2	1
	BC2-9017 (+) PVU1	hygro	600	9	4	0	3	1	1	1	0
	Total		1400	147	79	71	3	5	5	3	1

[1] kana = resistant to kanamycin
[2] hygro = resistant to hygromycin

content higher than the BC1 or BC2 fusion parent, were considered as potential somatic hybrids. Flow cytometric analysis was carried out according to De Laat *et al.* (1987).

Crossing experiments

All potential somatic hybrids were grown in a greenhouse from February to October following the safety regulations stipulated by the Dutch Government for growing genetically modified organisms. Pollinations were performed with the tetraploid potato cultivars 'Bimonda', 'Désirée', 'Katahdin' and 'Mansour' and the tetraploid breeding clone AM66-42.

Berries of the potential somatic hybrids were harvested between three and five weeks after pollination for ovule rescue. Ovule culture was performed using HLH-medium according to Neal and Topoleski (1983). Ovules were transferred to fresh medium every fortnight.

Genomic in situ *hybridisation (GISH)*

Mitotic chromosome spreads of root tips on grease-free slides were made as described by Pijnacker and Ferwerda (1984). Root tips were collected from *in vitro* grown plants, ten days after propagation *in vitro*. GISH was performed according to Schwarzacher and Heslop-Harrison (1994). Modifications were mainly described by Jacobsen *et al.* (1995). Probe DNA was sonicated into fragments of 1-5 kb. Sonicated total genomic DNA was directly labelled with fluorescein-11-dUTP following the nick translation protocol. DNA used for blocking was autoclaved for 5 minutes into fragments of approximately 100 bp. For each preparation 50 ng probe and 3-5 mg blocking DNA was used. After hybridisation the chromosomes were counterstained with a mixture of DAPI (4'6-diaminidine-2-phenylindole) (2 µg/ml) and propidium iodide (1 mg/ml) for 10 min and afterwards mounted in one drop of antifade solution.

Results

Fusion experiments between BC1-6738 and 2x potato genotypes.

The number of calli that resulted from the five fusion combinations of BC1-6738 (+) 2*x* potato varied between 600 and 1150 (Table 1, A). At least 24% of these calli regenerated into plants. Between 65 and 88% of the plants of the five different fusion combinations were not analysed for hybrid nature because of poor *in vitro* growth. The remaining 307 regenerants were analysed by DNA content measurement with a flow cytometer. All genotypes with a higher DNA content than the BC1-6738 fusion parent were considered to be potential fusion hybrids.

Most of the analysed regenerants appeared to be the BC1 fusion parent (Table 1, A), as was expected since the BC1 genotype contained the kanamycin selection marker. The fusion combination that was carried out without kanamycin selection, also yielded 23 potato regenerants. Despite the use of kanamycin and hygromycin a few potato regenerants were also detected among the regenerants of the combination BC1-6738 (+) PVU1. Forty-nine regenerants were concluded to be potential somatic hybrids because they had a higher relative DNA content than the BC1 fusion parent. In four fusion combinations the percentage of somatic hybrids among the regenerants analysed was rather low, between

4 and 7%. As expected 90% from the regenerants of the combination BC1-6738 (+) PVU1 appeared to be somatic hybrids, because both fusion parents contained a selection marker.

All somatic hybrids were propagated *in vitro* and transferred to the greenhouse. Ten of the 49 somatic hybrids grew poorly *in vitro* and were also not able to grow well *in vivo*. Among the remaining 39 somatic hybrids with a good *in vitro* vigour, 20 genotypes were vigorous in the greenhouse and flowered as well. At least one flowering genotype per fusion combination could be selected but the majority of the selected genotypes, 15 hybrids, had resulted from the combination BC1-6738 (+) PVU1. Since there were three fusion hybrids of this combination that were moderately vigorous and also able to flower, the total number of flowering genotypes from this combination was 18 (Table 1, A).

Table 2. Results of crossing experiments of the somatic hybrids BC1-6738 (+) 2*x* potato with tetraploid potato genotypes.

Genotype	Fusion combination	Relative DNA-content	Number of pollinations	Number of berries (berry set)	Number of cultured ovules
F101-2	BC1-6738 (+) 6486-4	1.18	44	6 (14%)	0
F102-1	BC1-6738 (+) 6486-4	1.36	72	11 (15%)	1
F103-1	BC1-6738 (+) 052-1	1.30	4	0 (0%)	-
F104-2	BC1-6738 (+) 6487-9	1.49	16	5 (31%)	4
F108-1	BC1-6738 (+) PVU1[1]	1.12	11	0 (0%)	-
F108-2	" "	1.24	82	38 (46%)	14
F108-3	" "	1.25	10	0 (0%)	-
F108-4	" "	1.22	13	0 (0%)	-
F108-5	" "	1.74	2	0 (0%)	-
F108-6	" "	1.29	18	2 (11%)	0
F108-7	" "	1.29	81	1 (1%)	1
F109-1	BC1-6738 (+) PVU1[1]	1.30	45	1 (2%)	0
F109-2	" "	1.26	42	17 (40%)	0
F109-3	" "	1.28	90	3 (3%)	1
F109-4	" "	1.64	7	0 (0%)	-
F109-6	" "	1.28	33	2 (6%)	2
F109-7	" "	1.14	33	1 (3%)	0
F109-12	" "	1.04	48	11 (23%)	26
F109-15	" "	1.19	9	0 (0%)	-
F109-16	" "	1.27	81	9 (11%)	5

[1] Series F108 and F109 resulted from different experiments with the same combination of genotypes

None of the somatic hybrids was as vigorous as the BC1 fusion parent itself which could reach a height of two to three meters in the greenhouse. A majority of the twenty selected somatic hybrids was able to grow to a height of approximately 1.5 m. Some of the hybrids showed morphological abnormalities of leaves and flowers or dropped their flowers before anthesis.

Fusion experiments between BC2-9017 and 2x potato genotypes

Fusion experiments with BC2-9017 were less successful. No calli could be harvested from two of the five fusion combinations, namely BC2-9017 (+) RH90-052-1 and BC2-9017 (+) 6486-19. The fusion experiment with potato genotype 6486-4 resulted in 150 calli that did not regenerate into shoots (data not shown). Only from the combinations BC2-9017 (+) 6487-9 and BC2-9017 (+) PVU1 was a reasonable number of calli obtained, respectively 800 and 600 (Table 1, B). The first combination, BC2-9017 (+) 6487-9, resulted in 138 regenerants from which 75 plants could be analysed with the flow cytometer. Since no selection marker was used in this combination, regenerants of both fusion parents were expected as well. Four somatic hybrids were identified, whereas the remaining regenerants were BC2 plants. In the second combination, BC2 (+) PVU1, the potato fusion parent PVU1 contained the hygromycin selection marker. Only nine regenerants were obtained among which one somatic hybrid was identified. Three plants were potato regenerants.

All five somatic hybrids showed good *in vitro* vigour (Table 1, B). In the greenhouse three of these genotypes grew vigorously and, compared to the BC2 fusion parent, these three somatic hybrids showed improved vigour. The BC2 could grow to a height of about 1 m and showed early senescence of leaves, whereas the new somatic hybrids could reach a height of approximately 1.5 m without early senescence of their leaves. Only one of the selected somatic hybrids, which had resulted from the combination BC2-9017 (+) 6487-9, was able to produce flowers. Eighteen pollinations were made but no berries were obtained. The remaining two genotypes dropped their flowers before anthesis, like the BC2 fusion parent.

Crossing experiments with somatic hybrids of BC1-6738 (+) 2x potato.

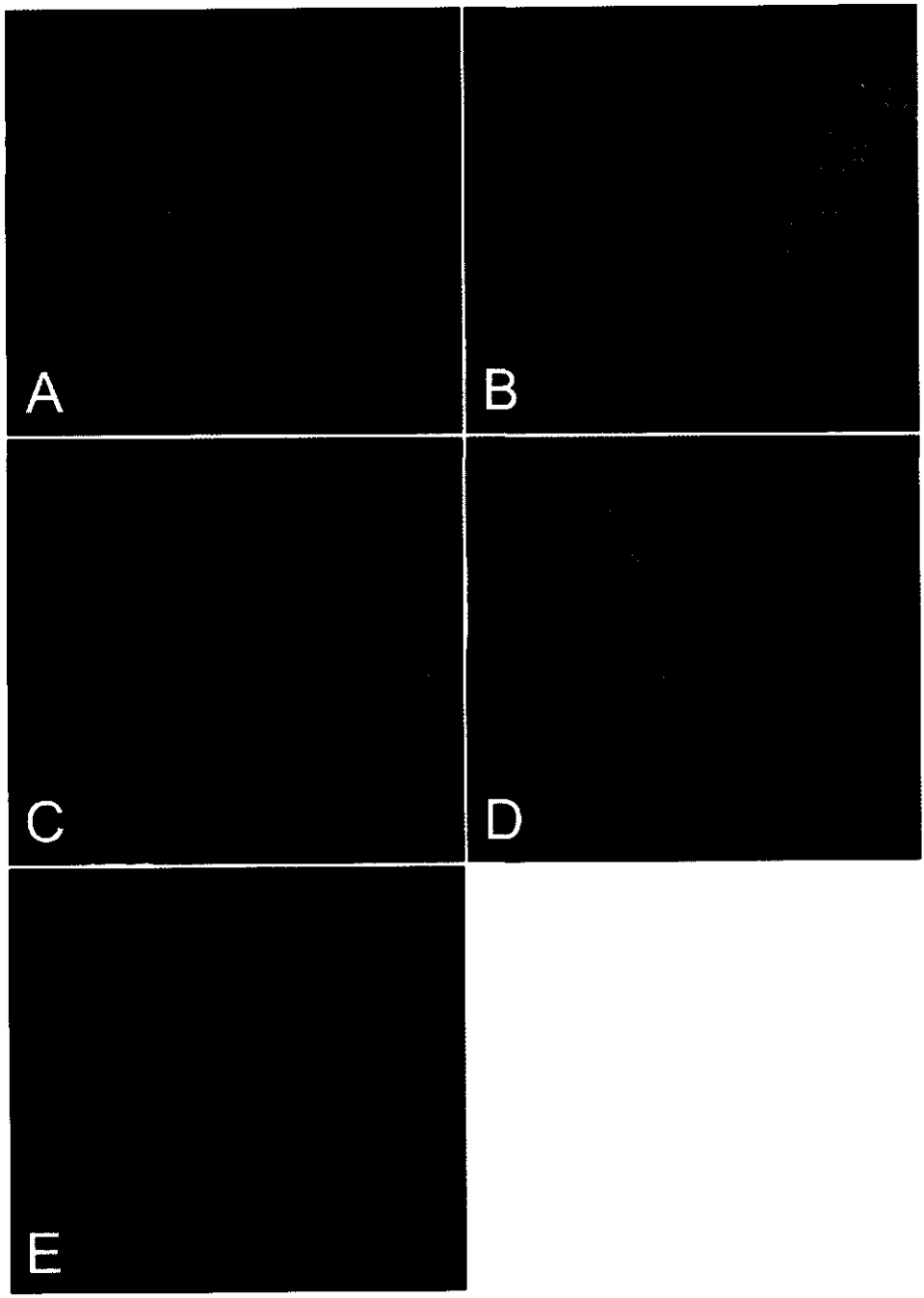
Twenty somatic hybrids that resulted from the fusion experiments between BC1-6738 (+) 2x potato were used in backcross experiments (Table 2). From each genotype two plants were put in the crossing greenhouse. Tetraploid potato genotypes such as 'Katahdin', 'Mansour' and 'Désirée' were used as male parents. Because some genotypes such as F103-1, F108-5 and F109-6 dropped most of their flower buds before they opened, only a few flowers could be pollinated and these did not result in the formation of berries. On thirteen somatic hybrids a total of fifteen pollinations or more was made. Genotypes F108-7, F109-1, F109-3, F109-6 and F109-7 had a very low berry set, between 1 and 6%. Some ovules could be rescued from the berries of three of these five genotypes. Genotypes F101-2, F102-1, F108-6, and F109-16 had a berry set between 11 and 15%. From the nine berries of F109-16 five ovules could be cultured, whereas only one ovule from the eleven berries of F102-1 and not a single ovule from the six

Table 3. Results of the GISH-analysis of *S. nigrum* (+) potato hybrids.

Genotype	Generation	Relative DNA-content	Number of *S. nigrum* chromo-somes	Number of *S. tuberosum* chromo-somes
AM10	initial potato fusion parent	0.28	-	24
SN14-0	initial *S. nigrum* fusion parent	1.00	72	-
F21-26	SN14-0 (+) AM10	1.27	72	24
BC1-6738	F21-26 x 4*x* potato (AM66-42)	1.00	36	36
6486-4, PVU1	2*x* potato fusion parents	0.27	-	24
F101-2	BC1-6738 (+) 6486-4[1]	1.18	42-45	41/42
F102-1	BC1-6738 (+) 6486-4[1]	1.36	36	60
F104-2	BC1-6738 (+) 6487-9	1.49	36-42	60
F108-2	BC1-6738 (+) PVU1[2]	1.24	36	60
F108-6	"	1.29	36	60
F108-7	"	1.29	36	58/59
F109-2	BC1-6738 (+) PVU1[2]	1.26	36	60
F109-3	"	1.28	36	60
F109-6	"	1.28	36	58-60
F109-12	"	1.04	22	58-60
F109-16	"	1.27	36	60

[1] Series F101 and F102 resulted from different experiments with the same combination of genotypes
[2] Series F108 and F109 resulted from different experiments with the same combination of genotypes

Figure 1. Genomic constitution of the initial somatic hybrid F21-26, BC1-6738 and three potential somatic hybrids of BC1-6738 (+) 2*x* potato. In figure 1A, F21-26, potato chromosomes fluoresce yellow whereas *S. nigrum* chromosomes fluoresce red. In the pictures 1B to 1E the *S. nigrum* chromosomes are yellow and the potato chromosomes are red. The number *of S. nigrum* chromosomes (*ngr*) and the number of *S. tuberosum* chromosomes (*tbr*) are given in parentheses. **A.** Initial somatic hybrid F21-26 (72 *ngr* + 24 *tbr*). **B.** BC1-6738 (36 *ngr* + 36 *tbr*). **C.** F109-12 (22 *ngr* + 58-60 *tbr*). **D.** F101-2 (42-45 *ngr* + 41/42 *tbr*). **E.** F102-1 (36 *ngr* + 60 *tbr*).


A
B
C
D
E

berries of F101-2 and the two berries of F108-6 was obtained.

The four remaining somatic hybrids, F104-2, F108-2, F109-2 and F109-12, had a berry set between 23 and 46%. Genotype F109-2 had a relatively high berry set of 40% but all berries were seedless. Sixteen pollinations on F104-2 did result in five berries that contained four ovules. F108-2 produced 38 berries (46%) from which 14 ovules could be rescued. F109-12 had a berry set of 23% but the number of ovules that could be rescued from the eleven berries was high: 26 in total. All genotypes on which at least 15 pollinations were possible and genotypes that produced berries from which ovules could be rescued, will be used in future crossing experiments.

GISH-analysis

The hexaploid *S. nigrum* genotype SN14-0 and the diploid potato genotype AM10, the initial fusion parents, were used in a control experiment. Mitotic chromosome spreads from root tips were hybridised with total genomic DNA of one of the species as a probe and DNA of the other species as blocking DNA. With GISH it was possible to differentiate clearly between *S. nigrum* and potato chromosomes. The original somatic hybrid, F21-26 (Horsman *et al.*, 1997), that had resulted from the somatic fusion of SN14-0 and AM10 was shown to contain the expected number of chromosomes of both species: 24 of potato and 72 of *S. nigrum*, respectively (Table 3, Figure 1A). The BC1 genotype BC1-6738, that was used as a fusion parent in the experiments described in this report, had resulted from a cross of the somatic hybrid F21-26 and the tetraploid potato genotype AM66-42. This BC1 genotype was expected to contain 36 chromosomes of both species. Counting of the differentially-stained chromosomes after GISH confirmed these chromosome numbers (Table 3, Figure 1B).

Based on the results of the crossing experiments, eleven hybrid genotypes were selected to be analysed with GISH. The selection consisted of all eight genotypes from which at least one ovule could be rescued and three genotypes that had produced more than one seedless berry (Table 2). Theoretically the somatic hybrids were expected to contain 96 chromosomes: 36 of *S. nigrum* and 60 of *S. tuberosum*. Six of the selected genotypes had this expected genomic constitution (Table 3, Figure 1E) and five genotypes deviated from this expectation. F108-7 and F109-6 were most likely missing one or two potato chromosome but both contained 36 chromosomes of *S. nigrum*. F104-2 had 60 chromosomes of potato origin but a higher number than 36 of *S. nigrum* chromosomes was identified. Between one and eight additional chromosomes or chromosome fragments of *S. nigrum* per cell were found in this genotype. F101-2 had a higher number of *S. nigrum* chromosomes than expected but the number of potato chromosomes was only slightly higher (41-42) than in the BC1-fusion parent (Table 3, figure 1D). Therefore it is doubtful that this genotype is a somatic hybrid. The fifth genotype with a deviating chromosome number was F109-12, the somatic hybrid with the highest number of ovules per berry. This genotype had only 22 *S. nigrum* chromosomes left but did contain 58 to 60 chromosomes of potato (Table 3, Figure 1C).

The relative DNA content of somatic hybrid F109-12 already indicated that several chromosomes might be missing since it was lower than the sum of the DNA-contents of the fusion parents. Most somatic hybrids with a

chromosome number that was nearly or exactly the expected chromosome number had a relative DNA content which equalled the sum of the parental DNA contents. DNA measurements of genotypes F102-1 and F104-2 were somewhat higher than expected. GISH-analysis proved ten of the eleven potential somatic hybrids that were selected after DNA content measurement and backcross experiments, to be real somatic hybrids. In these fusion experiments the combination of DNA-measurements and GISH has been shown to be a successful approach for the selection of fusion hybrids.

Discussion

The idea of using somatic fusion for adjustment of the genomic composition in order to solve backcross problems is new. In this contribution the first steps have been made to investigate the different factors of this approach, the most important one being the recognition of the desired somatic hybrids. Detection markers like isozymes were not useful in our case since they were not able to discriminate between the backcross hybrid and the potato fusion parent. A successful way to prescreen a large number of regenerants appeared to be flow cytometry. The combination of flow cytometry and GISH analysis was shown to be an effective method for the selection of the fusion hybrids.

In several publications, flow cytometry has been used for the analysis of DNA content of somatic hybrids. A high correlation between the 2C value and the number of chromosomes was found in fusion products of several Brassicaceae (Fahleson *et al.*, 1988). In the same plant family Sundberg and Glimelius (1991) established ploidy levels using flow cytometry. DNA content measurements have also been used fruitfully by several authors working with Solanaceae (Daunay *et al.*, 1993; Pijnacker *et al*, 1989), although in *S. brevidens* (+) *S. tuberosum* somatic hybrids a low correlation between nuclear DNA content and number of chromosomes was found (Valkonen *et al.*, 1994). In our case DNA, content measurements could be used successfully for the detection of somatic hybrids among regenerants, because of the differences in ploidy level of the fusion parents. The BC1-6738 and the BC2-9017 were 6*x* and approximately 5*x* respectively. This made it possible to distinguish between BC1-6738 or BC2-9017 regenerants, potato self-fusions and the desired fusion hybrids. The additional application of GISH indicated the reliability of flow cytometric observations. Ten of the eleven selected vigorous potential somatic hybrids appeared to be real fusion products.

GISH-analysis of the most important basic plant material showed the presence of the expected number of chromosomes in the initial fusion hybrid F21-26: 24 of *S. tuberosum* and 72 of *S. nigrum*. Observations of fusion hybrids of other combinations involving species from different genera such as *Solanum* (+) *Lycopersicon* showed that in many instances the expected number of chromosomes is not found in all hybrids. In 79% of the potato-tomato somatic hybrids analysed by Wolters and coworkers (1994) one or more chromosomes were missing. Garriga-Calderé *et al.* (1997) analysed three potato-tomato somatic hybrids with GISH and established the absence of one or two tomato chromosomes in two of these hybrids. Besides this, somatic recombination was also observed in potato-tomato somatic hybrids. (Wolters *et al.*, 1994; Garriga-Calderé *et al.*, 1997). The phenomenon

of missing chromosomes in somatic hybrids was also observed in other plant families. *Allium ampeloprasum* (4*x*) (+) *A. cepa* (2*x*) somatic hybrids were analysed with GISH by Buitenveld *et al.* (1998). All fusion hybrids lacked 3 to 7 chromosomes of the tetraploid *A. ampeloprasum* and one genotype had a lower number of *A. cepa* chromosomes as well.

The BC1 fusion parent BC1-6738, that was used successfully in backcross experiments, also possessed the expected number of potato and *S. nigrum* chromosomes: 36 of both species. Potato-tomato BC1 genotypes that had resulted from backcrosses with potato mostly did not contain the expected number of 12 tomato chromosomes. GISH-analysis of six BC1 plants revealed the presence of 10 to 12 tomato chromosomes. Identification of the individual tomato chromosomes with RFLP-markers and GISH revealed the presence of some tomato chromosomes in disomic condition. Therefore none of the six BC1 plants contained all twelve tomato chromosomes (Garriga-Calderé *et al.*, 1997). In the *S. nigrum*-potato BC1 this phenomenon of disomy could not be checked because of the absence of *S. nigrum*-chromosome-specific markers.

GISH-analysis of BC1-6738 (+) 2*x* potato showed that most of the selected potential fusion hybrids contained the sum of the number of parental chromosomes. Deviations were found in both genomes but not in such high frequencies as in the potato-tomato material earlier described. F109-12 was the most interesting somatic hybrid from the crossability point of view. In this hybrid a significant number of *S. nigrum* chromosomes was missing. It has to be checked whether or not this hybrid is still resistant to *P. infestans* like the BC1 fusion parent.

During each successive step of the selection scheme a substantial part of the material had to be removed. Over 1100 regenerants were obtained from the BC1 (+) 2*x* potato fusion combinations, but only 26% of the regenerants appeared to be vigorous *in vitro*. Among the 307 analysed plants 49 somatic hybrids were identified. Most of these hybrids were vigorous *in vitro* but only 20 hybrids grew vigorously in the greenhouse as well and were flowering. The selection of useful BC2-9017 (+) 2*x* potato somatic hybrids showed a similar pattern. Two vigorous and flowering genotypes were selected from 147 regenerants. Similar observations were made during the initial fusion experiments between *S. nigrum* related species and several potato genotypes (Horsman *et al.*, 1997). Results described in this contribution again showed the importance of producing a large quantity of starting material for the selection of somatic hybrids that were useful for crossing experiments. The next step will be more extensive backcrossing and embryo rescue experiments with the selected fusion products.

5

Prospects for the introgression of chromosomes from non-tuberous *Solanum nigrum* into *S. tuberosum*: a qualitative analysis of the meiosis through GISH and AFLP-analysis of backcross derivatives

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ABSTRACT

The chromosome constitution and meiotic behaviour of a somatic hybrid of *Solanum nigrum* ($2n=6x=72$) and diploid potato ($2n=2x=24$) and its BC1 and BC2 progeny were studied. The application of Genomic *in situ* Hybridisation (GISH) made it possible to distinguish clearly between *S. nigrum* and potato chromosomes in mitotic and meiotic chromosome spreads. The somatic hybrid F21-26 was found to be an octaploid with six genomes of *S. nigrum* and two of potato. A cross between F21-26 and a 4*x* potato genotype produced BC1-6738, a hexaploid with 36 chromosomes each of both species. With GISH it was impossible to determine whether the 36 chromosomes of *S. nigrum* represented three complete genomes. However, AFLP-data showed that none of the AFLP-specific markers that was amplified in the *S. nigrum* fusion parent and the somatic hybrid was missing in BC1-6738, which is an indication that no major chromosome elimination has taken place. A second backcross with 4*x* potato resulted in eleven BC2 genotypes with a near-pentaploid genomic constitution. Chromosome counts in nuclei of BC2 tetrad cells showed that transmission of alien *S. nigrum* chromosomes to BC3 progeny is likely since on average 9.1 *S. nigrum* chromosomes per BC2-microspore were detected. Meiotic analysis of metaphase I in BC1-6738 and in BC2-9019 indicated clearly that allosyndetic pairing occurs in these genotypes. Homoeologous pairing in both bivalent and trivalent formation was observed.

INTRODUCTION

The genus *Solanum* of the family of Solanaceae is a large one with more than 2000 species. It includes tuber-bearing (tuberous) and non-tuberbearing (non-tuberous) forms. With the aim to use the genetic variation that is present in this genus, extensive interspecific hybridisation has been carried out in the past. Generally, it is easy to sexually hybridise and subsequently make backcrosses within the group of tuberous *Solanum* species. Nevertheless, it is extremely difficult to cross tuberous and non-tuberous species. The first successful sexual hybrids of this kind were between *S. pinnatisectum* (tuberous) and species of the series Etuberosa, *S. etuberosum*, *S. brevidens* and *S. fernandizianum* (Hermsen and Taylor, 1979; Ramanna and Hermsen, 1981). Later on Watanabe *et al.* (1995) described the production of sexual hybrids between the same *Etuberosa* species and diploid *S. tuberosum*. However, in the cases mentioned above all species belong to the same section, namely Petota (Hawkes, 1978). Sexual hybrids between species of section Petota and species of the *S. nigrum* complex were made by Eijlander and Stiekema (1994). *S. nigrum* and *S. villosum* were crossed with *S. tuberosum* and *S. demissum* which resulted in only a limited number of hybrid genotypes.

Besides sexual hybridisation somatic hybridisation has been performed using more distantly related species (Wolters, 1994). Some drawbacks of those experiments are that 1) few attempts have been made to produce extensive backcrosses of the somatic hybrids; 2) hardly any cytological assessment has been undertaken with somatic hybrids and their backcross derivatives, when available. A few exceptions of hybrids that were investigated cytologically are the interspecific F1 hybrids of tuberous and non-tuberous *Solanum* species (Ramanna and Hermsen, 1979; Hermsen *et al.*, 1981) and the intergeneric hybrids of *Solanum* and *Lycopersicon* (Jacobsen *et al.*, 1994; Garriga-Calderé *et al.*, 1997). From these studies it was evident that a certain amount of homoeologous recombination occurs between the chromosomes of tuberous and non-tuberous *Solanum* species (Hermsen *et al.*, 1981) whereas only a highly restricted amount of intergeneric recombination occurs in the case of potato and tomato (Garriga-Calderé *et al.*, 1998).

With a view to introgress (a part of) chromosomes of *S. nigrum* into potato, somatic hybrids involving those species were produced and subsequently backcrossed to potato (Horsman *et al.*, 1997 and 1999). BC1 and BC2 progeny was obtained and in order to utilise these genotypes in further crossing experiments a cytological assessment was made applying the genomic *in situ* hybridisation (GISH) technique and a molecular analysis was carried out involving the AFLP (amplified fragment length polymorphism) technique. Special focus was on the possibilities of occurrence of recombination and the transmission of alien chromosomes to the progenies. In the light of these results the prospects for introgression are discussed.

MATERIALS AND METHODS

Plant material

All genotypes that were analysed were hybrids of *S. nigrum* and *S. tuberosum*. Somatic hybridisation experiments between *S. nigrum*

(2*n*=6*x*=72) genotype SN14-0 and diploid potato genotype AM10 gave rise to the octaploid (2*n*=8*x*=96) somatic hybrid F21-26 (Horsman *et al*, 1997). The diploid *S. tuberosum* genotype 87.1029/31, was an *amylose-free* (*amf*) mutant described by Jacobsen *et al.* (1989). AM10 was a transformant of clone 87.1029/31, containing the GUS-gene and the kanamycin resistance gene.

The BC1 genotype DJ93-6738, which will be referred to as BC1-6738, resulted from a cross between F21-26 and the tetraploid (2*n*=4*x*=48) breeding clone AM66-42. BC1-6738 was again crossed with several tetraploid cultivars which resulted in eleven BC2 genotypes that were able to grow *in vitro*. An overview of all genotypes and the parents of all BC2 genotypes can be found in Table 1.

Somatic hybrids and backcross genotypes were maintained *in vitro*. Plants were grown on basal MS medium with 30 g/l sucrose. All plants were grown in a greenhouse from February to October. The somatic hybrids and the backcross genotypes were grown according to government safety rules for genetically modified organisms.

Genomic in situ hybridisation (GISH)

For the analysis of the chromosome constitution mitotic chromosome spreads were made of root tips that were harvested from *in vitro* plants 7-10 days after propagation. Root tips were treated with 2 mM 8-hydroxyquinoline solution for 2.5 hours at 18°C after which they were fixed in ethanol-acetic acid 3:1. Preparations were made on grease-free slides according to Pijnacker and Ferwerda (1984). GISH was carried out with *S. nigrum* DNA as probe and *S. tuberosum* DNA as blocking or *S. tuberosum* DNA as probe and *S. nigrum* DNA as blocking following the procedure of Schwarzacher and Heslop-Harrison (1994), modifications described by Jacobsen *et al.* (1995) and details in Horsman *et al.* (1999). Per preparation 50 ng probe and 3-5 mg blocking DNA was used. After hybridisation the chromosomes were counterstained with a mixture of DAPI (4'6-diaminidine-2-phenylindole) (2 µg/ml) and propidium iodide (1 mg/ml) for 10 min and afterwards mounted in one drop of antifade solution.

Meiotic preparations were made of young anthers of flower buds that were collected between ten and twelve o'clock in the morning. One anther per flower bud was squashed in aceto-carmine and checked under a light-microscope in order to establish the meiotic stage. The remaining anthers of flowers with suitable meiotic stages were fixated in ethanol-acetic acid 3:1 for 30 minutes at room temperature. Enzyme treatment of the anthers was carried out with 0.5% pectolyase Y23, 0.5% cytohelicase and 0.5% cellulase RS in 10 mM citrate buffer pH 4.5 for two houres.

Preparations were made following the protocol of Pijnacker and Ferwerda (1984). GISH with *S. nigrum* DNA or *S. tuberosum* DNA as a probe was performed as described previously.

AFLP-analysis

The AFLP-analysis was performed according to Van Eck *et al.* (1995) which was based on the protocol as described by Zabeau and Vos (1993) and Vos *et al.* (1995). Three primer combinations, involving primers with three selective bases, were used:

1) E35/M48: Primer+ACA/Primer+CAC;
2) E35/M50: Primer+ACA/Primer+CAT;
3) E35/M58: Primer+ACA/Primer+CGT.

RESULTS

An important pre-requisite for cytological analysis of the somatic hybrid F21-26 and its backcross derivatives was that a distinction could be made between the chromosomes of *S. nigrum* and *S. tuberosum*. Genomic *in situ* hybridisation (GISH) made it possible to distinguish the genomes from each other unambiguously in both mitotic and meiotic preparations (Fig. 1).

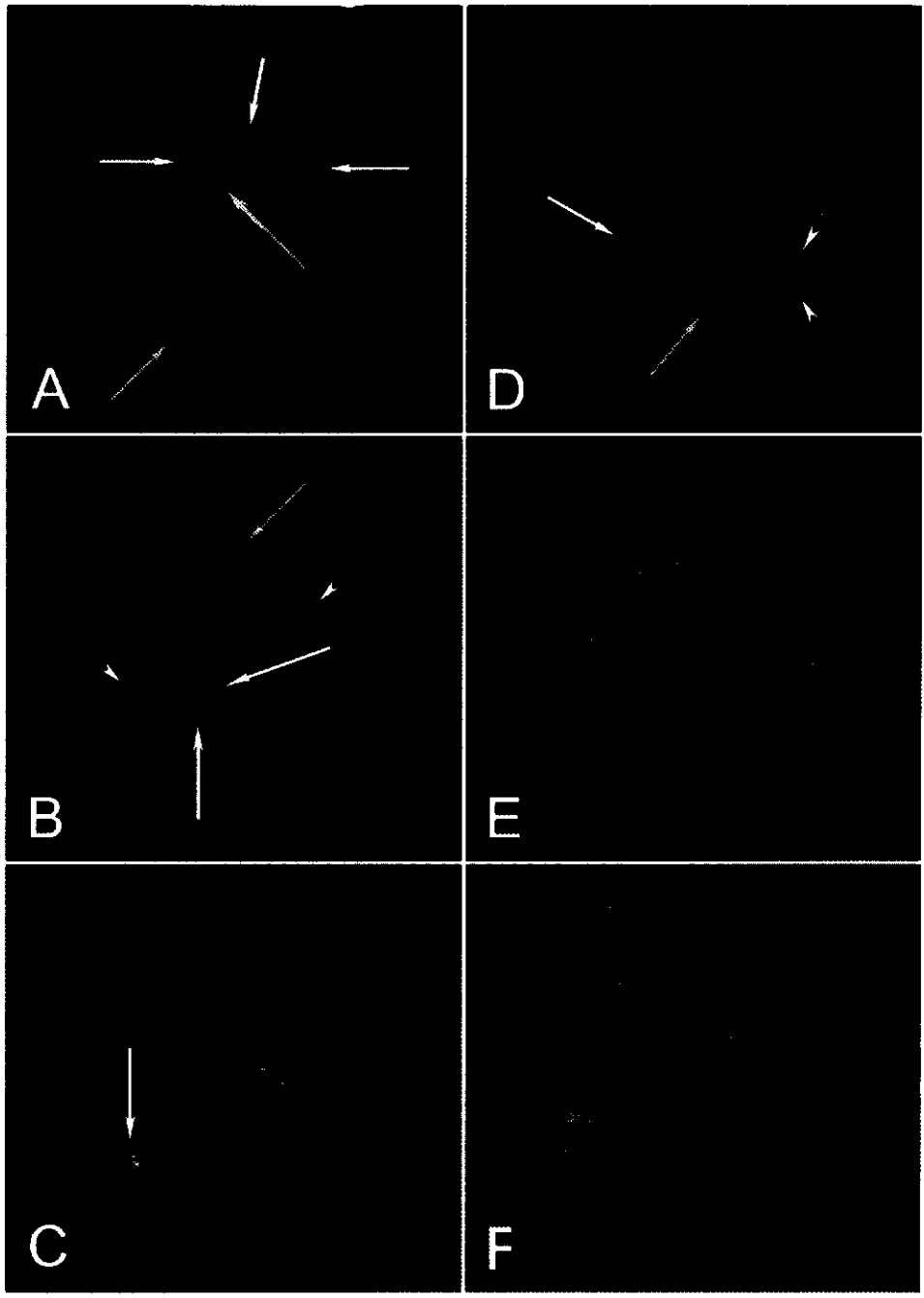
Somatic chromosome constitution of the fusion hybrid and BC1-genotype

Since the initial somatic hybrid was the result of a fusion between diploid *S. tuberosum* ($2n=2x=24$) and hexaploid *S. nigrum* ($2n=6x=72$) the chromosome number of the somatic hybrid was expected to be an octaploid ($2n=8x=96$). As indicated in Table 1 this was shown to be the case. A cross between the somatic hybrid and a tetraploid potato breeding clone gave rise to BC1-6738 which was shown to be a hexaploid. Thirty-six chromosomes of each of both *S. nigrum* and potato were counted in this genotype. The most logical explanation was that a normal gamete from the 8*x* somatic hybrid consisting of three genomes of *S. nigrum* and one genome of potato was involved in the fertilisation process. The fusion of such a gamete with two genomes of the 4*x* *S. tuberosum* backcross parent obviously resulted in a hexaploid BC1. However, since the individual chromosomes could not be identified in metaphase condition it was not possible to determine whether these 72 chromosomes represent six complete genomes. Theoretically it is possible that some chromosomes are overrepresented whereas others, in that case, have to be under-represented.

Pairing and transmission of chromosomes in BC1-6738

Being distant interspecific hybrids with high chromosome numbers it was not possible to obtain the chromosomes well spread out in cytological preparations of pollen mother cells. Therefore it was not possible to make a quantitative analysis of the extent of chromosome pairing during metaphase I

Figure 1. Meiotic behaviour in a first generation (BC1-6738, A-C) and a second generation (BC2-9019, D-F) backcross derivative of *S. nigrum* (+) potato somatic hybrids with tetraploid potato as revealed by GISH. In all pictures except C the *S. nigrum* chromosomes fluoresce yellow and the potato chromosomes red. In picture C coloration is reversed. "N" = *S. nigrum*, "T"= *S. tuberosum*, the colour of the letter is similar to the fluorescence signal of the chromosome. White arrows point at allosyndatic paired chromosomes, light blue arrows at autosyndatic pairing. White arrowheads indicate univalents. A-C: Metaphase I stage of BC1-6738 showing N-univalents, T-univalents, autosyndatic pairing of both N- and T-chromosomes, homoeologous bivalents and homoeologous trivalents in all four possible configurations: NNT, NTN, TTN and TNT. D: Metaphase I stage of BC2-9019 with a TTN-trivalent, several homologous bivalents and univalents. E: Late methaphase I, early anaphase I stage of BC2-9019 with lagging chromosomes of both potato and *S. nigrum*. F: Telophase II stage of BC2-9019.


A
D
B
E
C
F

stages. However, it was possible to analyse cells for meiotic chromosome configuration at metaphase I and to count chromosomes during anaphase I and telophase II in both BC1 and BC2 plants (Fig. 1). Meiotic preparations of the somatic hybrid F21-26 could not be made since no anthers with pollen mother cells undergoing meiotic division could be identified, despite the analysis of a large quantity of floral buds.

Meiotic analysis of the BC1-6738 genotype confirmed the genomic constitution established from the somatic cell analysis, i.e., three genomes each of both *S. nigrum* and potato were present. Because of the presence of this odd number of genomes (3*x* + 3*x*) in the BC1-6738, the chromosome pairing was complicated (Fig. 1A, B, C). Trivalents, bivalents and univalents were observed at metaphase I stages. Associations of more than three chromosomes could not be confirmed with certainty but trivalents were observed unequivocally. Because of the application of GISH two types of trivalents could be distinguished: those that involved only homologous chromosomes (autosyndetic pairing) and a second type that involved homoeologous chromosomes (allosyndetic pairing) (Fig. 1A, B, C). The latter types consisted of either one or two *S. nigrum* chromosomes with respectively two or one *S. tuberosum* chromosomes. Within the group of bivalents two types were distinguished as well: homologous and homoeologous pairs (Figure 1A, B). A considerable amount of univalents of both parents was also observed (Fig. 1A).

In view of the odd number of genomes of both parents in the BC1 plant, meiotic pairing and chromosome distribution during the first meiotic division was abnormal as expected. By analysing a limited number of telophase II stages, the number of *S. nigrum* chromosomes in individual nuclei was estimated. This number ranged from 13 to 19, the average being 16.7 per nucleus (Fig. 2) which is nearly half of the somatic number of *S. nigrum* chromosomes in this genotype (Fig. 2).

Because of the abnormal meiosis, BC1 plants showed a low fertility with a pollen stainability of 10-15%. BC1 pollen was not able to penetrate pistils of 4*x* potato genotypes.

Chromosome constitution, pairing and transmission in BC2

The chromosome constitution of eleven BC2 plants was estimated from counting their number in root tip cells. The total number of chromosomes ranged from 47-49 to 59-61 (Table 1) with a number of *S. nigrum* chromosomes that varied from 14 to 20. Except for KH94-9026 the chromosome numbers of the BC2 plants were concurrent with the pentaploid number of chromosomes that was expected from a 6*x*-4*x* cross.

Meiosis was abnormal in BC2 plants because of the pentaploid constitution (Fig. 1D, E). As in the BC1 genotype multivalents, bivalents and univalents were observed at metaphase I stages of BC2-9019 (Fig. 1D). Both bivalents and trivalents displayed autosyndetic as well as allosyndetic chromosome associations as in the BC1. Despite the unbalanced distribution of chromosomes during anaphase I stages (Fig. 1E), the second division seemed to occur normally giving rise to spore tetrads of microspores. A notable feature was that the chromosomes were well spread out in the nuclei of each of the pollen mother cells (Fig. 1F) so that it was possible to estimate the number of *S. nigrum* chromosomes which ranged from 7 to 12 with an average of 9.1 (Fig. 3)

Table 1. Determination of the number of *S. nigrum* and potato chromosomes in a somatic hybrid of both species and their first and second generation backcross hybrids using GISH.

Genotype	Fusion/cross	Generation	Number of *S. nigrum* chromosomes	Number of *S. tuberosum* chromosomes	Total number of chromosomes
F21-26	*S. nigrum* (+) 2*x S. tuberosum*	somatic hybrid	72	24	96
BC1-6738	F21-26 x Am66-42	BC1	36	36	72
KH94-9072	BC1-6738 x Désirée	BC2	14	33-35	47-49
KH96-9365	BC1-6738 x Mansour	BC2	14	40-42	54-56
KH96-9304	BC1-6738 x Frieslander	BC2	15	40	55
KH96-9345	BC1-6738 x Turbo	BC2	15	40	55
KH94-9026	BC1-6738 x Gloria	BC2	16	36-38	52-54
KH96-9320	BC1-6738 x Frieslander	BC2	16	38-40	54-56
KH94-9017	BC1-6738 x Katahdin	BC2	17	40-42	57-59
KH94-9084	BC1-6738 x Gloria	BC2	17	43	60
KH96-9380	BC1-6738 x Désirée	BC2	18	36-40	54-56
KH94-9019	BC1-6738 x Katahdin	BC2	19	37-41	56-60
KH96-9379	BC1-6738 x Désirée	BC2	20	39-41	59-61

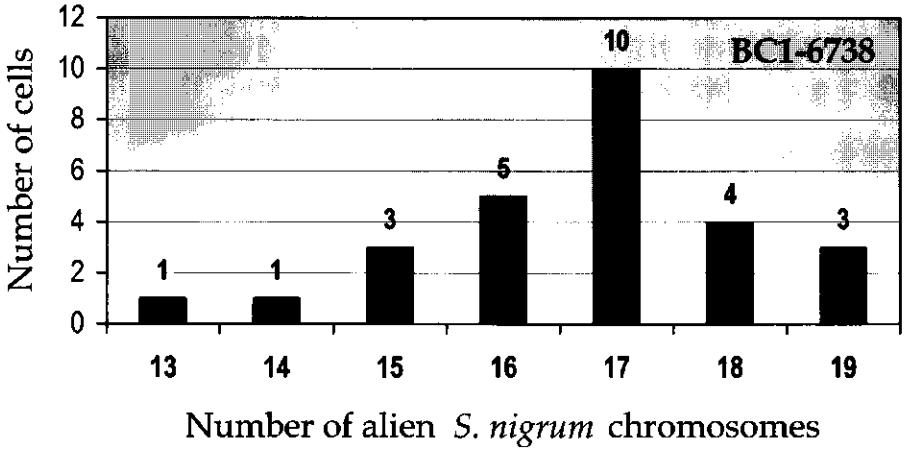


Figure 2. Chromosome counts in the nuclei of tetrad cells of BC1-6738. From a total of 27 tetrad cells the number of *S. nigrum* chromosomes was determined. On average 16.7 *S. nigrum* chromosomes were found per nucleus.

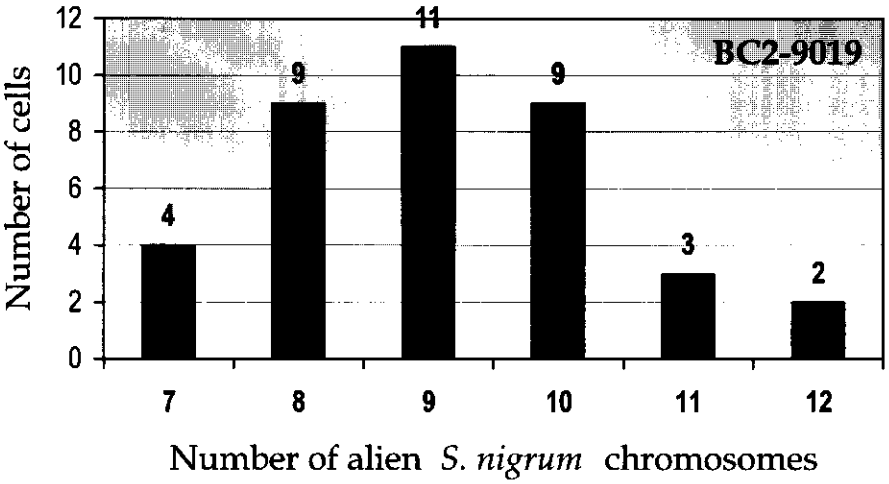


Figure 3. Chromosome counts in the nuclei of tetrad cells of BC2-9019. From a total of 38 tetrad cells the number of *S. nigrum* chromosomes was determined. On average 9.1 *S. nigrum* chromosomes were found per nucleus.

AFLP-analysis

For the identification of the degree of homozygosity in *S. nigrum* AFLP fragments of a selfing population with 48 descendants of the *S. nigrum* fusion parent were analysed. The initial *S. nigrum* fusion parent was found to be highly homozygous since only 4% of all amplified fragments appeared to be polymorphic. After a comparison of the AFLP-patterns of *S. nigrum*, the potato fusion parent and the potato backcross parents a total of 66 markers was selected from the remaining fragments for the analysis of the fusion product, the BC1 and BC2 derivatives.

All of the selected fragments were amplified in the somatic hybrid F21-26 as well as in the BC1-6738 (Table 2). Since the distribution of these AFLPs on the chromosomes of the *S. nigrum* genomes is unknown, this can not lead to any final conclusion about the presence of the complete *S. nigrum* genome in these genotypes. However, together with the observation of 72 *S. nigrum* chromosomes in F21-26 and 36 in BC1-6738, there was a strong indication that no major chromosome elimination had occurred during somatic hybridisation and the first backcross.

In the AFLP patterns of the ten BC2 genotypes that were analysed the percentage of *S. nigrum* specific markers that was detected varied from 48 to 71, indicating that, as expected, chromosome loss occurred in this generation (Table 2). BC2-9365, which was possessing the smallest number of *S. nigrum* chromosomes of all BC2's, showed only 50% of the *S. nigrum* bands whereas BC2-9379 with still 20 *S. nigrum* chromosomes had retained 71%. However, a linear relationship between the number of alien *S. nigrum* chromosomes in the BC2s and the number of *S. nigrum* markers could not be detected. Relatively large differences in the number of *S. nigrum* specific bands were found between BC2 genotypes with an equal number of *S. nigrum* chromosomes. For example, a difference of 17% in the number of *S. nigrum* specific fragments was shown between BC2-9304 and BC2-9345, both of which contain 15 chromosomes of *S. nigrum*. This could indicate a difference in the number of single chromosomes and induced diplo-chromosomes between those two genotypes.

Table 2. *S. nigrum* specific AFLP-bands in the descendants of *S. nigrum* (+) potato somatic hybrids.

Genotype	Number of *ngr*-chromosomen	Percentage of *ngr*-specific AFLP-bands present
F21-26	72	100 %
BC1-6738	36	100 %
BC2-9365	14	50 %
BC2-9304	15	65 %
BC2-9345	15	48 %
BC2-9026	16	52 %
BC2-9320	16	68 %
BC2-9017	17	58 %
BC2-9084	17	66 %
BC2-9380	18	60 %
BC2-9019	19	67 %
BC2-9379	20	71 %

Discussion

Several attempts have been undertaken to hybridise distantly related species within the Solanaceae (Wijbrandi, 1989; Wolters, 1994).

However, there have been relatively few studies to investigate backcrosses and monitor the backcross progeny extensively. The results reported in this article are an example in which the backcross progeny of a fusion hybrid has been analysed. As far as is known, it is the only successful attempt to backcross a *S. nigrum* (+) *S. tuberosum* hybrid (Horsman *et al.*, 1999). A similar study was carried out on potato (+) tomato somatic hybrids and their backcross progeny (Jacobsen *et al.*, 1994, Garriga-Calderé *et al.*, 1997). In that case the species belonged to different genera, viz. *Solanum* and *Lycopersicon*. Application of the GISH technique made it possible to distinguish between potato and tomato chromosomes (Jacobsen *et al.*, 1995). Fortunately, in our experiments it was also possible to discriminate between the chromosomes of *S. nigrum* and potato with GISH, although both species belong to the same genus, *Solanum*.

Two important aspects emerged from our cytological analysis of BC1-6738 and BC2-9019: 1) clear evidence was found for allosyndetic pairing, of importance for homoeologous recombination; 2) the *S. nigrum* chromosomes are transmitted in reasonable numbers from the somatic hybrid F21-26 to BC1-6738 and from BC1-6738 to the BC2 progeny. Looking at the number of *S. nigrum* chromosomes in telophase II nuclei of the BC2 plants, it seems to be likely that a relatively high frequency of the alien chromosomes will be transmitted to BC3 progeny as well.

Regarding the pairing between chromosomes of a tuberous and a non-tuberous species the only cytologically analysed *Solanum* hybrids were between *S. pinnatisectum* and the *Etuberosa* species *S. etuberosum*, *S. fernandizianum* and the hybrid *S. brevidens* x *S. etuberosum* (Ramanna *et al.*, 1979; Hermsen and Ramanna, 1981). In these biparental and trispecific triploid hybrids trivalents were observed in low frequencies (respectively 0-3 and 1-4 per cell) which indicated the occurrence of recombination between the species involved. Taxonomically these species are more closely related (Hawkes, 1978) than the species used in our investigation, *S. tuberosum* and *S. nigrum*, which belong to different sections of the Solanaceae. This means that the considerable amount of allosyndetic pairing observed in BC1-6738 and BC2-9019 indicates that allosyndetic pairing is much more widespread. It may also be noted that allosyndetic pairing in intergeneric hybrid derivatives, like BC1 and BC2 of potato (+) tomato somatic hybrids (Garriga-Calderé *et al.*, 1999), is much more restricted than in our hybrids. A quantitative analysis might be very useful for the establishment of phylogenetic trends. When backcross derivatives with lower ploidy levels will become available it might be possible to quantify the extent of pairing and show recombination. So far in none of the backcross derivatives recombination could be shown with GISH. This is probably because the euchromatic parts of chromosomes in solanaceous crops such as tomato (Ramanna and Prakken, 1967) and potato (Ramanna and Wagenvoort, 1976) are highly condensed. Because of this, crossover segments that are generally distal, cannot be made visible easily unless large segments of chromosomes are involved in crossingover.

No preferential elimination of *S. nigrum* chromosomes seemed to have occurred in the somatic hybrid and the BC1-6738 since the latter genotype contained exactly 36 *S. nigrum* chromosomes, half the number of *S. nigrum* chromosomes of the somatic hybrid. It is

possible that these 36 chromosomes do not represent three genomes exactly. One or more chromosomes could be overrepresented whereas others, in that case, have to be underrepresented. Besides, the AFLP data showed clearly that all *S. nigrum* specific markers that were present in the initial *S. nigrum* fusion parent could also be detected in the BC1-6738, which indicates that all parts of the chromosomes that were covered by the AFLPs are retained after the first backcross. Since it is unknown at present whether the markers used by us are dispersed over all chromosomes of the *S. nigrum* genome, no conclusions can be drawn about the completeness of this genome in BC1-6738. However, AFLP-analysis of the first potato-tomato BC1, which contained 50% of the twelve tomato chromosomes, showed that from the tomato-specific AFLP-markers also 50% could still be detected in this genotype (unpublished data).

In potato-tomato hybrids preferential elimination of tomato chromosomes was already observed in the BC1 plants (Garriga-Calderé *et al.*, 1997). None of the five BC1 plants that were the result of a cross between a potato-tomato somatic hybrid and 4*x* potato contained all twelve chromosomes of tomato. The transmission from BC1 to BC2 plants varied from 1.7 till 3.4 tomato chromosomes, depending on the chromosomal constitution of the BC1 genotype. The BC1 that contained 11 single tomato chromosomes transmitted, on average, 1.7 chromosomes to its BC2 descendants. Another BC1 mother plant with 8 single and 2 disomic tomato chromosomes (Garriga-Calderé *et al.*, 1998) transferred on average 3.4 alien chromosomes to the BC2 progeny. In our experiments we established a transmission of 14 to 20 *S. nigrum* chromosomes from BC1 to the BC2 genotypes.

The *S. nigrum*-complex is a highly polymorphic taxon with world wide distribution and the species are most successful as weeds. It is attractive to transfer the chromosomes of these species into potato in order to widen the genetic basis for some of the desirable characters like resistances against pests and diseases. In this context it is worthwhile to stably integrate alien *S. nigrum* chromosomes into potato, even if the traditional method of introgression has to be practiced. Fortunately it is possible to apply the GISH and AFLP techniques to the *S. nigrum*-potato hybrids which facilitates the identification of genotypes with desirable alien chromosomes among the backcross derivatives and thus enhances the chance of success.

6

General discussion

The use of taxonomically related species in plant breeding as a source of genetic variation is very common in plant breeding. Several methods are being used in order to make the genes of these species available, from which sexual hybridisation followed by recurrent backcrossing is most common. In this thesis the application of one of the alternative approaches was described, namely somatic hybridisation followed by recurrent backcrossing. The successive steps of our approach will be discussed in this chapter and some alternatives will be compared.

PRODUCTION OF SOMATIC HYBRIDS

In order to produce large populations of starting material for backcross experiments, four different species of the *S. nigrum* complex with different ploidy levels were somatically hybridised to three potato genotypes. Most of the fusion combinations resulted in somatic hybrids that were vigorous *in vitro* but appeared to be inviable after transferring to the greenhouse. Only from the three combinations *ngr* (+) 1029/31, *ngr* (+) AM10^{K} and *ame*$_{2x}$ (+) Désirée a substantial part of the hybrids was also vigorous in the greenhouse. Three other combinations resulted in a low percentage of relatively well growing hybrids: *chp* (+) 1029/31, *ame*$_{6x}$ (+) 1029/31 and *ame*$_{6x}$ (+) AM10^{K}. Among the hybrids of the remaining combinations, not a single plant was able to grow in the greenhouse.

The striking contrast between *in vitro* and *in vivo* vigour has not been described in detail in literature, however, it was observed earlier in some combinations within the Solanaceae. Several potato-tomato genotypic combinations resulted in somatic hybrids that performed very well in the greenhouse, whereas plants of other genotypic combinations all died *in vivo* (Garriga-Calderé *et al.*, 1997). Poor performance of potato (+) tomato somatic hybrids *in vivo* was also reported by Schoenmakers *et al.* (1993), but in their case also a low frequency of vigorous plants was obtained.

Difficulties with rooting and culturing of somatic hybrids were also encountered in Brassicaceae, mainly within intertribal combinations. *Brassica napus* (+) *Thlaspi perfoliatum* fusion products were difficult to transfer to the greenhouse (Fahleson *et al*, 1994b) but difficulties were even more pronounced in fusions between *B. napus* (+) *Barbarea vulgaris*, which never resulted in plants that could grow under greenhouse conditions (Fahleson *et al.*, 1994a). The difference with our somatic hybrids was, that the *B. napus* (+) *Barbarea vulgaris* were also difficult to culture *in vitro*, since their *in vitro* growth was rather slow whereas our somatic hybrids grew vigorously *in vitro*. Another troublesome combination was *B. napus* (+) *Lesquerella fendleri* from which only 5 of the 53 symmetric somatic hybrids could be established in the greenhouse (Skarzinskaya *et al.*, 1996). Partial elimination of DNA of the wild species using irradiation appeared to improve the *in vivo* performance of *B. napus* (+) *L. fendleri* fusion products since 32 % of the asymmetric hybrids rooted and grew *in vivo*. In fusion combinations of Solanaceae species, for instance *L. esculentum* (+) *L. peruvianum*, irradiation had a negative effect on vigour and fertility of the hybrids (Wijbrandi *et al.*, 1990)

The problem of establishing somatic hybrids that are able to grow vigorously in the greenhouse seems to occur mainly among combinations of more distantly related species.

However, the factor of relatedness can not explain the differences that we observed between the hybrids of the different fusion combinations since all of the species of the *S. nigrum*-complex are expected to have the same phylogenetic distance to potato. Our results indicate that there might be a major genotypic influence on the results of somatic hybridisation experiments. AFLP results showed that apparently the hexaploid *S. americanum* that was used in our experiments, is an accession of *S. nigrum*. Nevertheless its performance in somatic hybridisation experiments was quit different from the performance of the official *S. nigrum* accession that we used.

BACKCROSS EXPERIMENTS

Octaploid *S. nigrum* (+) potato somatic hybrids were backcrossed to both *S. nigrum* and potato. Considerable differences were observed between both crossing programmes since backcrosses with *S. nigrum* were rather easy to accomplish whereas backcrosses with potato had a very low success rate. Only 31 pollinations with *S. nigrum* pollen were necessary in order to obtain twelve BC1 plants from which one was already self-fertile. From these BC1 descendants BC2 progeny could easily be obtained. On the contrary over 4300 pollinations were necessary in order to obtain two BC1 plants with potato.

Potato (+) tomato somatic hybrids showed an equally low success rate in their crossing experiments with potato (Jacobsen *et al.*, 1994; Garriga-Calderé *et al.*, 1997). Both tetraploid somatic hybrids [2x potato (+) 2x tomato] and hexaploid ones [4x potato (+) 2x tomato] were crossed with 4x potato genotypes from which only the latter resulted in backcross progeny.

The main difference between the *S. nigrum* (+) potato and the potato (+) tomato backcross experiments with potato, was the ability to produce of BC2 and BC3 progeny. The *S. nigrum* (+) potato BC1 was again crossed with tetraploid potato genotypes, which resulted in 12 plants out of 3000 cultured ovules. In the greenhouse all BC2 genotypes were less vigorous than the BC1 parent and no BC3 progeny could be obtained. In the potato (+) tomato backcrossing programme BC2 and BC3 progeny from different BC1 genotypes could easily be obtained (Garriga-Calderé *et al.* 1997).

Differences in crossability were also observed between *S. brevidens* (+) potato somatic hybrids with different genomic constitutions. Ehlenfeldt and Helgeson (1987) were the first to produce backcross progeny from this combination. They used tetraploid hybrids with two genomes of both species and hexaploid hybrids that resulted from fusion experiments between *S. brevidens* and a tetraploid potato genotype. Tetraploid somatic hybrids crossed poorly with both 2*x* and 4*x* potato genotypes, whereas the hexaploid *S. brevidens* (+) potato somatic hybrids crossed very well with tetraploid potato. Jacobsen *et al.* (1993) showed similar results after backcrossing *S. brevidens* (+) potato somatic hybrids. In their experiments the hexaploid somatic hybrids with four genomes of potato crossed much better than the tetraploid fusion hybrids.

So the production of BC1 progeny was the main obstruction in the crossing experiments with potato genotypes of the potato (+) tomato as well as the *S. brevidens* (+) potato somatic hybrids, from which only the hexaploid somatic hybrids with an excess of potato genomes were successful. Crosses of the *S. nigrum* (+) potato

somatic hybrids with potato faced a new obstruction in every generation. This might be caused by the fact that during the backcross programme of the *S. nigrum* (+) potato hybrids the genomic ratios had to be reversed. The initial somatic hybrid contained a surplus of *S. nigrum* genomes and only two genomes of potato but the aim of the backcross program was to obtain potato material with only the *P. infestans* resistance of *S. nigrum*. The BC1 that resulted from a cross with 4*x* potato was expected to be a hexaploid genotype that contained three genomes of both species. The plants that resulted from a second backcross with 4*x* potato were most likely to be pentaploid, but aneuploid imbalanced genomes for potato as well as *S. nigrum* were obtained, which might be an explanation for their low vigour.

In the potato (+) tomato backcross programme the somatic hybrids as well as the BC1 and BC2 genotypes contained four genomes of potato and an additional number of tomato chromosomes that depended on the generation. All hybrids in every generation had a surplus of potato genomes containing sufficient balanced genetic information for the development of normally growing plants. In our crossing programme the *S. americanum* (+) Désirée hybrids had a genomic ratio that was comparable to the hexaploid potato (+) tomato somatic hybrids but, unfortunately, did not produce any berries after pollination.

In an attempt to improve the backcross ability of the BC1-genotype with potato, the genomic composition was modified by somatic fusion. Two potato genomes were added by somatic hybridisation in order to create a surplus of potato genomes in this genotype. Twenty somatic hybrids could be selected which were expected to contain five genomes of potato and three of *S. nigrum*. Subsequently the plants were used in backcross experiments in which they were pollinated with potato pollen. The first preliminary crossing results did not show a major improvement of the crossability of the somatic hybrids as compared to the BC1-fusion parent. Two genotypes had a higher berry set than the BC1-6738 (23%) but the number of ovules per berry of these two genotypes was lower. In fact, none of the BC1-somatic hybrids had a higher number of ovules per berry than the BC1-6738. Crossing experiments were continued with a selection of the BC1-somatic hybrids which have resulted in four backcross plants (unpublished results). Two of these plants were even obtained without ovule culture, directly from mature seeds. Unfortunately the backcross plants are less vigorous than the BC1-somatic hybrids from which they were obtained and berryset seems to be low. Until now one seed was obtained.

ASSESSMENT OF THE POSSIBILITIES OF INTROGRESSION

Our approach was aimed at introgression of interesting traits from *S. nigrum* into the potato genome. A prerequisite for success is the occurrence of recombination between chromosomes of the two species. Application of GISH showed homoeologous association in the BC1-6738 and BC2-9019. Despite a clear differentiation between the chromosomes of the two parental species it was not possible to establish whether or not there were *recombinant* chromosomes in the progenies. This failure to detect homoeologous recombination might be due to two reasons:

- There might be considerable recombination but it is difficult to detect.

- There is no (or very little) crossing-over between the chromosomes of potato and *S. nigrum*;

In species with large chromosomes like Lily, with one of the largest genomes in the plant kingdom with a 2C value of 72 pg, intergenomic recombination in the backcross derivatives can easily be monitored through GISH (Karlov *et al.*, 1999). In species with small genomes, like the ones that belong to the Solanaceae (2C value is approximately 2.0 pg), it is much more difficult to show introgression.

In backcross derivatives of potato (+) tomato somatic hybrids, in which the homoeologous chromosomes were equally well differentiated as in our study, no genetic recombination was detected between potato and tomato chromosomes. This might be expected because of the phylogenetic distance between potato and tomato since they belong to different genera, *Solanum* and *Lycopersicon* respectively. Potato and *S. nigrum* however, both belong to the genus *Solanum*, although being tuberous and non-tuberous species, respectively. In view of the taxonomic position relatively more homoeologous pairing and crossing-over is expected in potato (+) *S. nigrum* hybrids than in potato (+) tomato hybrids. This was indeed shown to be the case in our experiments. Other combinations of tuberous and non-tuberous species already showed a considerable amount of homoeologous chromosome pairing, in sexual hybrids of *S. etuberosum* and *S. pinnatisectum* (Ramanna and Hermsen, 1979; Ramanna and Hermsen, 1982) as well as in somatic hybrids involving *S. etuberosum* or *S. brevidens* for instance (Novy and Helgeson, 1994; McGrath *et al.*, 1994). In these cases, however, despite the occurrence of indirect evidence for homoeologous chromosome pairing and crossing-over, direct evidence of recombinant segments could not be demonstrated cytologically either in *Solanum* or *Lycopersicon* species hybrids. However, one exception is a report written by Parokonny *et al.* (1997) who detected recombinant segments in *L. esculentum* x *L. peruvianum* hybrids.

The question is raised why cytological detection of recombinant segments has not been possible in our *S. nigrum* (+) potato fusion hybrids. One problem in general seems to be the distribution of repetitive DNA on small chromosomes like those of *Solanum* and *Lycopersicon*. More than 77% of the repetitive DNA is situated in the so-called centromeric heterochromatin, occupying the proximal parts of the chromosomes (Peterson *et al.*, 1996). The distal euchromatic parts, where chiasmata and crossing-over have been shown to occur, have very little repetitive DNA so that it is difficult to differentiate these parts with GISH. This might be the cause for the detection of the alleged recombinant fragments in the *L. esculentum* x *L. peruvianum* hybrids (Parokonny *et al.*, 1997). Moreover, the euchromatic parts in both potato and tomato are extremely contracted during metaphase stages of mitotic chromosomes (Ramanna and Prakken, 1967; Ramanna and Wagenvoort, 1976).

Thus, in the absence of convenient cytological methods for the detection of recombination, the use of molecular mapping methods such as the RFLP and AFLP-technique appear to be the best alternatives.

ALTERNATIVES TO THE SOMATIC HYBRIDISATION APPROACH

If recombination between chromosomes of *S. nigrum* and potato does not occur it might be necessary to turn to an alternative approach like:

- Production of addition lines, combined with induced translocation;
- Sexual hybridisation, combined with the use of 2*n*-gametes.

The above options will be discussed in the following paragraphs.

Production of addition or substitution lines

One of the most successful examples of substitution of a chromosome is the substitution of the 1B chromosome of wheat by 1R of rye. Nowadays many commercial wheat varieties posses this 1R substitution or one of the 1AL/1RS, 1BL/1RS or 1D/1RS translocations which were derived from it. A survey in 1998 of 454 European wheat varieties found 1RS in 17% of the genotypes (Kazman *et al.*, 1998). Approximately 330 genotypes with 1RS from 35 different countries were identified by Rabinovich (1998). The short arm of 1R provides for resistance to yellow rust (*Puccinia striiformis*), leaf rust (*P. recondita*), stem rust (*P. graminis*) (Bartos *et al.*, 1973), powdery mildew (*Erysiphe graminis*) (Heun and Friebe, 1990) and insects (Martin *et al.*, 1976). Depending on the wheat genotype into which it is introduced, 1RS may also have a positive effect on yield (Villareal *et al.*, 1991; Schlegel and Meinel, 1994). Unfortunately 1RS can also have a negative effect on the bread making quality (Martin and Stewart, 1990) or on flour yield (McKendry *et al.*, 1996), depending on the genotype.

Substitution 1R was developed by extensive selection for several resistances in a wheat-rye backcross programme, combined with cytogenetic monitoring which first resulted in monosomic addition lines for the 1R-chromosome and later on in substitution lines. The translocation lines occurred either spontaneous or were induced by irradiation. The spontaneous translocation developed from breakages of wheat and rye univalent chromosomes at the centromeres, followed by exchange and fusion of the respective chromosome arms (Mettin *et al.*, 1978). Induced translocation was applied to backcross progeny of a cross between triticale and wheat, resulting in a translocation in the cultivar 'Amigo' (Sebesta *et al.*, 1994).

Within the Solanaceae some examples of the creation of addition lines can be found as well. A set of monosomic alien addition lines of *S. lycopersicoides* chromosomes in a tomato background was assembled with sexual methods (Chetelat *et al.*, 1998). The second example is the selection of a complete series of monosomic alien tomato addition lines in the background of the cultivated potato (Garriga-Calderé *et al.*, 1997; Haider Ali *et al.*, in press). Although both combinations involve a *Solanum* and a *Lycopersicon* species, it should be emphasised that *S. lycopersicoides* and *L. esculentum* are much more closely related to each other than *S. tubersoum* and *L. esculentum* (Rick, 1979) and therefore homoeologous pairing is occurring at a lower frequency in the latter combination than in hybrids of *S. lycopersicoides* and *L. esculentum*. As a result of this, diploid recombinants were identified in progenies of

the *S. lycopersicoides* addition lines, which demonstrated the possibility of transferring monogenic characters from *S. lycopersicoides* to tomato (Chetelat *et al.*, 2000). The tomato addition lines were established despite abnormal behaviour of the tomato chromosomes like preferential chromosome elimination, precocious disjunction and division. Despite these drawbacks transmission of the tomato chromosomes is fairly high, which indicates that the addition lines can be used for further introgression studies. So far introgression of tomato traits into a potato background was not observed, but in one case a reciprocal translocation was detected (Garriga-Calderé *et al.*, 1999).

Pairing between chromosomes of potato and chromosomes of tomato seems to occur less frequently than pairing of potato and *S. nigrum* chromosomes and no preferential chromosome elimination was observed in potato-*S. nigrum* hybrids. Therefore the prospects for spontaneous introgression of *S. nigrum* traits into the potato background from addition lines might be more positive. If, however, introgression is not detected, an alternative might be found in induced translocation. With this method the addition line of interest is irradiated with the aim of translocating the piece of alien chromosome containing the desirable character to another, preferably homoeologous chromosome. This technique, however, can only be used for simple, screenable characters and cannot be controlled since the translocating fragment can transfer to any other chromosome. Another disadvantage is that it is not unlikely that undesirable traits might be introduced as well.

Direct hybridisation and the use of 2n-gametes

A consequence of somatic hybridisation of two distantly related species is that the resulting hybrids will be allopolyploids. Experiences in the past have shown that the prospects for intergenomic recombination between alien chromosomes in allopolyploids is minimal because of preferential pairing during meiosis in the fusion hybrid. Sexual hybridisation between diploid potato ($2n=2x=24$) and *S. nigrum*, or one of the other species of the *S. nigrum*-complex, might therefore be an attractive alternative because the resulting hybrids have the advantage of having only a homoeologue as a pairing partner for each chromosome so preferential pairing cannot occur. Such distant allodiploid hybrids can be especially useful when they produce 2*n*-gametes, as has been recently shown in *Lilium* (Lim *et al.*, 2000). An LA-hybrid of *Lilium longiflorum* (L) and an Asiatic Lily hybrid (A) that produced 2*n*-gametes, was used as a pollinator to produce backcross progeny to the Asiatic Lily hybrid (A), resulting in ALA-BC1 progeny. Three to four homoeologous crossovers were observed in some chromosomes besides the assortment of alien chromosomes during the process of sexual polyploidisation. Another progeny of *somatically* polyploidised *Lilium*-hybrids was analysed as well, showing no evidence of intergenomic recombination at all (Karlov *et al.*, 1999). Comparable results were found in *Alstroemeria* (Kamstra *et al.*, 1999). *Alstroemeria aurea* x *A. inadora* hybrids were backcrossed to *A. inadora* which resulted in six BC1 plants: two aneuploids ($2n=2x+1$) and four triploids ($2n=3x=24$). Recombinant chromosomes were found in all BC1 genotypes, indicating homoeologous recombination during meiosis in

the species hybrid. Analysis of the meiosis in the triploid BC1-genotypes showed preferential pairing of *A. inodora* hybrids.

Finally, while considering the alternatives for using the species of the *S. nigrum* complex for introgression of desirable traits into potato, it should be pointed out that the present study has demonstrated that homoeologous pairing between *S. nigrum* and potato chromosomes does occur. Because the alien chromosomes are transmitted through the gametes, introgression might be possible. An example of intergeneric hybrids in which homoeologous pairing occurred just as in our BC1-6738 are those of *Lolium* and *Festuca* (King *et al.*, 1998; Zwierzykowski *et al.*, 1998). In the allopolyploid hybrids of these species homoeologous pairing has led to introgression of characters between these genera. It also illustrates the relevance of improving the crossability of the complex hybrids to the recurrent parent and of improving the efficiency of monitoring introgression and selection.

Despite the extreme difficulty of achieving success through sexual hybridisation, the possibility cannot be ruled out. There are several examples in which successful hybridisation was achieved through long years of trials. Crosses between tuberous and non-tuberous species of the section Petota for example were achieved unexpectedly (Hermsen and Taylor, 1979). Sexual hybrids of *S. lycopersicon* and *S. lycopersicoides* were successfully backcrossed to *Lycopersicon* after 40 years, first to *L. pennelli* (Rick, 1951; Rick *et al.*, 1986, Rick *et al.*, 1988), later on to *L. esculentum* (Chetelat *et al.*, 1997). In the case of potato-tomato fusion hybrids, although produced by Melchers *et al.* in 1978, they were backcrossed to potato 15 years later (Jacobsen *et al.*, 1994). Sexual hybridisation between *S. nigrum* x potato was shown to be possible (Eijlander and Stiekema, 1994) but was extremely laborious. These examples illustrate that the discovery and use of appropriate genotypes can enable us to achieve success even in the case of crosses that were supposed to be extremely difficult or impossible. So whatever approach will be chosen for the introgression of traits from *S. nigrum* into potato, it will most likely be a matter of long breath.

SUMMARY

The species of the *Solanum nigrum*-complex are wild relatives of the cultivated potato and potentially interesting sources of genetic variation. The traditional method of introgressing a specific trait from a related species is sexual hybridisation followed by recurrent backcrossing but often one or more reproductive barriers have to be overcome in this process. The discovery of the fusion of protoplasts opened new prospects for further broadening of the genetic base of potato because this technique offered the possibility to use distantly related species.

Fusion experiments were performed between diploid ($2n=2x=24$) or tetraploid ($2n=4x=48$) potato genotypes and four species belonging to the *S. nigrum* complex, namely *Solanum nigrum* ($2n=6x=72$), *S. villosum* ($2n=4x=48$), *S. chenopodioides* ($2n=2x=24$) or *S. americanum* ($2n=2x=24$ and $2n=6x=72$). All five accessions of the four species of the *S. nigrum*-complex were able to form fusion hybrids with at least one of the potato genotypes but some combinations were more successful than others. It was shown that the ploidy level as well as the genotype were factors that influenced the somatic combining abilities. In some combinations the cell-selectable markers kanamycin or hygromycin resistance were used but they did not influence the somatic combining abilities considerably. However, such markers can be useful to improve efficient selection of somatic hybrids in sufficient numbers. Almost half (373) of the 761 somatic hybrid plants performed well *in vitro*, which was in striking contrast with their performance *in vivo*. Only 60 genotypes out of 761 somatic hybrids were vigorous in the greenhouse and were able to flower.

Vigorous somatic hybrids of the fusion combinations 2*x* *S. americanum* (+) Désirée, *S. chenopodioides* (+) 2*x* potato and *S. nigrum* (+) 2*x* potato were used in backross experiments. Only the somatic hybrids of *S. nigrum* (+) 2*x* potato were successfully backcrossed to both potato and *S. nigrum*. First and second generation backcross progeny with *S. nigrum* could easily be obtained. Self-fertility was already restored in one of the BC1 genotypes. Backcrosses with potato had a much lower success rate. Only pollinations with tetraploid potato resulted in seed containing berries. Two BC1 genotypes were obtained after 5000 pollinations from which 505 ovules were cultured. The first genotype, BC1-6738, was a vigorously growing genotype, both *in vitro* and in the greenhouse, and flowered abundantly. The second genotype, BC1-9001, showed many abnormalities and dropped its flowers before anthesis. BC1-6738 was again crossed with tetraploid potato and also in this generation the success rate was low. Over 5000 pollinations resulted in 1750 berries from which over 3000 ovules were obtained. Twelve plants germinated from these ovules, which were not as vigorous *in vitro* and *in vivo* as the BC1 parent. Some of the BC2 genotypes were used for further backcrosses but so far no BC3 plants could be obtained.

The application of genomic *in situ* hybridisation (GISH) made it possible to distinguish clearly between *S. nigrum* and potato chromosomes in mitotic and meiotic chromosome spreads. It was used to determine the genomic constitution of the somatic hybrid F21-26 and its backross progeny. F21-26 was found to be an octaploid with six genomes of *S. nigrum* and two of potato. BC1-6738, the result of a cross between F21-26 and 4*x* potato, appeared to be a hexaploid with 36 chromosomes of each of both species. Unfortunately it was impossible to determine with GISH whether the 36 chromosomes of *S. nigrum* represented three complete genomes. However, AFLP-data

showed that none of the AFLP-specific markers that were amplified in the *S. nigrum* fusion parent and in the somatic hybrid was missing in BC1-6738, which is an indication that no major chromosome elimination has taken place. Most of the eleven BC2 genotypes that were analysed had a near-pentaploid genomic constitution with 14-20 chromosomes of *S. nigrum* and between 33 and 43 chromosomes of potato.

The meiotic behaviour of BC1-6738 and BC2-9019 was also studied with GISH. Chromosome counts in nuclei of BC2 tetrad cells showed that transmission of alien *S. nigrum* chromosomes to BC3 progeny is likely. An average of 9.1 *S. nigrum* chromosomes per microspore in BC2-9019 was observed. Meiotic analysis of metaphase I in BC1-6738 and in BC2-9019 indicated clearly that allosyndetic pairing in both bivalent and trivalent formation occurs in these genotypes, which is of importance for homoeologous recombination. So far in none of the backcross derivatives recombination could be shown with GISH.

BC1-6738 and eight BC2 genotypes that resulted from the backcross program with potato were tested for their resistance to *Phytophthora infestans*. The BC1 genotype was as resistant as the *S. nigrum* fusion parent but among the eight BC2 genotypes that were scored, six were resistant whereas two genotypes showed lesions on the inoculated leaves, indicating they were susceptible.

Since no progeny was obtained from the BC2 genotypes, alternative approaches were sought to overcome this sexual crossing barrier. With the aim to improve the crossability of the backcross derivatives, it was attempted to alter the genomic composition of BC1-6738 and BC2-9017 by adding two haploid potato genomes by somatic hybridisation. Five diploid potato genotypes were used in these fusion experiments, of which one contained the hygromycin resistance gene. All vigorous regenerants that resulted from the fusion experiments were used for the estimation of nuclear DNA content through flow cytometry. Plants with a DNA content higher than that of the BC1 or BC2 genotypes were considered potential somatic hybrids. A total of forty-nine potential somatic hybrids resulted from fusion experiments with BC1-6738, from which 20 grew vigorously in the greenhouse and did flower. Eight genotypes produced seeded berries and five genotypes gave seedless berries after pollination with several potato cultivars. Only five potential somatic hybrids were detected among the 79 flow-cytometrically analysed regenerants from BC2-9017 (+) 2*x* potato fusion experiments. Two of these hybrids were rather vigorous and did flower, but pollinations with potato did not yet give any berry set.

Eleven potential somatic hybrids of BC1-6738 (+) 2*x* potato were selected for GISH-analysis to determine their genomic composition. Theoretically the somatic hybrids were expected to contain 96 chromosomes: 36 of *S. nigrum* and 60 of *S. tuberosum*. Six of the selected genotypes had this expected genomic constitution and five genotypes deviated from this expectation. Two of these five, F108-7 and F109-6, were most likely missing one or two potato chromosomes but contained 36 chromosomes of *S. nigrum*. One genotype, F104-2, had 60 chromosomes of potato origin but between one and eight additional chromosomes or chromosome fragments of *S. nigrum* per cell were found. The fourth genotype, F101-2, had a higher number of *S. nigrum* chromosomes than expected (42-45) but the number of potato chromosomes was only slightly higher (41-42) than in the BC1-fusion parent which makes it doubtful that this genotype is a true somatic

hybrid. The fifth genotype with a deviating chromosome number was F109-12 which had only 22 *S. nigrum* chromosomes left but did contain 58 to 60 chromosomes of potato.

Crossing experiments were continued with selected BC1-somatic hybrids, which resulted in four backcross progeny plants. Two of these plants were even obtained without ovule culture, directly from mature seeds. Unfortunately these backcross plants were less vigorous than the somatic hybrids from which they were derived and berry set seemed to be low.

It can be concluded that traits from *S. nigrum* have become available for the cultivated potato with the aid of protoplast fusion. However, introgression of these traits by repetitive backcrossing with potato is much more complicated then initially expected although it seems to be possible. Large numbers of starting material and a lot of work and time seem to be necessary to accomplish the introgression of *S. nigrum*-traits into the cultivated potato.

SAMENVATTING

De soorten van het *Solanum nigrum*-complex zijn wilde verwanten van onze cultuuraardappel en zijn in potentie interessante bronnen van genetische variatie. De traditionele manier om een bepaalde eigenschap van een verwante soort te introduceren in een cultuurgewas is het kruisen van de twee soorten gevolgd door het maken van herhaalde terugkruisingen. In veel gevallen wordt deze aanpak bemoeilijkt door het vóórkomen van verschillende kruisingsbarrières De ontdekking van de mogelijkheid van het fuseren van protoplasten bood nieuwe perspectieven voor het verbreden van de genetische basis van aardappel omdat deze techniek het mogelijk maakte om ook minder verwante soorten te gebruiken.

Vier soorten uit het *S. nigrum* complex, namelijk *Solanum nigrum* (2*n*=6*x*=72), *S. villosum* (2*n*=4*x*=48), *S. chenopodioides* (2*n*=2*x*=24) of *S. americanum* (2*n*=2*x*=24 and 2*n*=6*x*=72) zijn gebruikt in fusie-experimenten met diploïde (2*n*=2*x*=24) en tetraploide (2*n*=4*x*=48) aardappelgenotypen. Alle vijf accessies van de vier eerder genoemde soorten waren in staat om fusieprodukten te vormen met minstens één van de aardappelgenotypen maar sommige combinaties bleken succesvoller te zijn dan andere. Het ploidieniveau en het genotype waren factoren die van invloed waren op de resultaten van de fusie-experimenten, aangeduid als de somatische combinatie geschiktheid. In sommige fusie-experimenten is gebruik gemaakt van kanamycine en/of hygromycine resistentiegenen waarmee fusieprodukten op celniveau geselecteerd konden worden. Deze selectiemerkers hadden geen effect op de somatische combinatie geschiktheid maar bleken wel nuttig te zijn voor het verbeteren van de efficiëntie van de selectie van grote aantallen fusieprodukten. Van de 761 fusieprodukten groeide bijna de helft goed *in vitro* (373) maar in de kas bleken de meeste planten niet in staat om te overleven. Slechts 60 hybriden waren in staat om in de kas te groeien en om bloemen te vormen.

Groeikrachtige fusieprodukten van de combinaties 2*x* *S. americanum* (+) Désirée, *S. chenopodioides* (+) 2*x* aardappel en *S. nigrum* (+) 2*x* aardappel zijn gebruikt om terugkruisingen te maken. Alleen de *S. nigrum* (+) aardappel hybriden leverden nakomelingen op, zowel na kruising met aardappel als na kruising met *S. nigrum*. Vooral de terugkruisingen met *S. nigrum* bleken eenvoudig te zijn want er waren slechts enkele bestuivingen nodig voor de eerste BC1 en BC2-planten. Bij één van de BC1-planten was de mogelijkheid tot zelfbevruchting alweer hersteld. Terugkruisingen met aardappel waren veel minder succesvol. Alleen bestuiving met tetraploide aardappelgenotypen leidde tot bessen met zaad. In totaal waren er meer dan 5000 bestuivingen nodig waaruit 505 zaadknoppen werden uitgeprepareerd voor het verkrijgen van twee BC1-nakomelingen. Het eerste genotype, BC1-6738, was een groeikrachtige plant zowel *in vitro* als in de kas en bloeide uitbundig. De tweede BC1, BC1-9001, had veel afwijkingen en liet vroegtijdig zijn bloemen vallen.

Ook BC1-6738 werd met tetraploide aardappel teruggekruist en wederom was het slagingspercentage laag. Meer dan 5000 bestuivingen resulteerden in 1750 bessen waaruit 3000 zaadknoppen uitgeprepareerd konden worden. Hieruit zijn twaalf BC2-planten voortgekomen die geen van allen zo groeikrachtig waren als de BC1-ouder zelf. Met een aantal van de BC2-genotypen is getracht een volgende generatie terugkruisingsnakomelingen te maken maar tot op dit moment is dat niet gelukt.

Met behulp van genomische *in situ* hybridisatie (GISH) was het mogelijk om onderscheid te maken tussen *S. nigrum*- en aardappelchromosomen in mitotische en meiotische chromosoompeparaten. Daardoor kon de genomische samenstelling van fusieprodukt F21-26 en zijn nakomelingen worden bepaald. F21-26 bleek een octaploid te zijn met zes genomen van *S. nigrum* en twee genomen van aardappel. BC1-6738, een nakomeling van F21-26 en een tetraploid aardappel genotype, bleek een hexaploid te zijn met 36 chromosomen van elk van beide soorten. Helaas was het met GISH niet mogelijk om te bepalen of de 36 chromosomen van *S. nigrum* drie volledige genomen vertegenwoordigden. AFLP-gegevens hebben echter aangetoond dat alle AFLP-specifieke merkers die in de *S. nigrum*-fusieouder voorkwamen, ook in het fusieprodukt en BC1-6738 werden geamplificeerd, wat aangeeft dat er waarschijnlijk geen grote chromosoom-eliminaties hebben plaatsgevonden. Het grootste deel van de elf geanalyseerde BC2-genotypen had een chromosoomaantal dat ongeveer overeenkomt met dat van een pentaploid met 14-20 *S. nigrum*-chromosomen en tussen de 33 en 43 chromosomen van aardappel.

Van BC1-6738 en BC2-9019 is ook de meiose bestudeerd met behulp van GISH. Chromosoomtelling in nuclei van BC2 tetraden lieten zien dat transmissie van *S. nigrum* chromosomen van de BC2 naar BC3-nakomelingen zeer waarschijnlijk is. Gemiddeld werden 9.1 *S. nigrum* chromosomen geteld per microspore van het genotype BC2-9019. Meiotische analyse van de metafase I van zowel BC1-6738 als BC2-9019 toonde aan dat allosyndetische paring regelmatig voorkomt in deze genotypen. Zowel bivalenten als trivalenten werden waargenomen waarbij zowel trivalenten van twee aardappelchromosomen en één *S. nigrum*-chromosoom werden gevonden als trivalenten van twee *S. nigrum*-chromosomen met één aardappelchromosoom. Allosyndetische paring is een belangrijke voorwaarde voor homeologe recombinatie. In geen van de terugkruisingsnakomelingen is echter een recombinant chromosoom m.b.v. GISH waargenomen.

BC1-6738 en acht BC2-nakomelingen uit het terugkruisingsprogramma met aardappel zijn getoetst op hun resistentie tegen *Phytophthora infestans*. BC1-6738 was even resistent als de *S. nigrum*-fusieouder maar de resistentie splitste uit in de BC2-generatie. Van de acht BC2-genotypen bleken er zes resistent te zijn maar op de geïnoculeerde bladeren van de overige twee genotypen werden lesies waargenomen waaruit geconcludeerd werd dat ze vatbaar waren.

Omdat tot op heden geen nakomelingen zijn verkregen uit terugkruisingen van de BC2-genotypen met aardappel, is naar een methode gezocht om deze kruisingsbarrière te doorbreken. In een poging om de kruisbaarheid te verbeteren, is getracht de genomische samenstelling van BC1-6738 en BC2-9017 te veranderden door via protoplastenfusie twee extra haploide aardappelgenomen toe te voegen. In totaal zijn vijf diploïde aardappelgenotypen, waarvan er één het hygromycineresistentiegen bezat, gebruikt in deze fusie-experimenten. Van alle groeikrachtige regeneranten die daaruit resulteerden, is het DNA-gehalte bepaald met behulp van flow cytometry. Alle planten met een hoger DNA-gehalte dan BC1-6738 of BC2-9019, werden beschouwd als potentiële fusieprodukten. Uit de fusie-experimenten met BC1-6738 zijn in totaal 49 potentiële

fusieprodukten voortgekomen, waarvan er twintig goed groeiden in de kas en bloemen vormden. Na bestuiving met verschillende aardappelgenotypen werden van acht genotypen bessen met zaad geoogst en gaven vijf genotypen zaadloze bessen. Onder de 79 regeneranten van de BC2-9017 (+) 2*x* aardappel fusie-experimenten werden slechts vijf potentiële fusieprodukten gevonden. Twee van deze hybriden waren redelijk groeikrachtig en bloeiden maar bestuivingen met aardappelpollen resulteerden niet in bessen.

Van elf potentiële fusieprodukten met BC1-6738 als fusieouder is de genoomsamenstelling bepaald met behulp van GISH. Theoretisch werd verwacht dat zij 96 chromosomen zouden hebben: 36 van *S. nigrum* en 60 van *S. tuberosum*. Zes genotypen bleken exact deze verwachtte genoomsamenstelling te hebben maar de andere vijf weken daar vanaf. Twee van deze vijf genotypen, F108-7 en F109-6, misten hoogstwaarschijnlijk een of twee aardappelchromosomen maar hadden wel 36 chromosomen van *S. nigrum*. Eén genotype, F104-2, had het verwachtte aantal van 60 aardappelchromosomen maar er werden tussen de 1-8 extra *S. nigrum*-chromosomen en/of chromosoomfragmenten gevonden. Het vierde genotype, F101-2, had een hoger aantal *S. nigrum*-chromosomen (42-45) maar bevatte slechts een beperkt aantal extra aardappelchromosomen dan de BC1-fusieouder waardoor het twijfelachtig is dat dit daadwerkelijk een fusieprodukt is. Het vijfde genotype met een van het verwachte aantal afwijkend aantal chromosomen had slechts 22 *S. nigrum*-chromosomen en 58-60 aardappelchromosomen.

De terugkruisingsexperimenten zijn voortgezet met een selectie van de BC1-fusieprodukten. Tot nu toe hebben die geresulteerd in vijf nakomelingen waarvan er twee zelfs zijn verkregen zonder zaadknopcultuur direct uit afgerijpt zaad. Helaas waren ook deze terugkruisingsnakomelingen minder groeikrachtig dan de somatische hybriden waaruit ze zijn verkregen en is de besvorming slecht.

Geconcludeerd kan worden dat m.b.v. protoplastenfusie de eigenschappen van *S. nigrum* nu voor de aardappel beschikbaar zijn. Introgressie van deze eigenschappen d.m.v. herhaald terugkruisen van fusieprodukten lijkt mogelijk maar is lastiger dan oorspronkelijk verwacht. Veel uitgangsmateriaal, inspanning en geduld lijken nodig om de *S. nigrum*-eigenschappen daadwerkelijk over te brengen naar de cultuuraardappel.

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NAWOORD

Eindelijk klaar! Iedereen die mij een beetje kent weet dat dat een hele opluchting is. Mijn contract als Aio was tenslotte al een aantal jaren geleden afgelopen maar door een aantal "vertragende factoren" heeft het verschijnen van dit proefschrift nogal op zich laten wachten. Eerst even terug naar het begin.

September 1993 ben ik als Aio begonnen bij Plantenveredeling nadat ik een jaar bij het CPRO had gewerkt als NOP-per (NOP staat voor Na-doctoraal Onderzoeksproject) en tot de conclusie was gekomen dat ik toch echt verder wilde in het onderzoek. Die kans kreeg ik dus en vol enthousiasme ben ik aan de slag gegaan. Het project werd gefinancierd door de "Stichting Bevordering Veredelingsonderzoek" waarin de aardappelkweekbedrijven Karna, Stet Holland en Hettema participeerden en waarvan prof. Hermsen adviseur was. Twee keer per jaar werd er vergaderd met de kwekers wat altijd heel gezellig was en waar ik altijd kon rekenen op belangstelling voor mijn resultaten. Het was iedere keer echter ook duidelijk dat mijn materiaal nog ver van de praktijk afstond.

Mijn Aio-onderzoek maakte deel uit van een groter project waar ook een postdoc in zat, Ronald Hutten. Ik had nog nooit iemand zo enthousiast over aardappels en de aardappelveredeling horen praten als hij en al snel was ik ingewijd in de wereld van de pollenpreparaten, de veldproeven, de 2x-4x-kruisingen, de knollenlijsten, het chips bakken, de 1e, 2e etc. jaars en ga zo maar door. Ondertussen was ik bezig met plantjes in glazen potjes en labproefjes maar het was duidelijk dat mijn werk een hoger doel had: de perfecte pieper!

Die plantjes in die glazen potten dat waren fusieprodukten (als alles goed was gegaan), stuk voor stuk gemaakt door Marjan Bergervoet. Marjan, eigenlijk zou je voor de gein eens uit moeten rekenen hoeveel glazen potten en buizen je door je handen hebt laten gaan en hoeveel liter medium daarmee gemoeid was in al die projecten die je van plantmateriaal hebt voorzien. Het zou me niet verbazen als je daar een zwembad mee kunt vullen en dan bedoel ik niet zo'n pierebadje maar eentje van wedstrijdformaat.

In het lab brak de FISH en GISH-periode in volle hevigheid uit op het moment dat ik aan mijn project begon. De eerste beginselen hadden we opgepikt bij Erfelijkheidsleer bij Hans de Jong maar als snel konden we bij Plantenveredeling ook aan de slag. In het begin was het nog een beetje pionieren met o.a. een waterbad dat op zolder onder het stof vandaan was gehaald maar Anja, Silvan en Fransesc wisten daar in rap tempo verandering in te brengen en zo ontstond er een heus FISH-lab dat garant stond voor zeer kleurrijke resultaten. Tanya, thank you for helping me to paint my material.

En toen werd het zomer. "Kom", zei Dirk-Jan Huigen, "we gaan de kas in, kruisingen maken." En zo stond ik ineens labeltjes te schrijven, bloemetjes te bestuiven en alles netjes bij te houden in mijn kruisingsboekje. Dat klinkt allemaal romantisch maar oh wee als het echt zomer werd, dan was het nauwelijks meer uit te houden en ging de lol er snel vanaf. De suggestie van Dirk-Jan was om dan wat eerder op de ochtend te beginnen. Een klein probleempje, voor 9 uur 's ochtends bevind ik mij nog in mijn "sub-optimale" periode, om maar eens een understatement te gebruiken. Er zat dus weinig anders op dan om even flink af te zien. Gelukkig heb ik daar ook wel wat hulp bij gehad: Richard Fratini en

Andro Tjin Wong Joe hebben beiden tijdens hun afstudeervakken ook menige druppel gezweet in de kas.

Na een dag op het lab of in de kas was het lekker om in de aardappelkamer even na te praten en een potje "bommen te vegen". Met Heleen, Herman en de twee Ronalden heb ik menig nuttig en nutteloos gesprek gevoerd. Herman altijd even kritisch, Ronald E. geïnteresseerd in alles, Ronald H. "nu even niet want het is half één en dus tijd om te klaverjassen" en Heleen altijd spontaan en gezellig. We hebben toch heel wat afgelachen en dat was zeer heilzaam tegen stress en neerslachtige buien.

En dan moest er natuurlijk ook met enige regelmaat serieus wetenschappelijk gediscussieerd en gepland worden. Evert, jij was altijd in staat om mij in korte tijd weer bij de les te krijgen. Wat ik erg bewonderd heb is dat hoe druk je het ook had, je altijd binnen een paar dagen reageerde op plannen en concept-artikelen. Dr. Ramanna, thank you for your help, especially during the final stages of writing this thesis. You were always an inspiration. Professor Hermsen, bedankt voor alle adviezen en aanmoedigingen bij het schrijven van de verschillende hoofdstukken.

En toen was mijn contract afgelopen en liep ik tegen de eerste vertragende factor aan, de geboorte van onze dochter. Lieve Robin, van alle vertragende factoren was jij absoluut de leukste. Na jouw geboorte werd het een stuk moeilijker om aan schrijven toe te komen want spelen met jouw was veel leuker. Daarnaast had ik als nieuwbakken ouder geen idee "what hit me" en waren zaken als gebroken nachten niet echt bevorderlijk voor de schrijverij. Maar op jouw manier heb je me ook geholpen. Op de moeilijke momenten was jij altijd in de buurt om me even af te leiden en niets werkt zo therapeutisch als twee kleine armpjes om mijn nek, een natte zoen op m'n wang en een lach van oor tot oor die je me geeft zonder dat ik er iets voor hoeft te doen

De tweede vertragende factor diende zich aan in de vorm van een baan toen Robin bijna een jaar oud was. En nu werk ik op het Bestuurscentrum en ben ik een van die ambtenaren in "het witte gesticht". Ik moet jullie vertellen, ik heb het er ontzettend naar mijn zin. Leuke collega's die ook allemaal erg mee hebben geleefd bij de laatste fase van mijn promotie, leuk werk, wat wil je nog meer! In tegenstelling tot wat veel mensen denken, is het een bewuste keuze van mij geweest en ben ik er niet per ongeluk terechtgekomen. Iedereen die niet begrijpt "waar die lui in het Bestuurscentrum de hele dag mee bezig zijn" wil ik uitnodigen om een keer bij mij langs te komen, ik leg het graag een keer uit. Daarmee zeg ik niet dat iedereen moet weten wat er centraal allemaal gebeurt want wat wij doen daar gaat het niet om. Onderzoek en onderwijs, daar gaat het binnen Wageningen UR om en ook centraal wordt dat heus wel begrepen.

Maar het schrijven moest doorgaan en het moge duidelijk zijn dat dit boekje er niet had gelegen zonder de steun van een aantal mensen.

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duidelijkheid, je hoeft me niet meer achter m'n vodden aan te zitten en samen te spannen met Evert want het is KLAAR! Pap, iedereen is jaloers op zo'n handige vader als jij. Nog bedankt voor die prachtige badkamer. Heb je de bouwtekening voor de erker, de uitbouw achter en de dakkapellen al klaar? Niet stiekem beginnen als wij er niet zijn!

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Karin

CURRICULUM VITAE

Karin Horsman werd op 14 november 1966 in Arnhem geboren als dochter van Jan Theunis Horsman en Kitty Aalders. Vanaf 1979 bezocht zij het VWO in Zevenaar alwaar zij in 1985 haar diploma haalde. In dat zelfde jaar startte zij haar studie Plantenveredeling aan de Landbouwhogeschool te Wageningen. Haar afstudeerrichting waren somatische celgenetica en virologie. Haar stage in de tomatenveredeling bracht ze door in Israël bij het veredelingsbedrijf Hazera Seeds in Kiriat Gat. In 1991 studeerde ze af aan de Landbouwuniversiteit Wageningen waarna ze parttime schermdocente werd bij dezelfde instelling. In 1992 werkte ze bij de afdeling Moleculaire Biologie van het CPRO-DLO aan de mapping van nematodenresistentiegenen in aardappel. In 1993 begon zij bij de vakgroep Plantenveredeling van de Landbouwuniversiteit Wageningen aan het promotieproject waarvan het resultaat hier voor u ligt in het proefschrift getiteld "Somatic hybrids of *Solanum tuberosum* and species of the *Solanum nigrum*-complex and their backcross progeny". Sinds 1999 is zij als beleidsmedewerker in dienst van Wageningen Universiteit en Research Centrum, in eerste instantie bij de afdeling Onderzoek- en Onderwijsbeleid maar sinds januari 2000 bij de stafafdeling Onderzoekstrategie.

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