MARKER ASSISTED ELUCIDATION OF THE ORIGIN OF 2N-GAMETES IN DIPLOID POTATO

HET GEBRUIK VAN MARKERGENEN OM DE ONSTAANSWIJZE VAN 2N-GAMETEN IN DE DIPLOÏDE AARDAPPEL AAN TE TONEN

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Keywords

potato, *Solanum spp.*, sexual polyploidization, unreduced gametes, 2n-eggs, multilocus analysis, restitution mechanism, gene-centromere mapping, recombination

STELLINGEN

1 Genetische analyse met één proximaal en één distaal markergen per chromosoom maakt het mogelijk om van iedere 2n-gameet het restitutiemechanisme te achterhalen.
Dit proefschrift

2 Post-meiotische verdubbeling van de gereduceerde megaspore wordt ten onrechte als "Second Division Restitution" (SDR) beschreven en dient als het additionele mechanisme Post-Meiotische Restitutie (PMR) beschouwd te worden.
Douches DS, CF Quiros (1988) Euphytica 38:247-260
Dit proefschrift

3 Alhoewel verschillende restitutiemechanismen per kloon mogelijk zijn tijdens de 2n-gameetvorming, kunnen klonen geselecteerd worden waarin er slechts één werkzaam is.
Dit proefschrift

4 Voor het nauwkeurig schatten van recombinatiefrequenties zijn FDR en SDR 2n-gameten superieur ten opzichte van de normaal gereduceerde gameten.
Dit proefschrift

5 Voor het bewijzen van absolute chiasma-interferentie, zoals aangetoond in de nakomelingen van 2n-gameten in diverse vissoorten en aardappel, is het essentieel om de overkruisingen per individuele meiose te kunnen analyseren.
Thorgaard GH, Allendorf FW, Knudsen KL (1983) Genetics 7:524-530
Guo X, Allen SK, Jr (1996) Biol Bull 191:145-148
Dit proefschrift

6 De doelgerichtheid van cytologische analyses naar de ontstaanswijze van 2n-gameten kan aanzienlijk verbeterd worden door eerst met een genetische analyse het restitutiemechanisme te bepalen.

7 De fluorescente *in situ* hybridisatie techniek belooft een kleurrijke toekomst voor cytogenetici.

8 Iedere functie kan in deeltijd worden uitgevoerd.

9 Hoe 'gebruikersvriendelijker' het computerprogramma, hoe vaker men tijdens het gebruik beschuldigd wordt van het illegaal handelen of het maken van fatale fouten.

10 Het grootste nadeel van een overtuiging is dat er geen ruimte overblijft voor een andere waarheid.

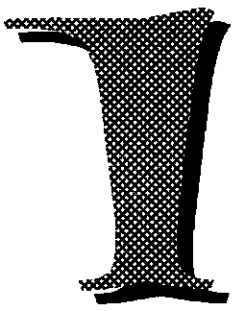
11 De generatie die in de zestiger jaren op de barricades stond en intussen zijn huisje, boompje en beestje dik voor elkaar heeft, stopt nu de oude generatie met een uitgeklede AOW weg en houdt de jonge generatie, die moeilijk aan de bak komt, flexibel.

Stellingen behorende bij het proefschrift 'Marker assisted elucidation of the origin of 2n-gametes in diploid potato', Heleen J.M. Bastiaanssen, Wageningen, 8 oktober 1997.

ABSTRACT

This thesis describes the selection and evaluation of diploid potato clones (2n=2x=24) that produce unreduced or 2n-gametes with 24 chromosomes instead of the normal reduced n-gametes with 12 chromosomes. To elucidate the modes of origin of the 2n-gametes, the progenies derived from such gametes were analysed for different marker loci of one chromosome. Besides the already known mechanisms of First and Second Division Restitution (FDR and SDR) in potato, the multilocus analysis showed the additional mechanism of Post-Meiotic Restitution (PMR) resulting in completely homozygous 2n-gametes. The FDR and SDR 2n-gametes were used for gene-centromere mapping. Multilocus analysis of SDR 2n-gametes showed the map position of the centromere of chromosome 8 in relation to the RFLP-loci. These genetic analyses also demonstrated the occurrence of a single crossover per chromosome arm (high level of chiasma interference). In the light of these findings, it is possible to construct more critical genetic maps of potato.

CONTENTS



General introduction

GENERAL INTRODUCTION

RELEVANCE OF SEXUAL POLYPLOIDIZATION

Some of the most important crop plants, such as wheat, oat, cotton, sugarcane, tobacco, and banana, are all polyploids. Also the cultivated potato, *Solanum tuberosum* L., is one of them. In general, polyploids can originate through the doubling of the number of chromosomes (somatic doubling) as in the case of colchicine doubling, or through the functioning of numerically unreduced gametes (2n-gametes). The latter of these processes is also referred to as meiotic doubling or sexual polyploidization and has received considerable attention in recent years. This is because sexual polyploidization has the advantages to occur spontaneously and to generate genetic variability (Mendiburu and Peloquin 1976, 1977a; Bingham 1980; Hermsen 1984; Peloquin *et al.* 1989a; Watanabe and Peloquin 1991; David *et al.* 1995; Tai and de Jong 1997).

Besides the relevance of 2n-gametes for sexual polyploidization, they are a prerequisite for asexual reproduction in which embryos develop from egg cells without fertilization (diplosporic apomixis) (Koltunow 1993). Induction of diplosporic apomixis in a vegetatively propagated crop like potato is highly attractive because many of the diseases, especially those caused by viruses, are not transmitted through true seeds (Jongedijk *et al.* 1985).

Having reconsidered the importance of 2n-gametes for sexual polyploidization and diplosporic apomixis, it is relevant to consider the use of 2n-gametes in practical breeding. Different extensive surveys (Harlan and de Wet 1975; Veilleux 1985; Bretagnolle and Thompson 1995) have shown that 2n-gametes occur in a large number of plant species, both wild and cultivated, in highly variable frequencies. Also in *Solanum* spp., these 2n-gametes occur frequently (Den Nijs and Peloquin 1977). The first suggestion to use 2n-gametes for the improvement of the cultivated potato (*Solanum tuberosum*) was made by Chase (1963), who proposed the so-called 'analytic breeding' method. According to this, the ploidy level of the autotetraploid potato (2n=4x=48) is reduced to the dihaploid condition (2n=2x=24)

and breeding is carried out at the diploid level. As compared to the tetraploid level breeding at the diploid level is attractive, because of the simple genetics, the requirement of smaller populations and the possibility for introgression of genes directly from the diploid wild *Solanum* species. Once the desirable diploid genotypes are selected, the tetraploid condition is restored with the help of 2n-gametes through either unilateral (4x.2x- or 2x.4x-crosses) or bilateral (2x.2x-crosses) sexual polyploidization (Mendiburu and Peloquin 1977b).

Subsequent to the suggestion of Chase (1963), considerable knowledge has been generated on the modes of origin of 2n-gametes (Lam 1974; Ross and Langton 1974; Ramanna 1979, 1983; Mok and Peloquin 1975a, b; Mendiburu and Peloquin 1976, 1977a, b; Taylor 1978; Veilleux *et al.* 1982; Iwanaga 1984; Stelly and Peloquin 1986a, b; Douches and Quiros 1987, 1988a, b; Werner and Peloquin 1987, 1991; Watanabe and Peloquin 1989, 1993; Jongedijk *et al.* 1985, 1991b; Werner *et al.* 1992; Conicella *et al.* 1991; Barone *et al.* 1995). Consequently, the 2n-gametes are becoming increasingly relevant in the breeding of potato (Peloquin *et al.* 1989b; Iwanaga *et al.* 1989; Ortiz *et al.* 1991a, b, 1994; Frusciante *et al.* 1992; Watanabe *et al.* 1992, 1995; Hutten *et al.* 1994a, 1995; Clulow *et al.* 1995; Qu *et al.* 1996). The utilization of 2n-gametes in breeding programmes is also becoming increasingly popular in other crops such as alfalfa (*Medicago* spp.: Veronesi *et al.* 1986; Bingham 1990; Tavoletti *et al.* 1991; Motzo *et al.* 1994; Barcaccia *et al.* 1995), banana (*Musa* spp.: Ortiz and Vuylsteke 1995), blueberry (*Vaccinium* spp.: Ortiz *et al.* 1992; Qu and Hancock 1995), red clover (*Trifolium pratens*: Parrott and Smith 1986; Taylor and Wiseman 1987), sugarcane (*Saccharum officinarum*: Bhat 1985), grasses (*Dactylis glomerata:* Sato *et al.* 1993; *Lolium/Festuca*: Morgan *et al.* 1995), rye (*Secale cereale*: Lelley *et al.* 1987), oats (*Avena sativa*: Katsiotis and Forsberg 1995) and cassava (*Manihot esculenta*: Nassar 1992).

MODES OF ORIGIN OF 2N-GAMETES

In general, 2n-gametes can originate through pre-meiotic doubling, meiotic nuclear restitution, post-meiotic doubling or the development of egg cells from a somatic cell (apospory) (Rhoades and Dempsey 1966; Peloquin 1989a). Most unreduced gametes

originate from meiotic disturbances in chromosome pairing, centromere division, spindle formation or cytokinesis (Veilleux 1985). These aberrations include suppression of the first meiotic division, an extra chromosomal replication during interkinesis between the first and second meiotic divisions, omission of the second meiotic division, and fusion of nuclei (Rhoades and Dempsey 1966; Pfeiffer and Bingham 1983). Some examples of mutant genotypes with premeiotic doubling have been reported in maize, *Brassica* (Veilleux 1985) and oat (Katsiotis and Forsberg 1995), among others. An example of post-meiotic doubling is the endoduplication in tetrads as described in sugarcane (Bremer 1961; Bhat and Gill 1985). The aposporic development of egg cells is reported in grasses (Asker and Jerlin 1992; Koltunow 1993).

In potato, Lam (1974) described the occurrence of premeiotic chromosome doubling at a very low frequency in a *Solanum chacoense* trisomic. Except for this report, the only two modes of origin of 2n-gametes that have been demonstrated so far in potato are the first division restitution (FDR) and second division restitution (SDR) during meiosis (Peloquin 1989a). In the case of FDR, the entire diploid complement divides equationally (Mok and Peloquin 1975a) and the non-sister chromatids are included in each restitution nucleus (Fig.1). Genetically, FDR 2n-gametes are expected to retain the parental gene combinations nearly intact, including heterozygosity and epistatic gene interactions (Mendiburu and Peloquin 1976, 1977a; Peloquin 1983; Ramanna 1979). In the case of SDR, the products of the reduction division are restituted and the sister chromatids are included in one and the same restitution nucleus. Genetically, SDR gametes have disrupted parental gene combinations and a high degree of homozygosity (Fig.1). Therefore, SDR gametes give rise to highly heterogeneous populations of 2n-gametes (Mendiburu and Peloquin 1976, 1977a; Hermsen 1984). It is important to realize that the definitions of FDR and SDR are more commonly used to describe the genetic composition of the 2n-gametes rather than the cytological stage in which the nuclear restitution occurs. For example, abnormalities like parallel and fused spindle formation during metaphase and anaphase II give rise to gametes that are equivalent to FDR (Mok and Peloquin 1975a; Ramanna 1979).

For the purpose of breeding, both 2n-pollen and 2n-eggs can be utilized albeit for different purposes. Whereas 2n-pollen originate predominantly through FDR and are useful

for transferring a high degree of heterozygosity to the progeny, 2n-eggs are mostly of SDR origin and facilitate the transfer of a high degree of homozygosity to the progeny. This is because in the microsporogenesis of potato the absence of reductional cell wall formation subsequent to telophase I and the formation of fused spindles at metaphase II favour FDR origin (Ramanna 1979). On the other hand, the successive cell wall formation in the megasporogenesis of potato favours the SDR 2n-egg formation (Jongedijk 1985).

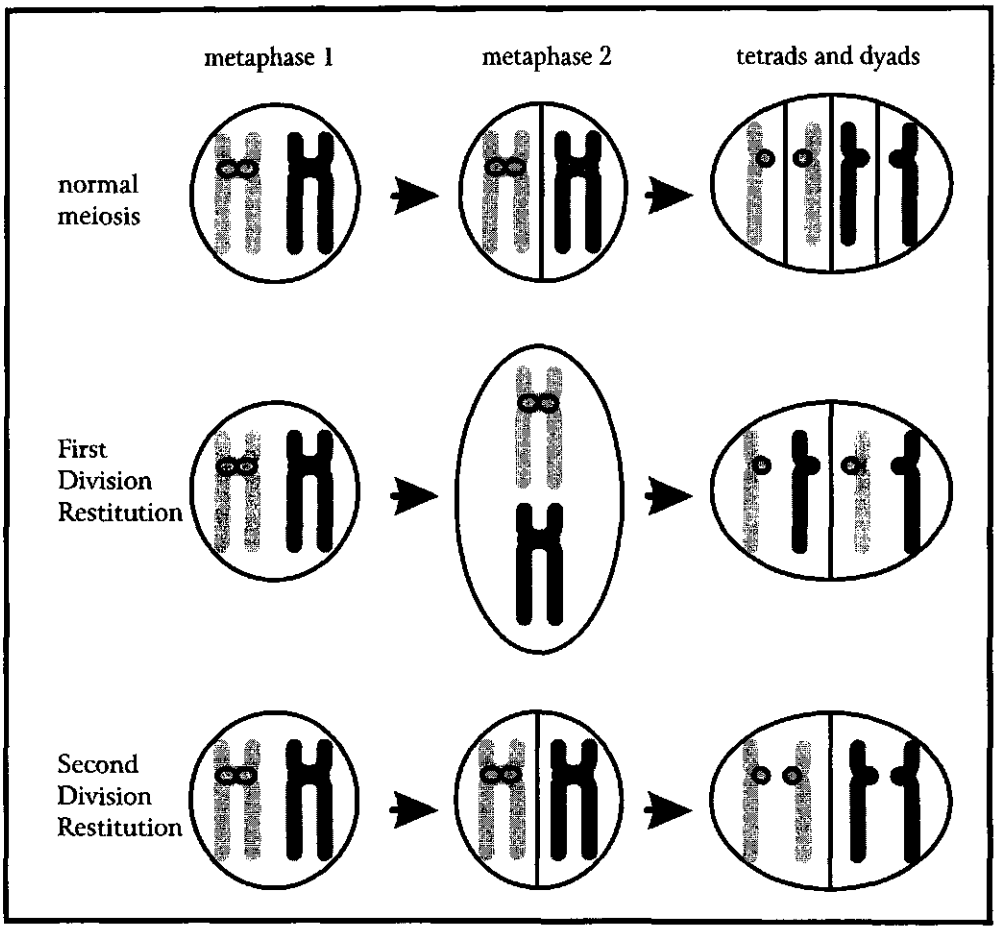


Figure 1. Generation of four n-spores (tetrads) following normal meiosis, and two 2n-spores (dyads) following first and second division restitution in a diploid hybrid.
Maternal and paternal chromosomes are indicated in light and dark grey, respectively.

Two cytologically characterized mechanisms of FDR 2n-pollen formation are the pseudo-homotypic (mitotic like) division of the chromosomes at metaphase I following inhibited chromosome pairing (Gustafsson 1935; Ramanna 1983), and the occurrence of parallel or fused spindle formation during metaphase and anaphase II (Mok and Peloquin 1975a; Ramanna 1979). Cytological studies of megasporogenesis have shown the omission of the second meiotic division as the most common mechanism of SDR 2n-egg formation (Werner and Peloquin 1987, 1991; Jongedijk *et al.* 1991b; Conicella *et al.* 1991). However, the cytological elucidation of the nuclear restitution mechanisms is complicated since different meiotic aberrations can occur simultaneously in one plant, resulting in a mixture of FDR-SDR 2n-pollen (Mok and Peloquin 1975a; Oliviera *et al.* 1995) or 2n-egg cells (Douches and Quiros 1988b; Conicella *et al.* 1991; Werner and Peloquin 1987, 1991). Only when synaptic mutants with a failure of chromosome pairing during meiotic prophase are used, it is possible to select for clones with equational division of all univalents, resulting in the exclusive occurrence of FDR 2n-gametes (Iwanaga and Peloquin 1979; Okwuagwu and Peloquin 1981; Ramanna 1983; Douches and Quiros 1988a; Jongedijk *et al.* 1989, 1991b).

INFLUENCE OF CROSSING-OVER ON THE GENETIC COMPOSITION OF 2N-GAMETES

The nuclear restitution mechanism is not the only factor that determines the genetic composition of 2n-gametes. This is because the degree of heterozygozity of the FDR and SDR 2n-gametes is also dependent on the extent of crossing-over in the germ cells, preceding the nuclear restitution. In FDR gametes, all genetic loci situated in the region between the centromere and the first cross-over will stay heterozygous, whereas 50 percent of those that are distal to the cross-over will be homozygous. On the contrary, in SDR gametes, all loci between the centromere and the first cross-over will be homozygous, whereas the distal ones will be heterozygous (Mendiburu and Peloquin 1976; Hermsen 1984). In the case of asynaptic or desynaptic mutants, in which the homologous chromosomes fail to pair or have reduced amounts of chiasma formation respectively, the degree of crossing-over is reduced (Jongedijk *et al.* 1989). Since viable 2n-gamete formation cannot occur through the SDR

mechanism in desynaptic mutants (Ramanna 1983; Jongedijk 1985), the 2n-gametes are of exclusively FDR origin. As a result of the FDR mechansim and the reduced recombination, the 2n-gametes of synaptic mutants will be highly heterozygous and homogeneous (Douches and Quiros 1988a; Jongedijk *et al.* 1991a; Okwuagwu and Peloquin 1981; Ramanna 1983).

Besides the interest of generating different degrees of heterozygosity, the 2n-gametes are useful for the study of the extent of crossing-over in the germ cells, preceeding the nuclear restitution, for the purpose of genetic mapping. To this end, the FDR or SDR 2n-gametes of normal synaptic clones, in which the recombination frequency is high, are genotyped at different marker loci and used in linkage analysis. The special feature about this linkage analysis is that it can be used for determining the map distances between the centromeres and marker loci, the so-called 'gene-centromere mapping' (Rhoades and Dempsey 1966; Mendiburu and Peloquin 1979). This is based on the fact that the 2n-gametes comprise two of the four chromatids of each bivalent. The FDR gametes allow the monitoring of the non-sister chromatids of each half-bivalent, whereas the SDR gametes allow the inspection of the sister chromatids of each half-bivalent. Since the chance of a crossover is dependent on the distance of a locus to the centromere, the distal marker loci in FDR gametes tend to be homozygous more frequently than the proximal loci. In contrast, the distal marker loci in SDR gametes tend to be heterozygous more frequently than the proximal loci. In this way, 2n-gametes offer a valuable method to map loci relative to the centromeres, which is often referred to as half-tetrad analysis.

EXPLOITATION OF FDR AND SDR 2N-GAMETES

The two types of 2n-gametes can be useful for different purposes. Especially in the case of desynaptic clones, the FDR gametes can transfer a high degree of heterozygosity and uniformity to the progeny. This pollen can be exploited in 4x.2x-crosses to produce true potato seeds for the generation of highly heterozygous and uniform progenies (Ortiz and Peloquin 1991a; Frusciante *et al.* 1992; Clulow *et al.* 1995).

In the case of normal synaptic clones, where the recombination frequency is reduced,

the SDR-gametes can facilitate the creation of relatively high degree of homozygosity. Such a situation is described in *Chrysantthemum* (Dowrick 1953), and can also be expected to occur in interspecific hybrids comprising one genome of a cultivated species and the other one from a related wild species. Each pair of chromosomes in SDR 2n-gametes of such F_1 hybrids will be derived from either the cultivated or the alien genome alone. Therefore, these SDR 2n-gametes are expected to possess variable number of chromosomes of the alien genome (Fig. 2). If the alien parental genotype of the interspecific hybrid carries a desired character, the SDR 2n-gametes can be exploited in 2x.4x-crosses for the generation and selection of progeny plants in which only the desired character is present in combination with nearly the complete genome of the cultivated species. This method offers the possibility to eliminate the undesirable genes and chromosomes of the alien genome rapidly, and thus they will be highly useful for introgression programmes in breeding.

As mentioned before, both the FDR and SDR 2n-gametes of normal synaptic clones can be exploited for gene-centromere mapping (Mendiburu and Peloquin 1979). The assignment of the positions of the centromeres in genetic maps will allow the identification of both the short and the long arm of each chromosome. This will enable to identify the number of crossovers per chromosome arm to study chiasma interference at different parts of chromosomes, which can be important for more fundamental genetics of potato.

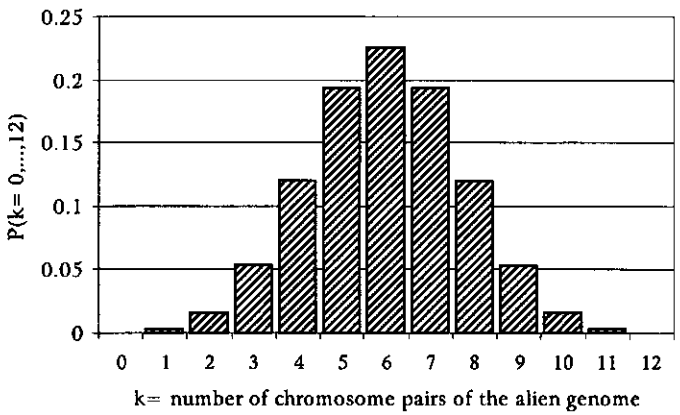


Figure 2. Expected genetic composition of the SDR 2n-gametes of a cultivated-wild species F_1 hybrid in which the recombination frequency is highly restricted.

USE OF GENETIC MARKERS FOR THE ASSESSMENT OF THE GENOTYPES OF 2N-GAMETES

In order to utilize the 2n-gametes effectively for sexual polyloidization or gene-centromere mapping, an exact knowledge regarding their origin and genetic composition is essential. The analysis of the genotypes of 2n-gametes requires progeny plants that are derived from these 2n-gametes. This is possible by inducing the parthenogenetic development of 2n-eggs by prickle pollinations using pollen of another species or genus. It results in maternal-like or matromorphic plants, such as described in *Rubus* (Dowrick 1966), *Brassica* (Eenink 1974), barley (Devaux 1992), and onion (Campion *et al.* 1995).

Alternatively, progeny plants from 2n-gametes can be generated through a 2x.4x-cross, or its reciprocal (Hanneman and Peloquin 1968). This is especially convenient, because only the tetraploid embryos that are derived from the functioning of a 2n-gamete (2n=2x) of the diploid parent and a n-gamete (n=2x) of the tetraploid parent develop, while triploids derived from the functioning of two n-gametes (n[=x] + n[=2x]) abort, which is called the 'triploid block' in *Solanum* (Marks 1966). To genotype the 2n-gametes of the diploid parent different genetic markers can be used to analyse the tetraploid progeny through half-tetrad analysis (Mendiburu and Peloquin 1979; Douches and Quiros 1987, 1988a, b; Jongedijk *et al.* 1991b; Wagenvoort and Zimnoch-Guzowska 1992; Werner *et al.* 1992; Barone *et al.* 1995).

If appropriate genetic markers with known map positions are used, half-tetrad analysis can be applied to elucidate the mode of origin of 2n-gametes, to assess the extent of crossing-over during micro- and megasporogenesis, and to estimate the level of homo- and heterozygosity of 2n-gametes. For simultaneous elucidation of the restitution mechanism and the number of crossovers per chromosome of each individual 2n-gamete, it is necessary to monitor the segregation of marker loci on different parts of a chromosome, including the proximal regions. Only if the combinations of alleles at linked loci on a pair of chromosomes in individual 2n-gametes can be determined, it will be possible to demonstrate whether only one or a mixture of different restitution mechanisms occur in a clone. The multilocus linkage analysis of tetraploids, derived from 2x.4x-crosses, requires markers with suitable

polymorphisms for genotyping at the 4x level, and random distribution along the whole length of one chromosome.

There are relatively few morphological and isozyme markers available in potato (Douches and Quiros 1988c; Jacobs *et al.* 1995). However, most of them are not convenient for genetic studies at the tetraploid level. In contrast, the biochemical marker *amylose-free (amf)*, which is new in the genetics of potato, is very useful at the tetraploid level (Jacobsen *et al.* 1989, 1991; Flipse *et al.* 1996). Moreover, this marker allows the analysis of the extent of crossing-over in diploid 2n-gamete producers, since its locus is assigned to the distal part of chromosome 8 of potato (Gebhardt *et al.* 1991; Jacobs *et al.* 1995). Besides, the RFLP markers in potato (Bonierbale *et al.* 1988; Tanksley *et al.* 1992; Gebhardt *et al.* 1991; Jacobs *et al.* 1995; Barone *et al.* 1995) are expected to be most advantageous for half-tetrad analysis because of the high number of loci available, and the potential to identify all different alleles per locus in tetraploids.

THE MAIN AIM AND SCOPE OF THE THESIS

To gain a deeper understanding of the modes of origin of 2n-gametes in diploid potato, as well as in order to assess their implications for genetic studies, both 2n-pollen (Chapter 2) and 2n-egg formation (Chapters 3,4,5 and 6) have been investigated.

Chapter 2 describes half-tetrad analysis using FDR 2n-pollen to estimate the extent of crossing-over in both synaptic and desynaptic genotypes of diploid potato. The focus of this chapter is on the use of the *amylose-free (amf)* locus on chromosome 8 and the isozyme *alcohol dehydrogenase* (*Adh-1*) locus on chromosome 4 as pollen marker loci for the analysis of both the 2n-pollen directly as well as the genotypes of the tetraploid progenies of 4x.2x-crosses, in which the diploid male parent is heterozygous for these markers.

In view of the differences between the male and female meiosis in potato, it is expected that the 2n-egg formation will also be different from 2n-pollen. As a result of the successive cell wall formation in female meiosis, the 2n-eggs are expected to originate through SDR, whereas the 2n-pollen can originate through FDR as well as SDR. In this context, the

selection of 2n-egg producing genotypes is considered to be the prerequisite to investigate and exploit SDR 2n-gametes. In contrast to the easy selection of genotypes that produce high frequencies of 2n-pollen by monitoring the occurrence of large (2n) and small (n) pollen grains in stained pollen preparations, it is much more difficult to select genotypes that produce sufficient numbers of 2n-eggs. In the absence of clear cytological methods and criteria for monitoring the egg cells in ovules, 2x.4x-crosses can be used to select genotypes with sufficient number of 2n-eggs. Chapter 3 describes the results of an extensive 2x.4x-crossing programme to select such 2n-egg producers. The female parents that have been used in these crosses represent a large genetic diversity to allow the selection of diploid clones with distinct modes of 2n-egg formation. In this crossing programme, special attention is paid to the selection of 2n-egg producers that are heterozygous for the *amf* marker.

Once the 2n-egg producers are available, the next step in the study of 2n-egg formation is the the generation of plant material, that can be used for the genetic analysis. The ideal situation is the generation of a progeny that has been directly derived from the unfertilized 2n-eggs. To this end, the induction of the parthenogenetic development of 2n-eggs is investigated as a first option. Alternatively, 2x.4x-crosses can be used to generate tetraploid progenies. Chapter 4 provides data on the generation of diploid parthenogenetic progeny through prickle pollinations of 2n-egg producers as well as the generation of tetraploid hybrid progenies from 2x.4x-crosses. In addition, the diploid 2n-egg producing clones and the tetraploid male parents are analysed for RFLP markers to determine the polymorphisms, that will be informative for the multilocus analysis of 2n-eggs, using the diploid parthenogenetic and tetraploid hybrid progenies.

The success of the approach to select informative RFLP probes is demonstrated in Chapter 5, in which RFLP-analysis of a tetraploid progeny of a diploid 2n-egg producer has shown the complete homozygosity of the 2n-eggs. This analysis provides evidence for a new restitution mechanism of 2n-egg formation in potato. In Chapter 6, the same multilocus analysis approach is applied in the tetraploid progeny of another diploid 2n-egg producer to elucidate the restitution mechanism and the extent of crossing-over.

Finally, the results and implications of genetic analysis of 2n-gametes are dicussed in Chapter 7.

Pollen markers for gene-centromere mapping in diploid potato

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POLLEN MARKERS FOR GENE-CENTROMERE MAPPING IN DIPLOID POTATO

ABSTRACT

The utility of two pollen genetic markers for estimating the extent of meiotic recombination between the centromere and a marker gene was tested in 2n-pollen of diploid potato clones. One of these markers was the distal locus *amylose-free* (*amf*) on chromosome 8 and the other was the isozyme locus *alcohol dehydrogenase* (*Adh-1*) on chromosome 4. In the case of the *amf* locus, the gene-centromere distance was estimated in a normal synaptic and a desynaptic genotype. In both cases the genetic analysis was confined to: (1) a direct estimation of the phenotypic (blue vs red) segregation ratios in FDR (first division restitution) 2n-pollen and (2) a classification of the 4x progeny from 4x (nulliplex *amf*) × 2x (*Amf/amf*) crosses into duplex, simplex and nulliplex classes. The recombination frequency between the centromere and the *amf* locus in the normal synaptic genotype B92-7015-4 corresponded to a gene-centromere distance of 48.8 cM, whereas this distance amounted to 13.3 cM in the desynaptic genotype RS93-8025-1. Hence desynapsis reduced crossing-over by 73%. The observed genetic distance of 48.8 cM in the normal synaptic clone, B92-7015-4, is the highest gene-centromere distance reported so far in potato and this could be explained on the assumption of absolute chiasma interference. For the *Adh-1* locus, it was found that heterozygous 2n-pollen grains could be detected in pollen samples of the diploid clones, because of the occurrence of a heterodimeric band of the isozyme. Unlike the *amf* locus, the gene-centromere distance for the *Adh-1* locus was estimated only on the basis of the duplex, simplex and nulliplex classes in the progenies from 4x (nulliplex $Adh\text{-}1^2$) × B92-7015-4 ($Adh\text{-}1^1/Adh\text{-}1^2$) crosses and was found to be 19.4 cM. Because the accurate positions of centromeres in relation to other loci are not available in the existing genetic maps of potato, which are saturated with molecular markers, half-tetrad analysis is a promising additional approach to the basic genetics of this crop.

INTRODUCTION

As in many other crops, genetic maps of the diploid (2n=2x=24) cultivated potato, *Solanum tuberosum*, have become available in recent years (Bonierbale *et al.* 1988; Gebhardt *et al.* 1991; Tanksley *et al.* 1992; Jacobs *et al.* 1995; Van Eck *et al.* 1995). Despite the localization of numerous molecular and some morphological markers on the 12 possible linkage groups of potato, the maps do not accurately indicate the positions of the centromeres in relation to these marker loci. Information about centromere positions is important for providing fixed points in the linkage groups of genetic maps, distinguishing both chromosome arms, identifying proximal and distal marker genes, and investigating interference. One method of localizing centromeres in relation to marker loci is through 'half-tetrad analysis' (HTA), or 'gene-centromere mapping', which has been successfully used in some plants (Rutishauser 1956; Nel 1975; Qu and Hancock 1995) as well as in fishes (Thorgaard *et al.* 1983; Allendorf *et al.* 1986; Seeb and Seeb 1986; Liu *et al.* 1992; Johnson *et al.* 1996) and mammals (Eppig *et al.* 1983; Jarrell *et al.* 1995). However, the application of this method requires that the organisms studied produce numerically unreduced (2n) gametes or that their first-meiotic-division products can be isolated, and that the cytological mode of origin of 2n-gametes is clearly known. In potato, 2n-gametes occur frequently (Den Nijs and Peloquin 1977), and their cytological modes of origin have been elucidated as first division restitution (FDR), second division restitution (SDR), or a mixture of both types (Peloquin *et al.* 1989a). One of these modes of origin is fused spindle formation during metaphase 2 and anaphase-II which gives rise to FDR gametes (Mok and Peloquin 1975a; Ramanna 1979).

So far, HTA has been carried out in potato by monitoring segregation ratios in the tetraploid (2n=4x=48) progenies derived from crosses between 4x (nulliplex) × 2x (heterozygotes), or vica versa (Mendiburu and Peloquin 1979; Douches and Quiros 1987, 1988a; Jongedijk *et al.* 1991a, Wagenvoort and Zimnoch-Guzowska 1992; Werner *et al.* 1992; Barone *et al.* 1995). Instead of analysing the 4x progenies, it would be more efficient to monitor segregation for marker genes in the 2n-gametes themselves. The combination of pollen-specific markers in diploid clones that produce 2n-pollen through

fused spindle formation offers the possibility of analyzing large samples of FDR 2n-gametes in potato.

In the present investigation we have tested the utility of two pollen markers for such an approach. The first involves the monogenic recessive mutant *amylose-free* (*amf*), with a modified potato starch composition (Jacobsen *et al.* 1989). There are a number of advantages in using this genetic marker: (1) iodine-potassium iodide staining of the microspores enables unambiguous scoring of the wild-type (blue) and mutant (red) phenotypes; (2) large samples of both n- and 2n-microspores can be easily scored (Jacobsen *et al.* 1991): (3) at the tetraploid level duplex, simplex, and nulliplex genotypes can be identified (Flipse *et al.* 1996), and (4) its locus is assigned to the distal part of chromosome 8 of potato (Gebhardt *et al.* 1991; Jacobs *et al.* 1995). The second genetic marker is the dimeric isozyme alcohol dehydrogenase (ADH), which is expressed in the pollen of potato and is found to be a proximal marker (Douches and Quiros 1987). In tomato, the ADH isozyme in mature pollen grains (Tanksley *et al.* 1981) is known to be encoded by the *Adh-1* locus on chromosome 4 (Tanksley *et al.* 1992). In the present investigation, the nomenclature *Adh-1* used in tomato has been retained for potato, because the locus that encodes ADH isozyme in the mature pollen of potato has also been localized on chromosome 4 (Jacobs *et al.* 1995).

In order to test the effectiveness of gene-centromere mapping, the segregation data from 2n-microspores in diploid heterozygotes were compared with those from progenies of 4x (nulliplex) × 2x (heterozygotes) crosses. The specific aims of the HTA were: (1) to estimate the genetic distance between the *amf* locus and the centromere; (2) to determine the effect of desynapsis (*ds-1*) (Jongedijk and Ramanna 1989) on the extent of crossing-over between *amf* and the centromere; and (3) to verify the proximal position of the *Adh*$_1$ 1 locus.

MATERIALS EN METHODS:

Plant material

Three diploid (2n=2x=24) and five tetraploid (2n=4x=48) genotypes were generated from complex crosses between diploid *S. tuberosum* (*tbr*), *S. phureja* (*phu*), *S. microdontum* (*mcd*), and *S. verrucosum* (*ver*). The choice of the diploid genotypes was based on the following criteria: (1) allelic differences for amylose free (*amf*) starch resulting from the defective granule-bound starch synthase (*GBSS*) locus on chromosome 8 (Gebhardt *et al.* 1991; Jacobs *et al.* 1995), (2) normal (*Ds-1/.*) versus desynaptic (*ds-1/ds-1*) chromosome pairing behaviour, and (3) the presence or absence of 2n-pollen. The genotypes at the *Adh-1* locus on chromosome 4 (Tanksley *et al.* 1992, Jacobs *et al.* 1995) of the three selected clones were identified based on differences in the electrophoretic migration of ADH-1 proteins, demonstrating the presence of the alleles *Adh-1*^{1}, *Adh-1*^{2}, and *Adh-1*^{3}. The genotypes and parentages of each of the three diploids are as follows:

1. HB93-7108-8: *Amf/amf; Adh-1*^{2}*/Adh-1*^{3}; *Ds-1/.*. This clone is an interspecific hybrid between clone B16 of *mcd* (BGRC 18568), and clone 87-1031-29 of *tbr* (Jacobsen *et al.* 1989), and produces only n-pollen. It was used as a control for monitoring the segregation of the *amf* and *Adh-1* markers in n-pollen.
2. B92-7015-4: *Amf/amf; Adh-1*^{1}*/Adh-1*^{2}; *Ds-1/.*. This clone produces a high frequency of 2n-pollen. It is a hybrid between clone 880004-2 of predominantly *tbr* origin (Jacobsen *et al.* 1991) and clone IVP101 of predominantly *phu* origin (Hutten *et al.* 1994a). B92-7015-4 was used for monitoring the segregation of the *amf* and *Adh-1* markers in both n- and 2n-pollen.
3. RS93-8025-1: *Amf/amf; Adh-1*^{2}*/Adh-1*^{2}*; ds-1/ds-1*. This clone was derived from the cross between clones 880004-11 and EC322, both of which are heterozygous for desynapsis *(Ds-1/ds-1)* (Jacobsen *et al.* 1991). In a population with 112 normal synaptic (*Ds-1/.*) and 28 desynaptic (*ds-1/ds-1*) plants, the clone RS93-8025-1 was the only one that expressed the desired four characters, viz., heterozygosity for *amf*, homozygosity for *ds-1*, male fertility and the formation of 2n-pollen.

The five tetraploid genotypes were all nulliplex for the *amf* marker. The clones J90-6001-

25, J90-6011-3, J90-6020-17, J90-6020-22 were derived from *amf/amf/amf/amf* × *Amf/amf* crosses (Jacobsen *et al.* 1991), whereas clone HB93-7133-3 was derived from selfing of a duplex genotype, involving the diploid clone BE1050 (Hutten *et al.* 1995) and the tetraploid clone J90-6011-3. Among the tetraploid clones, only clone HB93-7133-3 was homozygous for the *Adh-1* marker (*Adh-1*^{2}/*Adh-1*^{2}/*Adh-1*^{2}/*Adh-1*^{2}) ; all the others were heterozygous.

Plants were grown in the greenhouse either on bricks or as grafts on tomato root-stocks in order to induce flowering. The tetraploid female parents were emasculated 2-3 days before the opening of the flowers and pollinated when stigma were receptive. Berries were harvested about 6 weeks after pollination. The 4x progenies derived from 4x (nulliplex) × 2x (heterozygous) crosses were used to classify the duplex, simplex and nulliplex genotypes. Hereafter, these 4x progenies will be indicated as 4x.2x progenies.

Cytological analysis of 2n-pollen formation

For the study of n- and 2n-microsporogenesis young anthers were fixed in a 3:1 mixture of ethanol and propionic acid (saturated with ferric acetate), and squashed in a 2% aceto-carmine solution. In these preparations, the number of bivalents was estimated in at least 50 cells. Since fused spindles give rise to FDR 2n-pollen formation in both normal synaptic and desynaptic clones (Jongedijk *et al.* 1991b), the frequency of fused spindle formation was investigated in 100-600 cells per clone. For the estimation of pollen stainability and the frequency of 2n-pollen, 500-1000 mature pollen grains were counted after staining with lactophenol acid fuchsin. Within the sample of stainable pollen, 2n-pollen was distinguished from normal reduced pollen on the basis of grain size and number of germ pores (Jacobsen 1976; Ramanna 1979).

Iodine staining for starch phenotypes (*amf*-marker)

Anthers of flower buds were collected about 1 day before anthesis, and two anthers per bud were squashed in a drop of Lugol/chloral hydrate (1:2) (Jacobsen *et al.* 1991; Flipse *et al.* 1996). Microspores with blue or red starch were counted. In the clones B92-7015-4 and RS93-8025-1, the number of blue and red microspores were determined

separately in the large 2n- and in the smaller n-microspores. In tetraploids, about 50 microspores were scored for the confirmation of nulliplex (100% red) genotypes, and 100-500 microspores were scored to distinguish the simplex (blue: red = 1:1) and duplex (blue: red = 5:1) genotypes. Segregation data of similar and homogeneous ($P_{homogeneity} > 0.05$) 4x.2x progenies were pooled. Chi square tests were used to test the goodness of fit to the expected ratios, significant at the 0.001 level.

Isozyme analysis of ADH-1

For ADH-1 isozyme electrophoresis mature anthers of three flowers were collected and ground in a 1% solution of 2-mercaptoethanol in order to extract proteins. Electrophoresis through 12.5% poly-acrylamide gel (Phast System) and staining were both carried out according to Jacobs *et al.* (1995). Based on the electrophoretic mobility of ADH-1, three different homodimeric variants were detected. In the order of decreasing mobility, these variants were designated as ADH-$1^{1/1}$, ADH-$1^{2/2}$, and ADH-$1^{3/3}$, encoded by the alleles *Adh-1*^{1}, *Adh-1*^{2}, and *Adh-1*^{3} respectively. In tetraploids, the simplex genotypes were distinguished from the duplex genotypes on the basis of intensity of the homodimeric bands (Douches and Quiros 1987).

Gene-centromere mapping

Assuming complete chiasma interference, gene-centromere distances were estimated according to the formulae of Mendiburu and Peloquin (1979) using the frequencies (f) of the different genotypes in the segregating population. For the *amf* marker gene in FDR 2n-pollen the gene-centromere distance was estimated as 2.f(*amf*/*amf*).100 cM. From the analysis of 4x.2x progenies, the gene-centromere map distance was estimated as f(nulliplex+duplex).100 cM, for both the *Adh-1* and the *amf* marker. The 95% binomial confidence interval $[f_i, f_j]$ for the frequency (f) of nulliplex genotypes in a population of n individuals was calculated according to Fruend (1971) as:

$$[f_i, f_j] = n/(n+(1.96)^2) \cdot [f+((1.96)^2/2n) \pm 1.96 \cdot \sqrt{\{f(1-f)/n + ((1.96)^2/4n^2)\}}].$$

The corresponding confidence interval for the estimated gene-centromere distance was derived by substituting f_i and f_j in the relevant mapping formulae.

RESULTS

Microsporogenesis in diploids

Microsporogenesis and pollen formation were examined in the three diploid clones to investigate the level of chromosome pairing and the restitution mechanism of 2n-pollen formation. The control diploid, HB93-7108-8, and the 2n-pollen-forming clone, B92-7015-4, exhibited normal bivalent pairing and a high percentage of pollen stainability (Table 1).

Table 1. Meiotic behaviour (bivalent formation and percentage of fused spindles), and pollen formation (percentages of stainable pollen and 2n-pollen) in three diploid clones HB93-7108-8 (control), B92-7015-4 (normal synaptic) and RS93-8025-1 (desynaptic).

Diploid clone (*Amf/amf*):	Average number of bivalents per cell	Percentage of		
		Fused spindles (per 100 pollen mother cells)	Stainable pollen (per 100 pollen grains)	2n-pollen (per 100 stainable pollen grains)
HB93-7108-8 (*Ds-1/.*)	12	0	90	0
B92-7015-4 (*Ds-1/.*)	12	75	72	63
RS93-8025-1 (*ds-1/ds-1*)	2.7	12	12	100

Unlike these, the desynaptic clone RS93-8025-1 had an average of about three bivalents per cell and, consequently, pollen stainability was very low. The characteristic feature of the control genotype, HB93-7108-8, was that fused spindles were completely absent at the metaphase 2 stages and only stainable (n) pollen grains of uniform size (± 23 μm diameter) were formed. On the other hand, B92-7015-4 produced both n- and 2n-pollen grains. The 2n-grains were distinctively large in size (far exceeding 23 μm) and amounted to 63% of the stainable pollen. In the desynaptic clone, RS93-8025-1, only the 2n-pollen grains were stainable. There was a correspondence between fused spindle and functional 2n-pollen formation in both B92-7015-4 and RS93-8025-1 (Table 1). This

indicates that the 2n-pollen in both clones originated through FDR.

Segregation for the *amf* marker in diploids and estimates of its map distance to centromere

Based on starch phenotypes and size, the microspores of both diploid and tetraploid clones could be unambiguously classified (Fig. 1A-J). All three diploid clones segregated for the blue and red microspore phenotypes. The n-pollen of the control clone, HB93-7108-8, segregated 1 blue : 1 red (Fig. 1D), as expected. In B92-7015-4, which formed both n- and 2n-pollen, the segregation ratios for the two classes were different (Fig. 1E). Whereas the n-microspores segregated approximately 1 blue : 1 red, as expected, the 2n-microspores showed a segregation ratio of 3 blue: 1 red (Table 2). This 3:1 segregation of 2n-microspores could be explained by assuming one crossover between the centromere and the *amf* locus in all pollen mother cells, followed by random assortment of the crossover and non-crossover chromatids during the restitutional (fused spindle) anaphase 2 stage. As a result of this, the three genotypes of 2n-microspores, viz., *Amf/Amf; Amf/amf* and *amf/amf*, were expected to occur with a ratio of 1:2:1 respectively, which is in agreement with the observed value of 3 blue (*Amf/.*) to 1 red (*amf/amf*). Based on the microspore segregation data of this clone, the gene-centromere distance for the *amf* locus was estimated as 48.8 cM [42.5-55.6 cM]. Although the desynaptic clone RS93-8025-1 also produced 2n-microspores through fused spindles (i.e. FDR as in B92-7015-4), the segregation of blue: red microspores deviated significantly from the expected 3:1 ratio (Table 2; Fig. 1F). This result could be explained on the basis of the reduction of chiasma formation due to desynapsis in this clone. The map distance between the centromere and the *amf* locus in RS93-8025-1 was estimated as 13.3 cM [10.9-16.2 cM].

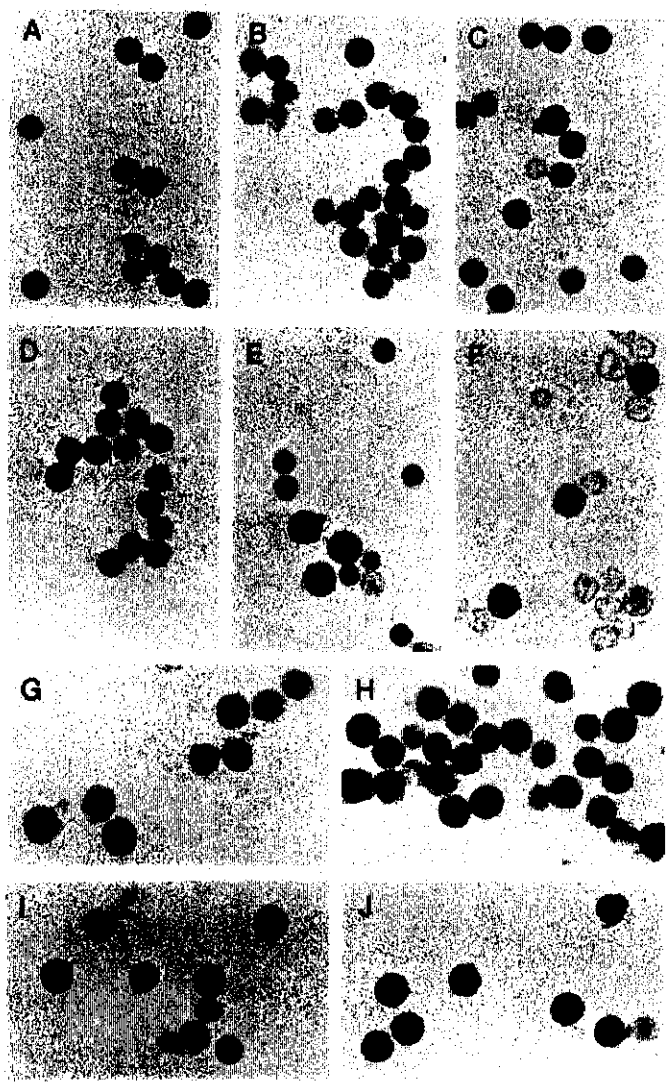


Figure 1. Starch phenotypes of blue and red in n- and 2n-microspores in diploid (**A-F**) and tetraploid (**G-I**) genotypes. **A-C** n-microspores in three diploid genotypes; **A**= wild-type *Amf/Amf*; **B**= heterozygous genotype *Amf/amf*; and **C**= mutant genotype *amf/amf*. Note the 1 blue: 1 red segregation in **B** as compared to solely blue and red in **A** and **C** respectively.**D-F** n- and 2n-microspores in heterozygous diploid clones. **D** shows the microspores of the control clone, HB93-7108-8, containing only small (n-) microspores with blue and red starch phenotypes. **E** shows the segregation for small (n-) and large (2n-) microspores in the normal synaptic clone, B92-7015-4, in which both types segregate for blue and red phenotypes. **F** shows the microspores of the desynaptic clone, RS93-8025-1, showing large functional 2n-microspores (blue and red) and the aborted ones.**G-J** microspore segregation pattern in the wildtype tetraploid genotype (**G**) and in the three genotypic classes of 4x-progenies derived from 4x (nulliplex *amf*)×2x *(Amf/amf)*-crosses (**H-J**). **G** = quadruplex *(Amf/Amf/Amf/Amf)* genotype, all blue; **H**= duplex *(Amf/Amf/amf/amf)*, 5 blue: 1 red; **I**= simplex *(Amf/amf/amf/amf)*, 1 blue: 1 red and **J**= nulliplex *(amf/amf/amf/amf)*, all red.

Table 2. Segregation ratios of n- and 2n-microspores for blue and red phenotypes in three diploid clones HB93-7108-8 (control), B92-7015-4 (normal synaptic) and RS93-8025-1 (desynaptic).

Diploid clone (*Amf/amf*)	n-microspores			2n-microspores		
	blue (*Amf*)	red (*amf*)	$P_{1:1}$	blue (*Amf/.*)	red (*amf/amf*)	$P_{3:1}$*
HB93-7108-8 (*Ds-1/.*)	232	222	>0.6	-	-	
B92-7015-4 (*Ds-1/.*)	365	296	>0.005	496	160	>0.6
RS93-8025-1 (*ds-1/ds-1*)	-	-		1305	93	<0.0005

* assuming FDR and complete chiasma interference

Classification of 4x.2x progenies for *amf* marker segregation

The segregation of the *amf* marker in 2n-microspores allowed a distinction of only two phenotypic classes, viz., blue (*Amf/.*) and red (*amf/amf*). In order to distinguish all three expected classes, *Amf/Amf*; *Amf/amf* and *amf/amf*, it was necessary to generate 4x progenies from 4x (nulliplex *amf*) × 2x (*Amf/amf*) crosses and classify these 4x.2x progenies into duplex, simplex and nulliplex genotypes. Such a classification, based on the segregation for starch phenotypes of microspores in tetraploids (Figs. 1G-J) was carried out for two population types in which the normal synaptic (B92-7015-4) and the desynaptic (RS93-8025-1) clones, respectively, were used as male parents on a set of four different female parents (Table 3). In both types of population, about 450 plants of the 4x.2x progenies could be classified into duplex, simplex and nulliplex (Figs. 1H-J) genotypes. Since both the duplex and nulliplex genotypes originated from a crossover between the centromere and the *amf* locus, followed by FDR in pollen mother cells, these two classes were expected to occur at equal frequencies. The observed numbers of duplex and nulliplex genotypes (83 and 97 in the 4x.2x progenies of B92-7015-4; 25 and 30 in the 4x.2x progenies of RS92-8025-1) in both cases fit a 1:1 ratio (χ^2= 1.09 and 0.45 respectively). The map distance between *amf* and the centromere was estimated as 39.5 cM [35.1-44.0 cM] in the normal synaptic clone B92-7015-4, and as 12.0 cM [9.2- 15.2 cM] in the desynaptic clone RS93-8025-1.

Table 3. Genotype classes of duplex, simplex and nulliplex 4x progenies derived from 4x (nulliplex *amf*)× 2x (*Amf/amf*) crosses. Both the normal synaptic diploid parent B92-7015-4 and the desynaptic diploid parent RS93-8025-1 produced 2n-pollen through fused spindles (FDR).

4x-female parent	2x-male parent	4x.2x-progeny (4x)			
(amf/amf/amf/amf)	*(Amf/amf)*	# duplex (%) *(Amf/Amf/amf/amf)*	# simplex (%) *(Amf/amf/amf/amf)*	# nulliplex (%) *(amf/amf/amf/amf)*	total
HB93-7133-3	B92-7015-4	8 (17)	28 (60)	11 (23)	47
J90-6001-25	(*Ds-1/.*)	32 (26)	62 (51)	27 (22)	121
J90-6011-3		19 (15)	84 (65)	27 (21)	130
J90-6020-22		24 (15)	102 (65)	32 (20)	158
Total*		83 (18)	276 (61)	97 (21)	456
HB93-7133-3	RS93-8025-1	1 (2.0)	45 (90)	4 (8.0)	50
J90-6011-3	(*ds-1/ds-1*)	14 (8.6)	134 (83)	13 (8.1)	161
J90-6020-17		1 (4.5)	21 (95)	0 (0.0)	22
J90-6020-22		9 (4.0)	202 (90)	13 (5.8)	224
Total*		25 (5.5)	402 (88)	30 (6.6)	457

* 4x.2x-progenies are homogeneous, P(χ^2 homogeneity)>0.05

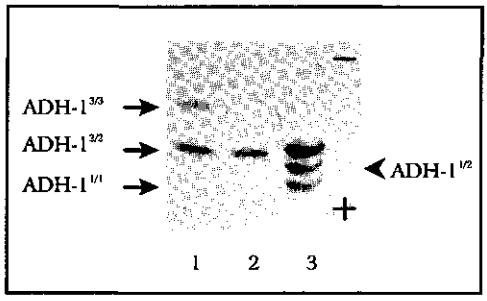
A heterodimeric band of ADH-1 as indication of FDR 2n-pollen

Isozyme analysis of the *Adh-1* marker gene in the diploid clones that were used for the analysis of n and 2n-pollen revealed different numbers of bands per clone (Fig. 2). The control HB93-7108-8 (*Adh-1*^{2}/*Adh-1*^{3}) showed two homodimeric bands encoded by the alleles *Adh-1*^{2} and *Adh-1*^{3}. Since the desynaptic clone RS93-8025-1 was homozygous (*Adh-1*^{2}/*Adh-1*^{2}), it showed only the homodimer encoded by the *Adh-1*^{2} allele. The characteristic feature of the normal synaptic clone B92-7015-4 (*Adh-1*^{1}/*Adh-1*^{2}) was that it showed not only the two homodimeric bands ADH-1$^{1/1}$ and ADH-1$^{2/2}$, but also an extra band of the heterodimeric protein ADH-1$^{1/2}$.

The presence or absence of the heterodimer was investigated in relation to pollen production in these clones. The control clone was not able to produce any 2n-pollen and showed only the homodimeric bands. Obviously, the pollen mixture comprised only the haploid genotypes *Adh-1*^{2} and *Adh-1*^{3}, so the two subunits of the isozyme were never present in the same cell to form an heterodimer. The clone B92-7015-4 produced 63% of

2n-pollen and was able to produce the heterodimeric protein ADH-$1^{1/2}$ (Fig. 2) in all heterozygous FDR 2n-pollen, as identified by the presence of both *Adh-1*^{1} and *Adh-1*^{2} alleles in the same cell. Since the heterodimeric band was strong, the pollen mixture had to include a lot of 2n-pollen with the *Adh-1*^{1}/*Adh-1*^{2} genotype. Assuming *Adh-1* to be a proximal marker gene, these genotypes confirmed the expected FDR origin of the 2n-pollen.

Figure 2. Zymogram of pollen samples of the three diploid clones. Lane 1 shows the homodimers ADH-$1^{2/2}$ and ADH-$1^{3/3}$ in a pollen sample of the control clone HB93-7108-8 (*Adh-1*^{2}/*Adh-1*^{3}). Lane 2 shows the homodimer ADH-$1^{2/2}$ in a pollen sample of the desynaptic clone RS93-8025-1 (*Adh-1*^{2}/*Adh-1*^{2}). Lane 3 shows the homodimeric bands ADH-$1^{1/1}$ and ADH-$1^{2/2}$, and the extra band of the heterodimer ADH-$1^{1/2}$ in a pollen sample of the normal synaptic clone B92-7015-4 (*Adh-1*^{1}/*Adh-1*^{2}), indicating the presence of 2n-pollen grains of genotype *Adh-1*^{1}/*Adh-1*^{2}.



Classification of 4x.2x progeny for *Adh-1* marker segregation

Pollen of 36 tetraploid offspring plants from the 4x.2x cross between HB93-7133-3 (*Adh-1*^{2}/*Adh-1*^{2}/*Adh-1*^{2}/*Adh-1*^{2}) and B92-7015-4 (*Adh-1*^{1}/*Adh-1*^{2}) were used for the classification of the genotype for the isozyme marker *Adh-1*. The nulliplex (*Adh-1*^{2}/*Adh-1*^{2}/*Adh-1*^{2}/*Adh-1*^{2}) genotypes were identified based on the absence of the homodimer ADH-$1^{1/1}$, while the simplex (*Adh-1*^{1}/*Adh-1*^{2}/*Adh-1*^{2}/*Adh-1*^{2}) genotypes were distinguished from the duplex (*Adh-1*^{1}/*Adh-1*^{1}/*Adh-1*^{2}/*Adh-1*^{2}) genotypes based on the intensity of each of the bands of the homodimers ADH-$1^{1/1}$ and ADH-$1^{2/2}$. The 4x.2x progeny segregated into three genotypic classes in the ratio of nulliplex: simplex: duplex= 5 : 29 : 2 respectively. The gene-centromere map distance of the *Adh-1* marker in clone B92-7015-4 was estimated as 19.4 cM [9.7 - 35.0 cM].

DISCUSSION

The utility of the *amf* marker for studying the extent of crossing-over

This investigation demonstrates the usefulness of the pollen marker *amf* for a study of basic genetics in potato. Besides the clear-cut identification of the phenotypes in large populations of n-microspores (Fig. 1), it is possible to estimate the extent of crossing-over between the *amf* locus and the centromere by the segregation patterns in 2n-microspores. For example, a clear difference between the segregation ratios of n- and 2n-microspore samples was observed in clone B92-7015-4 (Table 2). Its n-microspores segregated 1 blue : 1 red (monogenic), as in the control clone HB93-7108-8, whereas the 2n-microspores displayed a ratio fitting 3 blue : 1 red. Because the 2n-microspores of clone B92-7015-4 were produced after fused spindle formation, they have a FDR origin. Therefore, the occurrence of 24.4% red 2n-microspores in this clone should be interpreted as due to a high frequency of pollen mother cells with one crossover between the *amf* locus and the centromere, resulting in a gene-centromere map distance of 48.8 cM [42.5-55.7 cM]. Indeed, this marker has been localized on the most distal part of chromosome 8 in genetic linkage maps of potato (Gebhardt *et al.* 1991; Jacobs *et al.* 1995). However, the latter investigations revealed no information about the gene-centromere distance. The 3:1 ratio of blue (*Amf/.*) and red (*amf/amf*) 2n-microspores was also found by Jacobsen *et al.* (1991) in 2n-microspores of three normal synaptic diploid clones, which are closely related to clone B92-7015-4. For the parental clone 880004-2, which forms fused spindles, they observed 178 blue (*Amf/.*) and 56 red (*amf/amf*) microspores. From their data a gene-centromere map distance of 47.9 cM [37.8-59.6 cM] can be calculated. This is very close to the estimation of 48.8 cM in the present investigation.

In 2n-microspores, only two of the expected three genotypic classes can be detected, because *amf/amf* genotypes are red, and both the *Amf/Amf* and *Amf/amf* genotypes are blue. The only method of classifying all three genotypes of 2n-microspores is via identification of duplex, simplex and nulliplex genotypes in 4x progenies from nulliplex tetraploids × heterozygous diploids, as demonstrated in Table 3. The numbers of nulliplex and duplex genotypes fit the expected 1:1 ratio, but a slight shortage of duplex

genotypes is observed. Owing to this shortage, the *amf*-centromere distance of 39.5 cM as calculated from the 4x.2x progenies data of clone B92-7015-4 (Table 3), is lower than the distance of 48.8 cM calculated from the 2n-microspores (Table 2). The shortage of duplex genotypes may be due to misclassification of simplex and duplex genotypes in the 4x.2x progeny, rather than to any other putative cause. The rationale of this assumption is the fit of 359 (duplex + simplex) : 97 (nulliplex) to 3 blue : 1 red (χ^2=3.38), as observed also in 2n-microspores (Table 2). Pooling of the simplex and duplex genotypes resulted in a gene-centromere distance of 42.5 cM [35.5-50.7 cM]. In view of the overlapping confidence intervals, the estimates of *amf*-centromere map distances derived from 2n-microspore segregations and those from the segregations in 4x.2x-progenies are not significantly different.

Indications for one crossover per arm

Assuming complete chiasma interference in FDR 2n-gametes, a distal marker (*A/a*) is expected to segregate *A.*: *aa* = 3:1 or *AA : Aa : aa*= 1:2:1 and, therefore, its gene-centromere distance is estimated as 2.(f(*aa*)= 1/4).100 cM = 50 cM (Mendiburu and Peloquin 1979). Assuming random multiple exchanges per chromosome arm, the segregation of a distal marker is expected to be independent of the centromere, and should be *A.*: *aa* = 5:1 or *AA : Aa : aa*= 1:4:1 (Mather 1935). In this model, the frequency of *aa* gametes will not exceed 1/6 for distal genes. In the model of Mendiburu and Peloquin (1979), a frequency of *aa* gametes of 1/6 corresponds to a gene-centromere distance of 2.(f(*aa*)= 1/6).100 cM = 33.3 cM. Therefore, estimates of gene-centromere distances exceeding 33.3 cM are in favour of the complete chiasma interference model of Mendiburu and Peloquin (1979). However, marker genes with a gene-centromere distance of more than 33.3 cM are rarely found in HTA experiments in potato (Douches and Quiros 1987; Jongedijk 1991a; Wagenvoort and Zimnoch-Guzowska 1992). Consequently, Jongedijk (1991a) supported the genetic model for random multiple exchanges per chromosome arm (Mather 1935). Since our estimates of the gene-centromere distance of the *amf* marker in FDR 2n-pollen and 4x.2x progenies clearly exceeded 33.3 cM, our data indicate that multiple crossing-overs do not occur between

the centromere and the *amf* locus. This corroborates the model of Mendiburu and Peloquin (1979). The occurrence of one crossover per arm has been further confirmed through RFLP-analysis of 2x.4x-progenies in potato (Chapter 6). This genetic evidence for a high degree of chiasma interference agrees with cytological observations of 13.3-14.0 chiasmata per cell (or 1.2 per bivalent) in diploid potato (Jongedijk and Ramanna 1989) and 17.1 ± 2 chiasmata per cell in tomato (Sherman and Stack 1995). In line with this situation, gene-centromere mapping in different fish species (Allendorf *et al.* 1986; Liu *et al.* 1992; Seeb and Seeb 1986; Thorgaard *et al.* 1983) revealed evidence for complete chiasma interference.

Reduction of crossing-over due to desynapsis

A genotype combining pollen fertility, FDR 2n-microspore formation, the *amf* marker gene in heterozygous condition *(Amf/amf)*, and desynapsis *(ds-1/ds-1)*, could be used for demonstrating the effect of desynapsis on the extent of crossing-over through HTA. In the population RS93-8025 with 140 plants only one such genotype was selected. The fertile pollen (12%) of this selected desynaptic clone RS93-8025-1 comprised only FDR 2n-pollen (Table 1). This FDR origin of 2n-microspores enabled a comparison of the extent of crossing-over between the centromere and the *amf* locus in the desynaptic clone RS93-8025-1 with that in the normal synaptic clone B92-7015-4. Whereas the gene-centromere distance was found to be 48.8 cM in the normal synaptic clone, it was only 13.3 cM in the desynaptic clone. This was confirmed both in 2n-microspores and in 4x.2x progenies. This reduction of crossing-over (73%) nearly equals the observed reduction (78%) in bivalent formation from 12.0 bivalents per cell in the normal synaptic clone to 2.7 bivalents per cell in the desynaptic clone (Table 1). Severe reduction of recombination of more than 70% in desynaptic potato clones was also found in other HTA studies, in which different markers were used (Douches and Quiros 1988a; Jongedijk *et al.* 1991a).

Segregation of *Adh-1* in 2n-pollen and in 4x.2x progenies

Although the isozyme ADH-1 can be used as a pollen marker, the phenotypes of

2n-pollen grains, unlike those for the *amf* marker, cannot be distinguished individually in the present material. However, this isozyme is helpful for an assessment of the occurrence of FDR 2n-pollen because of the presence of heterodimeric bands. Such heterodimeric bands occur only in cells where two different alleles are expressed as in the case of heterozygous 2n-pollen grains. Owing to its relatively proximal position, the large amount of heterozygous 2n-pollen found in clone B92-7015-4 (Fig.1) and the 80.6 % of simplex (*Adh-1*^{1}/*Adh-1*^{2}/*Adh-1*^{2}/*Adh-1*^{2}) 4x.2x-progeny plants confirmed the FDR origin of this 2n-pollen. The corresponding map distance between the centromere and the *Adh-1* locus was estimated to be 19.4 cM [9.7 - 35.0 cM] and is comparable with the distance of 15.8 cM that was found by Douches and Quiros (1987) for the ADH isozyme which is expressed in the pollen of potato.

Gene-centromere mapping through pollen markers

If a null allele at the *Adh-1* locus becomes available, it will be possible, as was demonstrated in tomato (Wisman *et al.* 1991), to distinguish individual pollen phenotypes just as in the case of the *amf* marker. Also transformation of diploid potato clones producing FDR 2n-pollen with reporter genes that are expressed in pollen, like the cloned wild-type *Amf* allele (Flipse *et al.* 1996) or the β-glucuronidase (GUS) gene, can be used to increase the number of genetic loci for the identification of individual pollen phenotypes. The possibility to use transgenics with pollen specific marker loci at all 12 chromosomes would be of great importance for efficient gene-centromere mapping and defining the extent of crossing-over in potato. Although tomato has been used for extensive genetic mapping, HTA has not been exploited in this model plant species because 2n-gametes occur very rarely, if ever (Ramanna, unpublished). Information about the positions of centromeres in relation to molecular marker loci in tomato is only available for chromosomes 6, 7 and 9, obtained from mapping in deletion and trisomic lines (Van Wordragen *et al.* 1994, Frary *et al.* 1996). In view of the synteny of the genetic maps of potato and tomato (Bonierbale *et al.* 1988; Tanksley *et al.* 1992), the exploitation of 2n-gametes for gene-centromere mapping in potato can be highly relevant to tomato as well.

Selection of diploid tuberous *Solanum* hybrids for 2n-egg formation using 2x.4x-crosses

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SELECTION OF DIPLOID TUBEROUS *SOLANUM* HYBRIDS FOR 2N-EGG FORMATION USING 2X.4X-CROSSES

ABSTRACT

Diploid families of Tuberosum hybrids as well as Tuberosum-wild species F_1 hybrids were generated to select 2n-egg producing genotypes of different genetic backgrounds. Plants were selected if they produced more than four seeds per berry after pollination with tetraploid males (2x.4x-crosses). From the nine families of Tuberosum hybrids that were derived from one or two 2n-egg producing parents, 12 out of 82 (15%) 2n-egg producing plants were selected. From the 32 families of Tuberosum-wild species F_1 hybrids, 21 of the 274 (8%) 2n-egg producing plants were selected. The level of 2n-egg formation was estimated in 25 selected diploid hybrids and five control clones (three high, one moderate and one low 2n-egg producers) on the basis of seed set following 2x.4x-crosses using 13 tetraploid males in four crossing years. Besides the effect of the diploid 2n-egg producing female on the seed set following 2x.4x-crosses (P=0.0001), there was a significant effect of the tetraploid male (P=0.0001), whereas the effect of the crossing year (P=0.0688) was less significant. On the basis of differences in the seed set following 2x.4x-crosses as compared to the control clones for low, moderate and high level of 2n-egg formation, the level of 2n-egg formation in two of the newly selected hybrids was classified as very high, four were high, sixteen were moderate and three were low. The significance of the selected hybrids for the genetic analysis of various restitution mechanisms of 2n-egg formation is discussed.

INTRODUCTION

The breeding of the cultivated potato, *Solanum tuberosum,* is difficult because of the tetrasomic inheritance of characters (2n=4x=48). Another complication in the breeding of potato is the difference in ploidy level with some related species that hampers the introgression of desirable characters from these wild *Solanum* species into the cultivated potato. A relatively simple alternative is to breed potato at the diploid level (2n=2x=24), where the pattern of inheritance is expected to be more straight forward, the process of selection will be more efficient, and the introgression of characters from diploid wild *Solanum* spp. will be possible. To this end, Chase (1963) proposed the so-called 'analytic breeding', in which the breeding and selection are carried out at the diploid level and, finally, the tetraploid condition is restored by using numerically unreduced gametes (2n-gametes) having the sporophytic number of chromosomes. For this sexual polyploidization, both the 2n-eggs and the 2n-pollen can be used in either unilateral (2x.4x- or 4x.2x-) or bilateral (2x.2x-) crosses (Mendiburu and Peloquin 1977b). Several studies have shown that both 2n-pollen and 2n-eggs occur widely in diploid breeding lines of potato, wild tuberous *Solanum* species and interspecific hybrids (Den Nijs and Peloquin 1977; Iwanaga and Peloquin 1982; Veilleux 1985; Stelly and Peloquin 1986b; Werner and Peloquin 1987, 1991; Watanabe and Peloquin 1989, 1993; Ortiz *et al.* 1991b; Barone *et al.* 1993). Despite the fact that the frequencies of 2n-gametes vary widely, it is possible to select diploid genotypes that produce high frequencies of 2n-gametes that can be used in practical breeding (Ramanna 1983; Jongedijk *et al.* 1991b; Ortiz and Peloquin 1992; Hutten *et al.* 1994a; Qu *et al.* 1996).

In practice, the screening of diploid clones for 2n-pollen is easier than for 2n-egg formation. Pollen grains can be directly mounted in a drop of staining solution (lactophenol-acid fuchsin), and monitored for 2n-pollen based on size differences as well as the number of germ pores (Jacobsen 1976; Ramanna 1979). In contrast, the ploidy status of the eggs can only be studied either after sectioning or clearing of the ovules. In these preparations, the 2n-eggs can be identified on the basis of the nucleolar size of female gametophyte nuclei (Stelly and Peloquin 1986b; Jongedijk *et al.* 1991b; Conicella *et al.* 1991). Alternatively, the diploid clones that produce 2n-eggs can be selected on the basis of the seed set following pollination

with a tetraploid parent (Hanneman and Peloquin 1968; Werner and Peloquin 1987). In this type of cross, the seeds comprise almost exclusively tetraploid embryos derived from fertilized 2n-eggs, since the triploid embryos derived from fertilized n-eggs are aborted because of the so-called 'triploid block' in *Solanum* (Marks 1966). Therefore, the seed set following 2x.4x-crosses has been frequently used to estimate the level of 2n-egg formation (Den Nijs and Peloquin 1977; Iwanaga and Peloquin 1982; Veilleux 1985; Stelly and Peloquin 1986b; Werner and Peloquin 1987, 1991; Jongedijk *et al.* 1991b; Conicella *et al.* 1991; Barone *et al.* 1993).

Although the selection of 2n-egg producing genotypes is more complicated, they can be useful in breeding for different purposes. For example, 2n-egg producing genotypes can be used in bilateral sexual polyploidization. Furthermore, the genetic compositions of 2n-eggs and 2n-pollen can be distinct as a result of different modes of origin. Whereas 2n-pollen originate predominantly through the first division restitution, or FDR, and are useful for transferring a high degree of heterozygosity to the progeny (Mok and Peloquin 1975a; Jacobsen 1976; Ramanna 1979), 2n-eggs originate mostly through the second division restitution, or SDR, and facilitate the transfer of a high degree of homozygosity to the progeny (Jongedijk 1985; Stelly and Peloquin 1986a, b; Werner and Peloquin 1987, 1991; Conicella *et al.* 1991). Because of these differences in the genetic composition, it is possible that, in some cases, the 2n-eggs will be more attractive to transfer characters to the tetraploid progeny than the 2n-pollen. To elucidate the restitution mechanism(s), the genetic composition of 2n-eggs can be studied in segregating tetraploid progenies, raised from crosses between a diploid 2n-egg producing clone and a tetraploid male in the so-called 'Half-Tetrad Analysis (HTA)'(Mendiburu and Peloquin 1979; Stelly and Peloquin 1986a; Douches and Quiros 1988b; Jongedijk *et al.* 1991a; Werner *et al.* 1992; Barone *et al.* 1995).

The aim of this study was the selection of diploid clones of different genetic backgrounds for the formation of 2n-eggs to enable analysis of various restitution mechanisms. To this end, diploid families of both Tuberosum hybrids and Tuberosum-wild species F_1 hybrids were generated and used in 2x.4x-crosses to select the 2n-egg producing genotypes. The selected genotypes were crossed with different pollinators in different years

to estimate the level of 2n-egg formation. To enable cytological investigations in the future, special effort was made to select diploids with a high frequency of 2n-egg formation. To this end, the Tuberosum hybrids comprising diploid families that were derived from two 2n-egg producing parents were used. Since the *amylose free (amf)* marker gene was successfully used in HTA of 2n-pollen (Chapter 2), special effort was made to use both diploid and tetraploid *amf* mutants in the crossing programme to generate suitable plant materials for genetic analysis of 2n-eggs. This recessive marker gene is coding for a modified starch composition and can be analysed in microspores (Jacobsen *et al.* 1989). The selected 2n-egg producing clones that were heterozygous *(Amf/amf)* were crossed with nulliplex tetraploids *(amf/amf/amf/amf)* to generate tetraploid progenies allowing HTA of the 2n-eggs of the diploid parent.

MATERIALS AND METHODS

Plant material

Diploid parents

Fifteen diploid (2n=2x=24) parents of predominantly *S. tuberosum* origin, were crossed for generating diploid families, that were called 'Tuberosum hybrids'. The Tuberosum parents were also crossed with diploid genotypes of wild *Solanum* species to generate families, that were called 'Tuberosum-wild species F_1 hybrids'. Both types of families were used for the selection of clones that produced 2n-eggs. The genotypes, pedigrees and levels of 2n-egg formation of each of these Tuberosum parents are described in Table 1. The three diploids BE1050, CD1015, and CD1045 were used as parents, because of their high level of 2n-egg cell formation (> 15 seeds per berry). Also the parent ED1045 was known to produce 2n-eggs, since the ensuing progeny of the cross ED1045 × CD1045 comprised both tetraploid and diploid genotypes (unpublished data). However, its frequency of 2n-eggs was unknown, since the clone could not be tested in 2x.4x-crosses because of virus infection. The two parents, B92-7014-7, and RH89-023-18 had moderate levels of 2n-egg formation (5-15 seeds per berry), whereas the four clones B92-7015-3, B92-7015-4, RH87-175-1, and RH89-024-3

were used because of their low level of 2n-egg cell formation (< 5 seeds per berry). The three diploids 87-1029-31, 87-1030-9, and 87-1031-29 were homozygous for the amylose free (*amf*) starch marker, defective for the granule-bound starch synthase (*GBSS*) gene on chromosome 8 (Gebhardt *et al.* 1991; Jacobs *et al.* 1995). These *amf/amf* parents were crossed with wildtype diploids *(Amf/Amf)* to generate 2n-egg producing diploids, that were heterozygous for this marker gene. The two diploids SUH2293, and SUH3711 (originally selected by van Suchtelen, Wageningen) were used as parents because of the good fertility and tuberization, at the diploid level.

Table 1. The diploid Tuberosum parents; their pedigrees, levels of 2n-egg formation and genotypes for the *amf* marker gene

Code	Pedigree (♀ × ♂)[1]	No. of seeds per berry in 2x.4x-crosses[1]	Level of 2n-eggs	Genotype *amf* marker	Reference
87-1029-31	87-1017-5 × 87-1024-1	n.d.	-	*amf/amf*	Jacobsen et al., 1989
87-1030-9	87-1016-1 × 87-1017-5	n.d.	-	*amf/amf*	Jacobsen et al., 1989
87-1031-29	87-1017-5 × 87-1024-1	n.d.	-	*amf/amf*	Jacobsen et al., 1989
B92-7014-7	(*mga* × SH82-59-22) * IVP101[3]	5-15	moderate	*Amf/Amf*	unpublished
B92-7015-3	880004-2 * IVP101 [3]	0-1	low	*Amf/amf*	unpublished
B92-7015-4	880004-2 × IVP101	1-4	low	*Amf/amf*	Chapter 2; unpublished
BE1050	USW5295.7 × 77-2102-37	19-35	high[5]	*Amf/Amf*	Hutten et al., 1994a
CD1015	USW5337.3 × USW7589.2	15-22	high[5]	*Amf/Amf*	Hutten et al., 1994a
CD1045	USW5337.3 × USW7589.2	20-34	high[5]	*Amf/Amf*	Hutten et al., 1994a
ED1045	77-2102-37 × USW7589.2	n.d.[4]	high	*Amf/Amf*	unpublished
RH87-175-1	*Mcd*18302-36 × SH82-59-223	1-2	low	*Amf/Amf*	unpublished
RH89-023-18	*Vrn*24732-7 × SH82-70-297	5-6	moderate[5]	*Amf/Amf*	unpublished
RH89-024-3	CHI-EP46 × *Vrn*24732-10	1-3	low[5]	*Amf/Amf*	unpublished
SUH2293	SH72-200-34 × SH74-97-2046	n.d.	-	*Amf/Amf*	Suchtelen, Wageningen
SUH3711	H491 × SH-mixture	n.d.	-	*Amf/Amf*	Suchtelen, Wageningen

[1] codes for diploid clones of wild species indicate plant number following abbreviation for species name and BGRC-accesion number; *mga= S. megistacrolobum, mcd= S. microdontum, vrn= S. vernei*

[2] n.d.= not determined

[3] derived from spotless seed following prickle pollination with IVP101.

[4] known to produce 2n-eggs; its frequency cannot be determined because of virus infection

[5] used as control clones in 2x.4x-crossing programme

The diploid wild *Solanum* species (including the BGRC accession numbers), that were used for generating Tuberosum-wild species F_1 hybrids were *S. ajanhuiri* (the primitive cultivar Yari), *S. chacoense* (16998, 18618), *S. megistacrolobum* (27262), *S. microdontum* (18302, 18568), *S. phureja*, *S. sparsipilum* (8206), *S. spegazzinii* (15458), *S. tarijense* (24717), and *S. vernei* (15451, 24732).

Tetraploid parents

The pedigrees of the 13 tetraploid genotypes that were used as males in the 2x.4x-crosses are described in Table 2. The four tetraploids J90-6011-3, J90-6020-17, J90-6020-22, and J90-6021-5 were nulliplex for the marker gene *amf*. These nulliplex tetraploid males *(amf/amf/amf/amf)* were crossed with heterozygous diploids *(Amf/amf)* to generate the tetraploid progenies allowing indirect genotyping of the 2n-eggs of the diploid parent for this marker gene.

Table 2. Pedigrees of the tetraploid clones, used as pollinator in the 2x.4x-crosses

Code	Pedigree (♀ × ♂)	Genotype *amf* marker	Reference
J90-6011-3	87-1030-9-lc3 (4x) × 880004-3	*amf/amf/amf/amf*	Jacobsen *et al.*, 1991
J90-6020-17	87-1031-29-lc2 (4x) × 880004-3	*amf/amf/amf/amf*	Jacobsen *et al.*, 1991
J90-6020-22	87-1031-29-lc2 (4x) × 880004-3	*amf/amf/amf/amf*	Jacobsen *et al.*, 1991
J90-6021-5	87-1031-29-lc3 (4x) × 880004-2	*amf/amf/amf/amf*	Jacobsen *et al.*, 1991
HB93-7133-6	(BE1050 × J90-6021-5)-7 ⊗	*amf/amf/amf/amf*	
105ESC90-32	VE71-105 × Escort	*Amf/Amf/Amf/Amf*	
Adora	Primura × Alcmaria	*Amf/Amf/Amf/Amf*	
Atrela	Mara × VTN62-33-3	*Amf/Amf/Amf/Amf*	
Desirée	Urgenta × Depesche	*Amf/Amf/Amf/Amf*	
Frieslander	Gloria × 74A3	*Amf/Amf/Amf/Amf*	
Gineke	Ultimus × Record	*Amf/Amf/Amf/Amf*	
Hertha	DHS61-133-3 × KONST62-374	*Amf/Amf/Amf/Amf*	
Katahdin	USDA24642 × USDA40568	*Amf/Amf/Amf/Amf*	
Kondor	KONST61-333 × Wilja	*Amf/Amf/Amf/Amf*	

Control clones for 2n-egg formation

Six diploid clones with known levels of 2n-egg formation were included as control clones for the seed set following the 2x.4x-crosses in the crossing programme of the selected diploid hybrids with the tetraploid males. The three parental clones BE1050, CD1015, and CD1045 (Hutten *et al.* 1994a), were used as control clones for a high level of 2n-egg formation. The clone RH89-023-18 was the control for a moderate level of 2n-egg formation, and the clone RH89-024-3 was the control for a low level of 2n-eggs (Table 1).

Crosses

Plants were grown in the greenhouse either on bricks or as grafts on tomato root-stocks in order to induce flowering. The tetraploid pollinators were planted in an early, moderate and late series to guarantee the availability of pollen during the whole crossing season. The female parents were emasculated 2-3 days before the opening of the flowers and pollinated when the stigmas were receptive. Berries were harvested about 6 weeks after pollination. After softening, they were cleaned individually for the extraction and counting of the seeds per berry.

Selection of 2n-egg producing hybrids

For the selection of 2n-egg producing genotypes of different genetic backgrounds, 41 families of diploid hybrids were used in 2x.4x-crosses. Nine of these families comprised Tuberosum hybrids, and the other 32 families comprised Tuberosum-wild species F_1 hybrids. Already in the first year after sowing, the hybrids were used for both maintenance and 2x.4x-crosses. For the production of tubers, the seedlings were grown in the greenhouse, of which the Tuberosum-wild species F_1 hybrids were grown under short day conditions. For the 2x.4x-crosses, at least ten seedlings per family were grafted on to tomato root-stocks and transferred to the crossing greenhouse to induce flowering. The flowers of each plant were pollinated with at least three tetraploid males, using only one pollinator per inflorescence, and selected for 2n-egg cell formation if it produced more than 4 seeds per berry.

Data analysis

To estimate the degree of 2n-egg cell formation in the diploid clones, the seed set following 2x.4x-crosses was tested for the diploid females (five controls and the selected hybrids) and the 13 tetraploid males during a period of four years. Since not all possible crossing combinations were made, the data set was incomplete. The effects of the female, male and crossing year were investigated using the General Linear Models Procedure in SAS 6.11 (SAS 1989). To this end, the log-transformed average number of seeds per berry of all 2x×4x×year crossing combinations (seed set) were analysed, using the number of berries as weights.

The model was $\ln(\text{seed set}_{ijk} + 1) = \mu + \alpha_i + \beta_j + \gamma_k + e_{ijk}$, with μ the general level, α_i (i=1, ..., 30) the effects of the females, β_j (j=1, .., 13) the effects of the males, γ_k (k=1,..,4) the effects of the years, and e_{ijk} normally distributed random variables with zero expectation and common variance σ^2. The least squares means of the ln(seed set +1) following 2x.4x-crosses were calculated for the individual diploid females to obtain unbiased estimates of the degree of 2n-egg formation. The least squares means of the ln(seed set +1) per male were calculated to estimate the influence of the tetraploid males in 2x.4x-crosses. The least squares means of the ln(seed set +1) in the four crossing years were calculated to estimate the effect of different growing seasons. Finally, the least square means of the ln(seed set +1) and their 95% confidence limits were back transformed to estimate the average number of seeds per berry. The seed sets of the newly selected females were tested for differences as compared to the low, the moderate, and the average of the three high 2n-egg producing controls to classify the level of 2n-egg formation in these clones.

RESULTS

Selection of 2n-egg producing hybrids

For the selection of diploid 2n-egg producers of different genetic backgrounds, both Tuberosum hybrids and Tuberosum-wild species F_1 hybrids were used in 2x.4x-crosses. From the nine families of diploid Tuberosum hybrids with 2n-eggs producing parents, 12 out of

82 (15%) plants produced more than four seeds per berry (Table 3). The frequency of selected plants varied from 0 to 25% per family. The frequency of selected plants in the three families, B92-7017, HB94-7201, and HB94-7202, that were derived from crosses between two parents with a high level of 2n-egg formation (high × high) was not superior as compared to the families that were derived from high × moderate, high × low, or even low × low crosses (Table 3). To investigate whether the level of 2n-egg formation of the parental clones had an effect on the level of 2n-egg formation of the hybrids, the 2x.4x-crossability of the selected clones was further tested in crosses with different males in different crossing years.

Table 3. Selection of Tuberosum hybrids that were derived from 2n-egg producing parents and produced more than 4 seeds per berry following 2x.4x-crosses

Family	Pedigree (♀ × ♂)	Type of cross[1]	Number of clones		
			tested	selected (%)	tuberized[2]
B92-7017	ED1045 × CD1045	high × high	23	4 (17)	3
HB94-7201	BE1050 × CD1015	high × high	4	1 (25)	0
HB94-7202	CD1045 × CD1015	high × high	4	0 (0)	0
HB94-7274	BE1050 × RH89-023-18	high × moderate	6	0 (0)	0
HB93-7004	87-1029-31 × CD1015	low × high	9	2 (22)	2
HB93-7076	CD1015 × RH87-175-1	high × low	10	1 (10)	1
HB94-7220	CD1045 × RH89-024-3	high × low	6	1 (17)	0
HB93-7060	B92-7015-4 × B92-7014-7	low × moderate	10	1 (10)	1
HB93-7012	RH87-175-1 × B92-7015-3	low × low	10	2 (20)	1
		Total	82	12 (15)	8

[1] The levels of 2n-egg formation of the parents were classified on the basis of seed set following 2x.4x-crosses (Table 1)

[2] Some selected clones failed to produce tubers in the maintenance, and could not be used in further 2x.4x-crosses

From the 32 families of Tuberosum-wild species F_1 hybrids, 21 of the 274 (8%) plants produced more than four seeds per berry (Table 4). The frequency of selected plants varied from 0 to 40% per family. As compared to the Tuberosum-wild species F_1 hybrids involving

the species *S. megistacrolobum, S. microdontum, S. sparsipilum, S. spegazzinii, S. tarijense,* and S. *vernei*, the frequencies of selected Tuberosum-*S. chacoense* F_1 hybrids (23%) and Tuberosum-*S. ajanhuiri* (cv. Yari) F_1 hybrids (25%) were relatively high. The family HB93-7108 that was derived from the clone B16 of *S. microdontum* (BGRC 18568) and the genotype 87-1031-29 comprised six (35%) plants, that produced more than four seeds per berry following 2x.4x-crosses (Table 4). This frequency was remarkably high, since not a single plant could be selected in two other families of Tuberosum- *S. microdontum* F_1 hybrids, involving another parental plant (B5) of the same *S. microdontum* accession (Table 4). The effect of the low, moderate or high level of 2n-egg formation of the Tuberosum parent on the frequency of selected Tuberosum-wild species F_1 hybrids was analysed by pooling the families that were derived from the *Solanum* spp. × high/moderate, and *Solanum* spp. × low crosses. The *Solanum* spp. × high/moderate families comprised 69 Tuberosum-wild species F_1 hybrids, of which 13 (19%) produced sufficient seeds following 2x.4x-crosses, whereas there were only eight of the 205 (4%) Tuberosum-wild species F_1 hybrids of the *Solanum* spp. × low families, that were successful in the 2x.4x-crosses.

Four of the 12 selected Tuberosum hybrids and four of the 21 selected Tuberosum-wild species F_1 hybrids did not tuberize, and therefore, could not be maintained. The remaining 25 selected diploid hybrids and six control clones were used in further 2x.4x-crosses to estimate the level of 2n-egg formation more precisely.

Table 4. Selection of Tuberosum-wild species F_1 hybrids, that produced more than four seeds per berry following 2x.4x-crosses

Family	Pedigree[1] (♀ × ♂)	Type of cross[2]	Number of clones		
			tested	selected (%)	tuberized[3]
HB94-7403	Yari × 87-1030-9	*ajh* × low	3	0	0
HB93-7130	Yari × B92-7014-7	*ajh* × moderate	5	2 (40)	2
HB94-7211	*Chc*16998-B07 × CD1015	*chc* × high	6	2 (33)	2
HB94-7239	*Chc*16998-B05 × 87-1029-31	*chc* × low	7	1 (14)	1
HB94-7299	CD1045 × *Chc*16998-B02	*chc* × high	8	2 (25)	2
HB94-7252	*Chc*18618-B08 × CD1015	*chc* × high	8	3 (38)	3
HB94-7276	CD1045 × *Chc*18618-B11	*chc* × high	6	2 (33)	2
HB94-7335	*Chc*18618-B06 × 87-1031-29	*chc* × low	8	0	0
HB94-7316	BE1050 × *Mga*27262-B06	*mga* × high	2	0	0
HB94-7292	87-1029-31 × *Mga*27262-B06	*mga* × low	4	0	0
HB93-7008	87-1031-29 × *Mcd*18302-B15	*mcd* × low	7	0	0
HB93-7085	CD1045 × *Mcd*18302-B15	*mcd* × high	10	0	0
HB93-7096	*Mcd*18302-B1 × 87-1029-31	*mcd* × low	11	0	0
HB93-7097	*Mcd*18302-B1 × B92-7015-3	*mcd* × low	9	0	0
HB93-7098	*Mcd*18302-B15 × CD1045	*mcd* × high	9	0	0
HB93-7099	*Mcd*18302-B15 × SUH3711	*mcd* × low	13	0	0
HB93-7100	*Mcd*18302-B17 × 87-1029-31	*mcd* × low	25	0	0
HB93-7102	*Mcd*18302-B4 × 87-1029-31	*mcd* × low	22	0	0
HB93-7103	*Mcd*18302-B4 × CD1045	*mcd* × high	4	1 (25)	0
HB93-7101	*Mcd*18568-B5 × 87-1029-31	*mcd* × low	26	0	0
HB93-7108	*Mcd*18568-B16 × 87-1031-29	*mcd* × low	17	6 (35)	4
HB93-7110	*Mcd*18568-B5 × CD1045	*mcd* × high	2	0	0
HB93-7006	87-1029-31 × *Spl*08206-B18	*spl* × low	9	0	0
HB93-7009	87-1031-29 × *Spg*15458-B9	*spg* × low	6	0	0
HB93-7054	B92-7015-3 × *Spg*15458-B5	*spg* × low	10	1 (10)	1
HB94-7336	BE1050 × *Tar*24717-B04	*tar* × high	2	0	0
HB94-7392	*Tar*24717-B02 × 87-1030-9	*tar* × low	10	0	0
HB94-7250	*Vrn*15451-B04 × CD1015	*vrn* × high	6	1 (17)	0
HB94-7307	*Vrn*15451-B09 × 87-1029-31	*vrn* × low	5	0	0
HB94-7308	*Vrn*24732-B05 × 87-1029-31	*vrn* × low	7	0	0
HB94-7309	*Vrn*24732-B02 × SUH2293	*vrn* × low	6	0	0
HB94-7326	*Vrn*24732-B04 × CD1015	*vrn* × high	1	0	0
	Total		274	21 (8)	17

[1] Codes for diploid clones of wild species indicate plant number (B..) following abbreviation for species name and BGRC-accesion number; *ajh*= *S. ajanhuiri*, *chc*= *S. chacoense*, *mga*= *S. megistacrolobum*, *mcd*= *S. microdontum*, *spl*= *S. sparsipilum*, *spg*= *S. spegazzinii*, *tar*= *S. tarijense*, and *vrn*= *S. vernei*.

[2] The levels of 2n-egg formation of the parents were classified on the basis of seed set following 2x.4x-crosses (Table 1)

[3] Some selected clones failed to produce tubers in the maintenance, and could not be used in further 2x.4x-crosses

Estimation of the level of 2n-egg formation in the selected diploid hybrids based on the seed set following 2x.4x-crosses

In total, 182 female×male×year crossing combinations were made. These crossings resulted in 1561 berries and 18667 seeds. The average number of seeds per berry per crossing combination (seed set) ranged from 0 to 104, with an overall mean of 12.0 seeds per berry. Transformation of the 182 seed set data into ln(seed set + 1) enabled a standard analysis of the transformed data. The effects of the female and male were highly significant (P=0.0001), whereas the effect of the crossing year was less significant (P=0.0688). Because the interaction effects were not significant, they were not included in the model. Figure 1 shows the least squares means and their standard errors of the ln(seed set + 1) following 2x.4x-crosses for the 30 diploid females, the 13 tetraploid males and the four crossing years. The overall mean of the ln(seed set + 1) data was 2.230, corresponding to 8.3 seeds per berry. This overall mean was lower than the overall mean of the untransformed data (12 seeds per berry) as a result of the log-transformation needed for the analysis of variance, in which the low values for the seed set had larger effects on the mean than in the mean of the untransformed data.

More details and the back-transformed results of the seed set per crossing year, tetraploid male and diploid female are presented in Table 5.

Table 5. Seed set following 2x.4x-crosses per year, tetraploid pollinator, and diploid female parent

	No. of 2x×4x×year combinations	No. of berries	Average number of seeds per berry per combination			
			Min, Max	Mean and 95% confidence interval[1]		Mean and 95% confidence interval for subset of pollinators[1,2]
A. Years						
1992	11	50	2, 18	8.1	[4.4 ; 14.4]	
1993	44	231	0, 51	4.8	[3.6 ; 6.4]	
1994	71	579	0, 92	5.8	[4.7 ; 7.2]	
1995	56	701	2, 104	7.1	[5.6 ; 9.0]	
Total:	182	1561	0, 104			
B. Tetraploid pollinators						
amf/amf/amf/amf genotypes						
J90-6011-3	15	85	0, 18	4.6	[3.1 ; 6.7]	
J90-6020-17	5	19	2, 9	2.9	[1.0 ; 6.4]	
J90-6020-22	22	142	0, 16	5.0	[3.5 ; 6.9]	
J90-6021-5	4	19	2, 8	4.0	[1.6 ; 8.5]	
Amf/Amf/Amf/Amf genotypes						
Adora	21	220	1, 51	11.0	[8.3 ; 14.4]	
Atrela	3	9	0, 5	2.0	[0.2 ; 6.6]	
Desirée	29	230	0, 47	9.0	[6.9 ; 11.7]	
105ESC90-32	27	269	3, 55	10.5	[8.0 ; 13.5]	
Frieslander	18	232	5, 92	12.1	[9.1 ; 15.9]	
Gineke	2	10	2, 4	4.3	[1.1 ; 12.5]	
Hertha	19	183	1, 104	6.8	[5.1 ; 9.2]	
Katahdin	6	96	5, 27	12.5	[8.2 ; 18.9]	
Kondor	11	70	0, 39	8.5	[5.4 ; 13.1]	
Total:	182	1561	0, 104			

Table 5. continued

	No. of 2x×4x×year combinations	No. of berries	Average number of seeds per berry per combination				
			Min, Max	Mean and 95% confidence interval[1]		Mean and 95% confidence interval for subset of pollinators[1,2]	
C. Diploid females							
Control clones							
BE1050 (high)	10	45	8, 47	14.1	[8.8 ; 22.2]	16.3	[7.0; 36.1]
CD1015 (high)	7	41	4, 55	9.5	[5.7 ; 15.5]	15.6	[7.0; 33.3]
CD1045 (high)	7	54	7, 45	12.6	[8.1 ; 19.2]	20.4	[11.3; 36.3]
RH89-023-18	5	21	3, 25	6.4	[3.0 ; 12.5]	7.8	[0.95; 38.9]
RH89-024-3	3	16	2, 7	3.1	[1.0 ; 7.3]	6.8	[1.6; 22.8]
Tuberosum							
B92-7017-44	4	5	2, 21	5.8	[1.0 ; 22.0]	7.1	[-0.23; 84.9]
B92-7017-46	3	23	2, 19	5.7	[2.7 ; 11.2]	19.1	[6.0; 56.5]
B92-7017-63	5	36	14, 104	32.7	[19.8 ; 53.6]	47.4	[21.7; 103]
HB93-7004-6[3]	7	43	4, 16	5.2	[2.9 ; 8.8]	6.4	[1.3; 22.8]
HB93-7004-9[3]	5	17	0, 5	3.1	[1.0 ; 7.6]	7.3	[0.50; 45.1]
HB93-7012-35[3]	4	24	0, 6	2.1	[0.7 ; 4.5]	-	-
HB93-7060-4	9	41	3, 21	6.0	[3.4 ; 10.0]	8.7	[3.5; 20.0]
HB93-7076-18	3	20	0, 9	4.7	[1.9 ; 10.2]	-	-
Tuberosum-wild species F_1 hybrids							
HB93-7054-3[3]	9	55	0, 15	5.4	[3.2 ; 8.7]	5.2	[0.37; 27.5]
HB93-7108-7[3]	3	40	2, 11	6.0	[3.3 ; 10.4]	11.7	[5.6; 23.3]
HB93-7108-8[3]	14	186	4, 33	11.1	[8.3 ; 14.6]	16.8	[11.3; 24.8]
HB93-7108-10[3]	5	15	2, 5	4.5	[1.6 ; 10.6]	5.8	[0.64; 27.1]
HB93-7108-15[3]	5	14	2, 30	12.8	[5.6 ; 28.0]	25.4	[8.0; 76.5]
HB93-7130-1	9	47	5, 64	29.3	[18.7 ; 45.6]	58.4	[29.0; 116]
HB93-7130-4	5	24	11, 22	14.0	[7.3 ; 26.5]	27.4	[6.1; 112]
HB94-7211-3	5	42	2, 13	8.0	[4.6 ; 13.6]	8.1	[0.69; 47.8]
HB94-7211-8	6	72	2, 11	4.3	[2.7 ; 6.6]	7.0	[3.6; 12.6]
HB94-7239-5[3]	6	113	3, 8	3.8	[2.5 ; 5.7]	6.2	[2.4; 14.7]
HB94-7252-2	6	58	0, 15	4.3	[2.6 ; 6.9]	8.0	[4.4; 14.1]
HB94-7252-8	6	92	1, 15	5.5	[3.6 ; 8.2]	9.2	[5.7; 14.4]
HB94-7252-10	5	87	6, 40	10.3	[6.9 ; 15.0]	16.5	[9.7; 27.6]
HB94-7276-13	6	57	1, 16	4.6	[2.7 ; 7.5]	6.4	[3.3; 11.8]
HB94-7276-17	7	89	0, 9	2.9	[1.8 ; 4.5]	5.6	[2.7; 10.9]
HB94-7299-3	6	84	1, 5	1.5	[0.7 ; 2.5]	2.2	[0.68; 4.9]
HB94-7299-15	7	100	2, 7	2.6	[1.6 ; 4.0]	4.0	[1.9; 7.7]
Total:	182	1561	0, 104				

[1] Back transformed least squares means and confidence limits of the logarithm transformed seed set

[2] The subset comprised the pollinators Adora, 105ESC90-32, Frieslander and Katahdin

[3] Heterozygous for *amf* marker *(Amf/amf)*

The effect of the crossing year on the seed set following 2x.4x-crosses

In contrast to the least squares means of the ln(seed set + 1) following 2x.4x-crosses, that varied largely between the 30 diploid females as compared to the males, the least square means of the four different crossing years showed low variation (Fig.1). Because of the use of sub-optimal tetraploid males, all 2x.4x-crossing combinations yielded less than 18 seeds per berry in the first crossing year (Table 5A). As a result of the selection of more efficient tetraploid pollinators, as well as diploid hybrids with a better 2x.4x-crossability, the seed set increased to 104 seeds per berry for the best combination in 1995. In the same period, the number of berries that were derived from 2x.4x-crosses increased from 50 to 701 per crossing year.

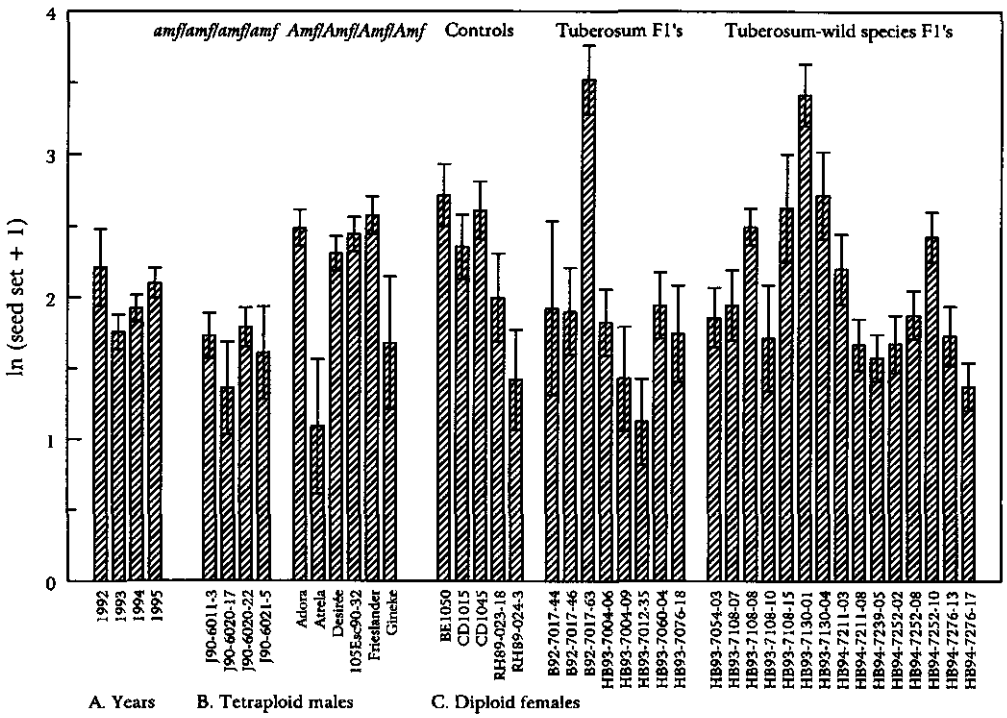

Figure 1. Least squares means and standard deviations (error bars) of the ln-transformed seed set following 2x.4x-crosses per crossing year (A), tetraploid pollinator (B) and diploid female parent (C).

The efficiency of the tetraploid pollinators on the seed set following 2x.4x-crosses

For the 13 male parents, the unbiased estimates of the mean seed set following 2x.4x-crosses varied from 2.0 to 12.5 seeds per berry. The seed sets induced by the four male parents that were nulliplex for the *amf* marker were below average (Fig. 1B and Table 5B). These nulliplex pollinators were not discarded, because they were valuable for the generation of tetraploid progenies allowing the genetic analysis of the 2n-eggs based on the segregation for the *amf* marker of the tetraploid progeny plants. The highest number of seeds per berry (104) was obtained in the crossing combination of B92-7017-63 × Hertha in 1995. In general, the clones Adora, Frieslander, Katahdin and 105ESC90-32 induced the highest seed set following 2x.4x-crosses, of more than 10 seeds per berry (Table 5B). As a result of this, the seed set per diploid female was higher, when only the crossing combinations with these four pollinators were analysed (Table 5C).

The effect of the diploid female on the seed set following 2x.4x-crosses

Within the 30 female parents, the estimates of the mean seed set following 2x.4x-crosses varied from 1.5 to 32.7 seeds per berry (Table 5C). As expected, the mean seed sets of the diploid control clones for a high level of 2n-egg formation, BE1050, CD1015, and CD1045, were above average. The seed set of the control for a moderate level of 2n-eggs, the clone RH89-023-18, was around average, and that of the control for a low level of 2n-eggs, the clone RH89-024-3, was below average (Fig. 1C, Table 5C).

In order to compare the seed sets of the 25 newly selected hybrids with the low, moderate and high levels of the control clones, the least squares means of the ln(seed set + 1) following 2x.4x-crosses of the 25 hybrids were tested for contrasts with the clones RH89-023-18 (low), and RH89-024-3 (moderate) and the average of the clones BE1050, CD1015 and CD1045 (high). In addition to the control clones with a high level of 2n-egg formation, six hybrids showed seed sets following 2x.4x-crosses that were higher than the control clone with a low level of 2n-egg formation (Table 6). These hybrids were the Tuberosum hybrid B92-7017-63, the two Tuberosum-*S. microdontum* F_1 hybrids HB93-7108-8, and HB93-7108-15, the two Tuberosum-*S. ajanhuiri* F_1 hybrids HB93-7130-1, and HB93-7130-4, and the Tuberosum-*S. chacoense* F_1 hybrid HB94-7252-10. Two of

them, the clones B92-7017-63 and HB93-7130-1, showed very high seed sets of 32.7 and 29.3 seeds per berry respectively, that even exceeded the high level of the three control clones. The four other hybrids and the three controls showed the high level of 10 to 14 seeds per berry. In contrast, there were two Tuberosum-*S. chacoense* F_1 hybrids (HB94-7299-3 and 15), and one Tuberosum hybrid (HB93-7012-35) with lower seed sets (mean of 1.5 to 2.6 seeds per berry) than the moderate control clone. The seed set of the remaining 16 hybrids (mean of 2.9 to 8.0 seeds per berry) was not different from the level in the moderate control clone (Table 6).

The high seed sets following 2x.4x-crosses became more pronounced when only the crossing combination with the four pollinators, Adora, 105ESC90-32, Frieslander, and Katahdin, that induced seed sets of more than 10 seeds per berry, were analysed. With these pollinators, the average seed set was 47 to 58 seeds per berry for the clones with very high frequencies of 2n-eggs, 16 to 27 seeds per berry for the clones with high frequencies of 2n-eggs, 5 to 19 seeds per berry for the clones with low/moderate frequency of 2n-eggs, and less than 4 seeds per berry for the ones with a low frequency of 2n-eggs (Table 5C).

Seed set of the selected hybrids in relation to their pedigree

From the two hybrids with a very high seed set following 2x.4x-crosses, one was derived from a high × high cross, whereas the other was derived from *S. ajanhuiri* and a moderate 2n-egg producing Tuberosum parent (Table 6). The four hybrids with a high seed set were Tuberosum-wild species hybrids that were derived from one low, one moderate and one high producing Tuberosum parent and three genotypes of the species *S. ajanhuiri*, *S. microdontum* and *S. chacoense*. The hybrids with moderate seed set comprised six Tuberosum hybrids that were derived from high × high, high × low, and moderate × high crosses, and ten Tuberosum-wild species F_1 hybrids that were derived from interspecific crosses involving both low and high Tuberosum parents and genotypes of *S. spegazzinii*, *S. microdontum*, and *S. chacoense*. Within the three hybrids with a low seed set following 2x.4x-crosses, there was one Tuberosum hybrid from a low × low cross, and two Tuberosum-*S. chacoense* F_1 hybrids with a high 2n-egg producing Tuberosum parent.

Table 6. Seed set following 2x.4x-crosses: Pairwise differences between the newly selected diploid hybrids and the control clones for high, moderate and low level of 2n-egg formation

	Type of cross[1]	P-value[2]			Estimated level of 2n-egg formation
		low	moderate	high	
Control clones					
RH89-024-3		-	0.2121	***0.0021***	low/moderate
RH89-023-18		0.2121	-	0.0793	moderate
BE1050/CD101		***0.0021***	0.0793	-	high
Tuberosum hybrids					
B92-7017-44	high × high	0.4757	0.9114	0.3025	moderate
B92-7017-46	high × high	0.2947	0.8225	***0.0427***	moderate
B92-7017-63	high × high	***0.0001***	***0.0001***	***0.0004***	very high
HB93-7004-6[3]	high × low	0.3423	0.6454	***0.0045***	moderate
HB93-7004-9[3]	high × low	0.9941	0.2328	***0.0047***	moderate
HB93-7012-35[3]	low × low	0.5223	***0.0414***	***0.0001***	low
HB93-7060-4	moderate × low	0.2143	0.8820	***0.0146***	moderate
HB93-7076-18	high × low	0.4938	0.5934	***0.0252***	moderate
Tuberosum-wild species F_1 hybrids					
HB93-7054-3[3]	*spg* × low	0.2784	0.7108	***0.0032***	moderate
HB93-7108-7[3]	*mcd* × low	0.2184	0.8871	***0.0193***	moderate
HB93-7108-8[3]	*mcd* × low	***0.0053***	0.1409	0.6560	high
HB93-7108-10[3]	*mcd* × low	0.5834	0.5439	***0.0313***	moderate
HB93-7108-15[3]	*mcd* × low	***0.0191***	0.2021	0.8824	high
HB93-7130-1	*ajh* × moderate	***0.0001***	***0.0002***	***0.0007***	very high
HB93-7130-4	*ajh* × moderate	***0.0061***	0.0947	0.6431	high
HB94-7211-3	*chc* × high	0.0735	0.5994	0.1687	moderate
HB94-7211-8	*chc* × high	0.5386	0.3358	***0.0001***	moderate
HB94-7239-5[3]	*chc* × low	0.6805	0.2157	***0.0001***	moderate
HB94-7252-2	*chc* × high	0.5408	0.3599	***0.0001***	moderate
HB94-7252-8	*chc* × high	0.2408	0.7163	***0.0005***	moderate
HB94-7252-10	*chc* × high	***0.0103***	0.2161	0.4879	high
HB94-7276-13	*chc* × high	0.4498	0.4591	***0.0003***	moderate
HB94-7276-17	*chc* × high	0.9156	0.0742	***0.0001***	moderate
HB94-7299-3	*chc* × high	0.2010	***0.0024***	***0.0001***	low
HB94-7299-15	*chc* × high	0.7193	***0.0384***	***0.0001***	low

[1] The levels of 2n-egg formation of the Tuberosum parents are presented in Table 1, the parental genotypes of wild species are indicated as follows: *ajh= S. ajanhuiri, chc= S. chacoense, mcd= S. microdontum, spg= S. spegazzinii*

[2] P-values < 0.05 (italics) indicate significant contrasts between lsmeans of the ln (seed set + 1) of hybrid and control

[3] Heterozygous for *amf* marker *(Amf/amf)*

DISCUSSION

Frequency of 2n-egg producing genotypes per family

The extensive 2x.4x-crossing programme allowed the selection of new hybrids with low to very high frequencies of 2n-eggs. The frequency of plants that produced more than four seeds per berry following 2x.4x-crosses varied between families. The frequency of selected plants was 15% for the Tuberosum hybrids with 2n-egg producing parents (Table 3). From the diploid families of Tuberosum-wild species F_1 hybrids with a high/moderate 2n-egg producing Tuberosum parent, 19% of the hybrids were selected. From the Tuberosum-wild species F_1 hybrids with a low 2n-egg producing Tuberosum parent, only 4% of the hybrids were selected (Table 4). The higher frequency of selected plants in families derived from diploid Tuberosum parents that produced 2n-eggs suggested a genetic basis for 2n-egg formation and possibilities for recurrent selection. Other reports also supported a genetic basis of 2n-egg formation, although there is no consensus about the inheritance (Werner and Peloquin 1987, 1990, 1991; Jongedijk *et al.* 1991b; Ortiz and Peloquin 1992; Barone *et al.* 1993).

The 2n-eggs are not restricted to *S. tuberosum*, but they can also occur in different *Solanum* spp. (Den Nijs and Peloquin 1977). In our 2x.4x-crossing programme, 10 of the 32 families of Tuberosum-wild species F_1 hybrids comprised genotypes that produced more than four seeds per berry following 2x.4x-crosses. These families of Tuberosum-wild species F_1 hybrids involved a genotype of the species *S. ajanhuiri, S. chacoense, S. microdontum, S. spegazzinii* or *S. verneii* as one of the parents (Table 4). Both the Tuberosum-*S. ajanhuiri* F_1 and the Tuberosum-*S. chacoense* F_1 hybrids showed high frequencies of 2n-egg producing plants as compared to the other *Solanum* spp. (25% versus <5%). Since we did not test the genotypes of the *Solanum* spp. themselves, their frequencies of 2n-egg producing genotypes were unknown. From other studies (Den Nijs and Peloquin 1977; Douches and Quiros 1988b; Werner and Peloquin 1991), it is known that the species *S. chacoense* as well as Tuberosum-*S. chacoense* F_1 hybrids comprise 2n-egg producing genotypes. Because of this 2n-egg formation and other desired characters, such as resistances to Colorado potato beetle (Bamberg *et al.* 1996), potato

leafroll virus (Brown and Thomas 1994), and root-knot nematodes (Janssen *et al.* 1996), the species *S. chacoense* has a high potential for introgression programmes in potato breeding.

The frequency of 2n-egg producing Tuberosum-*S. microdontum* F_1 hybrids was low, except for the family HB93-7108. This family was derived from a cross between the clone B16 of *S. microdontum* (BGRC 18568) and the Tuberosum parent 87-1031-29. The six (35%) selected plants in this family were not expected, since not a single 2n-egg producing plant was selected in any other family, involving either another genotype of the same *S. microdontum* accession or the Tuberosum clone 87-1031-29 as one of the parents (Table 4). Because of this, the genetic background of the clone *Mcd*18568-B16 or the specific combination of this clone with the clone 87-1031-29 seems to be favourable for 2n-egg formation.

The use of 2x.4x-crosses to estimate the level of 2n-egg formation in the selected hybrids

In addition to the identification of the frequency of 2n-egg producers in different families, the levels of 2n-egg formation of the selected hybrids were estimated in a 2x.4x-crossing programme over four years. Small differences were observed for the seed set of the 2x.4x-crosses between the four years (Fig. 1, Table 5). Also from other studies it is known that the environment can influence the expressivity of 2n-eggs (Veilleux 1985; Werner and Peloquin 1987).

Although the differences in seed set following 2x.4x-crosses were larger between the diploid females than between the tetraploid males (Fig.1, Table 5), the effect of the pollinator was highly significant (P=0.0001). Especially the seed set induction of the nulliplex males for the *amf* starch marker gene was low. Attempts are being made to develop nulliplex pollinators (*amf/amf/amf/amf*) with higher seed set following 2x.4x-crosses for an efficient generation of tetraploid progenies from 2n-eggs. Among the pollinators with wildtype starch (genotype *Amf/Amf/Amf/Amf*), the four pollinators Adora, Frieslander, Katahdin, and 105ESC90-32, induced seed sets of more than 10 seeds per berry (Table 5B). When only these good pollinators were used, the seed set per diploid female

increased (Table 5C). Therefore, it is important to use good pollinators in the 2x.4x-crossing programme to facilitate the generation of large tetraploid progenies, and to avoid underestimation of the frequency of 2n-eggs in the diploid females.

Since the use of the diploid clones BE1050, CD1015, and CD1045 in 2x.4x-crosses has been proven to generate large tetraploid progenies (Hutten *et al.* 1994a), it is demonstrated that the high seed set following 2x.4x-crosses correspond to high level of 2n-egg formation in these clones. Assuming the presence of an efficient triploid block in all 2x.4x-combinations, the variation in seed set following 2x.4x-crosses (2 to 58 seeds per berry for the subset of the four good pollinators) indicated large variability in the frequency of 2n-eggs among the 30 females, including the control clones, the Tuberosum hybrids and the Tuberosum-wild species F_1 hybrids (Table 5C). This variability is consistent with the reports of Den Nijs and Peloquin (1977), Veilleux (1985), Stelly and Peloquin (1986) and Werner and Peloquin (1987, 1991). This variation allowed us to identify two very high, four high, sixteen moderate and three low 2n-egg producing hybrids on the basis of differences in the seed set following 2x.4x-crosses between the newly selected hybrids and the control clones for low, moderate and high level of 2n-egg formation (Table 6).

The pedigrees of the selected hybrids

The two hybrids with a very high seed set following 2x.4x-crosses with the four good pollinators were the Tuberosum hybrid B92-7017-63 with a mean of 47 seeds per berry, and the Tuberosum-*S. ajanhuiri* F_1 hybrid HB93-7130-1 with a mean of 58 seeds per berry (Fig. 1C, Table 5C). The clone B92-7017-63 was derived from the parents ED1045 and CD1045 both of which have a high frequency of 2n-eggs (Table 3). In fact, this gene pool, of which the original clones were selected by Prof. S.J. Peloquin and associates (University of Wisconsin, Madison, WI) is shown to be a very important source of both 2n-egg and 2n-pollen producers in potato (Stelly and Peloquin 1986a; Jongedijk *et al.* 1991a, b; Barone *et al.* 1993; Hutten *et al.* 1994a). The other hybrid with a very high seed set following 2x.4x-crosses, the clone HB93-7130-1, originated from a quite different gene pool. This hybrid was derived from the cross between the clones Yari

(*S. ajanhuiri*) and B92-7014-7 (moderate frequency of 2n-eggs, and involving *S. megistacrolobum* in its pedigree).

Four Tuberosum-wild species F_1 showed an equally high (16 to 27 seeds per berry) seed set as the control clones with high frequencies of 2n-eggs (Table 5C, Table 6). Two of them, HB93-7108-8 and HB93-7108-15, were Tuberosum-*S. microdontum* F_1 hybrids that were heterozygous for the *amf* marker gene *(Amf/amf)*, because they were derived from the mutant Tuberosum parent 87-1031-29 *(amf/amf)* with a low level of 2n-egg formation. The third was the Tuberosum-*S. ajanhuiri* F_1 hybrid, HB93-7130-4, with a moderate 2n-egg producing Tuberosum parent. This hybrid was a full sib of the very high 2n-egg producing hybrid HB93-7130-1. The fourth hybrid HB94-7252-10 was a Tuberosum-*S. chacoense* F_1 from a high 2n-egg producing Tuberosum parent (Table 4). The sixteen moderate 2n-egg producing hybrids (5 to 19 seeds per berry) comprised both *Amf/Amf* and *Amf/amf* genotypes that were derived from intermated Tuberosum parents as well as interspecific Tuberosum × *Solanum* spp. crosses. These crosses involved low, moderate, and high 2n-egg producing Tuberosum parents and genotypes of *S. spegazzinii, S. microdontum*, and *S. chacoense*. Among the three hybrids with a low seed set following 2x.4x-crosses (less than 4 seeds per berry), there was one Tuberosum hybrid *(Amf/amf)* from a low × low cross, and two Tuberosum-*S. chacoense* F_1 hybrids with a high 2n-egg producing Tuberosum parent (Table 6).

Three important conclusions can be drawn from the pedigrees of the selected hybrids. Firstly, they indicated that 2n-egg formation is genetically determined but the inheritance is complicated. This is because diploid clones with very high or high levels of 2n-egg formation were obtained from different types of crosses, varying from crosses between two Tuberosum parents with both a high level of 2n-egg formation to direct crosses between a low 2n-egg producing Tuberosum parent and a genotype of a wild *Solanum* species. From the same types of crosses, also low and moderate 2n-egg producing hybrids were selected (Table 6). Secondly, the pedigrees showed that the 2n-egg producing clones can be obtained from highly different genetic backgrounds. Thirdly, both diploid Tuberosum hybrids and Tuberosum-wild species F_1 hybrids, that were heterozygous for the *amf* marker gene, could be selected from the 2x.4x-crosses. The

successful crosses of these *Amf/amf* genotypes with nulliplex males *(amf/amf/amf/amf)* indicated the possibility to generate tetraploid progenies segregating for this marker, facilitating the genetic analysis of 2n-eggs in potato.

Significance of the selected hybrids for genetic analysis of 2n-egg formation in potato

On the basis of the low to moderate seed set following 2x.4x-crosses in most selected hybrids, the frequency of 2n-eggs was expected to be too low for the cytological approach of analysing 2n-egg formation in potato. The alternative for the cytological approach in these hybrids was the generation of progenies for genetic analysis of the restitution mechanism(s) of 2n-eggs. From all 25 selected hybrids, it was possible to obtain a large number of berries per crossing combination and to produce sufficient seeds in order to generate large tetraploid progenies (Table 5C). Therefore, the frequency of 2n-eggs is not very relevant for the genetic analysis of 2n-eggs using the tetraploid progenies.

In view of the highly different genetic backgrounds, it is possible that different modes of origins of 2n-eggs will be present in the newly selected 25 hybrids. These restitution mechanisms can be elucidated by analysing the segregation of marker genes in the tetraploid progenies, that are derived from the 2x.4x-crosses. The newly selected hybrids with heterozygous genotype for the *amf* marker gene *(Amf/amf)*, that are successfully crossed with nulliplex males *(amf/amf/amf/amf)* can facilitate this genetic analysis of 2n-eggs in potato. Furthermore, the selected Tuberosum-wild species F_1 hybrids are expected to be highly heterozygous and to possess a high degree of polymorphisms for RFLP loci in the 2n-eggs. These polymorphisms are prerequisite for the genetic analysis of the 2n-eggs at different marker loci, using the tetraploid progenies of the 2x.4x-crosses. Besides the elucidation of the restitution mechanism(s), such as FDR and SDR, detailed genetic analyses with molecular markers will enable gene-centromere mapping and the estimation of the recombination frequencies in the selected hybrids.

The generation of progenies and the assessment of useful polymorphisms for multilocus RFLP analysis of 2n-eggs of diploid potato

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THE GENERATION OF PROGENIES AND THE ASSESSMENT OF USEFUL POLYMORPHISMS FOR MULTILOCUS RFLP ANALYSIS OF 2N-EGGS OF THE DIPLOID POTATO

ABSTRACT

In order to elucidate the restitution mechanism and number of crossovers per chromosome of individual 2n-eggs, it is necessary to monitor the genotype of each 2n-egg at marker loci on different parts of a chromosome, including the proximal regions. To pave the way for such multilocus RFLP-analysis of 2n-eggs of the diploid potato (2x=2n=24), both diploid parthenogenetic and tetraploid hybrid populations were generated. The diploid parthenogenetic progenies were generated through prickle pollination of six diploid 2n-egg producers. Eleven tetraploid hybrid progenies were generated from crosses between six diploid 2n-egg producing females and six tetraploid males (2x.4x-crosses). The parents of these progenies were analysed with RFLP markers of chromosomes 6 and 8 to identify the informative polymorphisms.

The frequency of RFLP loci that identified two separate alleles per locus in the 2n-egg producers and should be informative for multilocus analysis of 2n-eggs using the diploid parthenogenetic progenies was high (93%). The frequency of parthenogenetic diploids generated through prickle pollination of the six diploid 2n-egg producers was very low (less than 0.17 per berry). The generation of tetraploid hybrids from 2n-eggs was easier, since the 2x.4x-crosses yielded up to 11.0 tetraploids per berry. The RFLP patterns of the parents of the eleven 2x.4x-progenies were compared to identify marker loci with two unique alleles in the diploid 2n-egg producing parent. In the best three 2x.4x-combinations, the frequency of RFLP loci with two unique alleles in the diploid 2n-egg parent was 19-23%.

INTRODUCTION

The modes of origin of numerically unreduced (or 2n-) eggs in diploid potato (2n=2x=24) can be elucidated cytologically or genetically. In potato, the cytological approach is complicated, because the female meiosis is asynchronous and takes place in the inner layers of an ovule, as a single meiosis per ovule. Furthermore, 2n-egg producing diploid potato clones have a mixture of normal and abnormal megagametogenesis resulting in reduced (or n-), and unreduced (or 2n-) eggs, respectively (Jongedijk 1985; Stelly and Peloquin 1986b; Conicella *et al.* 1991).

The genetic approach is based on the fact that the two strands from each bivalent, that are recovered in the 2n-megaspores, constitute either sister or non-sister chromatids. Furthermore, the occurrence of crossovers between the non-sister chromatids of the bivalent results in different levels of heterozygosity at the proximal and distal parts of chromosomes (Rhoades and Demsey 1966; Nel 1975; Mendiburu and Peloquin 1979). In order to elucidate the restitution mechanism and number of crossovers per chromosome of each individual 2n-gamete, it is necessary to monitor the segregation for marker loci on different parts of a chromosome, including the proximal regions. Only if the combinations of alleles at linked loci on a pair of chromosomes in individual 2n-gametes can be determined, it will be possible to demonstrate either the exclusive occurrence of only one or a mixture of different restitution mechanisms per clone.

A drawback in the genetic approach is that individual 2n-eggs cannot be monitored for the segregation of the marker genes, such as in the genetic analysis of 2n-pollen (Chapter 2). However, it is possible to generate progenies from 2n-eggs enabling the analysis of their genetic compositions. The ideal situation for a genetic analysis of individual 2n-eggs is a diploid parthenogenetic progeny derived from unfertilized 2n-eggs. Such parthenogenetic development of 2n-eggs can be induced by 'prickle pollination' of the 2n-egg producers with the commonly used *Solanum phureja* pollinators, such as IVP48 and IVP101, which are homozygous for the embryo-spot marker (Hermsen and Verdenius 1973; Hutten *et al.* 1994b; Jacobsen *et al.* 1991). A second approach is the indirect genetic analysis of 2n-eggs using the tetraploid progeny derived from 2x.4x-crosses. In this type of crosses in *Solanum*,

the triploid embryos derived from fertilized n-eggs are aborted, which is called the 'triploid block' (Marks 1966). In the tetraploid progenies of 2x.4x-crosses, the genetic composition of 2n-eggs can only be identified when the 2n-egg producing diploid has unique alleles as compared to those of the male parent. In the past, different marker genes were successfully used for genetic analysis of 2n-eggs and 2n-pollen by analysing the tetraploid progenies that were derived from 2x.4x- or 4x.2x-matings, respectively (Douches and Quiros 1987, 1988a; Jongedijk *et al.* 1991a; Wagenvoort and Zimnoch-Guzowska 1992; Werner *et al.* 1992; Barone *et al.* 1995; Chapter 2). However, simultaneous determination of the restitution mechanism and the number of crossovers per chromosome of individual 2n-gametes was not possible, because there were too few markers available to genotype each 2n-gamete at both proximal and distal marker loci of one chromosome. This is only possible by means of multilocus linkage analysis of tetraploids, derived from 2x.4x-crosses, for markers with (1) suitable polymorphisms for genotyping at the 4x level and (2) random distribution along the whole length of one chromosome.

In the last ten years, multi-allelic RFLP markers with known map positions have become available in potato (Bonierbale *et al.* 1988; Tanksley *et al.* 1992; Gebhardt *et al.* 1989, 1991; Jacobs *et al.* 1995, Van Eck *et al.* 1994; Barone *et al.* 1995). In contrast to isozyme and morphological markers, representing only a few marker loci per chromosome, and molecular markers such as RAPDs or AFLPs, without clear multiple alleles per locus, the numerous multi-allelic RFLP markers are expected to meet the above mentioned requirements for multilocus analysis of 2n-eggs using the tetraploid progeny plants.

In order to develop plant materials with suitable polymorphisms for multilocus analysis of 2n-eggs, we used diploid 2n-egg producing hybrids of interspecific origin (Jacobsen *et al.* 1991; Hutten *et al.* 1994a; Chapter 3) for testing the following strategies: 1) generation of diploid parthenogenetic progenies from 2n-eggs by means of prickle pollination, 2) generation of tetraploid hybrid progenies from 2n-eggs by means of 2x.4x-crosses, 3) detection of the informative polymorphisms for the genotyping of 2n-eggs using the parthenogenetic diploid or tetraploid hybrid progenies, based on the RFLP-analysis of the diploid 2n-egg producers and the tetraploid male parents for markers on chromosomes 6 and 8.

MATERIALS AND METHODS

Plant materials

The diploid 2n-egg producers were derived from crosses between diploid *S. tuberosum* (*tbr*) and the species *S. ajanhuiri (ajh), S. chacoense (chc), S. megistacrolobum (mga), S. microdontum (mcd), S. phureja (phu), S. spegazzinii (spg)* and *S. verneii (vrn)*.

Six hybrids that produced low and high frequencies of 2n-eggs were used for inducing parthenogenetic diploids from 2n-eggs. The three hybrids with a low frequency of 2n-eggs were the *phu-vrn-tbr* hybrid 880004-11, (Jacobsen *et al.* 1991), and the two *mcd-tbr* hybrids RH87-175-1 (Chapter 3) and RH88-186-7 (CHI-EP5 × *Mcd*18302-10). The three hybrids with a high frequency of 2n-eggs were the *phu-vrn-tbr* hybrid CD1015, the *mcd-tbr* hybrid HB93-7108-8, and the *ajh-mga-tbr* hybrid HB93-7130-1 (Chapter 3). The six diploid 2n-egg producers were pollinated with IVP48 or IVP101, of (predominantly) *phu* origin. These diploid pollinators are homozygous for the dominant embryo-spot marker, caused by anthocyanin accumulation, and well known for their haploid induction ability in tetraploids (Hermsen and Verdenius 1973; Hutten *et al.* 1994b) as well as the the induction and selection of parthenogenetic diploids from 2n-eggs (Jacobsen *et al.* 1991).

Six diploid 2n-egg producers and six males were used in 2x.4x-crosses to generate eleven tetraploid progenies from 2n-eggs. The diploid female parents comprised the three previously mentioned 2n-egg producers CD1015, HB93-7108-8, and HB93-7130-1, the *phu-vrn-tbr* hybrid BE1050, the *spg-tbr* hybrid HB93-7054-3, and the *chc-tbr* hybrid HB94-7239-5 (Chapter 3). The six tetraploid male parents comprised the three cultivars Adora, Desirée, and Hertha, and the three genotypes 105ESC90-32, J90-6011-3, and J90-6020-22, which were all of *tbr* origin (Chapter 3).

Generation of diploid and tetraploid offsprings from 2n-eggs

Pollinations were made on emasculated flowers of 2n-egg producing diploid plants in the greenhouse during the spring and summer of 1992 to 1994. Six weeks after pollination, berries were harvested and allowed to soften for the extraction of seeds.

The IVP pollinators were used for the induction and selection of spotless seeds, that

developed parthenogenetically from n- and 2n-egg cells. The spotless seeds were separated from the spotted ones, counted and sown either in the greenhouse or *in vitro* in the early spring. In the greenhouse, batches of 100 seeds were sown in boxes of 10 x 10 holes. *In vitro* germination of seeds was carried out in Petri dishes on MS20 medium supplemented with 200 mg/l cefotaxime. Germination was monitored during a period of up to six months. All seedlings with 'nodal bands' (controlled by the same genes as embryo-spot) were discarded (Uijtewaal *et al.* 1987a).

From the seeds that were obtained in crosses between 2n-egg producing diploids and tetraploid male parents (Chapter 3), the seeds of eleven 2x.4x-crossing combinations were used for the generation of tetraploid progenies. These eleven 2x.4x-crossing combinations (indicated as A-K) were : (**A**) BE1050 × Hertha, (**B**) BE1050 × J90-6011-3, (**C**) CD1015 × J90-6011-3, (**D**) HB93-7130-1 × Adora, (**E**) HB93-7130-1 × Desirée, and (**F**) HB93-7130-1 × Hertha, (**G**) HB93-7108-8 × 105ESC90-32, (**H**) HB93-7108-8 × Adora, (**I**) HB93-7108-8 × J90-6020-22, (**J**) HB93-7054-3 × Desirée, and (**K**) HB94-7239-5 × Desirée. Germination of the seeds and raising of the seedlings were carried out in the greenhouse. In order to test the ploidy level of the progeny plants that were derived from 2x.4x-crosses, the DNA contents of the leaves were measured by flow cytometry (Plant Cytometry Services, Schijndel, The Netherlands).

RFLP-analysis

DNA was isolated from leaf samples according to Van der Beek *et al.* (1992). After precipitation, an extra washing of the DNA sample was carried out overnight with 10 mM ammonium acetate in 76% ethanol to improve the quality of the DNA. The six restriction enzymes *Eco*RI, *Eco*RV, *Hin*dIII, *Xba*I, *Dra*I, and *Hae*III (Gibco BRL) were used to digest the DNA samples. Southern hybridization was carried out according to Van Ooijen *et al.* (1994) with some minor modifications. DNA digests of 5 μg were separated overnight by electrophoresis through 0.8% agarose gels and transferred to nylon membranes (Hybound N^+, Amersham) by vacuum blotting. Inserts of tomato and potato DNA-clones were kindly supplied by SD Tanksley (Cornell University, Ithaca, NY, USA), C. Gebhardt (Max Plank Institute, Köln, Germany), R. Visser and P. Zabel (Wageningen Agricultural University, the

Netherlands). These inserts were amplified by the polymerase chain reaction (PCR) to generate probe DNA. After radio labelling of the probes using the Megaprime DNA labelling system (Amersham), the blots were prehybridized overnight in hybridization buffer containing 1% SDS, 1M NaCl, 10% dextran sulphate, 0.1 mg/ml herring sperm DNA (Boehringer Mannheim) in 50 mM Tris-HCl at pH 7.5. Hybridization was carried out overnight at 65°C with 5 ng/ml probe DNA in the same hybridization buffer. The blots were washed twice for one hour at 65°C in 0.5 x SSC and 1% SDS. For autoradiography, the blots were allowed to expose KODAK X-OMAT S films for 2-7 days.

Screening for fully informative polymorphisms

In order to monitor the genotypes of 2n-eggs at different loci of one chromosome, useful polymorphisms for RFLP markers were identified in Southern hybridization experiments with specific tomato and potato probes of chromosomes 6 and 8. For multilocus analysis of 2n-eggs in parthenogenetic diploids, all heterozygous (m^1m^2) RFLP loci in the diploid 2n-egg producers were considered as fully informative for genotyping the m^1m^1, m^1m^2, and m^2m^2 2n-eggs. In the case of multilocus analysis of 2n-eggs in 2x.4x-progenies, a RFLP marker was considered as fully informative, when the 2n-egg producing diploid was heterozygous (m^1m^2) at this marker locus, and when the two alleles were unique as compared to the alleles of the tetraploid male ($m^{\sigma}m^{\sigma}m^{\sigma}m^{\sigma}$) for at least one of the restriction enzymes tested. These fully informative polymorphisms were required for monitoring the $m^1m^1m^{\sigma}m^{\sigma}$, $m^1m^2m^{\sigma}m^{\sigma}$, and $m^2m^2m^{\sigma}m^{\sigma}$ genotypes in the 2x.4x-progeny at different loci, allowing the indirect genotyping of the m^1m^1, m^1m^2, and m^2m^2 2n-eggs of the diploid clone.

RESULTS

Generation of parthenogenetic diploids from 2n-eggs

In order to generate parthenogenetic diploids, six diploid 2n-egg producers were pollinated with the clones IVP48 or IVP101, homozygous for the embryo-spot marker.

Because of this dominant marker, spotted seeds were hybrids, whereas the spotless seeds were putative parthenogenetically developed eggs. Despite the large number of spotless seeds, only small progenies were obtained (Table 1).

Table 1. Generation of diploid progenies from 2n-eggs by means of prickle pollination of the diploid 2n-egg producers with the pollinators IVP48 or IVP101. The number of parthenogenetically developed plants (seedlings without nodal bands) per berry is compared to the seedset after 2x.4x crosses.

Diploid 2n-egg producing clone	Year of sowing	Results of the prickle pollinations in numbers of					Nr. of seedlings without nodal bands per berry	Nr. of seeds per berry in 2x.4x crosses[1]
		berries	seeds	spotless seeds	germinated spotless seeds	seedlings without nodal bands		
RH87-175-1	1993	434	>50000	1840	175	9	0.02	1
CD1015	1994	176	23371	524	190	28	0.16	10
880004-11	1994	53	8567	73	29	9	0.17	1
RH88-186-7	1994	49	5366	73	45	2	0.04	2
HB93-7130-1	1995	24	1563	95	42[2]	2	0.08	29
HB93-7108-8	1995	28	4269	29	24[2]	0	0.00	11

[1] Chapter 3 and unpublished data

[2] sown *in vitro*

The germination of the spotless seeds was very poor and, despite the absence of a clear embryo-spot on the seeds, most of the seedlings had to be discarded because of the presence of nodal bands, indicating the hybrid character of plants. This incomplete expression of the paternal anthocyanin marker at embryo stage was also found in other prickle pollination studies using the same pollinators (Uijtewaal *et al.* 1987a; Hutten *et al.* 1994b). Because of this, the number of seedlings without nodal band per berry, representing the frequency of parthenogenetically developed ovules, was very low for all six diploids tested. A notable feature was that these frequencies were not related to the seed set following 2x.4x-crosses indicating the level of 2n-egg formation. Furthermore, most seedlings without nodal bands died before the adult stage because of poor development and lack of vigour. Therefore, the

spotless seeds of 1995 were sown *in vitro*. Under sterile conditions, the seeds germinated better, but the only two seedlings without nodal band died in an early stage (Table 1). It should be noted that plants without nodal bands could be monoploids, if they were derived from parthenogenetically developed n-eggs. However, no effort was made to analyse the ploidy level of the plants without nodal bands, because all progenies were too small for proper genetic analysis.

Generation of tetraploid plants from 2n-eggs by means of 2x.4x-crosses

Table 2. Generation of tetraploid hybrids from 2n-eggs by means of 2x.4x-crosses

Code	Diploid 2n-egg producing parent	Tetraploid ♂ parent	Number of				Abberant genotypes
			berries	seeds	plants (%)	tetraploids	
A	BE1050	Hertha	9	250	233 (93)	nd	nd
B	BE1050	J90-6011-3	7	125	73 (58)	nd	nd
C	CD1015	J90-6011-3	11	130	113 (87)	nd	nd
D	HB93-7130-1	Adora	4	205	73 (36)	nd	nd
E	HB93-7130-1	Desirée	5	96	45 (47)	nd	nd
F	HB93-7130-1	Hertha	8	288	134 (47)	nd	nd
G	HB93-7108-8	105ESC90-32	16	165	130 (79)	128	2 triploids
H	HB93-7108-8	Adora	12	110	88 (80)	87	1 hexaploid
I	HB93-7108-8	J90-6020-22	19	114	89 (78)	89	0
J	HB93-7054-3	Desirée	11	167	121 (72)	121	0
K	HB94-7239-5	Desirée	10	80	66 (83)	62	1 aneuploid and 3 triploids

nd = not determined

The efficiency of generating tetraploid progenies from 2n-eggs by means of crossings between the diploid 2n-egg producers and tetraploid males was tested for eleven combinations (A to K) of the parents (Table 2). These 2x.4x-crosses yielded at least six seeds per berry. The frequency of plants that were obtained from these seeds was high (more than 58%), except for the 2x.4x-progenies D, E, and F, that were derived from the 2n-egg producer

HB93-7130-1. For five 2x.4x-progenies (G to K), the ploidy level of the plants was tested by flow cytometry. As expected, the great majority (487 of the 494 plants) was tetraploid. Only seven had aberrant ploidy levels, of which five were triploids, one hexaploid, and one aneuploid (Table 2). Comparison of the number of parthenogenetic diploids (Table 1) with that of the tetraploid plants in 2x.4x-progenies clearly indicated that the population size of the latter was much more suitable for multilocus analysis of 2n-eggs.

RFLP-analysis of the diploid 2n-egg producers to identify the markers that have predictive value for multilocus analysis of parthenogenetic diploids

If parthenogenetic diploids were available for indirect multilocus analysis of 2n-eggs, all heterozygous RFLP loci of the 2n-egg producing diploid would be informative. Therefore, the frequency of these heterozygous RFLP loci in diploid 2n-egg producers was analysed in the diploid parents of the 2x.4x-progenies, since the parthenogenetic development of the 2n-eggs in the six diploids tested was not successful. From the 18 RFLP loci of chromosome 8 tested in the diploid 2n-egg producers BE1050, CD1015, and HB93-7130-1, the numbers of heterozygous loci were 17, 16, and 17, respectively (Table 3). The 2n-egg producer HB93-7108-8 was heterozygous for 14 of the 15 loci of chromosome 6, and for all 13 loci tested of chromosome 8 (Table 4). From the 31 RFLP loci of chromosome 8, the diploid HB93-7054-3 was heterozygous for all loci, and the diploid HB94-7239-5 was heterozygous for 30 loci (Table 5). These data indicated that more than 93% of the RFLP loci in the six diploid 2n-egg producers (tested with five to six restriction enzymes) were heterozygous and, therefore, fully informative for the analysis of 2n-eggs using parthenogenetic diploids.

Table 3. Polymorphisms in three diploid parents as compared to four tetraploid parents of six 2x.4x-progenies.

Locus	Combination of 2x and 4x parent					
	A	B	C	D	E	F
	BE1050 (2x) versus Hertha (4x)	BE1050 (2x) versus J90-6011-3(4x)	CD1015 (2x) versus J90-6011-3 (4x)	HB93-7130-01 (2x) versus Adora (4x)	HB93-7130-01 (2x) versus Desirée (4x)	HB93-7130-01 (2x) versus Hertha (4x)
CD029	*Eco*RV	*Eco*RV, *Hae*III	***EcoRV, HaeIII***	*Eco*RV, ***HaeIII***	*Eco*RV, ***HaeIII***	*Eco*RV, ***HaeIII***
CT068	*Eco*RV	*Eco*RV, *Xba*I	*Eco*RV, *Xba*I	0	0	0
CT077	*Hae*III	*Hae*III	*Eco*RV, *Hae*III	*Hae*III	*Hae*III	*Hae*III
CT148	*Xba*I	***XbaI***	0	0	0	0
CT252	*Hae*III	*Eco*RV, *Hae*III	*Eco*RV, *Xba*I, *Hae*III	*Hin*dIII, *Hae*III	*Hin*dIII, *Hae*III	*Hae*III
GBSS	0	*Eco*RV	*Eco*RV	*Eco*RI, *Xba*I, ***HaeIII***	*Eco*RI, *Xba*I, ***HaeIII***	*Xba*I, ***HaeIII***
GP130	Monomorphic	Monomorphic	Monomorphic	*Eco*RI, *Dra*I	*Eco*RI, *Eco*RV	*Eco*RI, *Eco*RV
GP170	0	*Eco*RI, *Xba*I, *Hae*III	*Eco*RI, *Xba*I, *Hae*III	0	*Eco*RI	0
GP171	0	0	0	0	0	0
GP288	*Eco*RI	*Eco*RI	Monomorphic	0	0	0
SSP15	*Hae*III	*Xba*I, *Hae*III	*Xba*I	*Eco*RI, *Xba*I, *Hae*III	***EcoRI***, ***XbaI***, *Hae*III	***EcoRI***, *Xba*I, *Hae*III
SSP34	*Eco*RI	0	0	0	*Xba*I	0
TG176	*Eco*RI	0	0	0	0	0
TG294	0	*Dra*I	*Dra*I	*Hin*dIII	0	0
TG309	*Eco*RI	*Eco*RI	0	*Eco*RI, *Eco*RV, *Dra*I	*Eco*RI, *Eco*RV, *Dra*I	*Eco*RI, *Eco*RV, *Dra*I
TG402	0	0	0	*Hin*dIII, *Dra*I, *Xba*I	*Hin*dIII, *Dra*I, *Xba*I	*Hin*dIII, *Dra*I, *Xba*I
TG434	*Hin*dIII, *Hae*III	*Hin*dIII, *Hae*III	*Hin*dIII, *Hae*III	*Hin*dIII	*Hin*dIII	*Hin*dIII
TG496	*Hin*dIII, *Hae*III	*Hae*III	*Eco*RI	Monomorphic	Monomorphic	Monomorphic

The parents were tested for a set of 18 RFLP probes of chromosome 8 in combination with six restriction enzymes (*Eco*RI, *Eco*RV, *Hin*dIII, *Dra*I, *Xba*I, and *Hae*III). The polymorphisms found with these probe/enzyme combinations are listed per RFLP locus and indicated as follows:
'Monomorphic'; the diploid parent is homozygous (for all six probe/enzyme combinations)
'0'; the diploid parent is heterozygous (for at least one of the probe/enzyme combinations), but its alleles are not different from the tetraploid parent
Name restriction enzyme; the diploid parent is heterozygous with one unique allele as compared to the tetraploid parent
Name restriction enzyme; the diploid parent is heterozygous with two unique alleles as compared to the tetraploid parent

RFLP-analysis of the diploid 2n-egg producers and tetraploid males to identify the markers that have predictive value for multilocus analysis of the tetraploid hybrids

In view of the appropriate population sizes of the tetraploid progenies, that were derived from 2x.4x-crosses, for genetic analysis of 2n-eggs (Table 2), the possibilities for genotyping of tetraploids were investigated. Genotyping of the 2n-eggs using these tetraploid progenies was only possible at marker loci heterozygous in the 2n-egg producing diploid (m^1m^2) with two unique alleles as compared to those of the tetraploid male parent ($m^am^am^am^a$). Therefore, the diploid and tetraploid parents of the eleven 2x.4x-progenies (A to K) were tested for the presence of these so-called 'fully informative polymorphisms' using RFLP probes of chromosomes 6 and 8.

Fully informative polymorphisms between the parents of the six 2x.4x-progenies A, B, C, D, E, and F (Table 3), were found for 0 to 3 loci of the 18 RFLP loci of chromosome 8 tested (or < 17%). The diploid parent HB93-7108-8 and the three tetraploid parents of the 2x.4x-progenies G, H, and I, showed fully informative polymorphisms for 0 to 1 (or < 7%) markers of chromosome 6, and 0-2 (or < 16%) markers of chromosome 8 (Table 4). After extension of the number of RFLP probes from 15 to 40 chromosome 6 markers, and from 13 to 21 chromosome 8 markers for testing the parents of the 2x.4x-progeny G, still only 1 (3%) chromosome 6 marker, and an increased number of 4 (19%) chromosome 8 markers, were fully informative (data not shown). The number of chromosome 8 markers with fully informative polymorphisms between the diploid parents HB93-7054-3, and HB94-7239-5 and the tetraploid parent Desirée of the two 2x.4x-progenies J, and K were 6, and 7 (or 19, and 23%), respectively (Table 5).

Table 4. Polymorphisms in the diploid parent HB93-7108-8 as compared to the tetraploid parents of three 2x.4x-progenies.

Locus	Chr.	Combination of 2x and 4x parent		
		G	H	I
		HB93-7108-08 (2x) versus 105ESC90-32 (4x)	HB93-7108-08 (2x) versus Adora (4x)	HB93-7108-08 (2x) versus J90-6020-22 (4x)
H5G4	6	0	0	0
TG025	6	0	0	0
TG054	6	0	0	*Xba*I
TG115	6	*Eco*RV	0	0
TG118	6	*Eco*RI, *Eco*RV, *Hin*dIII	*Eco*RI, *Eco*RV, *Hin*dIII	*Eco*RV, *Dra*I, *Xba*I
TG220	6	*Eco*RV, *Hin*dIII, ***Xba*I**	*Xba*I	*Eco*RV, *Xba*I
TG231	6	*Dra*I	*Hin*dIII, *Dra*I	*Hin*dIII, *Dra*I
TG232	6	*Eco*RI, *Eco*RV, *Hin*dIII, *Dra*I, *Xba*I	*Eco*RV, *Hin*dIII, *Dra*I, *Xba*I	*Eco*RV, *Hin*dIII, *Dra*I, *Xba*I
TG275	6	*Eco*RI, *Eco*RV, *Dra*I	*Eco*RI, *Eco*RV, *Dra*I	*Eco*RI, *Eco*RV, *Hin*dIII, *Dra*I
TG292	6	*Eco*RI	*Eco*RI	*Eco*RI
TG436	6	*Hin*dIII	*Hin*dIII	0
TG444	6	Monomorphic	Monomorphic	Monomorphic
TG590	6	*Eco*RI, *Hin*dIII	*Eco*RI, *Hin*dIII, *Xba*I	*Hin*dIII, *Xba*I
TG593	6	*Dra*I, *Xba*I	*Dra*I, *Xba*I	*Dra*I, *Xba*I
TG642	6	*Dra*I	*Dra*I	0
CT252	8	*Eco*RV, *Hin*dIII, *Xba*I	*Xba*I	*Xba*I
GBSS	8	*Eco*RI, *Hin*dIII, *Dra*I	*Eco*RI, *Hin*dIII, *Dra*I	*Eco*RI, *Hin*dIII, *Dra*I
GP130	8	*Eco*RV, *Dra*I	0	*Eco*RV, *Hin*dIII, *Dra*I
GP170	8	0	0	*Eco*RI, *Hin*dIII, *Xba*I
GP171	8	0	0	0
GP173	8	0	0	0
GP288	8	0	0	0
TG045	8	*Hin*dIII, *Dra*I	*Hin*dIII, *Dra*I	*Hin*dIII, *Dra*I
TG294	8	*Eco*RV, *Xba*I	*Eco*RV, *Xba*I	*Eco*RV, *Dra*I, *Xba*I
TG346	8	*Eco*RI, ***Eco*RV**, *Hin*dIII, *Dra*I, *Xba*I	*Eco*RI, ***Eco*RV**, *Hin*dIII, *Dra*I, *Xba*I	*Eco*RI, *Eco*RV, *Hin*dIII, *Dra*I, *Xba*I
TG402	8	*Eco*RV, *Hin*dIII	*Eco*RV, *Hin*dIII	*Eco*RV, *Hin*dIII
TG434	8	***Hin*dIII**	*Hin*dIII	*Hin*dIII
TG496	8	*Eco*RI	0	*Eco*RI

The parents were tested for a set of 15 RFLP probes of chromosome 6, and 13 RFLP probes of chromosome 8 in combination with five restriction enzymes (*Eco*RI, *Eco*RV, *Hin*dIII, *Dra*I, and *Xba*I). The polymorphisms found with these probe/enzyme combinations are listed per RFLP locus and indicated as follows:
'Monomorphic'; the diploid parent is homozygous (for all six probe/enzyme combinations)
'0'; the diploid parent is heterozygous (for at least one of the probe/enzyme combinations), but its alleles are not different from the tetraploid parent
Name restriction enzyme; the diploid parent is heterozygous with one unique allele as compared to the tetraploid parent
Name restriction enzyme; the diploid parent is heterozygous with two unique alleles as compared to the tetraploid parent

Table 5. Polymorphisms in two diploid parents as compared to the tetraploid parent Desirée of two 2x.4x-progenies.

Locus	Combination of 2x and 4x parent	
	J	K
	HB93-7054-03 (2x) versus Desirée (4x)	HB94-7239-05 (2x) versus Desirée (4x)
CD040	*Eco*RI, *Eco*RV, *Xba*I, *Hae*III	*Eco*RI, *Eco*RV, ***HindIII***, ***XbaI***, *Hae*III
CT077	0	*Eco*RI
CT132	*Eco*RV	*Eco*RV, *Hae*III
CT148	*Dra*I	*Xba*I
CT241	*Eco*RI, *Eco*RV, *Hin*dIII, *Hae*III	*Eco*RV, *Hin*dIII, *Hae*III
CT29a	*Dra*I	*Dra*I
CT47	0	*Eco*RV, ***HindIII***, *Xba*I, ***HaeIII***
CT92	*Eco*RI, *Eco*RV, *Hin*dIII	*Eco*RI, *Eco*RV, *Dra*I, *Hae*III
CT228	*Xba*I	*Eco*RV, *Xba*I
GBSS	*Hae*III	0
GP130	*Eco*RI	Monomorphic
TG041	0	0
TG045	0	*Dra*I
TG072	*Eco*RI, *Eco*RV, *Hin*dIII	*Eco*RI, ***HindIII***
TG127	*Xba*I, *Hae*III	*Xba*I, ***HaeIII***
TG176	*Hae*III	*Dra*I, *Hae*III
TG228	*Eco*RI, ***HindIII***, *Dra*I, *Hae*III	*Eco*RI, *Eco*RV, *Hin*dIII, *Hae*III
TG294	0	0
TG302	*Hin*dIII	*Eco*RI
TG309	*Eco*RI	*Dra*I
TG346	***EcoRV***, *Hin*dIII, *Dra*I	***EcoRV***, *Hin*dIII
TG349	*Eco*RI, *Eco*RV, *Hin*dIII, *Dra*I	*Eco*RV, *Hin*dIII, *Dra*I
TG399	0	*Dra*I, *Xba*I
TG402	*Eco*RV, *Dra*I	*Hin*dIII, *Dra*I
TG434	***HindIII***	***EcoRI***, *Hin*dIII
TG481	*Eco*RI, *Eco*RV, ***HindIII***, *Hae*III	*Eco*RI, *Eco*RV, *Hin*dIII, ***HaeIII***
TG510	*Eco*RI, *Eco*RV	*Eco*RV, *Hin*dIII, *Hae*III
TG513	***EcoRI***, *Eco*RV, *Hin*dIII, *Dra*I, *Hae*III	*Eco*RI, *Eco*RV, *Dra*I
TG536	*Eco*RI, *Eco*RV, ***DraI***, ***XbaI***, *Hae*III	*Xba*I
TG553	*Hae*III	*Eco*RV, *Xba*I, *Hae*III
TG612	*Eco*RI, *Hin*dIII	*Eco*RI, *Hin*dIII, *Dra*I, *Hae*III

The parents were tested for a set of 31 probes of chromosome 8 in combination with six restriction enzymes (*Eco*RI, *Eco*RV, *Hin*dIII, *Dra*I, *Xba*I, and *Hae*III). The polymorphisms found with these probe/enzyme combinations are listed per RFLP locus and indicated as follows:

'Monomorphic'; the diploid parent is homozygous (for all six probe/enzyme combinations)

'0'; the diploid parent is heterozygous (for at least one of the probe/enzyme combinations), but its alleles are not different from the tetraploid parent

Name restriction enzyme; the diploid parent is heterozygous with one unique allele as compared to the tetraploid parent

Name restriction enzyme; the diploid parent is heterozygous with two unique alleles as compared to the tetraploid parent

These data clearly indicated that the number of fully informative RFLP markers between the 2x and 4x parents of the eleven progenies was generally low. A notable feature was that between the diploid parent HB93-7108-8 and the three male parents 105ESC90-32, Adora, and J90-6020-22, the frequency of fully informative loci was higher for markers of chromosome 8 than those of chromosome 6 (Table 4). The most polymorphic combinations were G) HB93-7108-8 versus 105ESC90-32, J) HB93-7054-3 versus Desirée, and K) HB94-7239-5 versus Desirée, with 19-23% fully informative markers of chromosome 8. This allowed the identification of 4, 6, and 7 loci of chromosome 8 with predicted value for multilocus analysis of 2n-eggs, using the 2x.4x-progenies G, J, and K, respectively.

Discussion

The generation of diploid parthenogenetic and tetraploid hybrid progenies from diploid 2n-egg producers

In order to generate plant materials for multilocus analysis, both the prickle pollinations to induce the parthenogenetic development of 2n-eggs, and the 2x.4x-crosses to generate hybrids from 2n-eggs were tested. The pollinations of the six diploid 2n-egg producers with IVP48 and IVP101 resulted in a low number of putatively parthenogenetic diploids. The frequency of seedlings without the paternal marker varied from 0.00 to 0.17 per berry for the six diploids and was not related with the level of 2n-egg formation as estimated in 2x.4x-crosses (Table 1). In fact, the frequencies of these parthenogenetically developed ovules were very similar to those of monoploids obtained from prickle pollinations of diploid clones without 2n-egg formation, using the same IVP pollinators (Uijtewaal *et al.* 1987a). Apparently, the 2n-egg containing ovules in the six diploids tested had no higher ability to develop parthenogenetically than the ovules with n-eggs. Because of this, the parthenogenetically derived progeny of the 2n-egg producers probably comprised both monoploids and diploids. In addition, an incomplete genome transfer of the IVP pollinator could result in aneuploid and diploid seedlings without nodal bands (Wilkinson *et al.* 1994). Unfortunately, it was not possible to analyse the ploidy level and genomic composition of the plants without nodal bands, because most plants died in an early stage. For the

parthenogenetically derived diploid plants this poor development and vigour could be the result of a high level of inbreeding depression resulting from homozygosity induced by the restitution mechanism of 2n-egg formation, whereas the weak performance of monoploids and aneuploids could be the result of their aberrant ploidy levels. In view of the possibilities for contaminations with monoploids and non-parthenogenetically developed plants, the limited sizes of the diploid progenies derived from parthenogenetically developed 2n-eggs of the six diploids (Table 1), were probably even overestimated. Therefore, prickle pollination of diploid 2n-egg producers was found to be too complex and elaborate for the generation of parthenogenetic diploids in order to analyse a large and representative sample of 2n-eggs.

Generation of tetraploid progenies from 2n-eggs by means of 2x.4x-crosses was more efficient than the generation of parthenogenetic diploids. The relatively large quantities of seeds generated in the eleven different combinations of 2x.4x-crosses (6 to 51 seeds per berry) resulted in a frequency of 36 to 93% plants (Table 2). From the 494 plants of five 2x.4x-progenies tested, only seven plants deviated from the expected 4x ploidy level (Table 2). The triploids were expected to arise sporadically from the zygotes originating from the fusion of n-eggs with the 2x-pollen of the tetraploid parent. Low numbers of triploids were frequently described in the progenies derived from crosses between diploid and tetraploid parents (Marks 1966; Hanneman and Peloquin 1968; Jongedijk *et al.* 1991b). The small number of five triploids derived from our 2x.4x-crosses indicated that the triploid block, as described by Marks (1966), was very effective. The observed hexaploid plant, probably originated from unreduced gametes of both the diploid and tetraploid parents. The DNA content of the only aneuploid was 12% less than that observed in the tetraploid genotypes indicating the presence of about 3.5 genomes. In view of the large numbers of tetraploid plants in the progenies, making 2x.4x-crosses was a straight-forward method to generate a progeny of large samples of 2n-eggs.

The informative polymorphisms for multilocus analysis of 2n-eggs using diploid parthenogenetic and tetraploid hybrid progenies

The generation of parthenogenetic diploids from 2n-eggs would be very effective for mulitilocus analysis. This is because more than 93% of the RFLP loci in the six diploids were

found to be heterozygous, and, therefore, predicted to be fully informative for multilocus analysis of parthenogenetic diploids (Tables 3, 4, and 5). The diploid 2n-egg producers were highly heterozygous as a result of their interspecific origin. High degrees of heterozygosity for RFLP markers in other diploid hybrids of potato were reported by Bonierbale *et al.*(1988), Gebhardt *et al.* (1989, 1991), Tanksley *et al.* (1992), Jacobs *et al.*(1995), and Barone *et al.* (1995). Because of this, it is attractive to use the parthenogenetic diploid progeny that are derived from diploid 2n-egg producers for multilocus analysis. However, the induction of parthenogenetic diploids from diploid 2n-egg producers needs to be improved.

Despite the fact that the diploid 2n-egg producers were heterozygous for more than 93% of the RFLP markers, the number of fully informative RFLP markers between the 2x and 4x parents of the tetraploid hybrid progenies was rather low. Five of the eleven combinations of 2x and 4x parents showed less than 10% fully informative RFLP markers of chromosome 8. The highest level of fully informative polymorphisms was 23%. This frequency of fully informative loci varied not only for the eleven combinations of 2x and 4x parents tested, but also for different chromosomes within one combination of parents. For example, only 1 of the 40 (2.5%) chromosome 6 markers, and 4 of the 21 (19%) chromosome 8 markers were fully informative for the 2x.4x-combination of HB93-7108-8 × 105ESC90-32.

In the 2x.4x-progenies, a minimum of three alleles per locus were required to identify two unique alleles of the 2n-egg producing diploid parent in the tetraploid progeny plants. Because less than 23% of the marker loci in the diploids tested had two unique alleles as compared to tetraploids (Tables 3, 4, and 5), and only rarely, six different alleles were found (data not shown), our data suggested a relatively low number of alleles per locus.

There is a strong assumption that a high degree of multi-allelism is important for maximizing heterotic effects in tetraploids as a result of increased intra- and inter locus interactions (Bingham 1980; Dunbier and Bingham 1975; Mendoza and Haynes 1974; Bonierbale *et al.* 1993; David *et al.* 1995). However, the data on the number of alleles per RFLP locus in potato are limited. In the study of Bonierbale *et al.* (1993), 15 to 41% of the RFLP loci in tetraploids were found to be homozygous, and the mean number of RFLP-alleles at heterozygous loci ranged from 2.4 to 3.0. Other RFLP-analyses of potato did not elucidate

the number of alleles per locus, because of the use of diploid BC1 populations, in which the parents share a common allele at each locus (Tanksley *et al.* 1992; Jacobs *et al.* 1995; Van Eck *et al.* 1994), or the presence of null alleles as a result of the length and separation of the DNA fragments in the RFLP-method developed by Gebhardt *et al.* (1989). The observed degree of multi-allelism in the 2x and 4x genotypes suggests that it is worthwhile to prescreen the polymorphisms between the diploid 2n-egg producers and the tetraploid males to select the most contrasting combinations of parents. Such directed 2x.4x-crosses will facilitate an efficient multilocus analysis of 2n-eggs using tetraploid progeny plants.

Post-Meiotic Restitution in 2n-egg formation of diploid potato

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POST-MEIOTIC RESTITUTION IN 2N-EGG FORMATION OF DIPLOID POTATO

ABSTRACT

The modes of origin of 2n-eggs were determined in four diploid Tuberosum-wild species F1 hybrids, involving the species *Solanum phureja*, *S. chacoense*, *S. spegazzinii*, and *S. microdontum*. To this end, the four hybrids (2n=2x=24) that were heterozygous for the genetic marker *amylose-free (Amf/amf)* were crossed with tetraploid (2n=4x=48) nulliplex *(amf/amf/amf/amf)* male parents. For this marker, which is known to be present on the distal position on chromosome 8 of potato, the resulting tetraploid progenies segregated into three clear-cut classes of nulliplex, simplex *(Amf/amf/amf/amf)* and duplex *(Amf/Amf/amf/amf)* genotypes. In three cases, the simplex genotypic class predominated and the mode of origin was explained as due to the Second Division Restitution (SDR). On the contrary, the progenies of the *S. microdontum* × *S. tuberosum (mcd-tbr)* hybrid comprised either nulliplex or duplex genotypes, indicating absolute homozygosity for this locus in the 2n-eggs. In order to test whether the 2n-eggs were homozygous also for other loci on the same chromosome, the tetraploid progeny of the *mcd-tbr* hybrid (2x) × potato clone 105ESC90-32 (4x) was analysed for four RFLP markers, specific to the chromosome 8 of potato. This analysis showed complete homozygosity for all 2n-eggs at all the loci investigated. In addition the occurrence of crossing-over between the two genomes of the *mcd-tbr* hybrid was evident. This indicated that the 2n-eggs of this hybrid originated through post-meiotic doubling of the chromosomes, which was reliably confirmed for the first time in potato. Similar to the terminology of First and Second Division Restitution (FDR and SDR), we proposed to characterize this post-meiotic doubling as a new restitution mechanism in potato, called Post-Meiotic Restitution (PMR). With the discovery of this genotype, a full spectrum of diploid clones that can produce 2n-eggs with complete homozygosity to a high degree of heterozygosity is now available.

INTRODUCTION

In plants, sexual polyploidization is an important feature in both nature and breeding programmes involving meiotic mutants with numerically unreduced gametes (or 2n-gametes). These 2n-gametes comprise the somatic number of chromosomes and may occur in all kinds of species (Harlan and de Wet 1975). In general, the 2n-gametes can originate through several types of nuclear division abnormalities that occur either before, during or after the completion of meiosis (Rhoades and Dempsey 1966; Eenink 1974; Nel 1975). In potato, however, the only two modes of origin of 2n-gametes that have been recognised and critically characterised so far are first division restitution (FDR), and second division restitution (SDR) mechanisms that occur during meiosis (Peloquin *et al.* 1989a; Veilleux 1985; Bretagnolle and Thompson 1995). In both cases, the 2n-gametes are derived from two of the four strands of each bivalent of a pair of homologous chromosomes. The FDR gametes originate through an equational division of all chromosomes, as a result of which the non-sister chromatids are included in one and the same gamete. In this case, the parental genotype is preserved largely intact and the gametes can be highly heterozygous. On the other hand, the SDR gametes originate from the restitution of chromosomes in the products of the first meiotic division, as a result of which the sister chromatids are included in one and the same 2n-gamete. Such gametes can be highly homozygous. Thus, depending on the knowledge whether the 2n-gametes have originated through FDR or SDR, it is possible to make a choice between highly heterozygous and homozygous gametes required in genetic studies as well as breeding programmes.

Through the use of appropriate meiotic mutants, it has been possible to select genotypes that produce highly heterozygous FDR to relatively homozygous SDR populations of 2n-gametes (Mok and Peloquin 1975a; Ramanna 1979 , 1983; Douches and Quiros 1987, 1988b; Jongedijk *et al.* 1991a; Wagenvoort and Zimnoch-Guzowska 1992; Werner *et al.* 1987, 1992; Barone *et al.* 1995; Chapter 2). In megasporogenesis, a cell wall is invariably formed between the daughter nuclei at telophase 1. If the nuclei restitute soon after telophase 1, the sister chromatids are included in one and the same

restitution nucleus which give rise to SDR gametes (Jongedijk 1985). However, it is believed that different meiotic aberrations can occur in one plant resulting in a mixture of FDR-SDR 2n-eggs (Conicella *et al.* 1991; Werner and Peloquin 1991). Especially in the ovules of potato, it is impossible to unequivocally demonstrate the cytological events that can elucidate the mode(s) of origin of 2-eggs.

In the absence of a reliable and convenient cytological method for determining the mode(s) of origin of 2n-eggs in potato, genetic methods are the alternatives (Rhoades and Dempsey 1966; Nel 1975; Mendiburu and Peloquin 1979). These methods make use of the fact that the FDR 2n-gametes comprise the non-sister chromatids of each homologous pair of chromosomes, whereas SDR 2n-gametes comprise the sister chromatids, except for the chromosomal parts that have been exchanged in crossing-over between non-sister chromatids. Based on the assumption of the frequent occurrence of one crossover between non-sister chromatids per chromosome arm, the model of Mendiburu and Peloquin (1979) is commonly used to explain the genetic compositions of FDR and SDR gametes in potato. According to this model, the FDR 2n-gametes will be 100% heterozygous at proximal marker loci, and 50% heterozygous at distal loci, if both combinations of non-sister chromatids occur randomly. In contrast; SDR 2n-gametes will be 100% homozygous at proximal marker loci, and 100% heterozygous at distal marker loci (Fig. 1).

Since the individual 2n-eggs of diploid clones are not amenable for direct analysis of most marker genes, it is routine to use 2n-egg producers in 2x.4x-crosses to generate tetraploid progeny plants for indirect analysis of the 2n-gametes. In this way, different types of genetic markers have successfully been used to analyse the level of heterozygosity of 2n-eggs (Douches and Quiros 1987, 1988b; Jongedijk *et al.* 1991a; Werner *et al.* 1992; Barone *et al.* 1995). These investigations, however, are lacking segregation data for several loci on a single chromosome so as to analyse the combinations of the maternal and paternal alleles at linked loci on a pair of chromosomes that constitute individual 2n-eggs. This information is essential in order to elucidate the degree of heterozygosity, the restitution mechanism and number of crossovers per chromosome in each individual 2n-egg. This can also facilitate to demonstrate either the exclusive occurrence of one

restitution mechanism or a mixture of several restitution mechanisms in a genotype. In a previous study, we have identified some RFLP markers of chromosome 6 and 8 of potato that are informative for multilocus analysis of tetraploid progenies that are derived from crosses between 2n-egg producing diploids and tetraploid males (Chapter 4).

In the present investigation, we firstly tested the utility of the distal biochemical marker *amylose-free* starch *(amf)* to demonstrate differences in the mode(s) of origin of 2n-eggs in four diploid clones on the basis of the segregation ratios in their tetraploid progenies. Secondly, we tested the utility of four RFLP markers dispersed over chromosome 8 for the multilocus analysis of a tetraploid progeny of one of these 2n-egg producers in order to elucidate the restitution mechanism(s) of 2n-egg formation, and to determine the extent of crossing-over.

MATERIALS AND METHODS

Plant materials

Four diploid clones that were derived from crosses between the diploid species *Solanum tuberosum (tbr)* and *S. phureja (phu), S. chacoense (chc), S. microdontum (mcd)* or *S. spegazzinii (spg)* were used for the analysis of 2n-egg formation. These four Tuberosum-wild species F1 hybrids were the following: 1) the *tbr-phu* hybrid HB93-7004-6; 2) the *chc-tbr* hybrid HB94-7239-5; 3) the *tbr-spg* hybrid HB93-7054-3; and 4) the *mcd-tbr* hybrid HB93-7108-8. All the four hybrids were heterozygous *(Amf/amf)* for the *amylose-free* starch marker and showed normal chromosome pairing during meiosis. The hybrids produced 2n-eggs in variable frequencies, as was evident from their seed sets following 2x.4x-crosses (Chapter 3).

These diploid hybrids were crossed with tetraploid males for the generation of tetraploid progenies. The three tetraploid clones that were used as nulliplex *(amf/amf/amf/amf)* male parents in the 2x.4x-crosses were J90-6020-22, J90-6011-3, and J90-6001-25 (Chapter 3). The tetraploid male parent 105ESC90-32 was quadruplex for

the *amf* marker *(Amf/Amf/Amf/Amf)*. This clone was the male parent of the selected 2x.4x-combination for multilocus RFLP-analysis of the 2n-eggs, because it possessed some RFLP loci of chromosome 8 with unique alleles as compared to the diploid *mcd-tbr* hybrid (HB93-7108-8). The tetraploid chromosome constitution of the progeny derived from this 2x.4x-cross was ascertained by flow cytometry (Chapter 4).

Classification of the tetraploid progenies based on the amf marker

The tetraploid progenies from the 2x *(Amf/amf)* × 4x *(amf/amf/amf/amf)* crosses were classified into the classes of duplex, simplex, and nulliplex genotypes for the *amf* marker on the basis of the starch phenotypes of microspores after staining in Lugol/chloral hydrate (1:2) (Jacobsen *et al.* 1989, 1991, Flipse *et al.* 1996, Chapter 2). The nulliplex genotypes in this tetraploid progeny were identified on the basis of only red microspores. To distinguish the simplex (blue: red = 1:1) and duplex (blue: red = 5:1) genotypes, 100-500 microspores were scored.

Multilocus RFLP-analysis of the tetraploid progenies

In the present investigation, the tetraploid progeny of the cross between the diploid *mcd-tbr* hybrid and the tetraploid male, 105ESC90-32, was analysed in more detail. At each of the four RFLP loci *TG536, TG482, TG434*, and *TG346* of chromosome 8, the *mcd-tbr* hybrid was heterozygous ($m^{mcd}m^{tbr}$) with unique alleles as compared to the tetraploid male ($m^{a}m^{a}m^{a}m^{a}$), allowing the full classification of the $m^{mcd}m^{mcd}m^{a}m^{a}$, $m^{mcd}m^{tbr}m^{a}m^{a}$, and $m^{tbr}m^{tbr}m^{a}m^{a}$ genotypes in the 2x.4x-progeny. The uniqueness of the polymorphisms for the four fully informative loci was established in earlier RFLP-analysis of both parents, using specific tomato and potato probes of chromosomes 6 and 8 (Chapter 4). The *TG536, TG482, TG434*, and *TG346* loci were very useful for genotyping of the tetraploid progeny, since their positions of respectively 6, 34, 83 and 91 cM in the tomato map of chromosome 8 enabled the investigation of the inheritance of a large part of this chromosome via 2n-eggs.

In order to identify the parental origin of each of the alleles *(mcd* or *tbr)* at the four loci both the *mcd* and the *tbr* parent of the 2n-egg producing hybrid were included in the

RFLP-analysis. This was essential to compare the origin of the alleles at the linked loci of the same chromosome within individual 2n-eggs in order to detect crossovers between the homoeologous chromosomes of the *mcd-tbr* hybrid. From the recombination frequencies between RFLP-loci a genetic map was constructed by using the computer package JoinMap 2.0 (supplied by P. Stam, Wageningen Agricultural University, The Netherlands).

RESULTS

Assessment of the restitution mechanism through monitoring the segregation for the *amf* marker

The distal marker locus *amf* was used to monitor the genotypes of 2n-eggs in four heterozygous *(Amf/amf)* diploid clones to detect differences in crossing-over or restitution mechanisms between the diploid clones. Because the 2n-eggs were not amenable for direct analysis of this marker, the four diploid *Amf/amf* genotypes were crossed with nulliplex males *(amf/amf/amf/amf)* to generate tetraploid progenies. Since all plants received two *amf* alleles from the nulliplex male parent, nulliplex progeny plants were derived from *amf/amf* 2n-eggs, whereas simplex plants originated from *Amf/amf* 2n-eggs, and duplex plants originated from *Amf/Amf* 2n-eggs of the heterozygous diploid females. Based on the segregation ratios for the starch phenotypes of the microspores in the tetraploid progeny plants, it was possible to classify these three distinct phenotypic classes as nulliplex, simplex and duplex and thus establish the genotypes for the *amf* marker (Table 1). The remarkable feature was that in the populations, where the three diploid 2n-egg producing *tbr-phu, chc-tbr*, and *tbr-spg* hybrids were involved, the simplex genotypic class far outnumbered the nulliplex and duplex classes (Table 1). This preponderance of simplex genotypes showed the high frequency of *Amf/amf* 2n-eggs in these three hybrids. On the contrary, the nulliplex and duplex classes far outnumbered the simplex class in the progeny of the *mcd-tbr* hybrid. This distortion in the segregation pattern of the *amf* genotypic classes was obviously due to a different mode of origin of the 2n-eggs in the

mcd-tbr hybrid as compared to the other three diploid females. To examine the relationship between the observed segregations for the *amf* locus and the probable meiotic nuclear restitution events that had occurred in the ovules, we considered the genetic consequences of the FDR and SDR mechanisms (Fig. 1).

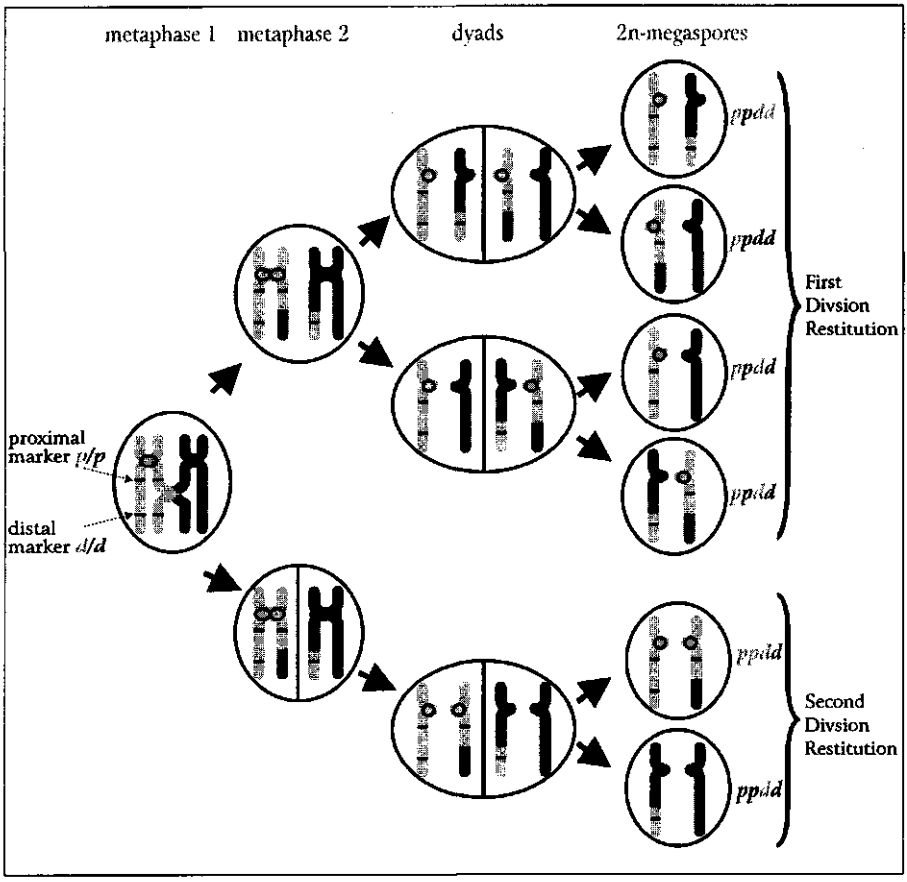


Figure 1: The genetic consequences of First Division Restitution and Second Division Restitution for a proximal marker locus (p) and a distal marker locus (d), assuming one crossover between these markers.

The maternal chromosomes and alleles are indicated in light grey, whereas the paternal chromosomes and alleles are indicated in dark grey (after Mendiburu and Peloquin 1979).

The *amf* locus was previously shown to be the most distal marker on chromosome 8 of potato, and it was suggested that in normal synaptic plants a single crossover

occurred between this locus and the centromere (Chapter 2) Because of this, the heterozygous 2n-eggs *(Amf/amf)* of heterozygous clones were the result from either the combination of both recombinant or both non-recombinant chromatids of non-sister origin (FDR), or the combination of the recombinant and non-recombinant sister-chromatids (SDR) of the bivalent of chromosome 8, as indicated for the distal locus in Fig. 1. In the case of FDR 2n-egg formation, the frequency of heterozygous *(Amf/amf)* 2n-eggs could not exceed 50%, except for clones with severely reduced recombination. In the case of SDR 2n-egg formation, the frequency of heterozygous *(Amf/amf)* 2n-eggs could be high in clones with the frequent occurrence of one crossover per arm. As a result of the successive cytokinesis in megasporogenesis in potato, only the sister-chromatids were expected to be included in one and the same restitution nucleus following the anaphase 1 stage (SDR 2n-egg formation) in normal synaptic clones. In view of this, it was expected that the high frequency of heterozygous 2n-eggs *(Amf/amf)* resulted from SDR following the frequent occurrence of one crossover between the *amf* locus and its centromere. This expectation was realised in the case of the 2n-eggs of the normal synaptic *tbr-phu*, *chc-tbr*, and *tbr-spg* hybrids (Table 1). In contrast, the highly homozygous *(Amf/Amf* and *amf/amf)* 2n-eggs of the *mcd-tbr* hybrid deviated from the expected segregation for the *amf* marker in SDR 2n-eggs of a normal synaptic clone.

The homozygous 2n-eggs from heterozygous clones *(Amf/amf)* were the result from either the combination of two recombinant chromatids of non-sister origin (FDR), or the combination of two non-recombinant sister-chromatids (SDR) of the bivalent of chromosome 8 (Fig. 1). In the case of FDR 2n-egg formation, the frequency of these *Amf/Amf* and *amf/amf* 2n-eggs could not exceed 50%. Therefore, it was impossible that the high frequency of homozygous *(Amf/Amf* and *amf/amf)* 2n-eggs of the normal synaptic *mcd-tbr* hybrid was the result of FDR. In the case of SDR 2n-egg formation, this frequency could be high in clones with severely reduced crossing-over between the *amf* locus and the centromere. Only if we assumed no crossing-over between the *amf* locus and the centromere, it might be expected to be due to SDR 2n-egg formation. In view of the most distal position of the *amf* locus on this chromosome, this would suggest the complete absence of crossing-over between the *mcd* and *tbr* genomes in the hybrid. Therefore, if the

2n-eggs of the *mcd-tbr* hybrid had originated through SDR, then totally homozygous 2n-eggs were only expected when there was no crossing-over at all between the *mcd* and *tbr* genomes.

Table 1. Segregation of the *amf* marker in progenies from *Amf/amf* × *amf/amf/amf/amf* crosses and its application to test the expected SDR mode of 2n-egg formation in four different Tuberosum-wild species F_1 hybrids

2n-egg producer (2x)	Genotypes[1] of the progeny (4x) from the *Amf/amf* × *amf/amf/amf/amf* crosses			Probability SDR[3]
Amf/amf	*amf/amf/amf/amf* (Expected)[2]	*Amf/amf/amf/amf* (Expected)[2]	*Amf/Amf/amf/amf* (Expected)[2]	
tbr-phu hybrid	5 (1.65)	16 (18.7)	1 (1.65)	0.107
chc-tbr hybrid	1 (3.45)	40 (39.1)	5 (3.45)	0.710
tbr-spg hybrid	1 (1.13)	12 (12.8)	2 (1.13)	0.588
mcd-tbr hybrid	34 (5.48)	2 (62.1)	37 (5.48)	0.000

[1] After iodine staining of the microspores, plants with only red microspores were classified as nulliplex genotypes, that were derived from homozygous *amf/amf* 2n-eggs. Plants with 1 blue : 1 red microspores were classified as simplex genotypes, that were derived from *Amf/amf* 2n-eggs. Plants with 5 blue : 1 red microspores were classified as duplex genotypes, that were derived from *Amf/Amf* 2n-eggs

[2] The expected frequencies of the nulliplex, simplex, and duplex genotypes for SDR were calculated with the formula of Mendiburu and Peloquin (1979):

freq(nulliplex+duplex)= 1 - 0.02(gene-centromere distance),

assuming one crossover per chromosome arm (Fig. 1) and a gene-centromere distance for the *amf* marker of 42.5 cM, as was determined using 4x.2x-progenies (Chapter 2)

[3] Based upon chi-square tests for simplex, and pooled nulliplex+duplex classes

Another explanation might be that the 2n-eggs of the *mcd-tbr* hybrid resulted from the doubling of chromosomes in the (haploid) products of normal meiosis. This would demonstrate the occurrence of an additional restitution mechanism in potato, that was not equivalent to either FDR or SDR. If post-meiotic doubling of the chromosomes had occurred in the *mcd-tbr* hybrid, the 2n-eggs were expected to be completely homozygous and to show crossover events between the *mcd* and *tbr* genomes.

In order to test these the possibilities of SDR and post-meiotic doubling, the level of homozygosity of the 2n-eggs for other loci as well as the presence or absence of crossing-over in the *mcd-tbr* hybrid were further investigated. To this end, the tetraploid

crossing-over in the *mcd-tbr* hybrid were further investigated. To this end, the tetraploid progeny that was derived from the cross *mcd-tbr* hybrid × 105ESC90-32 was used for multilocus RFLP-analysis.

RFLP analysis of the tetraploid progeny derived from the *mcd-tbr* hybrid and 105ESC90-32

All 138 tetraploid progeny plants of the cross *mcd-tbr* hybrid (2x) × 105ESC90-32 (4x) were genotyped at each of the four fully informative marker loci *TG536, TG481, TG434,* and *TG346* of chromosome 8. An example of a Southern blot showing polymorphisms for the probe *TG346* in the diploid *mcd-tbr* hybrid, its *mcd* and *tbr* parents, the tetraploid male parent (105ESC90-32) and some tetraploid progeny plants is illustrated in Fig. 2.

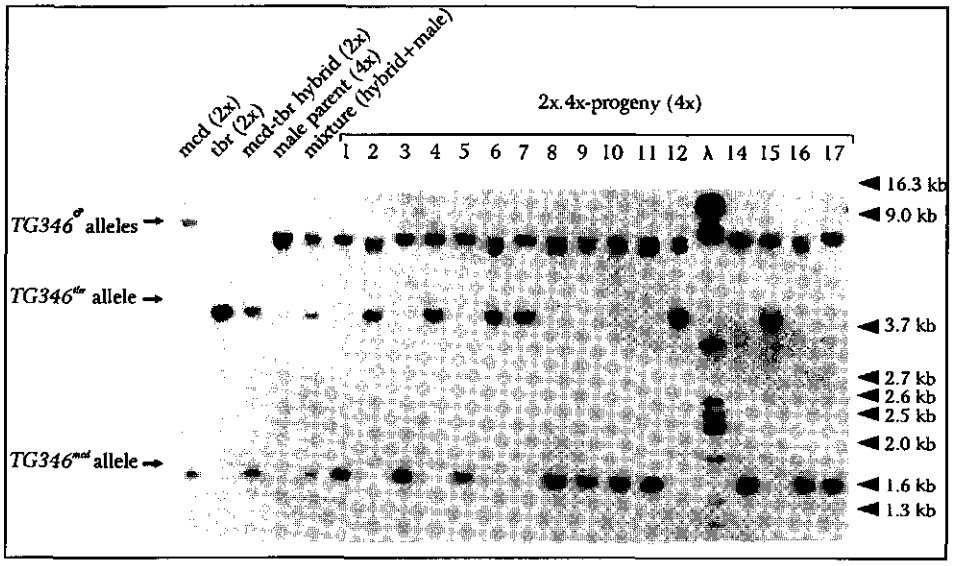


Figure 2: Southern blots showing the absence of tetraploid progeny plants with both alleles of the *mcd-tbr* hybrid

The parental clones are shown in lanes 1 to 5; *mcd* parent (lane 1) and *tbr* parent (lane 2) of the diploid *mcd-tbr* hybrid (lane 3), the tetraploid male parent 105ESC90-32 (lane 4), a mixture of *mcd-tbr* hybrid and 105ESC90-32 (lane 5). Sixteen plants of the tetraploid progeny of the cross *mcd-tbr* hybrid × 105ESC90-32 are shown in lanes 6-21. DNA samples were digested with *Eco*RV and hybridized to probe TG346. λ-DNA was used as a marker for the size of DNA-fragments (lane λ)

The marker loci *TG536, TG481, TG434,* and *TG346,* with their map positions of 6, 34, 83, and 91 cM on chromosome 8, respectively, covered 89% of the genetic length of the linkage map of this chromosome (Tanksley *et al.* 1992). Regarding these segregating markers, there were ten distinct classes of genotypes in the tetraploid progeny with two remarkable features (Fig. 3).

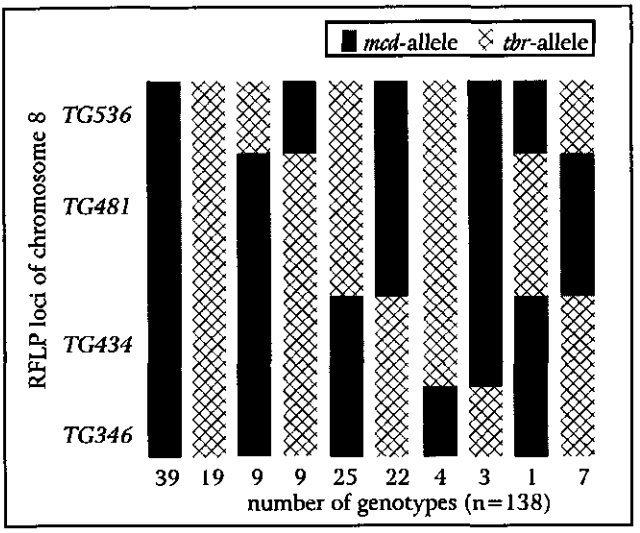


Figure 3 : Genotype frequency distribution of the tetraploid progeny derived from the cross *mcd-tbr* hybrid × 105ESC90-32 showing the allelic composition of the 138 plants at four RFLP loci of chromosome 8.

The order of the loci is in line with the tomato map of Tanksley *et al.* (1992). The bars represent the different genotypic classes, based on the presence of the *mcd* allele (solid black), or *tbr* allele (netted) at the four RFLP loci *TG536, TG481, TG434* and *TG346* of chromosome 8. The number under the bars represent the number of plants per genotypic class

Firstly, all plants displayed the presence of either the m^{mcd} allele or the allele m^{tbr} at all four loci. It indicated that all progeny plants were $m^{mcd}m^{mcd}m^{o}m^{o}$ or $m^{tbr}m^{tbr}m^{o}m^{o}$ and consequently originated from homozygous ($m^{mcd}m^{mcd}$ and $m^{tbr}m^{tbr}$) 2n-eggs. This homozygosity closely corresponded to the segregation pattern for the *amf* marker of the same chromosome with almost only duplex and nulliplex genotypes in the tetraploid offspring from the cross between the *mcd-tbr* hybrid and nulliplex males (Table 1). Secondly, most genotypes (80) showed the presence of both m^{mcd} alleles and m^{tbr} alleles in

homozygous condition for different markers of chromosome 8 (columns 3 to 10 in Fig. 3). This indicated the frequent crossing-over between the two genomes in the *mcd-tbr* hybrid. As a consequence of the observed crossing-over in the *mcd-tbr* hybrid, the homozygous 2n-eggs had originated through a restitution mechanism that was different from FDR, SDR, or a mixture of both.

The 2n-eggs of the *mcd-tbr* hybrid comprised 58 non-recombinant genotypes (columns 1 and 2 in Fig. 3) , 72 genotypes with one crossover (columns 3 to 8 in Fig. 3), and 8 genotypes with two crossovers (columns 9 and 10 in Fig. 3). The logical explanation for these genotypes was that the chromosomes in products of normal meiosis were doubled in order to give rise to 2n-eggs in the *mcd-tbr* hybrid. Assuming doubling of the chromosome 8 after the completion of normal meiosis, each crossover was expected to give rise to 50% of recombinant genotypes (Fig. 4), and the gametes were expected to comprise two identical copies of each chromosome, such as in the case of doubled haploids.

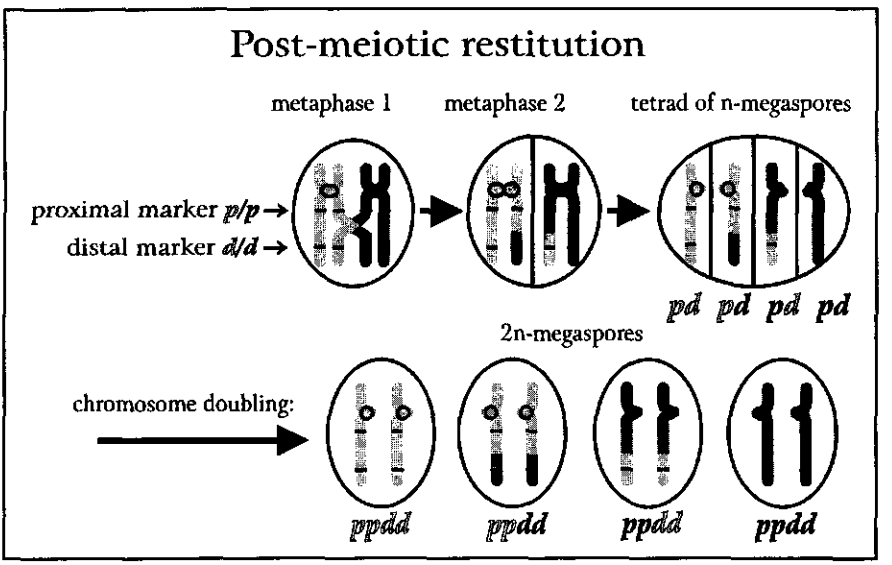


Figure 4: The genetic consequences of Post-Meiotic Restitution for a proximal marker locus (p) and a distal marker locus (d), assuming one crossover between these markers.
The generation of homozygous 2n-gametes by chromosome doubling following crossing-over and completion of normal meiosis. The maternal chromosomes and alleles are indicated in light grey, whereas the paternal chromosomes and alleles are indicated in dark grey.

Therefore, the genotypes of these 2n-eggs were expected to be similar to the genotypes of doubled haploids. To test this possibility, the genetic distances between the four RFLP loci of chromosome 8 in *mcd-tbr* hybrid were estimated on the basis of the observed recombination frequencies, using the doubled haploid option of the computer package Joinmap. The order of genes and the genetic distances were in line with the tomato map of Tanksley *et al.* (1992) (Fig. 5).

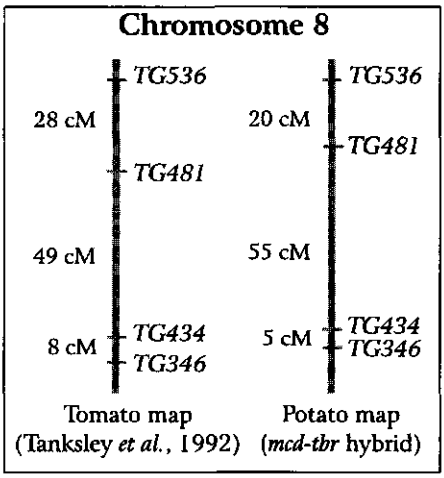


Figure 5: Genetic map distances of chromosome 8 in tomato and the *mcd-tbr* hybrid

DISCUSSION

Detection and confirmation of different restitution mechanisms of 2n-eggs

With the use of multiple genetic markers for the chromosome 8 of potato, we have demonstrated that the diploid *mcd-tbr* hybrid produced completely homozygous 2n-eggs. For the detection of this genotype, the *amf* marker was highly useful for two reasons. Firstly, the starch phenotypes of the microspores in the tetraploid progeny of *Amf/amf* × *amf/amf/amf/amf* crosses allowed full classification of the nulliplex, simplex and duplex genotypes. Secondly, the segregation ratio for the expected SDR origin of 2n-eggs was predictable, because of the distal position of the *amf* locus and the occurrence of a high

degree of chiasma interference (Chapter 2). This enabled us to detect the homozygous 2n-eggs of the *mcd-tbr* hybrid, that clearly deviated from the segregation expected for SDR 2n-egg formation (Table 1). The homozygosity of the 2n-eggs of this hybrid was demonstrated for 71 of the 73 progeny plants of the cross *Amf/amf* × *amf/amf/amf/amf*, and further confirmed in RFLP-analysis of another progeny of the same hybrid using four appropriate markers covering a major part of the chromosome 8. In view of the complete homozygosity of all progeny plants at all four RFLP loci tested, the two simplex *(Amf/amf/amf/amf)* plants were probably due to errors in the classification of duplex genotypes.

The suggested SDR 2n-egg formation in the other three hybids (Table 1) will be further confirmed for the *tbr-spg* hybrid through RFLP-analysis of a tetraploid progeny of (Chapter 6). These RFLP-analyses demonstrate the usefulness of the *amf* marker to facilitate the selection of clones with distinct restitution mechanisms.

Discovery of completely homozygous 2n-eggs

In potato, highly heterozygous to relatively homozygous 2n-eggs, that originated through FDR and SDR respectively, have been detected in the past (Iwanaga and Peloquin 1979; Stelly and Peloquin 1986a; Douches and Quiros 1988b; Jongedijk *et al.* 1991a; Werner *et al.* 1992; Barone *et al.* 1995). Nevertheless, diploid genotypes with completely homozygous 2n-eggs were not available. The present discovery of the *mcd-tbr* hybrid (HB93-7108-8) fills the gap and a complete spectrum of genotypes giving rise to highly heterozygous to 100% homozygous 2n-eggs are now available. Since the complete homozygosity of 2n-eggs can only be demonstrated with the use of multiple markers on a single chromosome, this discovery was possible on the account of the use of four informative RFLP marker loci (Chapter 4) covering a large part of the genetic length of the linkage map of chromosome 8 (Tanksley *et al.* 1992).

Evidence for post-meiotic restitution in 2n-egg formation

Besides homozygosity at all four RFLP loci of chromosome 8, the multilocus analysis of the 2n-eggs of the *mcd-tbr* hybrid demonstrated the frequent occurrence of

crossing-over between the two genomes in this hybrid. These recombinant and completely homozygous genotypes could not be the result of FDR or SDR 2n-egg formation, or a mixture of both. Assuming doubling of the chromosome 8 after the completion of normal meiosis in the *mcd-tbr* hybrid (Fig. 4), the recombination frequencies between the four RFLP loci were calculated to estimate the genetic distances. They closely corresponded with the expected map distances (Fig. 5), and confirmed the doubling of chromosome 8 following normal meiosis in the *mcd-tbr* hybrid (Fig. 4).

In sugarcane, Bremer (1959, 1961) presented cytological evidence for the occurrence of endomitotical division of chromosomes in a reduced nucleus during megasporogenesis, which is expected to be genetically equivalent to post-meiotic doubling of reduced gametes. In potato, genetic indications for the occurrence of post-meiotic doubling of reduced megaspores, which is expected to generate completely homozygous 2n-eggs, were reported by Stelly and Peloquin (1986a) as well as Douches and Quiros (1988b). In these genetic analyses, however, it was not possible to rule out the possibility of SDR 2n-egg formation in which no crossovers had occurred between the marker locus and its centromere, because of the use of a single marker gene per chromosome. Douches and Quiros (1988b) described the suggested post-meiotic doubling of the haploid products of normal meiosis as a type of SDR 2n-gamete formation. Since both the cytological events and the genetic consequences of post-meiotic doubling of the unreduced megaspores are very distinct from omission of the second division of the megaspore resulting in SDR 2n-eggs, we prefer to characterize this post-meiotic doubling as an additional mechanism called 'Post-Meiotic Restitution (PMR)' (Fig. 4).

The presence of only PMR 2n-egg formation in the *mcd-tbr* hybrid

In addition to the discovery of a new restitution mechanism, the multilocus analysis of the 2n-eggs of the *mcd-tbr* hybrid elucidated the number of restitution mechanisms involved. Only because of the complete homozygosity of the 2n-eggs for the four markers of chromosome 8, it was possible to conclude that all 2n-eggs were derived from PMR. In this way, we demonstrated the presence of a diploid clone with only one restitution mechanism of 2n-egg formation. This absence of a mixture of restitution

mechanisms could not have been demonstrated in a genetic analysis based on a single marker locus per chromosome.

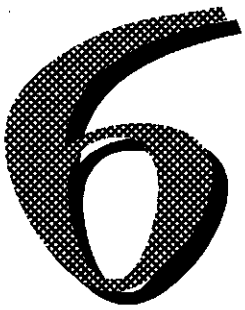
In order to confirm the presence of (only) post-meiotic restitution, it was not possible to use cytological methods, because the ovules in potato are not amenable for a critical study of meiosis and post-meiotic events. Alternatively, two indirect approaches could be conceived: 1) to analyse a tetraploid progeny that was derived from a cross between the diploid 2n-egg producer and a tetraploid male parent, as was done in this investigation; and 2) to analyse a diploid parthenogenetic progeny that was derived from 'prickle' pollinations of the 2n-egg producer (Hermsen and Verdenius 1973). The disadvantage of the first approach was that it required fully informative RFLP markers that distinguished a heterozygous pair of alleles in the diploid 2n-egg producer from the alleles of the tetraploid parent. Although labourious, it was possible to identify such markers (Chapter 4), and the present investigation showed the usefulness of these markers to elucidate the restitution mechanism of 2n-egg formation. The second approach of producing parthenogenetic progeny was attractive from the point of view that the genetic analysis of the diploid progeny would be more direct as compared to the analysis of the tetraploid progeny (Chapter 4). Moreover, parthenogenetic development of 2n-eggs of PMR-origin would allow the generation of completely homozygous genotypes.

Implications of PMR 2n-gametes

For the breeding of potato at the diploid level, it is important to realize that 2n-eggs are not always of SDR origin. This is because the genetic consequences of PMR are quite distinct from those of SDR (Fig. 1 and Fig. 4). The PMR 2n-eggs can be highly relevant for potato genetics because of the possibility to generate diploid homozygous genotypes. Since the cultivated potato is a highly heterozygous crop with a high degree of inbreeding depression and sterility, it is difficult to achieve homozygosity through repeated selfings. Alternatively, homozygous genotypes can be generated through the production of doubled monoploids by in vitro culture of anthers (Meyer *et al.* 1993; Veilleux *et al.* 1995), or stem explants of monohaploids (Uijtewaal *et al.* 1987b). Since

these genotypes are fixed, they can be propagated sexually as well as vegetatively. They can be used as tester lines in inheritance studies for analysing the extent of meiotic recombination, and for the generation of hybrids with maximal level of heterozygosity. Further more, the generation of both anther culture derived and PMR 2n-egg derived progenies would allow the use of gametic samples for genetic mapping to analyse sex differences in recombination (Rivard *et al.* 1996). Finally, the PMR 2n-eggs might be especially attractive when a F1 hybrid involving wild species, such as the ***mcd-tbr*** hybrid, is to be crossed with a tetraploid male and the undesirable genes are to be eliminated rapidly. For example, it is relatively easy to detect genotypes that are homozygous for a desired locus of ***mcd***, in which a substantial amount of the chromosomes are derived from *S. tuberosum* (Fig. 3).

Also in other crops, it is often desirable to produce homozygous genotypes through post-meiotic restitution (or doubling of haploids). These genotypes can serve different purposes, such as: 1) reduction of time required to obtain pure lines for hybrid seed production in, for example, onion (Campion *et al.* 1995) and maize (Wu 1986; Saisingtong *et al.* 1996) or for the direct selection of cultivars in barley (Devaux 1992); 2) fixed genotypes that can be propagated indefinitely by sexual means for replicated experiments; 3) mapping studies in, for example, *Brassica* (Ferreira *et al.* 1994), barley (Devaux *et al.* 1995), and pepper (Levebvre *et al.* 1995); 4) introgression programmes in breeding, such as nobilization in sugarcane (Bhat and Gill 1985), development of improved cultivars in *Brassica* (Chen and Beversdorf 1990), and production of alien addition, substitution and translocation lines, using the post-meiotic doubled microspores of intergeneric hybrids of wheat/rye (Wang *et al.* 1996).



Gene-centromere mapping and the detection of a single crossover per chromosome arm in potato

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GENE-CENTROMERE MAPPING AND THE DETECTION OF A SINGLE CROSSOVER PER CHROMOSOME ARM IN POTATO

ABSTRACT

Some fungi, such as *Neurospora* and *Sordaria*, produce spores in linearly grouped tetrads, reflecting the assortment of the four chromatids of each bivalent. Multilocus analysis of these individual spores resulting from a single meiosis enables the localisation of genetic loci in relation to the centromere and the assessment of the number of crossovers per chromosome arm. This tetrad analysis is not applicable in higher plants, because linear tetrads amenable for direct genetic analysis are lacking. However, plants occasionally produce gametes with sporophytic rather than gametophytic chromosome numbers. Since these numerically unreduced or 2n-gametes comprise two of the four chromatids of each bivalent, they can be exploited for both gene-centromere mapping and accurate determination of the extent of crossing-over. Based on this rationale, we have utilized a 2n-egg producing genotype of potato to map the centromere in relation to nine marker loci of chromosome 8. Our data provide critical estimates of recombination frequencies and strong evidence that not more than one crossover occurs in the long arm of this chromosome.

INTRODUCTION

The use of 2n-eggs is advantageous for genetic mapping in potato, because it allows the inspection of the sister chromatids of each half-bivalent. The reason for the presence of sister-chromatids is that, in female meiosis, the half-bivalents resulting from the reductional division are invariably separated by a cell wall during telophase 1. When nuclear restitution follows, only sister chromatids are included in one and the same 2n-megaspore, which is called Second Division Restitution (SDR) (Mok and Peloquin 1975; Ramanna 1979). As a result of crossing-over between non-sister chromatids at pachytene stage, the two chromatids of the SDR 2n-megaspores are identical from the centromeres till the site of the first crossover, whereas they are different at the parts distal to this crossover. Consequently, the 2n-eggs of a heterozygous genotype will be homozygous for loci near the centromere, and heterozygous for loci distal to the first crossover point (Fig. 1). Since the chance of a crossover is dependent on the distance to the centromere, the distal marker loci are more frequently heterozygous than the proximal loci. Based on these reasoning, SDR 2n-eggs of potato offer a suitable model system to map loci relative to the centromeres (Mendiburu and Peloquin 1979).

An additional advantage of using SDR 2n-eggs is the accurate identification of the number of crossovers per chromosome arm. Each single crossover and 50% of the double crossovers per arm can be identified in the SDR 2n-gametes by using markers that are evenly dispersed over the chromosome. This is twice as efficient as in the reduced (n-) gametes from normal meiosis, since single and double crossovers per arm can be detected in only 50% and 25% of the n-gametes, respectively (Fig. 1).

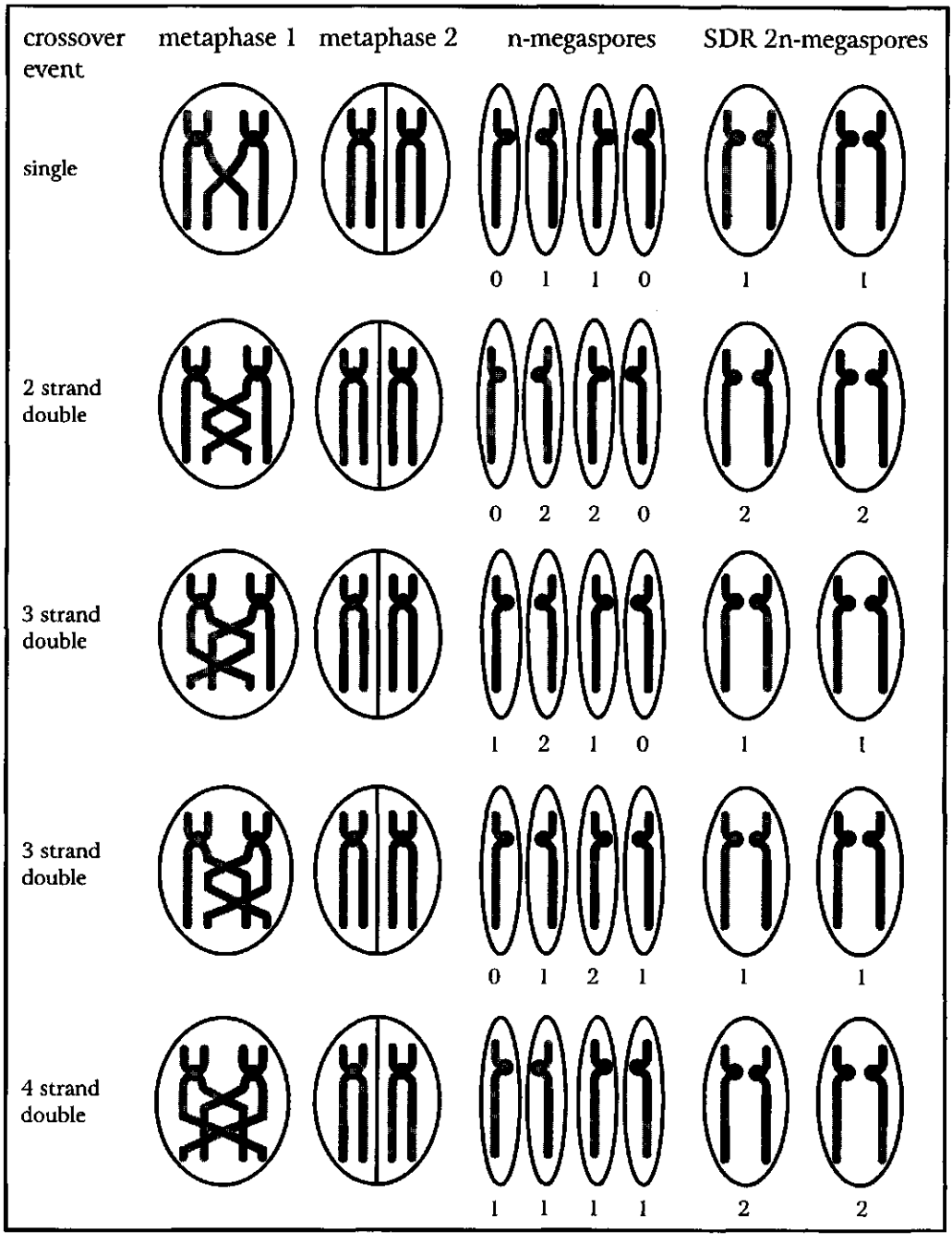
crossover event
metaphase 1
metaphase 2
n-megaspores
SDR 2n-megaspores
single
0 1 1 0
1 1
2 strand double
0 2 2 0
2 2
3 strand double
1 2 1 0
1 1
3 strand double
0 1 2 1
1 1
4 strand double
1 1 1 1
2 2

Figure 1. Identification of the centromeric region in SDR 2n-megaspores and the efficiency of crossover detection in multilocus analysis of n- and SDR 2n-megaspores.

At metaphase I, the homologous chromosomes of a diploid are grouped in bivalents. Each bivalent has two pairs of sister chromatids, one indicated in dark grey, the other in ligh grey. Each half-bivalent comprises both sister-chromatids, except for the regions that were involved in crossing-over between non-sister chromatids. As a result of this, the proximal part of a half-bivalent including the centromere is originated from sister chromatids, whereas the distal parts can be of non-sister origin. The ensuing SDR 2n-megaspores comprise the chromatids of these half-bivalents. Detection of the region that is derived from the sister-chromatids and that comprises the centromere is possible with the help of multilocus analysis of these SDR 2n-gametes. In this way, the position of the centromere can be localised in relation to other marker loci. When the position of the centromere is known, the number of crossovers per chromosome arm can be identified in both n- and SDR 2n-megaspores. However, not all crossover events have an effect that can be detected by means of multilocus analysis. The figures 0, 1, or 2 below the megaspores indicate the number of crossovers that can be detected after single and double crossover events. Single crossovers are detected in 50% of the n-megaspores, and in 100% of the SDR 2n-megaspores. Both crossovers of double crossover events are detected in only 25% of the n-megaspores, and in 50% of the SDR 2n-megaspores. So, the detection of crossovers in SDR 2n-megaspores is twice as efficient as in n-megaspores. Therefore, SDR 2n-gametes can be exploited for localisation of the centromere in relation to other marker loci, and the study of chiasma interference.

MATERIALS AND METHODS

RFLP-analysis of 2n-eggs in potato

The 2n-eggs from a diploid genotype of potato (*Solanum tuberosum)*, cannot directly be used for genetic analysis, but through diploid (2n=2x=24) × tetraploid (2n=4x=48) crossings, it is possible to generate progenies that can be used for genetic analysis (Swaminathan and Howard 1953). In potato, such a type of cross generally produces a tetraploid progeny because the triploid embryos that are derived from n-eggs of the diploid (n=x) and n-pollen of the tetraploid (n=2x) abort, whereas the tetraploid embryos that are derived from 2n-eggs (2n=2x) and n-pollen (n=2x) survive (Marks 1966). In this study, we used a 2n-egg producing interspecific hybrid (HB93-7054-3), that was selected from a cross between diploid *S. tuberosum* (*tbr*) × *S. speggazzinii* (*spg*) (Chapter 3). This *tbr-spg* hybrid was successful crossed with the tetraploid cultivar, Desirée, as male parent. Flow cytometric analysis confirmed the tetraploid level of all progeny plants (Chapter 3). Since each plant was derived from a 2n-egg (2x) of the *tbr-spg* hybrid and a n-pollen grain (2x) from the cultivar Desirée, genotyping of the 2n-eggs was only possible for marker loci that clearly distinguished the *tbr* and *spg* alleles of the diploid hybrid from the ♂ alleles of the cultivar Desirée.

Therefore, DNA samples of the *thr-spg* hybrid and the cultivar Desirée were tested for polymorphisms by using 31 RFLP markers of chromosome 8 in combination with six restriction enzymes. Five of them, the loci *TG536, TG228, TG513, TG434,* and *TG346,* showed both a unique *thr* and a unique *spg* allele of the hybrid (Chapter 3). These fully informative marker loci, previously mapped on chromosome 8 of tomato (Tanksley *et al.* 1992), were used for genotyping of 80 progeny plants. To facilitate the gene-centromere mapping, the progeny was also analysed for the tomato markers, *TG481* and *CT245a*, the potato and tomato marker *GP130,* and the marker gene for the *Granule-Bound Starch Synthase (GBSS)* in potato (Tanksley *et al.* 1992; Gebhardt *et al.* 1991). These four markers were only informative for one of the two alleles of the *thr-spg* hybrid.

RESULTS AND DISCUSSION

Gene-centromere mapping

Based on the genotypes for the *thr* and *spg* alleles at the five fully informative RFLP loci, the progeny could be divided into twelve classes (Fig. 2). These classes of 2n-eggs varied from complete homozygosity for all five markers up to different degrees of heterozygosity. The fully informative markers were ordered according to the frequency of plants that were heterozygous for the *thr* and *spg* alleles. The most remarkable marker was *TG228,* for which all 80 plants were homozygous. This indicated a very close linkage between the *TG228* locus and the centromere of chromosome 8. Seven plants were heterozygous for the marker *TG536,* and eleven plants were heterozygous for *TG513*. Since none of the plants were heterozygous at both *TG536* and *TG513*, these two loci were more closely linked to *TG228* than to each other. Therefore, *TG536* and *TG513* were localised on different arms of chromosome 8. The frequencies of plants that were heterozygous for *TG434* and *TG346* were 49 and 71, respectively. These two loci could be localised on the same arm as *TG513,* because all plants heterozygous for *TG513* were also heterozygous for *TG434* and for *TG346.*

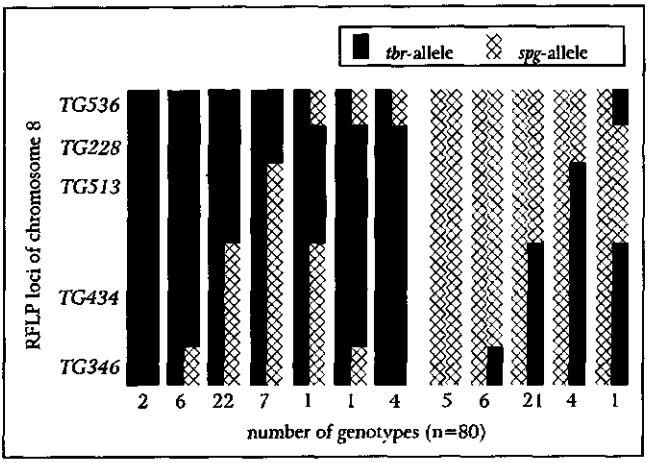

Figure 2. Twelve classes of 2n-eggs based on their genotypes for five selected markers of chromosome 8 showing the position of the centromere and not more than one crossover per chromosome arm. The RFLP loci, *TG536, TG228, TG513, TG434,* and *TG346* were found to be informative for the detection of both the *tbr* and the *spg* allele of the 2n-eggs of the *tbr-spg* hybrid in the tetraploid progeny derived from the cross between the *tbr-spg* hybrid and the cultivar Desirée. The map positions of these RFLP loci were known from the tomato map of Tanksley (1992). Because of the synteny between the potato and tomato maps, the order of the five markers in the tomato map was retained for presenting the genotypic classes of the 2n-eggs of the *tbr-spg* hybrid. The progeny plants from the cross *tbr-spg* hybrid × Desirée, representing 80 2n-eggs were analysed for all five markers. Twelve genotypic classes of SDR 2n-eggs of the diploid *tbr-spg* hybrid were found. These genotypes are illustrated in pairs of two bars, representing both chromatids of the original half-bivalent, in which the parts of *tbr* origin are indicated in solid black, and the parts of *spg* origin are netted. The figure below each pair of bars, represents the number of 2n-eggs per genotypic class.
At RFLP locus *TG228,* all 2n-eggs were homozygous for either the *tbr* or the *spg* allele, whereas the number of heterozygotes increased according to the more distal position of the RFLP locus tested. This implied a position of the centromere of chromosome 8 very close to *TG228* with *TG513, TG434,* and *TG346* on the long arm, and *TG536* on the short arm. In all genotypes, not more than one crossover was observed at both sides of locus *TG228,* indicating the occurrence of a single crossover per chromosome arm.

The four markers that identified either the *tbr* or the *spg* alleles were ordered in relation to the fully informative markers on the basis of the detectable homozygotes. Both *TG481* and *GP130* cosegregated with *TG513*, and *CT245a* was localised between *TG513* and *TG434*. Finally, the locus *GBSS* was shown to occupy the most distal position on this arm. The distal position of the *GBSS* locus has also been demonstrated earlier by means of gene-centromere mapping in 2n-pollen (Chapter 2). The order of loci in our gene-centromere mapping neatly fitted with the order of the synteny maps for tomato and potato (Bonierbale *et al.* 1988; Tanksley *et al.* 1992; Gebhardt *et al.* 1991; Jacobs *et al.* 1995).

This is the first report that proves the close linkage between the centromere of chromosome 8 and the RFLP locus *TG228*. This proximal position of *TG228* has been confirmed in other linkage analyses including DNA sequences that localized around the putative tomato centromeres (Broun and Tanksley 1996; Presting *et al.* 1996). The orientation of the RFLP linkage map with the cytological map of chromosome 8 in tomato (Tanksley *et al.* 1992) showed that the loci *TG513, TG481, GP130, CT245a, TG434*, and *TG346* were localised on the long arm of chromosome 8. Because of linkage to these markers, *GBSS* could be identified as a distal marker on the long arm of chromosome 8 of potato. This locus seems to coincide with the locus of the *Waxy* (*Wx*) gene in tomato. Since both loci are involved in starch metabolisms, they may refer to the same gene. The identification of the centromere and the long arm in the genetic map of chromosome 8 of potato enabled to identify the RFLP marker *TG536* as a marker on the short arm of this chromosome.

The number of crossovers on the long arm of chromosome 8

The multilocus data presented in Fig. 2 enable an estimation of the number of crossovers per chromosome arm. This was possible because of the observed homozygosity of all 80 2n-eggs for the locus *TG228*, that allowed to assign the centromere of chromosome 8 within a genetic distance of 1 cM (100/(2×80)) from this locus. Surprisingly, there were no genotypes indicating the occurrence of more than one crossover per chromosome arm. Because the traditional genetic analyses assume more than one crossover per chromosome arm, more attention is given to the data of Fig. 2 to present evidence for the occurrence of at most one crossover in our material. To this end, we consider the consequences of double crossovers.

Firstly, two strand double crossovers between *TG228* and *TG346* would have resulted in genotypes homozygous for either the *spg* or the *tbr* allele at both *TG228* and *TG346*, and heterozygous loci in between (Fig. 1). These genotypes were not observed. Despite the fact that such genotypes may have been hard to identify in our data set because of the gap between *TG513* and *TG434* (49 cM, according to the tomato map of Tanksley *et al.* 1992), the absence of these genotypes is remarkable. (Genotypes resulting from three strand doubles cannot be distinguished from those arising from single crossover bivalents, and, therefore, are

not informative in detecting double crossovers.)

Secondly, the genotypes resulting from four strand double crossovers should be homozygous for the *spg* allele at locus *TG228* and homozygous for the *thr* allele at locus *TG346* or vice versa. Also these genotypes were not observed (Fig. 2), whereas they were expected to occur in 25% of the double crossover 2n-gametes (Fig. 1). According to the formula described by Jongedijk *et al.* (1991), we have calculated that the expected frequency of such genotypes equals 4.6% over a distance of 77 cM (distance between *TG228* and *TG346* in the tomato map of Tanksley *et al.* 1992). The probability of its total absence in a sample of 80 plants is less than 0.025.

Thirdly, if multiple crossovers per arm were the rule rather than the exception, the frequency of genotypes heterozygous at the most distal marker would be between 50 and 75% (67% being the limiting frequency as the number of crossovers increases; Jongedijk *et al.* 1991). We observed 86% SDR 2n-eggs being heterozygous at *TG346* (Fig. 2), which is well above this theoretical range.

In addition to these three pieces of evidence from the fully informative markers, it should be noticed that all segregation data for the four markers that were only informative for the presence of either the *thr* or the *spg* allele also supported the observation of at most one crossover per arm. Taken together, this research represents strong evidence that a single crossover has occurred in the long arm of chromosome 8.

Estimating map distances

Multilocus analysis of 2n-eggs allows the mapping of marker loci in relation to the centromere rather than relative to the most distal marker on the short arm (Fig. 3). The use of the centromere as the origin of a genetic map reflects more the permanent reference point of the chromosome and has also the advantage that its position is independent from the mapping of new marker loci. In contrast, a map in which the most distal marker on the short arm is used as genetic origin needs to be revised each time when a more distal marker is identified.

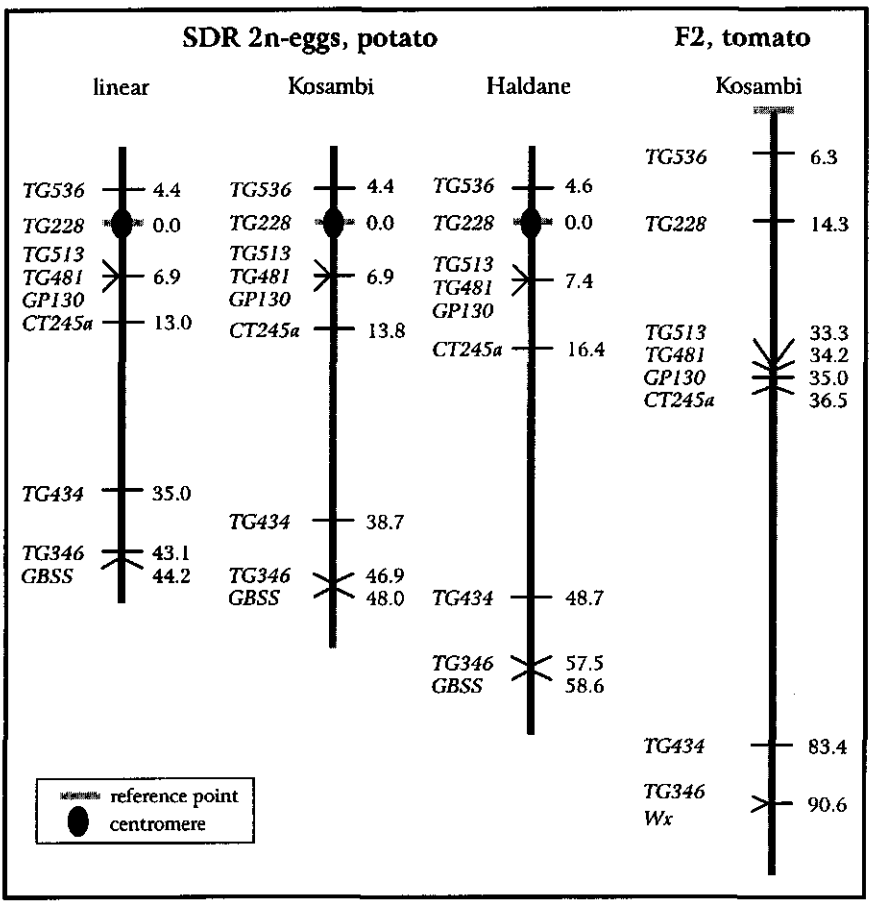

Figure 3. Genetic maps of chromosome 8, applying different mapping functions to the estimates of recombination frequency between adjacent markers in the 2n-eggs of the *thr-spg* hybrid, as compared to the tomato map of Tanksley *et al.* (1992).
In the maps based on 2n-eggs, all marker loci are localised in relation to the centromere (*TG228*). In the map based on n-gametes (F_2 plants), the markers are localised relative to the most distal marker on the short arm.

Also the complete chiasma interference hypothesised for chromosome 8 has consequences for the genetic map. At most one crossover per arm results in a linear mapping function, as opposed to the curvilinear mapping functions that are generally used for map construction from recombination data. For small recombination frequencies, however, the discrepancy is small. Therefore, constructing a map by adding small adjacent distances will not seriously influence the estimation of the total map length. Our data allow the estimation

of pairwise recombination frequencies for the adjacent markers of chromosome 8. Figure 3 shows the difference between the maps obtained by applying the (correct) linear mapping function, Kosambi's function, and Haldane's mapping function. The latter assumes the absence of interference. Since the genetic distances based on Kosambi's mapping function were smaller for the SDR 2n-eggs of the *tbr-spg* hybrid than for the F_2 population of tomato of Tanksley (1992), we concluded that the recombination rate in the *tbr-spg* hybrid was lower.

Chiasma interference

Multi-allelic marker loci that cover a whole chromosome arm were not used in earlier genetic investigations of 2n-gametes. Therefore, our multi locus analysis of SDR 2n-egg cells is the first report about the genetic assessment of the number of crossovers in potato. In one of the earlier analyses of 2n-eggs in potato, Ross and Langton (1974) showed a high frequency of 2n-eggs heterozygous for the *Rx* locus. They concluded that it was unlikely that all these genotypes resulted from SDR 2n-eggs formation with crossing over between the centromere and this locus. Later genetic and cytological studies in potato, however, provided evidence for the SDR origin of 2n-eggs (Jongedijk 1985, Stelly and Peloquin 1986a; Douches and Quiros 1988b). In addition, only 1 to 2 chiasmata per bivalent were observed in chiasma counts in diploid potato (Swaminathan and Howard 1953; Jongedijk and Ramanna 1989), suggesting a high level of chiasma interference (Sybenga 1996). Comparable to potato, a high level of chiasma interference was demonstrated to occur in different species of fishes and molluscs, as revealed by multi locus analysis of parthenogenetic progenies that were derived from 2n-gametes (Thorgaard *et al.* 1983; Liu *et al.* 1992; Guo and Allen 1996). These studies suggested that complete chiasma interference resulting invariably in a single crossover per arm in all gametes is more common than indicated by Ross and Langton (1974).

In our study, the position of the crossover seems to be random, but when a crossover event takes place it prevents any other crossover on the same arm. In mollusc, however, these sites of the single crossover seem to be preferentially located in recombination hot-region proximal to the centromere (Guo and Allen 1996). It remains intriguing what mechanism is responsible for only one crossover per chromosome arm. In order to establish the prevalence

of complete chiasma interference in other genotypes of potato and in different organisms, it is essential to analyse the segregation products derived from single meioses for a set of markers per chromosome. If the position of the centromere is known, both n- and 2n-gametes can be used for analysing the number of crossovers per chromosome arm. Since each half-bivalent equals two chromatids, the estimates of recombination frequencies in 2n-gametes are more accurate.

Implications of multilocus analysis of 2n-gametes

Unreduced gametes occur or can be induced in different crop plant species, such as orchard grass, maize, sugarcane, clover, cassava, rye, alfalfa, lilly, banana, and blueberry (Veilleux 1985). In addition, in animals, it is possible to exploit the ovarian teratomas (Ott *et al.* 1976; Christian *et al.* 1995), the isolated first polar bodies or second oocytes in mammals (Cui *et al.* 1992; Jarrell *et al.* 1995), and the parthenogenetic progenies derived from 2n-gametes in several fish species (Thorgaard *et al.* 1983; Liu *et al.* 1992; Johnson *et al.* 1996). This general prevalence of 2n-gametes allows improvement of genetic maps in many organisms, as has been demonstrated in this study for chromosome 8 of potato. Known map positions of centromeres are implicit for investigating the structure and functions of the centromeric regions. Furthermore, multilocus analysis of 2n-gametes allows accurate estimates of recombination frequencies at different parts of chromosomes that are essential for the refinement of the genetic distances. Finally, 2n-gametes offer favourable genetic material to study the intriguing mechanism of crossover-interference.

General discussion

GENERAL DISCUSSION

The occurrence of gametes with the somatic (or sporohytic) chromosome number in potato, as the result of meiotic aberrations, is already known for more than sixty years (Müntzing 1933, Oppenheimer 1933, Swaminathan and Howard 1953, Rees-Leonard 1935). These numerically unreduced gametes or 2n-gametes are the prerequisite for sexual polyploidization. This is also widely recognized as the origin of the polyploid species in the Solanums (Harlan and De Wet 1975, Den Nijs and Peloquin 1977, Iwanaga and Peloquin 1982) and allows interploidy crosses between diploid wild *Solanum* species and the tetraploid cultivated potato (Chase 1963; Peloquin 1983). Furthermore, 2n-eggs are important for diplosporic apomixis, *i.e.* the formation of embryos from egg cells without fertilization (Koltunow 1993). Since 2n-gametes are equivalent to half-tetrads, they can also be used for the purpose of gene-centromere mapping (Rhoades and Dempsey 1966; Mendiburu and Peloquin 1979). This thesis describes the genetic analysis of different types of 2n-gametes, such as First Division Restitution (FDR) 2n-gametes of a diploid clone with normal chromosome pairing and bivalent formation (Chapter 2), FDR 2n-gametes of a synaptic mutant with highly reduced recombination (Chapter 2), Post-Meiotic Restitution (PMR) 2n-gametes (Chapter 5), and Second Division Restitution (SDR) 2n-gametes (Chapter 6). These different types of 2n-gametes have been detected with the help of a strategic use of marker genes. In this chapter, the significance of using different genetic markers for the elucidation of the modes of origin of 2n-gametes will be discussed. Furthermore, the utility of the different types of 2n-gametes for genetics will be highlighted.

GENETIC ANALYSIS OF 2N-GAMETES

Genotyping of 2n-gametes using a single marker gene

The gene for *amylose-free starch (amf)* in potato was used as a genetic marker for the analysis of 2n-gametes, because of its excellent possibilities to genotype tetraploids, that were derived from diploid *(Amf/amf)* × tetraploid *(amf/amf/amf/amf)* crosses. This is because the

expression of this gene in microspores allowed to monitor, after iodine staining, the segregation of microspores with blue *(Amf/.)* and red *(amf/amf)* staining starch of tetraploid progenies. This segregation enabled to classify the nulliplex, simplex and duplex genotypes, that were derived from the *amf/amf, Amf/amf,* and *Amf/Amf* 2n-gametes of the diploid parent, respectively. It was even possible to directly monitor the blue *(Amf/.)* and red *(amf/amf)* 2n-microspores of diploid clones, since the unreduced (n-) microspores could be distinguished by size (Chapter 2).

Another reason for using the *amf* marker gene was its distal position on chromosome 8 of potato, allowing the investigation of the consequences of crossing-over on the genetic composition of 2n-gametes. The distal position of the *amf* marker gene on chromosome 8 of potato was already known from the RFLP-maps of Gebhardt *et al.* (1991) and Jacobs *et al.* (1995). They published the *GBSS* locus of the *amf* marker gene on top of the genetic map of chromosome 8. However, it was not known if this marker gene was localised on the short or long arm of chromosome 8, because the orientation of the map was unknown. In tomato, the orientation of the RFLP-map of chromosome 8 was known, since it was integrated with the available classical map in which some markers were localised on the short and long arms using deletion mapping (Khush and Rick 1968; Tanksley *et al.* 1992). For this chromosome, it was not possible to align the potato maps of Gebhardt *et al.* (1991) and Jacobs *et al.* (1995) with the tomato map of Tanksley *et al.* (1992), because there were very few markers in common. In this thesis, the distal position of the *amf* marker was confirmed in different gene-centromere mapping experiments. The gene-centromere distance was estimated as 48.8 cM using the FDR 2n-microspores of the clone B92-7015-4, and as 39.5 cM using the 4x.2x-progenies of this clone (Chapter 2). From the genotypes of the SDR 2n-eggs of the three clones HB93-7004-6, HB94-7239-5, and HB93-7054-3 (Chapter 5), the gene-centromere distance was estimated as 41.0 cM. In the RFLP-analysis of the SDR 2n-eggs of the diploid Tuberosum- *S. spegazzinii* hybrid, HB93-7054-3, the *GBSS* locus was mapped at a gene-centromere distance of 44.2 cM (Chapter 6). Using also seven tomato markers of chromosome 8, the *GBSS* locus was assigned to the long arm of chromosome 8 (Chapter 6). This information indicates that the orientation of the chromosome 8 in the RFLP maps of Gebhardt *et al.* (1991) and Jacobs *et al.* (1995) as well as in the AFLP map of Van Eck *et al.*

(1995) should be reversed in order to make it concurrent with the normal convention (the short arm at the top).

Because of the distal position of the *GBSS* locus on the long arm of chromosome 8 and the possibilities to identify all three genotypic classes of 2n-gametes, the *amf* marker gene was very convenient to show the genetic consequences of the restitution mechanism and the extent of crossing-over. In the analysis of FDR 2n-gametes of a desynaptic clone and the FDR, SDR and PMR 2n-gametes of normal synaptic clones, the genotypes for the *amf* marker varied from almost completely homozygous to completely heterozygous (Fig. 1).

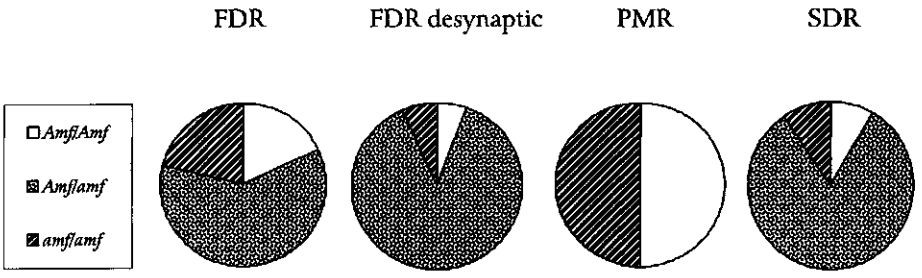

Figure 1. Proportion of *AmfAmf, Amfamf* and *amfamf* genotypes in different types of 2n-gametes of heterozygous *(Amfamf)* clones.
The genotypes of the 2n-gametes were analysed using the tetraploid progenies of *Amfamf* × *amfamfamfamf* and *amfamfamfamf* × *Amfamf* crosses.

In order to identify different types of 2n-gametes and to show the level of heterozygosity of 2n-gametes at a locus, it is essential to distinguish the heterozygous 2n-gametes from the homozygous ones. In contrast to many isozyme and morphological markers, it is possible to use the *amf* marker in tetraploid progenies of 2x.4x- or 4x.2x-crosses to indirectly identify the three classes of genotypes of the 2n-gametes of the diploid parent. In view of the distinctly different frequencies of the *AmfAmf, Amfamf,* and *amfamf* genotypes

in different types of 2n-gametes the *amf* marker was shown to be a strategic marker for genetic analysis of 2n-gametes (Fig. 1). Furthermore, the recombination percentages between the centromere and the *amf* marker of the FDR and SDR 2n-gametes in all four investigated clones with normal chromosome pairing were high, and indicated the frequent occurrence of a high level of chiasma interference in potato.

Genotyping of 2n-gametes for multiple markers per chromosome

Multilocus analysis of 2n-eggs using RFLP markers in 2x.4x-progenies

Having reconsidered the use of a single marker gene that can identify the proportions of the three genotypic classes of 2n-gametes of the diploid parent using the 2x.4x-progenies in order to distinguish different types of 2n-gametes, it is relevant to consider the advantages of multilocus analysis of 2n-gametes. To this end, the use of RFLP markers for the genetic analysis of the tetraploid progenies from 2x.4x- or 4x.2x-crosses to genotype the 2n-gametes of the diploid parent will be discussed.

For the genetic analysis of 2n-gametes it is essential that the 2n-gamete producing diploid is heterozygous at a locus (m^1m^2) and that both alleles can be identified in the progeny in order to classify all three genotypes (m^1m^1, m^1m^2, and m^2m^2) of 2n-gametes. To select such fully informative marker loci for the analysis of 2n-gametes, the RFLP patterns of the parents of eleven 2x.4x-progenies were analysed to identify the RFLP loci with two unique alleles in the diploid parent as compared to the tetraploid parent. In this way, three diploid parents were available for the analysis of 2n-eggs at four to seven fully informative RFLP loci of chromosome 8 in potato (Chapter 4). For each of these three 2x.4x-progenies, the informative RFLP loci were more or less evenly distributed on chromosome 8 and covered 84 cM of the total map length of 95 cM (Tanksley *et al.* 1992).

Two of the three tetraploid 2x.4x-progenies were analysed. The first one, the progeny of the *mcd-tbr* hybrid (HB93-7108-8) × 105ESC90-32, was analysed at four loci, whereas the second one, the progeny of the *tbr-spg* (HB93-7054-3) × Desirée, was anaysed at five fully informative loci of chromosome 8. These multilocus analyses demonstrated the PMR origin of the 2n-eggs in the *mcd-tbr* hybrid (Chapter 5), and the

SDR origin of the 2n-eggs in the *tbr-spg* hybrid (Chapter 6).

For further analysis of the 2n-eggs in the third hybrid, the *chc-tbr* hybrid, the progeny of the *chc-tbr* hybrid (HB94-7239-5) × Desirée with at least seven informative RFLP loci of chromosome 8 is available. Based on the segregation for the *amf* marker, the 2n-eggs of this hybrid are expected to be of SDR origin (Chapter 5). Therefore, additional multilocus analysis of the 2n-eggs in this hybrid can elucidate whether the SDR origin is the only restitution mechanism and whether there is a single crossover per arm, as was observed for the *tbr-spg* hybrid (HB93-7054-3).

The additional value of multilocus analysis of 2n-gametes

It is important to realize that only because of the use of multiple marker loci on the same chromosome for the analyses of 2x.4x-progenies it has been possible to elucidate the mode of origin of 2n-eggs in two diploid clones and to investigate the extent of crossing-over. In this context, the results of the RFLP-analyses of the PMR and SDR 2n-gametes will be discussed in more detail in order to demonstrate the relevance of the information about the genetic compositions at different loci. Also the theoretically expected genotypes of FDR 2n-gametes of a normal synaptic and an asynaptic clone for different marker loci, randomly distributed on the chromosome 8 of potato (Fig. 2), will be included in this discussion.

The distinct differences in genetic composition between the different types of 2n-gametes as shown for the *amf* marker (Fig. 1) are more pronounced when multiple markers per chromosome are included in the genetic analysis (Fig. 2). Whereas the genotypes for the *amf* marker of the SDR 2n-gametes of a normal synaptic clone are very similar to those of FDR 2n-gametes of a desynaptic clone, the genetic compositions are very distinct when proximal markers are analysed. The multiple markers per chromosome also allow to identify the level of heterozygosity at different parts of the chromosomes. It shows the possibilities to obtain completely homozygous (PMR) to completely heterozygous (FDR asynaptic) 2n-gametes.

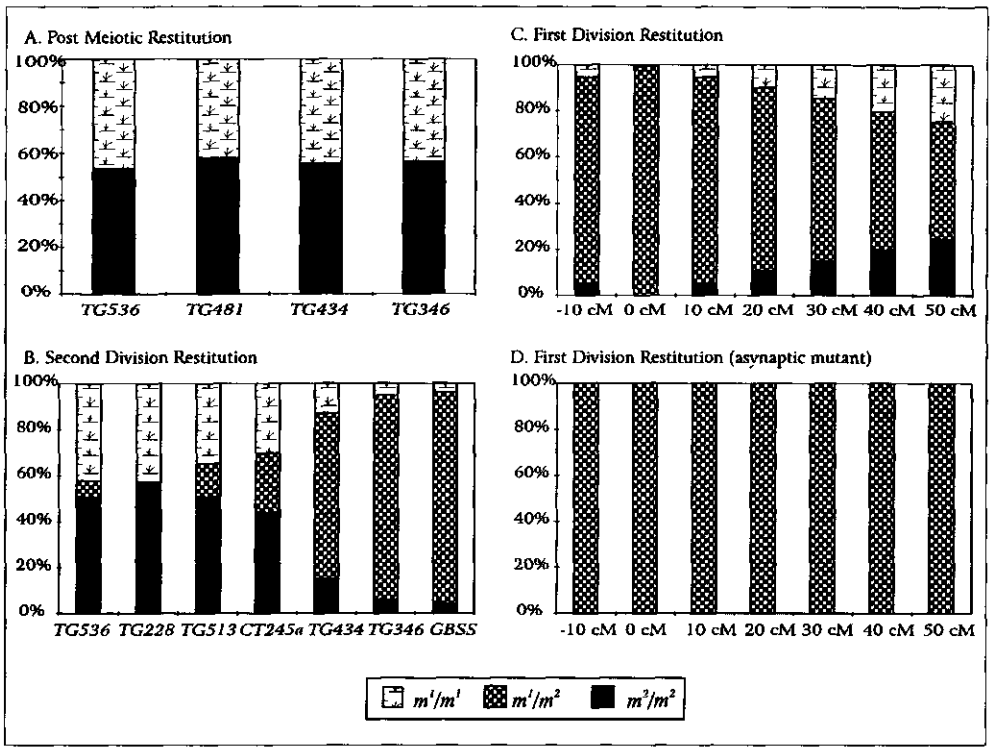

Figure 2. Observed (A,B) or theoretically (C,D) expected frequencies of m^1m^1, m^1m^2, and m^2m^2 genotypes in the different types of 2n-gametes of diploid (m^1m^2) clones for multiple marker loci on a same chromosome.

A) Genotypes of PMR 2n-gametes for RFLP loci of chromosome 8: experimental data

B) Genotypes of SDR 2n-gametes for RFLP loci of chromosome 8: experimental data

C) Genotypes of FDR 2n-gametes for multiple loci on one chromosome: theoretical model

D) Genotypes of FDR 2n-gametes of an asynaptic mutant for multiple loci on one chromosome: theoretical model

For the elucidation of the numbers of crossovers between loci, special attention was paid to the maternal or paternal origin of the alleles at the different loci per chromosome within the individual genotypes of 2n-gametes of the diploid genotypes. The presence or absence of crossovers is important to identify completely homozygous 2n-gametes as either SDR 2n-gametes where the recombination is severely reduced or PMR 2n-gametes (Chapter 5). Another important application of analysing the extent of crossing-over is the gene-centromere mapping of loci using FDR or SDR 2n-gametes of clones with meiotic

recombination (Rhoades and Dempsey 1966; Mendiburu and Peloquin 1979; Chapter 6). More over, the analysis of the genetic composition for the maternal and paternal alleles at different loci per chromosome allows to elucidate the restitution mechanism of individual 2n-gametes of the diploid clone. To distinguish a FDR from a SDR 2n-gamete the proximal markers that show no crossovers between the locus and the centromere are the most informative (Fig. 2). In this context, the RFLP locus *TG228* has been very important to conclude that all 2n-eggs of the *mcd-tbr* hybrid originated through SDR (Chapter 6). To distinguish a PMR from a SDR gamete, the distal markers are most informative.

The use of fully informative markers that can distinguish between the three genotypic classes of 2n-gametes (m^1m^1, m^1m^2, and m^2m^2) of heterozygous clones (m^1m^2) as well as the analysis of the genetic composition for the maternal and paternal alleles at multiple markers per chromosome are significant additions to the genetic analysis of 2n-gametes as compared to the earlier studies in potato (Ross and Langton 1974; Mendiburu and Peloquin 1976, 1977a, b; Taylor 1978; Iwanaga 1984; Stelly and Peloquin 1986a; Douches and Quiros 1987, 1988a, b; Werner and Peloquin 1987, 1991; Watanabe and Peloquin 1989, 1993; Jongedijk *et al.* 1991b; Werner *et al.* 1992; Barone *et al.* 1995). The essential difference is that multiple fully informative markers per chromosome can be used to unravel the processes of nuclear restitution and crossing-over. This is because the genetic composition of the 2n-gametes for the maternal and paternal alleles at the multiple loci per chromosome can demonstrate whether the pair of chromosomes in the 2n-gamete originated from the two sister chromatids (SDR), the two non-sister chromatids (FDR) or through duplication of a single chromatid (PMR). In genetic analysis using a single marker per chromosome, however, it is not possible to unravel the genetic consequences of crossing-over and the restitution mechanism. Also genetic markers that are not able to distinguish the maternal and paternal alleles of the 2n-gamete producing diploid should be avoided.

Increasing the number of informative loci for the genotyping of 2n-gametes

In view of the need for multiple marker loci per chromosome, the identification of the alleles using tetraploid progenies, and the importance of proximal markers to

distinguish between FDR and SDR 2n-gametes the utility of morphological, isozyme markers, and AFLP markers will be limited. The localisation of nine RFLP-markers of chromosome 8 in relation to the centromere using the SDR 2n-eggs of the *mcd-tbr* hybrid (Chapter 6) clearly demonstrated the utility of these RFLP markers for multilocus analysis of 2n-gametes. In most combinations of 2x and 4x crossing parents, however, the frequency of useful polymorphisms for the RFLP loci of chromosomes 6 and 8 was very low. Especially in the case of chromosome 6, only a low frequency of the RFLP loci tested was informative (Chapter 4). In order to localise the positions of the centromeres of all chromosomes in potato, also microsatellite markers are expected to be very useful for multilocus analysis of 2n-gametes. This is because microsatellite markers are codominant and multi-allelic. Furthermore, in tomato, the GATA microsatellites are mapped to clusters associated with centromeric regions (Broun and Tanksley 1996; Grandillo and Tanksley 1996).

Alternatively, another type of progeny can be generated in order to increase the number of marker loci that are informative for the analysis of 2n-gametes. The generation of progenies from unfertilized 2n-eggs through prickle pollination was not successful in the clones investigated. However, it is not unlikely that this method can be successful in other genotypes, since the induction of parthenogenesis is genotype depended (Hermsen and Verdenius 1973; Breukelen *et al.* 1977; Uijtewaal *et al.* 1987a). To generate progenies from unfertilized 2n-pollen in potato, anther culture can be considered (Meyer *et al.* 1993; Veilleux *et al.* 1995). It is also possible to generate another type of hybrid population from 2n-gametes. Instead of a tetraploid pollinator, for example, also a 2n-pollen producing diploid clone can be used as the male parent to generate a hybrid population from 2n-eggs. The advantage of using the progeny of a 2x.2x-cross instead of a 2x.4x-cross for monitoring the genotypes of the 2n-eggs of the diploid parent is that the alleles of the diploid parent has to be distinguished from two chromosomes of the diploid male parent instead of the four chromosomes of the tetraploid pollinator. Finally, if homozygous diploid clones with functional 2n-pollen or homozygous tetraploid clones with functional n-pollen become available in potato, these will be the perfect pollinators to be used in 2x.2x- or 2x.4x-crosses in order to generate progenies with a high number of informative polymorphisms to allow

multilocus analysis of the 2n-eggs of the diploid parent for all chromosomes.

EXPLOITATION OF THE DIFFERENT TYPES OF 2N-GAMETES IN GENETICS

The different types of 2n-gametes that are characterized in this thesis can be exploited for the generation of valuable information and materials for genetics. First of all, the study of the different meiotic mutants can increase the understanding of the meiotic processes. Secondly, the PMR 2n-gametes can be used to generate homozygous and fixed genotypes. Thirdly, the FDR and SDR 2n-gametes can be used for gene-centromere mapping. Fourthly, the genetic analysis of SDR 2n-gametes allows the accurate estimation of the extent of crossing-over.

The study of 2n-gamete formation to increase the knowledge of meiotic processes

Despite the fact that 2n-gamete producing genotypes show a mixture of normal and abnormal meioses, they are considered as meiotic mutants. One of the most intriguing features about the 2n-egg formation in both the *mcd-tbr* hybrid and the *tbr-spg* hybrid in this context is the formation of n- as well as 2n-eggs in combination with the discovery that all functional 2n-gametes are derived from the same restitution mechanism. Therefore, the identification of the restitution mechanism(s) per clone using multilocus analysis of 2n-gametes can be the first step in the procedure to investigate the expression of genes involved in the occurrence of nuclear restitution. The second step is the cytological characterization of the meiotic aberration(s). The third step is the assessment of the inheritance of the nuclear restitution through the use of test crosses, and the mapping of the genes involved. Finally, the genes involved in nuclear restitution have to be cloned and characterized.

The seed set following 2x.4x-crosses (Chapter 3) indicated a high level of 2n-egg formation in the *mcd-tbr* hybrid that produced PMR 2n-eggs (Chapters 5), and a moderate level of 2n-egg formation in the *tbr-spg* hybrid that produced SDR 2n-eggs (Chapter 6). In view of the pureness of the restitution mechanism in both clones as demonstrated in multilocus analysis, these levels of 2n-egg formation might be sufficient to allow the next step in the characterization of the meiotic mutants, *i.e.*, the cytological elucidation of the

deviations in megasporogenesis or megagametogenesis. One option to cytologically investigate the occurrence of nuclear restitution in 2n-egg formation is to clear the ovules and measure the nucleolus sizes of nuclei in megaspores and female gametophytes (Stelly & Peloquin 1986b; Werner & Peloquin 1987; Conicella *et al.* 1991; Jongedijk *et al.* 1991b).

Since the multilocus analysis showed the PMR origin of the 2n-eggs in the *mcd-tbr* hybrid, the moment of chromosome doubling during the 2n-egg formation in this clone could be directed to the stages following the second meiotic division. In this context, a preliminary cytological study was established in which cleared ovules of the *mcd-tbr* hybrid were analysed and compared with those of a diploid control as well as a tetraploid control clone. As expected, normal megasporogenesis was observed in the *mcd-tbr* hybrid. However, the measurements of nucleolus sizes of the 1-8 nucleate embryo sacs in the hybrid as well as in the diploid and tetraploid control clones varied too much in order to distinguish between reduced (n) and unreduced (2n) embryo-sacs (unpublished results). Because this method was inaccurate, it was not possible to analyse the moment of chromosome doubling in the *mcd-tbr* hybrid. For a more accurate analysis of the ploidy level of nuclei, it would be worthwhile to apply specific DNA stains to quantify the DNA content of the nuclei in the 1-8 nucleate embryo sacs.

Since both the parents and full sibs of the *mcd-tbr* hybrid (HB93-7108-8) are still available also investigation of the inheritance of PMR 2n-egg formation will be possible. From the 2x.4x-crosses (Chapter 3) it is known that both genotypes that produce 2n-eggs and genotypes without 2n-eggs are present in the family HB93-7108. In order to obtain information about the inheritance of PMR 2n-egg formation, the *mcd* parent (*Mcd*18568-B16) and the *tbr* parent (87-1031-29) as well as the progenies from crosses between genotypes of the HB93-7108 family with and without 2n-egg formation have to be tested in 2x.4x-crosses to estimate the level of 2n-egg formation.

The use of PMR gametes to generate homozygous lines

To obtain homozygous genotypes from PMR 2n-gametes, further development of techniques that allow the generation of progenies from unfertilized 2n-gametes is required. Such homozygous genotypes would be very convenient to be used in test crosses to

investigate the inheritance of characters, for example, in the mentioned multilocus analysis of 2n-eggs using the progenies of 2x.2x- or 2x.4x-crosses. Furthermore, crosses between homozygous genotypes would allow the generation of highly heterozygous genotypes from True Potato Seeds.

The use of FDR and SDR 2n-gametes for gene-centromere mapping

Since both the FDR and the SDR 2n-gametes allow to monitor two of the four strands of each bivalent, they can be used for gene-centromere mapping (Rhoades and Dempsey 1966; Mendiburu and Peloquin 1979). The gene-centromere mapping of nine RFLP loci of chromosome 8 using the SDR 2n-eggs of the *mcd-tbr* hybrid showed the locus *TG228* to be within a genetic distance of 1 cM from the centromere (Chapter 6). This gene-centromere distance is the first direct evidence for the position of the centromere in the genetic map of chromosome 8 in potato. In view of the synteny between tomato and potato maps, this position will be relevant for the genetic map of tomato as well. The mapping of loci in relation to the centromeres is important to facilitate the consolidation of the number of linkage groups to the number of chromosomes (Johnson *et al.* 1996) and to identify permanent reference points in the genetic maps. The map positions of centromeres are also implicit for investigating the structure and functions of the centromeric regions. To this end, different methods, such as deletion mapping (Wordragen *et al.* 1994), dosage analysis of trisomics (Frary *et al.* 1996) and clustering of marker genes in the regions around the putative centromeres (Presting *et al.* 1996; Broun and Tanksley 1996; Grandillo and Tanksley 1996), have been used to obtain indirect indications for the positions of centromeres in the genetic maps. Despite the examples of multilocus gene-centromere mapping in animals (Cui *et al.* 1992; Jarrell *et al.* 1995; Da *et al.* 1995) and fishes (Allendorf *et al.* 1986; Estoup *et al.* 1993; Johnson *et al.* 1995), the saturated molecular maps that are available for many plant species and the possibilities for gene-centromere mapping using 2n-gametes in plants (Rhoades and Dempsey 1966; Nel 1975; Mendiburu and Peloquin 1979), the use of molecular markers for multilocus gene-centromere mapping in plants is rarely reported (Barone 1995). In view of the interest for the centromeric regions, the multilocus gene-centromere mapping, as shown for the chromosome 8 in potato, deserves more attention than it has received so far in genetic

mapping of plants.

The use of SDR 2n-gametes to estimate the extent of crossing-over

The multilocus gene-centromere mapping of the SDR 2n-eggs of the *mcd-thr* hybrid also shows its relevance for the investigation of the extent of crossing-over. This is because the 2n-gametes allow to study recombination frequencies between a locus and the centromere over a long distance as well as to identify the number of crossovers per arm. As compared to unordered n-gametes, the SDR 2n-gametes even allow a more accurate estimation of the recombination frequencies because of the higher number of crossovers that can be detected using multiple markers per chromosome (Chapter 6). Also the FDR 2n-gametes can be used in multilocus analysis to detect the number of crossovers per chromosome arm (Fig. 3). Following a single crossover between the locus and centromere, 50% of the n- or PMR 2n-gametes, 50% of the FDR 2n-gametes and 100% of the SDR are recombinant. Following double crossovers between the locus and centromere, 25% of the n- or PMR 2n-gametes, 50% of the FDR 2n-gametes and 50% of the SDR are identified as double recombinants, whereas 50% of the n- or PMR 2n-gametes, 25% of the FDR 2n-gametes and 50% of the SDR 2n-gametes are identified as single recombinants. These frequencies clearly show that the SDR type of 2n-gametes is the most efficient for investigating crossing-over.

In view of this, it should be mentioned that the differential staining of the maternal and paternal genomes in 2n-gametes with the help of genomic *in situ* hybridization (GISH) would be a potential method to detect more crossover events (Takahashi *et al.* 1997; Kamstra *et al.* in preparation) than that is possible in multilocus analysis of 2n-gametes. This is because in multilocus analysis some crossover events are not detectable because of heterozygosity at both sides of the crossover. However, the differential staining of the maternal and paternal genomes in 2n-gametes has the potential to visualize these crossover events (Fig. 3).

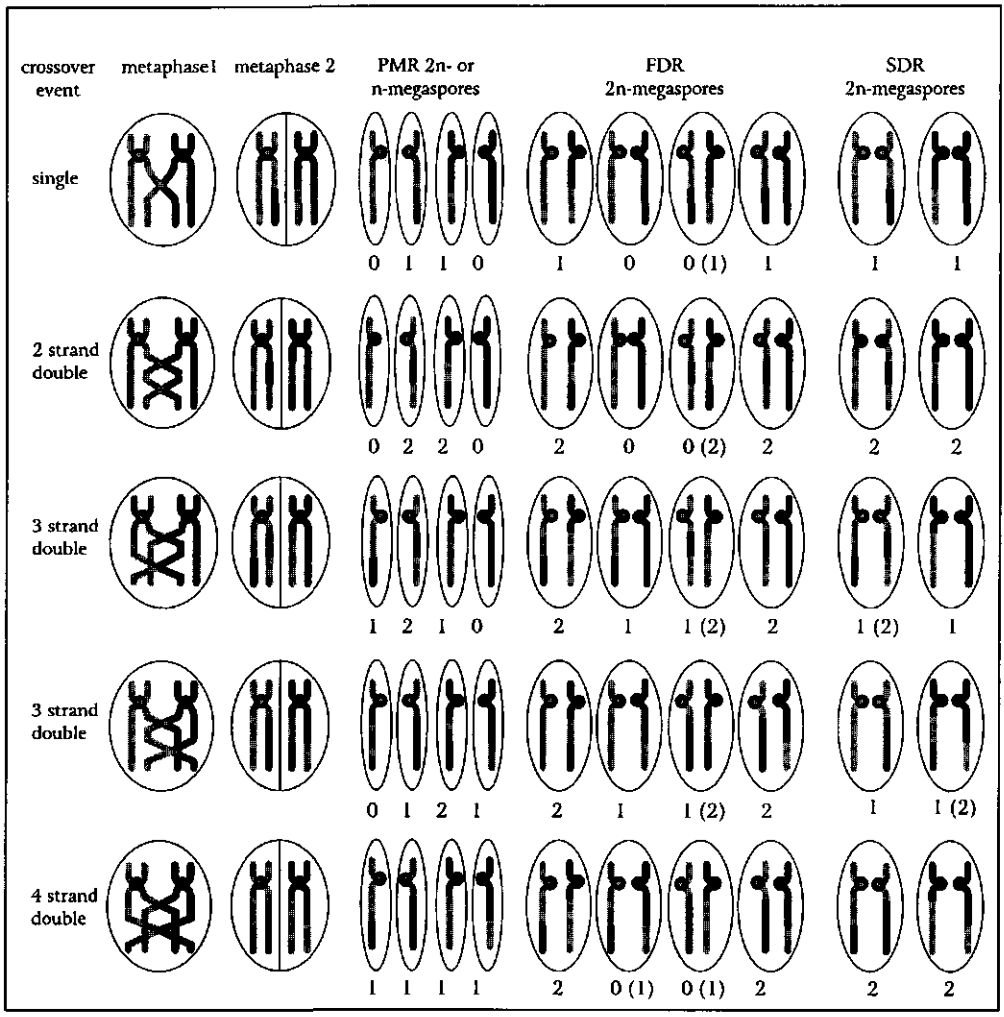

Figure 3. Detection efficiency of crossovers in n-, FDR 2n- and SDR 2n-gametes
The numbers below the megaspores indicate the number of crossovers that can be detected using multiple marker loci per chromosome. In some cases, GISH (see text) would be more efficient than multilocus analysis. In theses cases, the numbers between brackets indicate the numbers of crossovers that would be detected using GISH.

The observed occurrence of only one crossover per arm indicates a high level of chiasma interference. Surprisingly, numerous investigations on gene-centromere mapping using gynogenic lines in fishes and mollusc, also reported the occurrence of a high level of

chiasma interference (Thorgaard *et al.* 1983; Allendorf *et al.* 1986; Seeb and Seeb 1986; Guo and Allen 1996). Therefore, the number of crossovers per chromosome arm in some organisms might equal one crossover per arm more frequently than is assumed in the commonly used mapping functions to estimate genetic distances (Guo and Allen 1996; Chapter 6). A genetic map based on inbred lines will not be influenced by a high level of chiasma interference, since these maps reflect the crossover events of several cycles of meiosis. However, in progenies that are derived from single meioses, such as a F_1 or a backcross population, the occurrence of only one crossover per arm will result in reduced recombination rates and genetic distances.

CONCLUDING REMARKS

The use of multiple fully informative markers of one chromosome has contributed to a significant improvement of the evaluation of the genetic composition of 2n-gametes. This allowed the identification of a clone with post-meiotic doubling of the chromosomes (PMR), which is a new restitution mechanism of 2n-egg formation in potato. Furthermore, for both the *mcd-tbr* hybrid with PMR 2n-eggs, and *tbr-spg* hybrid with SDR 2n-eggs, it was demonstrated that there was only one restitution mechanism per clone.

The exploitation of SDR 2n-gametes for gene-centromere mapping was successful because of the RFLP locus *TG228* that demonstrated only SDR 2n-egg formation in this clone and also indicated the position of the centromere in the genetic map of chromosome 8. This allowed to analyse two of the four chromatids of a single meiosis in order to study chiasma interference. The gene-centromere mapping is considered as a valuable contribution to construct and refine genetic maps. The observed complete chiasma interference for chromosome 8 in the *mcd-tbr* hybrid can be compared with the situation in other chromosomes of potato as well as in other organisms in order to increase the understanding of crossing-over. The knowledge about restitution mechanisms and the level of crossing-over is important for sexual polyploidization, the exploitation of apomixis and genetic mapping, which are all relevant for breeding of the potato.

SUMMARY

A significant feature of the flowering plants (angiosperms) is that a majority of the species, including some of the important crop plants, have more than two sets of chromosomes (polyploids). One of these polyploid species is the cultivated potato, *Solanum tuberosum* L., with four sets of chromosomes (2n=4x=48). It is becoming increasingly evident that all polyploids, in nature as well as in cultivation, have predominantly originated through the functioning of numerically unreduced (2n-) gametes, which is called meiotic doubling or sexual polyploidization. Although this important fact has been well recognized, relatively few critical investigations have been made in the past on the exact modes of origin of 2n-gametes and the genetic consequences of using such gametes in the generation of polyploids. In some of the crop plants, it has already been shown that the levels of hetero- and homozygosity in the polyploid progenies can be effectively regulated through the appropriate use of different types of 2n-gametes. Furthermore, embryos can be generated from unfertilized 2n-eggs (diplosporic apomixis) from which maternal plants can be obtained. In addition, the occurrence of 2n-gametes in *Solanums* has allowed to transfer desired characters from diploid wild *Solanum* species (2n=2x=24) to the tetraploid cultivated potato. Moreover, in some plants and animals (including humans), 2n-gametes have been most effectively exploited for the refinement of genetic maps, through the method of gene-centromere mapping or half-tetrad analysis. In view of the implications of 2n-gametes for both fundamental and applied aspects in plant biology, the 2n-gametes deserve more attention than they have received so far.

In general, the 2n-gametes can originate through pre-meiotic doubling of chromosomes, meiotic nuclear restitution, post-meiotic doubling or aposporic development of the egg cells. Most 2n-gametes originate from meiotic disturbances in chromosome pairing, centromere division, spindle formation or cytokinesis. The various modes of origin result in different types of 2n-gametes with distinct genetic compositions. In potato, the only two modes of origin of 2n-gametes that have been demonstrated so far are the first division restitution (FDR) and the second division restitution (SDR). In the case of FDR, the chromosomal parts between the centromere and the first crossover will be derived from the

two non-sister chromatids of a bivalent (leads to heterozygosity), whereas in the case of SDR such regions will be derived from sister chromatids (leads to homozygosity). This type of assortment in FDR and SDR 2n-gametes can be exploited for the localisation of genetic loci in relation to the centromere (gene-centromere mapping) and the accurate assessment of the number of crossovers per chromosome arm. This thesis describes a strategic use of plant materials and genetic markers to evaluate the genetic composition of both 2n-pollen and 2n-eggs, that allows the identification of various modes of origin of 2n-gametes, the exploitation of FDR and SDR 2n-gametes for gene-centromere mapping and determination of the extent of crossing-over in potato.

For the evaluation of 2n-pollen, cytological analysis of microsporogenesis was applied to elucidate the FDR mechanism of 2n-pollen formation in a clone with normal chromosome pairing (normal synapsis) as well as in a clone with reduced chromosome pairing (desynapsis). Both clones were used as a pollinator in crosses with tetraploid female parents to generate tetraploid progenies through the functioning of 2n-pollen. Two pollen marker genes were used to analyse these progenies as well as the pollen samples of the two 2n-pollen producing clones. One of these markers was the distal locus *amylose-free* (*amf*) on chromosome 8 and the other one was the isozyme locus, *alcohol dehydrogenase* (*Adh-1*), on chromosome 4. In this experiment, the gene-centromere distances for the *amf* locus and the *Adh-1* locus were estimated as 48.8 cM and 19.4 cM, respectively. The observed genetic distance of 48.8 cM is the highest gene-centromere distance reported so far in potato and this could be explained on the assumption that a single crossover occurred on this chromosome arm (absolute chiasma interference). The recombination frequency between the centromere and the *amf* locus in the desynaptic clone was reduced by 73%.

The modes of origin of 2n--pollen can be monitored easily through cytological as well as genetic methods. However, it is much more difficult to determine the modes of origin of 2n-eggs cytologically and , therefore, genetic methods are essential. For this purpose, a four-step method for monitoring the genetic composition of 2n-eggs through a strategic use of plant material as well as appropriate genetic markers was developed. The four steps were the following: 1) the selection of diploid hybrids with 2n-egg formation, 2) the generation of

progenies from 2n-eggs, 3) the identification of polymorphic marker loci, which are informative for monitoring the genotypes of 2n-eggs, and 4) the monitoring of the genotypes of the progenies from 2n-eggs for multiple informative marker loci.

For the selection of 2n-egg producing genotypes of different genetic backgrounds (step 1), diploid Tuberosum hybrids as well as Tuberosum-wild species F_1 hybrids were generated. Plants were selected if they produced more than four seeds per berry after pollination with tetraploid males (2x.4x-crosses). From the Tuberosum hybrids that were derived from one or two 2n-egg producing parents, 12 out of 82 (15%) 2n-egg producing plants were selected. From the Tuberosum-wild species F_1 hybrids, 21 of the 274 (8%) 2n-egg producing plants were selected. The level of 2n-egg formation in 25 selected diploid hybrids and five control clones (three high, one moderate and one low 2n-egg producers) was estimated on the basis of seed set following 2x.4x-crosses using 13 tetraploid males in four crossing years. The level of 2n-egg formation in two of the newly selected hybrids was classified as very high (47-58 seeds per berry), four were high (16-27 seeds per berry), sixteen were moderate (5-19 seeds per berry), and three were low (2-4 seeds per berry). The two hybrids with very high level of 2n-eggs showed significantly higher seed set than the clones that were available before the selection programme.

To pave the way for the genetic analysis of the 2n-eggs of the selected diploid hybrids, both diploid parthenogenetic and tetraploid hybrid progenies were generated (step 2). The generation of parthenogenetic diploids through prickle pollination of six diploid 2n-egg producers was difficult (less than 0.17 plants per berry). The generation of tetraploid hybrids from crosses between six 2n-egg producing diploids as female parents and six tetraploids as male parents (2x.4x-crosses) was much easier (up to 11.0 tetraploids per berry).

In order to select plant materials with polymorphic markers that are informative for monitoring the genotypes of 2n-eggs (step 3) special attention was given to the generation and selection of 2n-egg producing hybrids that were heterozygous for the *amf* marker. The progenies of these hybrids were evaluated for the segregation of the *amf* marker to obtain indications about the mode of nuclear restitution of the 2n-eggs. For the elucidation of both the restitution mechanism and the number of crossovers per chromosome of individual 2n-eggs, multiple marker loci on different parts of a chromosome, including the proximal regions,

were required. To identify multiple marker loci that are informative for monitoring the genotypes of the progeny plants, the parents of the progenies of the diploid parthenogenetic and tetraploid hybrid populations were analysed for RFLP markers of the chromosomes 6 and 8. The frequency of RFLP loci that identified two separate alleles per locus in the 2n-egg producers was high (93%). All these loci were expected to be informative for the multilocus analysis of 2n-eggs using the diploid parthenogenetic progenies. For the evaluation of 2n-eggs using the progenies of 2x.4x-crosses, marker loci with two unique alleles in the diploid 2n-egg producing parent were required. These informative marker loci were identified through the comparison of the RFLP patterns of both the parents of the eleven 2x.4x-progenies. In the best three 2x.4x-combinations, the frequency of RFLP loci with two unique alleles in the diploid 2n-egg parent was 19-23%.

Using the *amf* marker, the mode of origin of 2n-eggs was determined in four diploid Tuberosum-wild species F_1 hybrids (step 4). The wild species involved were *Solanum phureja*, *S. chacoense*, *S. spegazzinii*, and *S. microdontum*. All four Tuberosum-wild species F_1 hybrids were heterozygous for the *amf* marker *(Amf/amf)* and had been crossed with tetraploid nulliplex *(amf/amf/amf/amf)* male parents.The resulting tetraploid progenies segregated into three clear-cut classes of nulliplex, simplex *(Amf/amf/amf/amf)* and duplex *(Amf/Amf/amf/amf)* genotypes. In three cases, the simplex genotypic class predominated and the mode of origin was explained as due to the SDR. On the contrary, the progenies of the *S. microdontum* × *S. tuberosum (mcd-tbr)* hybrid comprised either nulliplex or duplex genotypes, indicating absolute homozygosity for this locus in the 2n-eggs. In order to test whether the 2n-eggs were also homozygous for other loci on the same chromosome, the tetraploid progeny of the *mcd-tbr* hybrid (2x) × 105ESC90-32 (4x) was analysed for four RFLP markers, specific to the chromosome 8 of potato. This analysis showed complete homozygosity for all 2n-eggs at all loci investigated. In addition the occurrence of crossing-over between the two genomes of the *mcd-tbr* hybrid was evident. This indicated that the 2n-eggs of this hybrid originated through post-meiotic doubling of the chromosomes, which was reliably confirmed for the first time in potato. In line with the First and Second Division Restitution (FDR and SDR), this post-meiotic doubling was characterized as a new restitution mechanism in potato, the Post-Meiotic Restitution (PMR).

One of the three Tuberosum-wild species F_1 hybrids of which the mode of origin of 2n-eggs was explained as due to SDR on the basis of the segregation of the tetraploid progeny for the *amf* marker was the *S. tuberosum* × *S.spegazzinii (tbr-spg)* hybrid. The 2n-eggs of this hybrid were further analysed for nine RFLP markers of chromosome 8, using the progeny from the cross between the *tbr-spg* hybrid (2x) and the potato cultivar Desirée (4x). This analysis allowed to map the centromere in relation to nine marker loci of chromosome 8. The genotypes of the 80 progeny plants indicated that all 2n-eggs of the *tbr-spg* hybrid were homozygous at the locus *TG228*. This clearly demonstrated that all 2n-eggs had originated through SDR and that the genetic distance between *TG228* and the centromere of chromosome 8 was less than 1 cM. Consequently, seven RFLP markers were localised on the long arm of chromosome 8, whereas one marker was localised on the short arm. Furthermore, the multilocus analysis provided critical estimates of recombination frequencies and strong evidence that in this 2n-egg producing hybrid not more than one crossover occurred in the long arm of the chromosome 8.

In conclusion, the strategic use of plant materials and marker genes allowed to identify and evaluate different types of 2n-gametes, such as FDR 2n-gametes in a synaptic mutant with highly reduced recombination, and the FDR, SDR and PMR 2n-gametes in normal synaptic clones. The genetic compositions of these 2n-gametes were highlighted to demonstrate the different purposes of genetic analyses. Only one restitution mechanism of 2n-gamete formation per clone was observed. In the normal synaptic clones, there was evidence for complete chiasma interference. With the discovery of the diploid *mcd-tbr* hybrid that produces PMR 2n-eggs, a full spectrum of diploid clones that can produce 2n-eggs with complete homozygosity to a high degree of heterozygosity is now available. Finally, it was shown that SDR 2n-gametes are superior to detect the genetic consequences of single and double crossovers as compared to n- and FDR 2n-gametes.

SAMENVATTING

Een opmerkelijk groot deel van de bloeiende planten, ofwel bedektzadigen (angiospermen), is polyploïd. Dat wil zeggen dat het aantal kopieën van de basisset chromosomen in deze planten groter is dan twee. Tot de polyploïde soorten behoort een aantal belangrijke gewassen, waaronder de gecultiveerde aardappel, *Solanum tuberosum* L. Deze aardappel wordt als tetraploïd aangeduid, omdat ieder chromosoom in viervoud aanwezig is (2n=4x=48). Er zijn vele aanwijzingen dat polyploïde soorten ontstaan zijn uit gameten, waarin het aantal chromosomen niet gereduceerd is zoals gebruikelijk tijdens de meiose. Deze gameten hebben het somatische aantal chromosomen (2n) in plaats van het haploïde aantal (n) en worden daarom 2n-gameten genoemd. Het produceren van nakomelingen met een verhoogd ploïdieniveau met behulp van 2n-gameten staat bekend onder de term sexuele polyploïdisatie. Hoewel het belang van deze methode algemeen erkend wordt, is er relatief weinig onderzoek gedaan naar de precieze ontstaanswijze van 2n-gameten en de bijbehorende genetische consequenties voor het polyploïde nakomelingschap.

In een aantal belangrijke gewassen heeft men verschillende typen 2n-gameten gebruikt om de genetische samenstelling (mate van homo- en heterozygotie) van het nakomelingschap te manipuleren. Ook kunnen er embryo's ontwikkeld worden uit 2n-eicellen, zonder dat deze bevrucht zijn (diplospore apomixis). Op deze manier kan men zaad produceren, waaruit planten komen die alleen erfelijk materiaal van de moederplant bevatten. In het geval van de aardappel zijn de 2n-gameten ook belangrijk om eigenschappen uit diploïde *Solanum*-soorten (2n=2x=24), zoals bijvoorbeeld resistenties tegen ziekten, over te brengen naar de tetraploïde cultuuraardappel. Een heel andere toepassing van 2n-gameten is het verfijnen van genetische kaarten. Een genetische kaart is een weergave van de afstanden tussen de genen op de verschillende chromosomen van een bepaald organisme. Deze genetische afstanden zijn een maat voor het aantal overkruisingen tijdens de meiose. In tegenstelling tot de normale, gereduceerde (n) gameten bieden de 2n-gameten de mogelijkheid om, per chromosoom, de genetische afstanden tussen het centromeer en de genen in kaart te brengen. Deze methode wordt half-tetrade analyse genoemd en is toegepast in zowel plantaardige als dierlijke organismen, waaronder de mens. De 2n-gameten zijn dus bruikbaar voor zowel de toegepaste

als de fundamentele genetica en verdienen dan ook meer aandacht dan ze tot op heden gekregen hebben.

Algemeen beschouwd ontstaan 2n-gameten door pre- of postmeiotische verdubbeling van de chromosomen of restitutie van de kernen tijdens de meiose. Ook kunnen somatische cellen uitgroeien tot 2n-eicellen (aposporie). De meeste 2n-gameten ontstaan door restitutie (het herstellen van het aantal chromosomen door verdubbeling van het haploïde aantal) tijdens de meiose, vanwege afwijkingen in de paring van chromosomen, de deling van de centromeren, de vorming van de spoelfiguren of de aanleg van de celwanden. De diverse ontstaanswijzen hebben consequenties voor de genetische samenstelling van de 2n-gameten. In de aardappel zijn twee typen 2n-gameten bekend, die ontstaan zijn door restitutie tijdens de eerste of de tweede deling van de meiose. Deze worden aangeduid met respectievelijk 'First Division Restitution (FDR)' en 'Second Division Restitution (SDR)'. De FDR 2n-gameten omvatten de twee niet-zuster chromatiden van iedere bivalent en zijn heterozygoot, terwijl de SDR 2n-gameten juist de twee zuster chromatiden omvatten en homozygoot zijn. Dit geldt echter alleen voor het deel tussen het centromeer en de plaats van de eerste overkruising op beide chromosoomarmen. Genetische analyse van FDR of SDR 2n-gameten maakt zichtbaar waar de eerste overkruising ten opzichte van het centromeer is opgetreden. Op deze manier kunnen FDR and SDR 2n-gameten gebruikt worden voor het in kaart brengen van de genetische afstanden tussen de markergenen en het centromeer van ieder chromosoom en voor het bepalen van het aantal overkruisingen per chromosoomarm.

Dit proefschrift beschrijft een strategisch gebruik van plantmateriaal en markergenen om de genetische samenstelling van zowel 2n-pollen als 2n-eicellen te achterhalen. Deze methode is gebruikt voor het aantonen van verschillende ontstaanswijzen van 2n-gameetvorming in de aardappel, het lokaliseren van genen ten opzichte van het centromeer en het bepalen van het aantal overkruisingen per chromosoomarm.

De ontstaanswijze van 2n-pollen werd vastgesteld door cytologische bestudering van de mannelijke meiose en het ontstaan van de microsporen (microsporogenese). In zowel een diploïde aardappelkloon met normale paring van de chromosomen (normale synapsis) als een kloon met zeer beperkte paring van de chromosomen (desynapsis) bleek een FDR

restitutiemechanisme werkzaam te zijn. Beide klonen werden gebruikt voor het bestuiven van tetraploïde klonen om nakomelingschappen van deze 2n-pollen te genereren. Uit de 4x.2x-kruisingen kwamen tetraploïde nakomelingen voort (de triploïden aborteerden spontaan). Twee markergenen, die in stuifmeel tot expressie komen, werden gebruikt om zowel het stuifmeel als de tetraploïde nakomelingschappen van beide 2n-pollen producerende klonen te analyseren. Eén van deze twee markers was een distaal gen van chromosoom 8 coderend voor amylosevrij zetmeel *(amf)*. De andere marker was een gen van chromosoom 4 coderend voor het isozym alcohol dehydrogenase *(Adh-1)*. Op grond van de waargenomen recombinatiefrequenties werden de gen-centromeer-afstanden voor het *amf* en het *Adh-1* locus geschat als, respectievelijk, 48,8 en 19,4 cM. De *amf*-centromeer-afstand van 48,8 cM was de grootste gerapporteerde gen-centromeer-afstand in de aardappel en duidde op het veelvuldig voorkomen van precies één overkruising per chromosoomarm (absolute chiasma interferentie). In de desynaptische kloon bleek de recombinatiefrequentie tussen het centromeer en *amf* locus met 73% gereduceerd te zijn.

De 2n-gameetvorming is vaak bestudeerd in klonen met 2n-pollenvorming, omdat deze makkelijker te selecteren en te analyseren zijn dan klonen met 2n-eicelvorming. Vanwege een aantal verschillen tussen de mannelijke en vrouwelijke meiose is het verwachte restitutiemechanisme in 2n-pollen FDR, terwijl 2n-eicellen juist verwacht worden via SDR te ontstaan. Om te profiteren van de mogelijke toepassingen van SDR 2n-gameten in aardappel is in dit proefschrift een methode beschreven voor het zichtbaar maken van de genetische samenstelling van 2n-eicellen. Deze methode bestaat uit de volgende vier stappen: 1) het selecteren van diploïde klonen met 2n-eicelvorming, 2) het genereren van nakomelingschappen vanuit deze 2n-eicellen, 3) het identificeren van markergenen, die gebruikt kunnen worden om de genotypen van de 2n-eicellen van de diploïde ouder zichtbaar te maken in de nakomelingen, 4) het bepalen van de genetische samenstelling van de nakomelingen van de 2n-eicellen voor meerdere markergenen per chromosoom.

Zowel diploïde Tuberosum-hybriden als F_1's, afkomstig van diploïde Tuberosums en wilde *Solanum*-soorten werden gebruikt om diploïde klonen met 2n-eicelvorming te selecteren (stap 1). De planten werden geselecteerd indien zij na bestuiving met het stuifmeel van een tetraploïde kloon (2x.4x-kruisingen) meer dan vier zaden per bes produceerden. Er werden

12 van de 82 (15%) Tuberosum-hybriden en 21 van de 274 (8%) F_1's geselecteerd. Het niveau van de 2n-eicelvorming in de geselecteerde diploïde hybriden werd geschat door de gemiddelde zaadzetting na 2x.4x-kruising. Deze zaadzetting werd bepaald in een kruisingsprogramma met dertien tetraploïde bestuivers over vier jaar en vergeleken met vijf klonen met een bekend niveau van 2n-eicelvorming als controle (waarvan drie hoog, één middelmatig en één laag). In twee van de 25 geteste hybriden was het niveau van 2n-eicelvorming erg hoog (47-85 zaden per bes). Dit was beduidend hoger dan in de klonen die reeds beschikbaar waren bij aanvang van het kruisingsprogramma. Het niveau van 2n-eicelvorming in de overige hybriden werd als volgt ingeschaald: vier met een hoog niveau (16-27 zaden per bes), zestien met een middelmatig niveau (5-19 zaden per bes) en drie met een laag niveau (2-4 zaden per bes).

Voor het genereren van een nakomelingschap uit 2n-eicellen (stap 2) werd geprobeerd om zowel onbevruchte 2n-eicellen (parthenogenese) als bevruchte 2n-eicellen (kruisingen) uit te laten groeien tot planten. Het verkrijgen van een parthenogenetische nakomelingschap door middel van prikkelbestuivingen van diploïde klonen met 2n-eicelvorming verliep zeer moeizaam (minder dan 0,017 planten per bes). Het genereren van een tetraploïde nakomelingschap uit de 2x.4x-kruisingen daarentegen was veel eenvoudiger (tot 11,0 tetraploïden per bes).

Indicaties over de mogelijke ontstaanswijze van 2n-eicellen werden verkregen door de uitsplitsing voor het *amf* kenmerk te analyseren in de nakomelingen van heterozygote klonen *(Amf/amf)* met 2n-eicelvorming (stap 3). Voor het achterhalen van zowel het restitutiemechanisme als het aantal overkruisingen per chromosoomarm voor individuele 2n-eicellen van de diploïde ouder waren echter meerdere markergenen per chromosoom noodzakelijk. Een markergen was bruikbaar indien de 2n-eicel producerende diploïde ouder heterozygoot was voor dit gen èn de genetische samenstelling van de 2n-eicellen zichtbaar gemaakt kon worden in het nakomelingschap. Deze informatieve markers werden opgespoord door de genetische samenstelling (polymorfismen) van de ouders van zowel de parthenogenetische nakomelingschappen als de 2x.4x-kruisingspopulaties te analyseren voor moleculaire markergenen (RFLP loci) van de chromosomen 6 en 8. De frequentie RFLP markers met twee verschillende allelen per locus in de geteste diploïde klonen met 2n-

eicelvorming was hoog (93%). In een parthenogenetische nakomelingschap van 2n-eicellen waren al deze loci informatief. Voor de analyse van de 2x.4x-kruisingspopulaties waren de markergenen echter pas informatief indien de twee verschillende allelen in de diploïde ouder uniek waren ten opzichte van de allelen van de tetraploïde ouder. Deze informatieve markergenen werden opgespoord door de vergelijking van RFLP-patronen van de ouders van elf 2x.4x-nakomelingschappen. In de drie meest polymorfe oudercombinaties was de frequentie van RFLP loci met twee unieke allelen in de diploïde ouder 19-23%.

Voor vier diploïde interspecifieke hybriden werd de onstaanswijze van 2n-eicellen bepaald op basis van de uitsplitsing voor de *amf* marker in 2x.4x-nakomelingen. Deze interspecifieke hybriden waren afkomstig van de wilde *Solanum*-soorten *S. phureja, S. chacoense, S. spegazzinii* en *S. microdontum* en waren heterozygoot voor de *amf* marker *(Amf/amf).* Van deze hybriden waren bovendien 2x.4x-nakomelingen beschikbaar afkomstig van nulliplex *(amf/amf/amf/amf)* bestuivers. Deze 2x.4x-nakomelingschappen splitsten uit in drie verschillende klassen, te weten nulliplex, simplex *(Amf/amf/amf/amf)* en duplex *(Amf/Amf/amf/amf)* genotypen. In de nakomelingen van drie interspecifieke hybriden overheerste de simplex klasse, zoals verwacht op grond van SDR 2n-eicelvorming. In de nakomelingschap van de *S. microdontum* × *S. tuberosum (mcd-tbr)* hybride werden echter alleen nulliplex en duplex genotypen aangetroffen. Dit duidde op homozygotie voor de *amf* locus van alle 2n-eicellen van deze *mcd-tbr* hybride. Om na te gaan of de 2n-eicellen ook homozygoot waren voor andere loci van hetzelfde chromosoom werden de nakomelingen van de kruising *mcd-tbr* hybride (2x) × 105ESC90-32 (4x) geanalyseerd voor vier RFLP-markers van chromosoom 8. De genetische analyse maakte duidelijk dat alle 2n-eicellen homozygoot waren voor alle geteste loci. Bovendien bleken overkruisingen opgetreden te zijn tussen de twee genomen van de *mcd-tbr* hybride. Het ontstaan van volledig homozygote 2n-eicellen in combinatie met meiotische recombinatie kon verklaard worden door de verdubbeling van de chromosomen na de meiose. Dit was de eerste keer dat sluitend genetisch bewijs werd geleverd voor het optreden van post-meiotische verdubbeling van de chromosomen in de aardappel. In lijn met de terminologie van FDR en SDR werd dit nieuwe restitutiemechanisme aangeduid als 'Post-Meiotic Restitution (PMR)'.

Eén van de drie interspecifieke hybriden, waarin SDR 2n-eicelvorming werd

aangetoond op grond van de uitsplitsing voor de *amf* marker, was de *S. tuberosum* × *S. spegazzinii (tbr-spg)* hybride. De analyse van de 2n-eicellen van deze hybride werd voortgezet met behulp van het nakomelingschap van de 2x.4x-kruising tussen de *tbr-spg* hybride en het aardappelras 'Desirée'. De genetische analyse van dit tetraploïde nakomelingschap maakte het mogelijk om voor negen RFLP-marker loci van chromosoom 8 de gen-centromeer-afstanden te bepalen. Alle nakomelingen bleken homozygoot te zijn voor RFLP-locus *TG228*. Dit was een belangrijk gegeven om te kunnen concluderen dat alle 2n-eicellen ontstaan waren door SDR èn dat de genetische afstand tussen het centromeer van chromosoom 8 en de RFLP-locus *TG228* minder was dan 1 cM. In genetische analyse met meerdere markers per chromosoom (multilocus analyse) bleken de SDR 2n-gameten, in vergelijking met de PMR 2n-, FDR 2n- en de n-gameten, het meest nauwkeurig te zijn voor het schatten van de recombinatiefrequenties. Op deze manier kon de multilocus analyse van de SDR 2n-eicellen in de *tbr-spg* hybride ook gebruikt voor het aantonen van het zeer frequent optreden van precies één overkruising in de lange arm van chromosoom 8 (chiasma interferentie).

Een belangrijke conclusie van het in dit proefschrift beschreven onderzoek is dat door een strategisch gebruik van plantmateriaal en markergenen onderscheid gemaakt kan worden tussen de verschillende typen 2n-gameten. Door toepassing van deze methode is de genetische samenstelling van diverse typen 2n-gameten ontrafeld, zoals de FDR 2n-gameten van een synaptische mutant met sterk gereduceerde recombinatie en de FDR, SDR en PMR 2n-gameten van normaal synaptische klonen. Op grond hiervan kan geconcludeerd worden dat er in de geanalyseerde diploïde klonen slechts één restitutiemechanisme per kloon werkzaam is. Ook is bewijs geleverd voor een hoge mate van chiasma interferentie in normaal synaptische klonen. Een theoretische beschouwing van het gebruik van verschillende typen gameten in multilocus analyses heeft aangetoond dat de SDR 2n-gameten superieur zijn ten opzichte van de n-, PMR en FDR 2n-gameten voor het detecteren van recombinanten afkomstig van enkelvoudige en dubbele overkruisingen per chromosoomarm. Tot slot kan gesteld worden dat de ontdekking van de *mcd-tbr* hybride met de PMR 2n-eicellen het spectrum van beschikbare klonen met volledig homozygote tot volledig heterozygote 2n-gameten in de aardappel compleet heeft gemaakt.

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CURRICULUM VITAE

Helena Johanna Maria Bastiaanssen werd op 6 februari 1966 geboren te Breda. In 1984 behaalde zij het VWO-diploma aan het Strabrecht College in Geldrop. Aansluitend begon zij met de studie Plantenveredeling aan de Landbouwuniversiteit Wageningen (LUW). De doctoraal studie omvatte een stage bij het Franse onderzoeksinstituut ORSTOM in Ivoorkust en een tweetal afstudeervakken. Tijdens het eerste afstudeervak bij de vakgroepen Plantenveredeling en Plantencytologie en -morfologie deed zij onderzoek naar de gameetvorming en inductie van parthenogenese in aardappel. Tijdens het tweede afstudeervak werd een onderzoek uitgevoerd naar de transformatie van microsporen aan de Ecole Nationale Supérieure d'Agronomie de Toulouse (ENSAT) in het kader van het EG-uitwisselingsprogramma 'Erasmus'. In augustus 1990 rondde zij haar studie af en kwam in dienst als veredelaar van tarwe en gerst bij het bedrijf Van Der Have in Rilland. In april 1992 keerde zij terug naar Wageningen voor een aanstelling als Assistent in Opleiding bij de vakgroep Plantenveredeling van de LUW. De resultaten van het promotie-onderzoek staan beschreven in dit proefschrift.

NAWOORD

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Het juiste plantmateriaal kwam beschikbaar door langdurig verblijf in de soms oerwoudachtige omstandigheden in de kassen. Vooral Teus van de Brink, Daniëlla van der Gaag, Sjaak Jansen, Jan van Schaik en diverse studenten hebben meegewerkt bij het snoeien en kruisen van de planten en het schoonmaken van de (vaak lege) bessen. De *in vitro* instandhouding en vermeerdering van de belangrijkste klonen is uitgevoerd door Hilda Kranendonk, Zachi Sawor, Dirk-Jan Huigen en Marjan Bergervoet, waarna de plantjes in de kas vertroeteld werden door Jan Rijksten.

Samen met Prof. van Went van de vakgroep Plantencytologie- en morfologie en Bianca van Dorrestein werden diverse cytologische technieken gebruikt om de afwijkingen in de megasporevorming op een rijtje te zetten. De belangstelling voor de fluorescente en genomische *in situ* hybridisatie techniek (FISH en GISH) deelde ik met Hans de Jong van de vakgroep Erfelijkheidsleer en Dr. Ramanna. Dit leidde tot reisjes naar Engeland, waar Hans mij introduceerde bij de echte chromosoomliefhebbers en we ook in de pub veel ideeën uitwisselden. Ondanks een succesvolle introductie van de GISH-techniek op zowel de vakgroep Erfelijkheidsleer als Plantenveredeling bleek mijn aardappelmateriaal helaas niet exotisch genoeg voor flitsende chromosoomplaatjes in het proefschrift. Dit zal ruimschoots goed gemaakt worden in de proefschriften van Karin Horsman, Francesc Garriga en Silvan

Kamstra! Ook is er nog een artikel in de maak over het onderzoek naar GISH in somatische hybriden van *Solanum tuberosum* (+) *S. brevidens*, dat ik heb uitgevoerd samen met Miriam van der Wee en Marjan Bergervoet.

Een belangrijk contact was het samenwerkingsverband met het CPRO-DLO in het kader van het door de SSA gefinancierde onderzoek naar een snelle introduktie van resistentie tegen *Meloidogyne hapla* in aardappel. Hiervoor kwam drie maal per jaar een begeleidingscommissie bijeen met de aardappelveredelaars: W. Prummel, J.G.H. Vermeulen, P. Heeres, A.F.M. Heselmans en J.J.H.M. Allefs (waarvan alleen P. Heeres van begin tot eind van het project aanwezig was), de CPRO'ers: Coosje Hoogendoorn, Richard Janssen en Geert Janssen (AIO) en de IVP'ers: Evert Jacobsen en M.S. Ramanna. Ondanks het feit dat de IVP en CPRO-projecten niet veel gemeen hadden, leverden beide AIO-projecten genoeg resultaten op voor leuke discussies over de (on)mogelijke toepassingen in de praktische plantenveredeling. Geert en ik maakten er bovendien een goede gewoonte van om na iedere bijeenkomst samen te gaan lunchen om alle AIO-perikelen van ons af te praten en de IVP en CPRO-nieuwtjes uit te wisselen.

Na de komst van Pim Lindhout op de vakgroep werd hij als moleculair markerspecialist ingezet voor het RFLP-onderzoek naar de ontstaanswijze van 2n-gameten in aardappel. Zijn vragen waren voor mij een leerzame ervaring om de 2n-gameten problematiek helder te formuleren. Ook Petra van den Berg kon nu worden ingezet, zodat het trio met de kolossale benen een feit was. Petra nam alle blots voor haar rekening, waarmee tevens de belangrijkste resultaten uit dit onderzoeksproject behaald zijn. Vaak stonden we de autoradiogrammen al te analyseren, terwijl ze nog aan het waslijntje hingen te drogen!

Na jarenlang investeren in het selecteren van het juiste plantmateriaal en het vinden van de meest geschikte markergenen waren we zo enthousiast over de uitsplitsing van de RFLP-markers in de nakomelingen van 2n-gameten, dat we deze resultaten als eerste op papier hebben gezet. Hierdoor is het proefschrift van achter naar voren opgeschreven, wat achteraf misschien niet helemaal de meest efficiënte manier was. Wel hebben we de hoofdstukken goed op elkaar kunnen afstemmen en is er één gedenkwaardige week geweest waarin vier artikelen op de bus gingen. Voor het schrijven was er de onvermijdelijke

verbanning naar "de Terp", waar het overigens prima vertoeven was met de mede-AIO's in de laatste fase; Peter Arama, Hanneke Buiel, Anne Korststee, Johan Schut en Ronald Eijlander.

Er zijn een aantal mensen, waarvan ik de laatste jaren veel hulp heb gehad en die ik hier graag extra wil bedanken.

Ten eerste mijn promotor Evert Jacobsen, waarmee ik heel wat uurtjes discussie heb gevoerd. Evert; jij stimuleert mensen graag door ze uit te dagen met boute stellingen. Het liefst zie je daarop een uitgesproken mening als reactie. Op een enkele uitzondering na heeft deze aanpak wel gewerkt en heb ik veel waardering voor de manier waarop je me hebt gestimuleerd om al in een vroeg stadium van de data-analyse de belangrijkste boodschap boven water te halen. Ook wil ik je bedanken voor de hulp op de belangrijke momenten, waarvoor je steeds tijd wist vrij te maken in je overvolle agenda.

Dr. M.S. Ramanna, my co-promotor, you have learned me to enjoy science. We shared our enthusiasm about polyploids and the application of the FISH technique in cytogenetics. You had an excellent knowledge of the literature and gave me always good suggestions for references. I have appreciated the harmonious way we wrote and rewrote manuscripts, although it was time consuming. Most of all, you are a very warm person and it was a great pleasure to work with you.

Petra en Pim, ondanks menig melige werkbespreking hebben we productief samengewerkt. Petra; jij was altijd aanspreekbaar en bovendien perfectionistisch genoeg om alle blots uiteindelijk haarscherp te krijgen. Pim; bedankt voor je hulp bij het opzetten van de experimenten, het tot in de late uurtjes doornemen van mijn manuscripten en je steun op momenten dat (ex)-AIO zijn niet zo leuk was.

Karin, alhoewel wij lang niet iedere dag bij elkaar over de werkvloer kwamen, heb ik jouw nuchterheid en humor altijd gewaardeerd en hebben we vele persoonlijke en AIO-ervaringen uitgewisseld. Ik ben blij dat jij en Petra zo enthousiast reageerden om mijn paranimfen te zijn!

Tot slot wil ik Prof. Hermsen en Stam en de mede-auteurs bedanken voor de goede suggesties bij het schrijven van de manuscripten en alle medewerkers van de diverse vakgroepen voor de prettige samenwerking.

Uiteraard zijn er ook in mijn privé-leven een aantal mensen geweest die belangrijk waren tijdens deze AIO-periode. Voor de steun en belangstelling wil ik vooral "ons pap en ons mam" bedanken en ook mijn zussen, vrienden en in het bijzonder de Wageningse huisgenoten. Silvan; jou wil ik niet alleen bedanken voor je wezenlijke bijdrage aan het kaftje van dit boekje. Jij hebt mij diverse keren geholpen, toen ik met de keerzijde van het promoveren geconfronteerd werd. De eer en de computer is nu aan jou!

Heleen