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Postmeiotic restitution in *2n*-egg formation of diploid potato

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Four diploid ($2n = 2x = 24$) interspecific F_1 hybrids of tuberous *Solanum* species were tested for the modes of origin of 2*n*-eggs. The four hybrids were heterozygous for the genetic marker *amylose-free* starch *(Amf/amf)* on chromosome 8. By crossing these hybrids with tetraploid *S. tuberosum* parents ($2n = 4x = 48$) that were nulliplex for this marker, i.e. *Amf/amf* × *amf/amf/amf/amf* crosses, tetraploid progenies were generated and classified for starch phenotypes of microspores. Based on the segregation of the *amf* marker gene, the tetraploid progenies were classified into nulliplex, simplex and duplex genotypes. In the progenies of three F_1 hybrids, the simplex genotypes predominated and the origin of 2*n*-eggs could be explained as the result of second division restitution (SDR). But in the progeny of one F_1 hybrid (a *S. microdontum* × *S. tuberosum* hybrid), there were only nulliplex and duplex genotypes, indicating complete homozygosity of the 2*n*-eggs (*Amf/Amf* or *amf/amf*) for this locus. In order to genotype the 2*n*-eggs also for other loci on the same chromosome, a tetraploid progeny was generated from a cross between this hybrid and a tetraploid *S. tuberosum* parent (Tetra 4) and analysed for four RFLP loci on chromosome 8. This analysis showed that all 2*n*-eggs of the *S. microdontum* × *S. tuberosum* hybrid were homozygous for all four loci, as was observed for the *amf* locus. From the same analysis it was evident that crossovers had occurred between the two genomes of this F_1 hybrid. These homozygous and recombinant genotypes indicated that the 2*n*-eggs had originated from the doubling of the chromosome number in the normal haploid products of meiosis. Following the terminology of first and second division restitution (FDR and SDR), this new mode of origin of 2*n*-eggs in diploid potato is called post-meiotic restitution (PMR).

Keywords: 2*n*-gametes, postmeiotic doubling, restitution mechanism, RFLP analysis, sexual polyploidization, *Solanum*.

Introduction

In plants, sexual polyploidization by the formation of unreduced (2*n*) gametes is an important feature in both nature and breeding programs. In potato, FDR and SDR mechanisms have been shown to give rise to 2*n*-gametes (Veilleux, 1985; Peloquin *et al*., 1989; Bretagnolle & Thompson, 1995). In both cases, the 2*n*-gametes are derived from two of the four strands of each bivalent of a pair of homologous chromosomes (Fig. 1). The FDR gametes originate through an equational division of all chromosomes, as a result of which the nonsister chromatids are included in one and the same gamete. In this case, the 2*n*-gametes are highly heterozygous because homozygosity can occur only in the regions distal to chiasma formation. 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 2*n*-gamete. For SDR, homozygosity occurs in regions proximal to chiasma formation (Fig. 1). Thus, depending on the modes of origin, 2*n*-gametes with quite different genetic compositions can be generated.

Through the use of appropriate meiotic mutants in potato, it has been possible to select genotypes that produce highly heterozygous FDR to relatively homozygous SDR populations of 2*n*-gametes

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(Ramanna, 1983; Werner & Peloquin, 1987; Werner *et al.*, 1992; Douches & Quiros, 1988; Jongedijk *et al*., 1991; Bastiaanssen *et al*., 1996). It is believed that different meiotic aberrations can occur in one plant resulting in a mixture of FDR and SDR 2*n*-eggs (Conicella *et al*., 1991; Werner & Peloquin, 1991). However, in the ovules of potato, it is impossible to demonstrate cytological events that elucidate unequivocally the mode(s) of origin of 2*n*-eggs. In the absence of cytological methods for determining the mode(s) of origin of 2*n*-eggs in potato, genetic methods are employed (Mendiburu & Peloquin, 1979). In these methods it is assumed that homozygosity increases with genetic distances from the centromere in FDR, whereas it decreases in SDR (Fig. 1).

Genetic analysis of the tetraploid progeny from $2x \times 4x$ crosses can be used to deduce the mode of origin of 2*n*-eggs. Previous investigations (Douches & Quiros, 1987, 1988; Jongedijk *et al*., 1991; Werner *et al*., 1992; Barone *et al*., 1995) lacked segregation data for several loci on a single chromosome, preventing a detailed examination of the degree of heterozygosity, the restitution mechanism and the number of crossovers per chromosome in each individual 2*n*-egg. This also prevented firm conclusions on the exclusive occurrence of one restitution mechanism or a mixture of several restitution mechanisms in a genotype. In a previous study, RFLP markers of chromosomes 6 and 8 of potato were identified that are informative for multilocus analysis of tetraploid progenies that are derived from $2x \times 4x$ crosses (Bastiaanssen, 1997).

In the present investigation, the distal biochemical marker *amylose-free* starch (*amf*) was used to demonstrate differences in the mode(s) of origin of

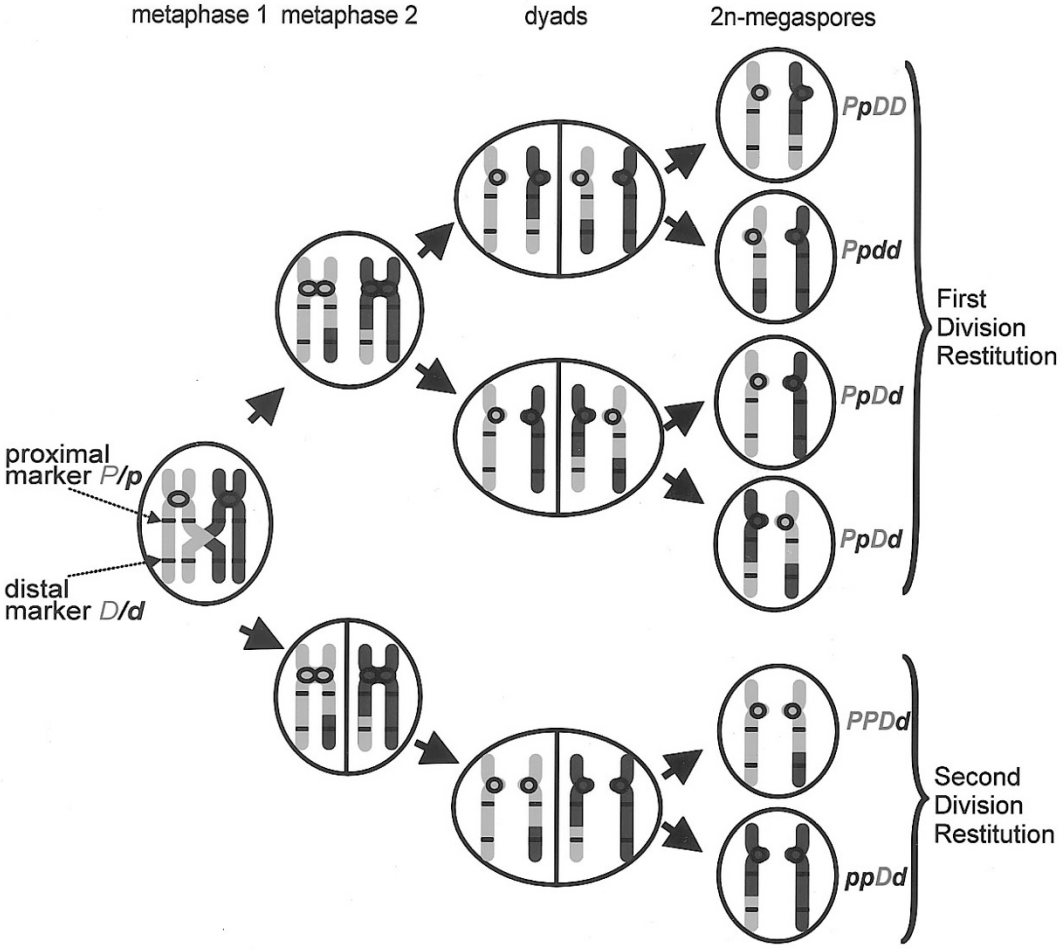

Fig. 1 The genetic consequences of first division restitution and second division restitution for a proximal marker locus (*P*/*p*) and a distal marker locus (*D*/*d*), assuming one crossover between these markers. The maternal chromosomes and alleles are indicated in light grey and upper case, whereas the paternal chromosomes and alleles are indicated in dark grey and lower case (after Mendiburu & Peloquin, 1979).

2*n*-eggs in four diploid clones of potato on the basis of the segregation ratios in their tetraploid progenies. Four RFLP loci on chromosome 8 were then used in a tetraploid progeny of one 2*n*-egg producing genotype to elucidate the restitution mechanism(s) of 2*n*-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 *S. tuberosum* (*tbr*) and *S. phureja* (*phu*), *S. chacoense* (*chc*), *S. microdontum* (*mcd*) or *S. spegazzinii* (*spg*) were used for the analysis of 2*n*-egg formation (Table 1). These four interspecific hybrids were heterozygous (*Amf*/*amf*) for the *amylose-free* starch marker and showed normal chromosome pairing during meiosis. The hybrids produced 2*n*-eggs in variable frequencies, as was evident from their seed sets following 2x × 4x crosses (Bastiaanssen, 1997).

The four diploid 2*n*-egg producing genotypes were crossed with four tetraploid *S. tuberosum* clones (Table 1) to generate progenies for genetic analysis. The tetraploid chromosome constitution of the progeny was ascertained by flow cytometry (Bastiaanssen, 1997).

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 (*Amf*/*Amf*/*amf*/*amf*), simplex (*Amf*/*amf*/*amf*/*amf*), and nulliplex (*amf*/*amf*/*amf*/*amf*) genotypes on the basis of the starch phenotypes of microspores after staining in Lugol/chloral hydrate (1 : 2) (Jacobsen *et al*., 1989, 1991; Bastiaanssen *et al*., 1996). The nulliplex genotypes in this tetraploid progeny were identified on the basis of only red (*amf*/*amf*) microspores. The microspores of the simplex genotypes were expected to segregate blue (*Amf*/*amf*) : red (*amf*/*amf*) in the ratio of 1 : 1. The microspores of the duplex genotypes were expected to comprise the genotypes *Amf*/*Amf* : *Amf*/*amf* : *amf*/*amf* in the ratio of 1 : 4 : 1, resulting in a segregation ratio of blue (*Amf*/−) : red (*amf*/*amf*) = 5 : 1. To distinguish the simplex and duplex genotypes, 100–500 microspores were scored.

Multilocus RFLP-analysis of the tetraploid progenies

In the present investigation, 138 tetraploid plants from a cross between the diploid *mcd* × *tbr* hybrid and Tetra 4 were analysed in more detail. The hybrid was heterozygous for four RFLP loci (*m* = *TG536*, *TG482*, *TG434*, *TG346*) on chromosome 8, and for each marker the alleles were distinguishable from the one to four different alleles (m^{δ}) of the tetraploid male (Bastiaanssen, 1997). The four selected markers covered a large part of chromosome 8. Both the diploid *mcd* and *tbr* parent of the 2*n*-egg-producing hybrid were included in the RFLP analysis to identify the parental origin of the alleles (m^{mcd} or m^{tbr}) at each of the four loci. This was essential for the classification of the $m^{mcd}m^{mcd}m^{\delta}m^{\delta}$, $m^{mcd}m^{tbr}m^{\delta}m^{\delta}$ and $m^{tbr}m^{tbr}m^{\delta}m^{\delta}$ genotypes in the tetraploid progeny, and for the detection of crossovers between the *mcd* and *tbr* genomes in the hybrid. A genetic map was constructed from the RFLP scores using the

Table 1 The clones that were used for 2x × 4x crosses to generate tetraploid progenies for genetic analysis of 2*n*-eggs

Solanum species involved	Clone*	Code	*Amf* genotype
Diploid 2*n*-egg producing female parents			
S. tuberosum × *S. phureja*	*tbr* × *phu* hybrid	HB93–7004–6	*Amf*/*amf*
S. chacoense × *S. tuberosum*	*chc* × *tbr* hybrid	HB94–7239–5	*Amf*/*amf*
S. tuberosum × *S. spegazzinii*	*tbr* × *spg* hybrid	HB93–7054–3	*Amf*/*amf*
S. microdontum × *S. tuberosum*	*mcd* × *tbr* hybrid	HB93–7108–8	*Amf*/*amf*
Tetraploid male parents			
S. tuberosum	Tetra 1	J90–6020–22	*amf*/*amf*/*amf*/*amf*
S. tuberosum	Tetra 2	J90–6011–3	*amf*/*amf*/*amf*/*amf*
S. tuberosum	Tetra 3	J90–6001–25	*amf*/*amf*/*amf*/*amf*
S. tuberosum	Tetra 4	105ESC90–32	*Amf*/*Amf*/*Amf*/*Amf*

**tbr*, *S. tuberosum*; *chc*, *S. chacoense*; *mcd*, *S. microdonitum*; *phu*, *S. phureja*, *spg*, *S. spegazzinii*.

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 four diploid *Amf*/*amf* genotypes were crossed with three nulliplex males (Table 1) to generate tetraploid progenies. Because all plants received two *amf* alleles from the nulliplex male parent, nulliplex progeny plants were derived from *amf*/*amf* 2*n*-eggs, whereas simplex plants originated from *Amf*/*amf* 2*n*-eggs, and duplex plants originated from *Amf*/*Amf* 2*n*-eggs. From the segregation ratios of starch phenotypes of the microspores in the tetraploid progeny plants, it was possible to classify them as nulliplex, simplex and duplex and thus establish the genotypes for the *amf* marker (Table 2). In the progenies from the *tbr* × *phu*, *chc* × *tbr* and *tbr* × *spg* hybrids, the simplex class far outnumbered the nulliplex and duplex classes (Table 2) showing a high frequency of *Amf*/*amf* 2*n*-eggs. In contrast, the nulliplex and duplex classes far outnumbered the simplex class in the progeny of the *mcd* × *tbr* hybrid. This clearly indicated a different mode of origin of the 2*n*-eggs in the *mcd* × *tbr* hybrid.

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 (Bastiaanssen *et al*., 1996). Heterozygous 2*n*-eggs *(Amf*/*amf)* could result from either FDR or SDR, as indicated for the distal locus in Fig. 1. Assuming normal frequency of crossovers, FDR would give rise to no more than 50% of heterozygous (*Amf*/*amf*) 2*n*-eggs. Thus, the most likely explanation for the high frequency of heterozygous (*Amf*/*amf*) 2*n*-eggs in the *tbr* × *phu*, *chc* × *tbr* and *tbr* × *spg* hybrids (Table 2) was SDR following the frequent occurrence of one crossover between the centromere and the *amf* locus. The suggested SDR 2*n*-egg formation was confirmed through RFLP analysis of a tetraploid progeny of the *tbr* × *spg* hybrid, in which homozygosity decreased with genetic distance from the centromere (Bastiaanssen, 1997).

The *mcd* × *tbr* hybrid behaved differently, producing a high frequency of homozygous (*Amf*/*Amf* and *amf*/*amf*) 2*n*-eggs. In the case of FDR 2*n*-egg formation, the frequency of these *Amf*/*Amf* and *amf*/*amf* 2*n*-eggs would not exceed 50%, whereas in the case of SDR 2*n*-egg formation, the observed high frequency could only occur with severely reduced crossing-over between the centromere and the *amf* locus. Another explanation might be that the 2*n*-eggs of the *mcd* × *tbr* hybrid resulted from the doubling of chromosomes in the haploid products of normal meiosis, a previously undescribed restitution mechanism in potato. If postmeiotic doubling of the chromosomes had occurred in the *mcd* × *tbr* hybrid, the 2*n*-eggs should be completely homozygous and show crossover events between the *mcd* and *tbr* genomes. In order to test this, the tetraploid progeny derived from the cross *mcd* × *tbr* hybrid × Tetra 4 was used for RFLP analysis.

Table 2 Segregation of the *amf* marker in progenies from *Amf*/*amf* × *amf*/*amf*/*amf*/*amf* crosses and its application to test the expected second division restitution (SDR) mode of 2*n*-egg formation in the four diploid clones of potato

	Genotypes of the 4x progeny plants from the *Amf*/*amf* × *amf*/*amf*/*amf*/*amf* crosses			
2*n*-egg producer (2x) *Amf*/*amf*	*amf*/*amf*/*amf*/*amf* (expected*)	*Amf*/*amf*/*amf*/*amf* (expected*)	*Amf*/*Amf*/*amf*/*amf* (expected*)	Probability SDR†
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

*The expected frequencies of the nulliplex, simplex and duplex genotypes for SDR were calculated with the formula of Mendiburu & 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 (Bastiaanssen *et al*., 1996).

†Based upon χ^2 tests for simplex and pooled nulliplex + duplex classes.

RFLP analysis of the tetraploid progeny derived from the mcd × tbr *hybrid and Tetra 4*

The 138 tetraploid plants from a *mcd* × *tbr* hybrid × Tetra 4 cross were genotyped at each of four fully informative marker loci (*TG536*, *TG481*, *TG434* and *TG346*) on chromosome 8. An example of a Southern blot showing polymorphisms for the probe *TG346* is shown in Fig. 2.

The marker loci *TG536*, *TG481*, *TG434* and *TG346* cover 89% of the genetic length of the linkage map of tomato chromosome 8 (Tanksley *et al*., 1992), and can be expected to give similar coverage in potato. The markers gave 10 distinct classes of genotypes in the tetraploid progeny with two remarkable features (Fig. 3). First, all plants had either the m^{mcd} allele or the allele m^{tbr} at all four loci. This indicated that all progeny plants were $m^{mcd}m^{mcd}m^{\delta}m^{\delta}$ or $m^{tbr}m^{tbr}m^{\delta}m^{\delta}$ and consequently originated from 2*n*-eggs in which chromosome 8 was completely homozygous. This homozygosity closely corresponded to the segregation pattern for the *amf* marker of the same chromosome (Table 2). Secondly, most genotypes (80) had m^{mcd} alleles and m^{tbr} alleles in homozygous condition for different markers of chromosome 8 (columns 3–10 in Fig. 3), showing frequent crossing-over between the two genomes in the *mcd* × *tbr* hybrid.

The 2*n*-eggs of the *mcd* × *tbr* hybrid comprised 58 nonrecombinant genotypes (columns 1 and 2 in Fig. 3), 72 genotypes with one crossover (columns 3–8 in Fig. 3), and eight genotypes with two crossovers (columns 9 and 10 in Fig. 3). The logical explanation for these genotypes is that chromosomes in haploid products of normal meiosis were doubled in order to give 2*n*-eggs in the *mcd* × *tbr* hybrid. This means that the genotypes of the 2*n*-eggs were equivalent to the genotypes of doubled haploids and that the genetic distances between the four RFLP loci of chromosome 8 in the *mcd* × *tbr* hybrid could be estimated using the doubled haploid option of the computer package JoinMap. The order of genes and the genetic distances were consistent with the tomato map of Tanksley *et al*. (1992) (Fig. 4).

Discussion

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

With the use of multiple genetic markers for chromosome 8 of potato, it has been demonstrated that the diploid *mcd* × *tbr* hybrid produced

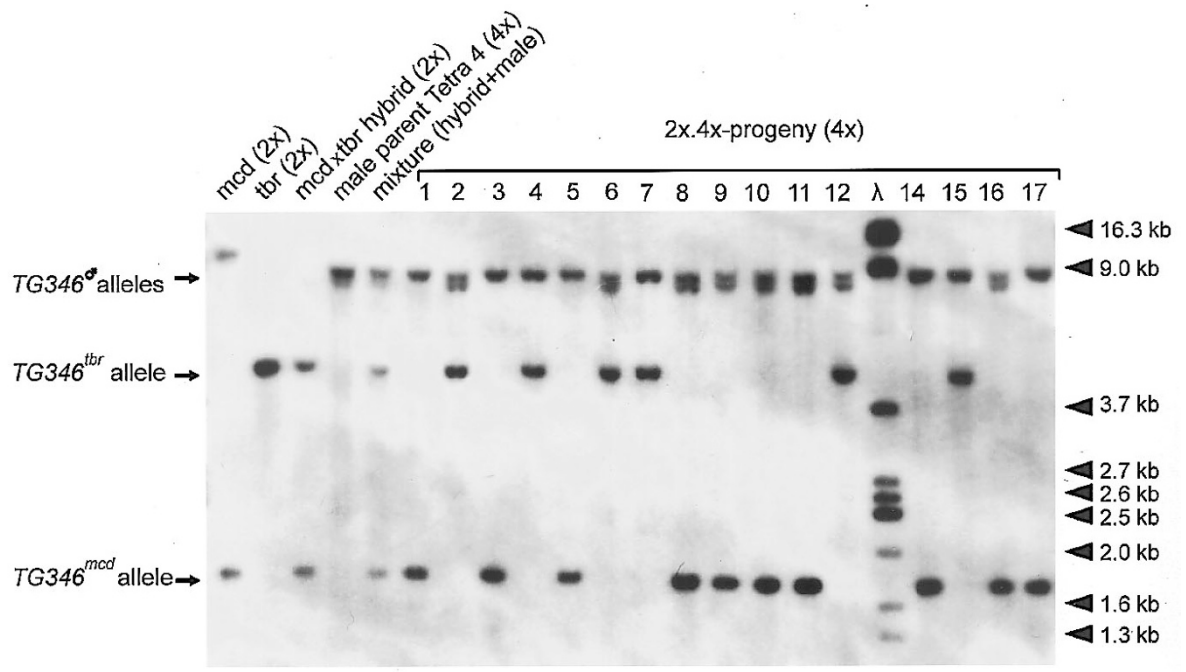

Fig. 2 Southern blots showing the absence of tetraploid progeny plants with both alleles of the *mcd* × *tbr* hybrid potato. The parental clones are shown in lanes 1–5; *mcd* parent (lane 1) and *tbr* parent (lane 2) of the diploid *mcd* × *tbr* hybrid (lane 3), the male parent Tetra 4 (lane 4), a mixture of *mcd* × *tbr* hybrid and Tetra 4 (lane 5). Sixteen plants of the tetraploid progeny of the cross *mcd* × *tbr* hybrid × Tetra 4 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).

completely homozygous 2*n*-eggs. The *amf* marker was very useful for the detection of this genotype, allowing quick assessment of the mode of 2*n*-egg formation, because of the full classification of the genotypes in the tetraploid progeny of *Amf/amf* × *amf/amf/amf/amf* crosses, and the prediction of the segregation ratio for different modes of origins of 2*n*-eggs based on the distal position of this marker locus and the occurrence of a high degree of chiasma interference (Bastiaanssen *et al*., 1996). This enabled detection of the homozygous 2*n*-eggs of the *mcd* × *tbr* hybrid, that clearly deviated from the segregation expected for SDR 2*n*-egg formation (Table 2). The homozygosity of the 2*n*-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 progeny plants of the same hybrid using four appropriate markers covering most of chromosome 8. In view of the complete homozygosity of all progeny plants at all four RFLP loci tested, the two simplex plants for the *amf* marker probably resulted from errors in the classification of duplex genotypes. The usefulness of the *amf* marker in facilitating the selection of clones

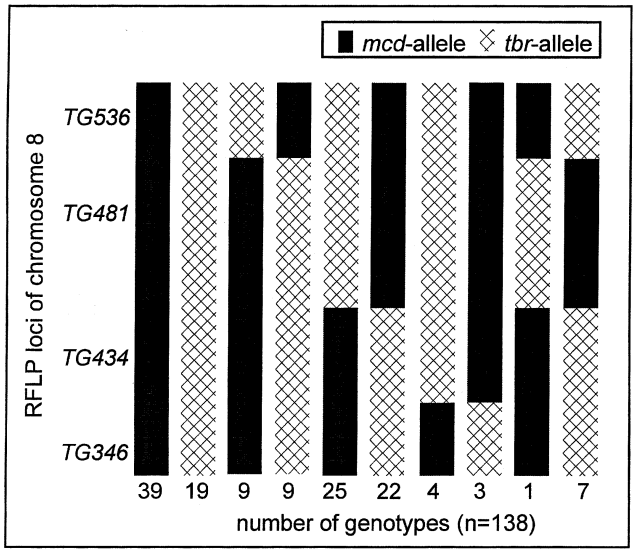

Fig. 3 Genotype frequency distribution of the tetraploid progeny derived from the potato cross *mcd* × *tbr* hybrid- × Tetra 4 to show the allelic composition of the 138 plants at four RFLP loci of chromosome 8. The order of the loci is the same as that on the tomato map of Tanksley *et al*. (1992). The bars represent the different genotypic classes, based on the presence of the m^{mcd} allele (solid black), or m^{tbr} allele (netted) at the four RFLP loci *TG536*, *TG481*, *TG434* and *TG346* of chromosome 8. The number under each bar represents the number of plants per genotypic class.

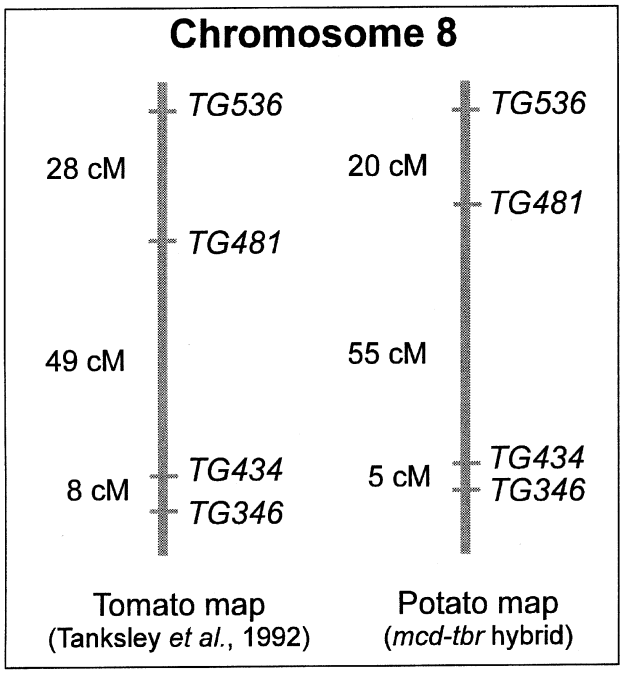

Fig. 4 Genetic map distances of chromosome 8 in tomato and the *mcd* × *tbr* hybrid.

with distinct restitution mechanisms was also demonstrated in progenies of the *tbr* × *spg* hybrid, in which the suggested SDR 2*n*-egg formation (Table 2) was confirmed through RFLP analysis (Bastiaanssen, 1997).

Discovery of completely homozygous 2n-eggs

In potato, highly heterozygous to relatively homozygous 2*n*-eggs that originated through FDR and SDR, respectively, have been detected previously (Iwanaga & Peloquin, 1979; Stelly & Peloquin, 1986; Douches & Quiros, 1988; Jongedijk *et al*., 1991; Werner *et al*., 1992; Barone *et al*., 1995). However, diploid genotypes with completely homozygous 2*n*-eggs have not been described before. The discovery of the *mcd* × *tbr* hybrid is therefore a valuable addition which completes a spectrum of genotypes giving rise to highly heterozygous to completely homozygous 2*n*-eggs.

Evidence for postmeiotic restitution in 2n-egg formation

In order to provide genetic evidence for postmeiotic doubling of chromosomes, it is essential to show genotypes of 2*n*-eggs that are recombinant as well as completely homozygous. These genotypes can only be shown in multilocus analysis, as was demon-

strated in the progeny of the *mcd* × *tbr* hybrid segregating for the m^{mcd} and m^{tbr} alleles at the four RFLP loci covering most of chromosome 8.

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

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

In addition to the discovery of a new restitution mechanism, the multilocus analysis of the 2*n*-eggs of the *mcd* × *tbr* hybrid elucidated the number of restitution mechanisms involved. Because of the complete homozygosity of the 2*n*-eggs for the four markers of chromosome 8, it was concluded that all 2*n*-eggs were derived from PMR. The absence of other restitution mechanisms could not have been demonstrated using a single marker locus per chromosome.

*Implications of PMR 2*n*-gametes*

For the breeding of potato at the diploid level, it is important to realize that 2*n*-eggs are not always of SDR origin. This is because the genetic consequences of PMR are quite distinct from those of SDR. Postmeiotic restitution 2*n*-eggs are highly relevant for potato genetics because of the possibility of generating homozygous diploid genotypes through, for example, 'prickle' pollinations (Hermsen & Verdenius, 1973) of PMR 2*n*-egg producers to induce parthenogenetic development of the homozygous 2*n*-eggs. Because 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*., 1987). Because 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. Furthermore, the generation of both anther-culture-derived and PMR 2*n*-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 2*n*-eggs might be especially attractive when a F_1 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).

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