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Early tapetal degeneration and meiotic defects are involved in the male sterility of *Solanum commersonii* (+) *S. tuberosum* somatic hybrids

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Abstract Somatic hybridization between *Solanum commersonii* and *S. tuberosum* resulted in the production of male-sterile hybrid plants, except for one fully male-fertile hybrid. The male-sterile hybrids exhibited a"pollen-less" phenotype, with rare pollen grains which were abnormal in shape and exine sculpture. Microsporogenesis and tapetal development were investigated both in male-sterile and male-fertile somatic hybrids to assess the cytological events that were involved in male sterility. The pattern of male sterility was complex, arising through mechanisms expressed at both sporophytic and gametophytic levels. Various abnormalities occurred first in the tapetum, and later during meiosis-II and cytokinesis. These caused the degeneration of the sporads and of the microspores when they were released. In the male-fertile hybrid, normal development of the tapetum and pollen mother cells was restored. The hypothesis that tapetal breakdown, meiosis-II and cytokinesis defects are related to each other, and depend on nuclear-mitochondrial interactions, is discussed. Because of the formation of multivalent chromosome configurations, it is likely that gene exchange between *S. commersonii* and *S. tuberosum* can occur in somatic hybrids, offering potential perspectives for the introgression of useful traits from *S. commersonii* into *S. tuberosum*.

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Introduction

Solanum commersonii is a tuber-bearing wild diploid species of potential value for potato breeding, because it possesses frost tolerance (Chen and Li 1980) as well as important resistances to biotic stresses (Hanneman and Bamberg 1986; Chavez et al. 1988). However, barriers to interspecific hybridization between *S. commersonii* ($2n = 2x = 24$, 1EBN) and *Solanum tuberosum* ($2n = 4x = 48$, 4EBN) exist due to endosperm balance number (EBN) diversity (Johnston et al. 1980). This causes embryo abortion (Smith and Desborough 1986), and thus prevents gene transfer from *S. commersonii* to *S. tuberosum*.

To overcome the interspecific incompatibility, somatic hybridization by protoplast electrofusion has been adopted (Cardi et al. 1993a). Alternatively, ploidy level and EBN value have been increased in *S. commersonii*, and attempts have been made to obtain hybrids after crossing of the resulting tetraploid clones ($2n = 4x = 48$, 2EBN) with dihaploid *S. tuberosum* ($2n = 2x = 24$, 2EBN) (Novy and Hanneman 1991; Carputo et al. 1995). But no pollen was produced by sexual triploid hybrids with *tuberosum* cytoplasm (Novy and Hanneman 1991) as well as by all but one of the somatic hybrids (Cardi et al. 1993b). On the other hand, sexual triploid hybrids with *commersonii* cytoplasm produced pollen, which was partially stainable (Carputo et al. 1995). Male sterility in somatic hybrids was maternally inherited (Cardi et al. 1993c) and therefore this can depend on negative nucleus-cytoplasm interactions, as found in other sexual interspecific hybrids (Hanneman and Peloquin 1981; Iwanaga et al. 1991).

Male sterility in somatic hybrids involving *S. tuberosum* has been reported also by other authors, but

microsporogenesis analysis did not allow the identification of the causes of male sterility (Ehlenfeldt and Helgeson 1987; Jacobsen et al. 1992; Mattheij et al. 1992; de Jong et al. 1993; Jacobsen et al. 1993; Wolters et al. 1994). By contrast, in other species, tapetal investigation in cytoplasmic male-sterile (CMS) genotypes has revealed its malfunctioning as a direct or indirect cause of CMS (Lee et al. 1979; Bino 1985a, 1985b; Holford et al. 1991; Kini et al. 1994; Zhang et al. 1994).

The present article reports detailed data on microsporogenesis and tapetal development both in male-sterile and male-fertile somatic hybrids between *S. commersonii* and *S. tuberosum* and describes the cytological events that are involved in male sterility.

Materials and Methods

Plant material

Nine *S. commersonii* (+) *S. tuberosum* somatic hybrids, produced by electrofusion (Cardi et al. 1993a), were grown from in vitro-plantlets and from tubers in an air-conditioned greenhouse (18–25°C) under natural daylight during spring and summer. Their morphology, chromosome number, fertility and frost tolerance were previously described (Cardi et al. 1993b). The fusion partners, i.e. diploid *S. commersonii* PI 243503 (designated CMM1) and dihaploid *S. tuberosum* DH81-7-1463 (SVP11), were grown from tubers under the same conditions as those of the hybrids. The genotype SVP11, which did not flower under the conditions mentioned above, was also grown in another location at a high altitude in a screenhouse during summer time.

Cytology

For microsporogenesis, 1.5–2.5-mm-size anthers were harvested from 3–6.5-mm-long floral buds, fixed in ethanol-propionic acid (3 : 1) with a small amount of ferric chloride at room temperature for at least 48 h and squashed in acetocarmine 2%. After coverslip removal by freezing with liquid N_2, the slides were made permanent by dipping them twice in butanol-acetic acid (1 : 1), once in absolute butanol, and finally by mounting the coverslip with Entellan.

Tapetum analysis

Two somatic hybrids, designated SH9A and SH9B, male-fertile and male-sterile respectively, were used for histological analysis. They originated from the same callus, and had the same chromosome number (2n = 48).

Floral buds and the isolated anthers were fixed in FAA (ethanol-formaldehyde-acetic acid 18 : 1 : 1) for 24–48 h, stored in 70% ethanol, dehydrated in a graded series and carried through the usual paraffin-embedding procedure. Serial sections (10–12 μm) were stained using Schiff's reagent and fast green. To stain callose, anther sections were treated with 0.1% aniline blue in 0.1 M potassium phosphate and observed under UV fluorescence.

Fertility

Pollen production was ascertained in somatic hybrids by squeezing anthers with a forceps, and pollen stainability was assessed using 2% acetocarmine. Pollen morphology was observed by electron microscopy after gold coating.

Results

Fusion partners

The donor parent *S. commersonii* (CMM1) exhibited a high percentage (98%) of pollen stainability and normal meiosis. The frequency of abnormalities, i.e. the occurrence of univalents (1.4 per cell) in microsporogenesis, was very low (Table 1). This resulted in cells with laggards (7%), and an asymmetric 13–11 distribution chromosomes to the two poles (1%) at anaphase-I, and telophase-II cells with micronuclei (4%) (data not shown). About 2% polyads occurred at the sporad stage (Table 2).

By contrast, the recipient parent *S. tuberosum* (SVP11) showed 5% pollen stainability only, and thus was characterised by highly sterile pollen grains of variable size with several abnormalities in microsporogenesis. The mean number of bivalents per cell was 10.8 (Table 1; Fig. 1a), but pairing failure occurred in 5% of pollen mother cells (PMCs), showing 24 univalents (Fig. 1b). At metaphase-I, precocious separation of bivalents was often observed (Fig. 1c). Lagging chromosomes, irregular disjunction, and asymmetric distribution to the two poles were seen as consequences of the occurrence of univalents (Fig. 1d, e, f). Nuclear restitution events were also observed: some prophase-II cells showed two nuclei, each containing 24 chromosomes (Fig. 1g). Irregular metaphases and anaphases occurred as a consequence of the equational division of a restitution nucleus with 24 chromosomes (Fig. 1h, i, j). In meiosis-II, degeneration of four-nucleated cells at telophase-II and lack of division of one nucleus were observed. In addition to tetrads, dyads and triads formed at the sporad stage in 35% and 37% of cases, respectively (Table 2).

Somatic hybrids

All somatic hybrids, except one, were male-sterile (Table 2). The rare pollen grains observed were abnormal. They were smaller and their surface was filled with bubble-like structures (Fig. 2). At meiosis, all somatic hybrids exhibited multivalent chromosome configurations (Table 1; Fig. 3a,b). Generally, one quadrivalent per PMC occurred in tetraploid ($2n = 48$) and hypotetraploid hybrids ($2n = 45$–47), and one hexavalent in hypohexaploid hybrids ($2n = 68$–70). The mean number of univalents per PMC differed among the various somatic hybrid clones, including the fertile (SH9A) and the sterile (SH9B) hybrids (Table 1; Fig. 3c). It ranged from 2.2 to 9.1, and was independent of ploidy level. In

Table 1 Chromosome associations at diakinesis in meiocytes of fusion partners (*S. commersonii*: CMM1, *S. tuberosum*: SVP11) and somatic hybrids of *S. commersonii* (+) *S. tuberosum*

Genotype	Chromosome number	Number of diakinesis cells	Range and mean number per diakinesis of				
			Univalents	Bivalents	Trivalents	Quadrivalents	Hexavalents
Fusion partners							
CMM1	24	42	1.4 (0–4)	11.3 (10–12)	0	0	0
SVP11	24	54	2.4 (0–24)	10.8 (0–12)	0	0	0
Somatic hybrids							
SH3A	48	22	3.0 (0–6)	20.3 (18–22)	0	1.1 (0–4)	0
SH9A	48	20	6.0 (0–16)	18.8 (15–23)	0	1.1 (0–3)	0
SH9B	48	25	2.2 (0–6)	20.5 (15–24)	0	1.2 (0–4)	0
SH2B	46	21	9.1 (6–14)	12.6 (7–19)	0.3 (0–1)	2.7 (0–5)	0
SH13D	45	16	3.6 (3–5)	19.3 (17–21)	0	0.7 (0–2)	0
SH16A	47	21	3.0 (1–7)	19.2 (14–23)	0	1.4 (0–4)	0
SH19A	46	20	4.8 (2–10)	18.2 (14–20)	0	1.2 (0–3)	0
SH5A	70	19	6.1 (4–8)	21.2 (11–32)	0.1 (0–1)	3.2 (0–8)	1.4 (0–4)
SH8B	68	22	8.5 (0–16)	19.6 (11–26)	0.7 (0–2)	2.6 (1–6)	1.3 (0–3)

Table 2 Frequency of sporads and pollen stainability in fusion partners (*S. commersonnii*: CMM1, *S. tubersonum*: SVP11) and somatic hybrids of *S. commersonii* (+) *S. tubersonum*; a = rare abnormal pollen grains

Genotype	Number of sporads	Sporad types (%)					Pollen stainability (%)
		Monad	Dyad	Triad	Tetrad	Polyad	
Fusion partners							
CMM1	106	0	0	0	98	2	98
SVP11	98	0	35	37	27	1	5
Somatic hybrids							
SH3A	62	16	74	10	0	0	a
SH9A	147	0	3	8	78	11	98
SH9B	119	17	75	8	0	0	a
SH2B	53	10	77	13	0	0	a
SH13D	116	5	77	18	0	0	a
SH16A	108	19	70	11	0	0	a
SH19A	66	8	80	12	0	0	a
SH5A	58	16	70	14	0	0	a
SH8B	81	16	68	15	1	0	a

general, the hypoploid hybrids showed a higher number of univalents, but some variation occurred within the hypotetraploids and tetraploids. The male-fertile hybrid SH9A showed a high frequency of unpaired chromosomes. At metaphase-I, precocious disjunction of bivalents and early movement of univalents were commonly observed in all hybrids (Fig. 3d). Telophase-I cells with micronuclei occurred at frequencies ranging from 1% to 61%. Meiosis-II was abnormal in all somatic hybrids, except in the fertile hybrid SH9A. After prophase-II, the other stages of meiosis-II were absent (Fig. 3e). The two nuclei of prophase-II frequently restituted to form a single nucleus. When meiosis-II proceeded, one nucleus did not divide, and the other often separated abnormally (Fig. 3f, g). At the sporad stage, tetrads were never observed in tetraploid hybrids, and they occurred only sporadically in hexaploid hybrids. Dyads occurred at a high frequency (68–80%) (Fig. 3h), when compared to monads or triads (Table 2). Moreover, dyads and triads exhibited

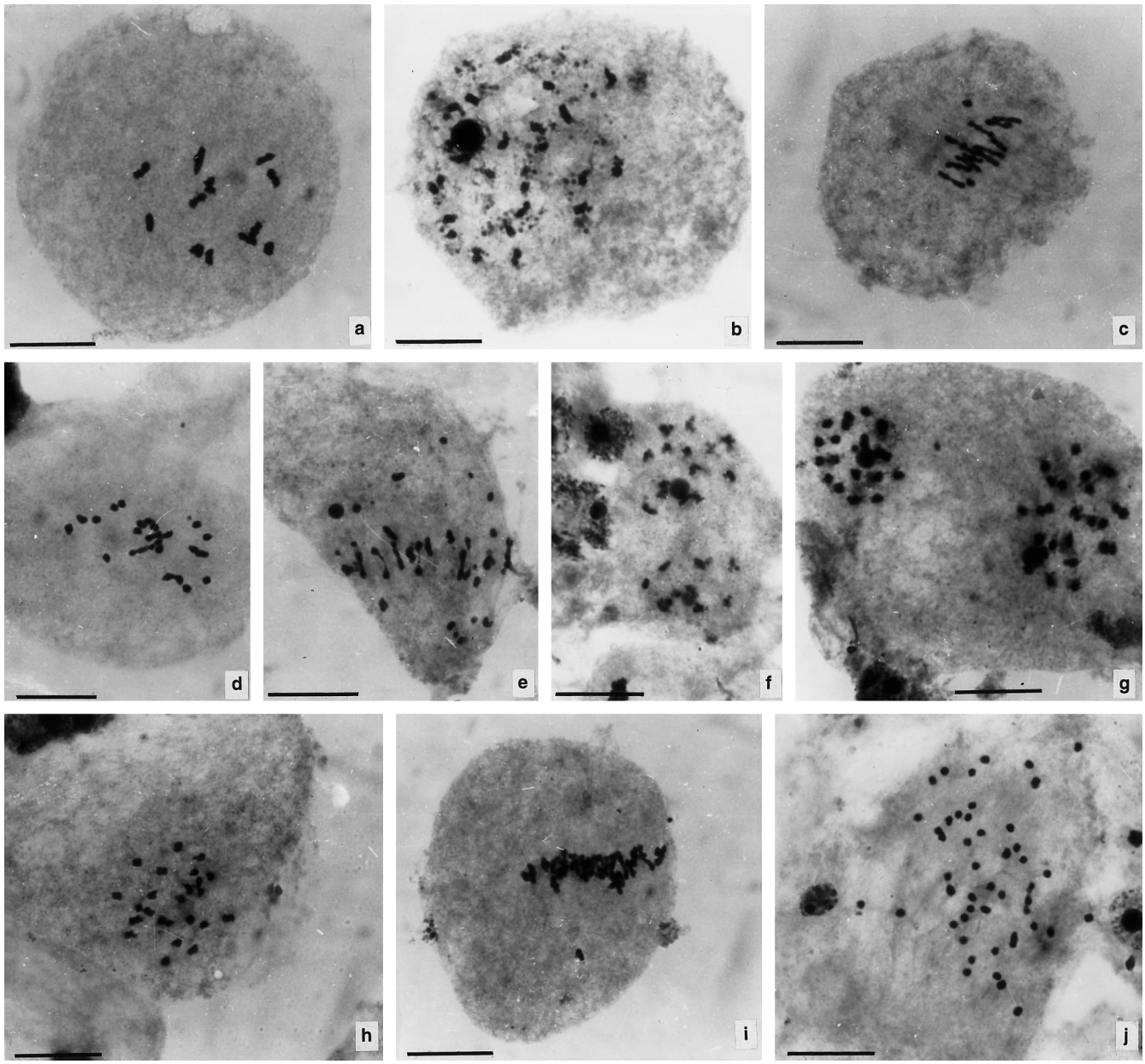


Fig. 1 a–j Microsporogenesis in the dihaploid *S. tuberosum* fusion partner SVP11. Chromosome associations showing 12 bivalents **(a)** and 24 univalents at diakinesis **(b)**; metaphase-I stage showing 12 bivalents in the equatorial plate, one precociously separated **(c)**, early movement of univalents **(d)**, reductional segregation of bivalents and equational segregation of univalents **(e)**; prophase-II stage showing an asymmetric 14–10 distribution of chromosomes at the two poles **(f)**, restitution nuclei each containing approximately 24 chromosomes **(g)**; late prophase-II showing a restitution nucleus with 24 chromosomes **(h)**; metaphase and anaphase stages in pollen mother cells (PMCs) with a restitution nucleus **(i, j)** (The bar represents 5 μm)

aberrant morphology due to abnormalities in cytokinesis, involving the occurrence of incomplete cell walls and persistent microtubules. When cytokinesis was absent or incomplete, the nuclei of the PMCs restituted to form a monad.

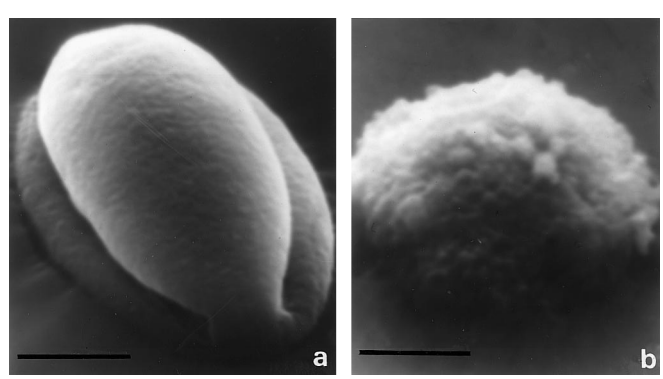


Fig. 2 Pollen grains observed by scanning electron microscopy in male-fertile somatic hybrid SH9A **(a)** and in male-sterile SH9B **(b)** (The bar represents 5 μm)

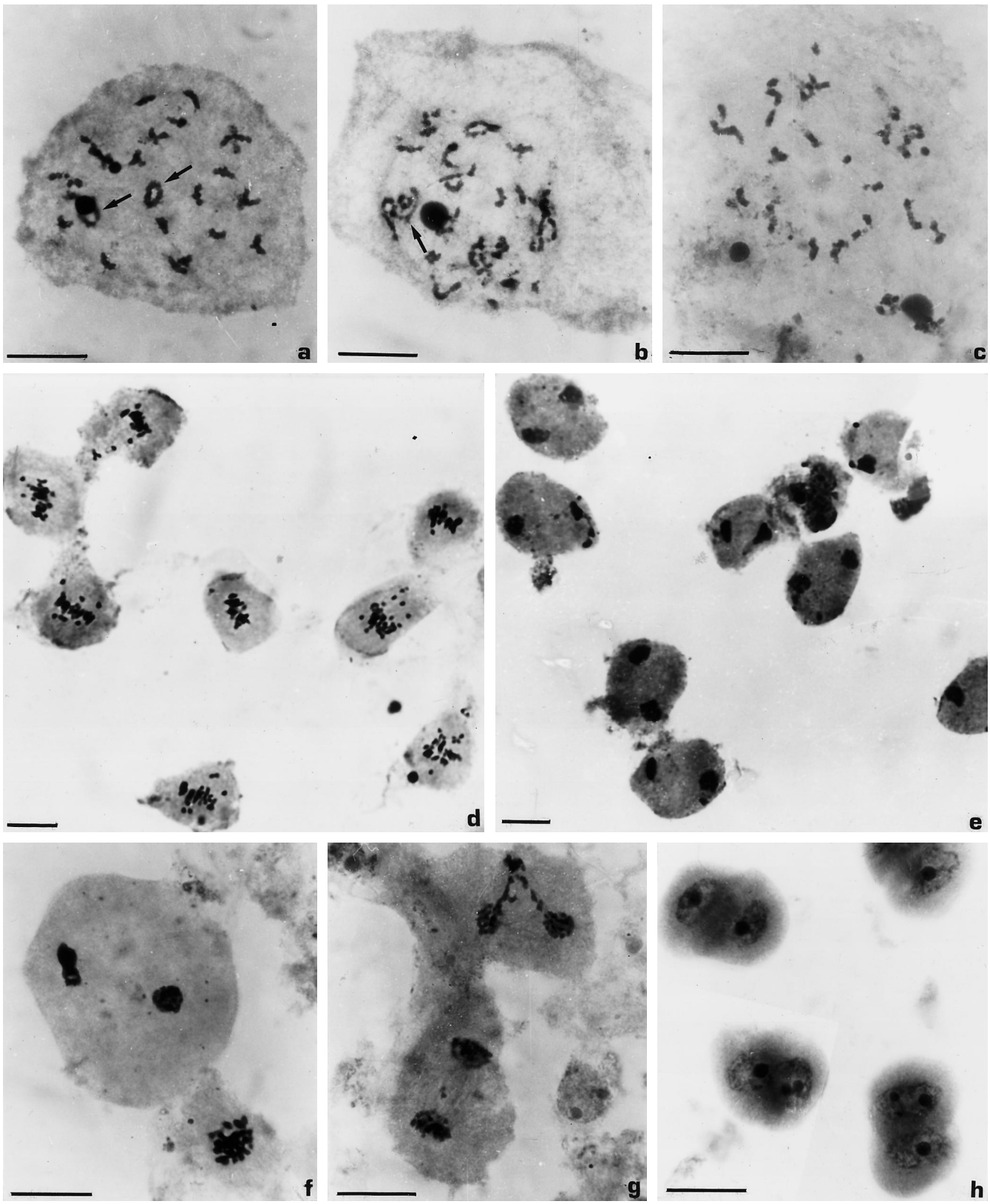


Fig. 3a–h Microsporogenesis in somatic hybrids (SH). Chromosome associations at late diplotene showing two quadrivalents (*arrows*) and 20 bivalents in a male-sterile tetraploid SH ($2n = 48$) **(a)**; one hexavalent (*arrow*), three quadrivalents, 23 bivalents and four univalents in a male-sterile hypohexaploid SH ($2n = 68$) **(b)**; 18 bivalents and 12 univalents in a male-fertile tetraploid SH ($2n = 48$) **(c)**; several metaphase-I PMCs showing early disjunction of bivalents in a male-sterile hypotetraploid SH **(d)**; PMCs showing lack of equational division and the occurrence of micronuclei in a male-sterile SH **(e)**; PMCs in meiosis-II showing irregular divisions and bridges in a male-sterile SH **(f, g)**; sporad stage showing dyads in a male-sterile SH **(h)**. (The bar represents 5μm)

The male-fertile somatic hybrid SH9A exhibited a normal meiosis-II and a high frequency (78%) of tetrads (Table 2). The lagging chromosomes and micronuclei, resulting from the unpaired chromosomes, caused the formation of some polyads (11%). Parallel spindles, dyads and 2n pollen (7%) were also observed in SH9A.

Tapetal development

Prior to meiosis, the male-sterile somatic hybrid SH9B and male-fertile SH9A did not show any difference in anther morphology. The sporogenous cells were surrounded by a continuous layer of tapetum, that was differentiated into an inner (connective side) and outer tapetum (external side). During the early meiotic stages, a large vacuole occurred predominantly in the inner tapetal cells of SH9B, whereas it was absent in SH9A cells. The outer tapetal cells of SH9B were comparable with those of SH9A. After prophase-I, aberrant development became evident in the inner tapetum of the male-sterile hybrid SH9B: the vacuole occupied most of the cell volume and the nucleus was flattened against the wall. However, the outer tapetum was similar in SH9A and SH9B (Fig. 4a, b). During meiosis-II, the inner tapetum of the sterile SH9B degenerated first, and outer tapetum later (Fig. 4d). The tapetal cells were disrupted; droplets and vesicles were detected in the loculus. The time of tapetum breakdown varied from the end of meiosis-I to the sporad stage. However, it always occurred earlier in the sterile hybrid SH9B than in the fertile hybrid SH9A (Fig. 4c). The tapetum degenerated in SH9A after the release of microspores (Fig. 4e). At anthesis, anthers of normal morphology contained an empty locule in the sterile SH9B (Fig. 4f).

Callose was deposited around the meiocytes during meiosis in SH9B, but it is not clear if it degenerated earlier in SH9B than in SH9A (Fig. 4g, h).

Discussion

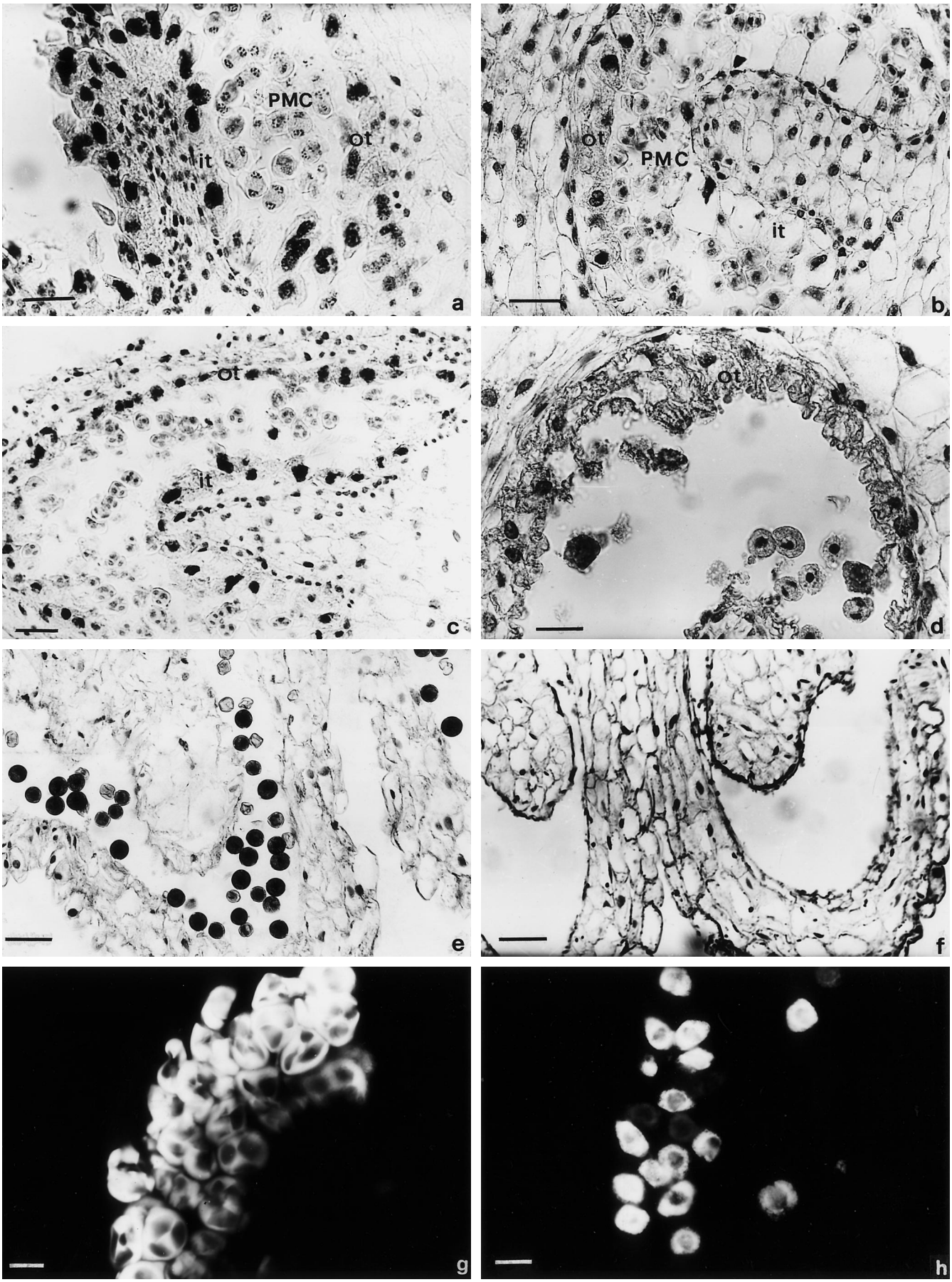
Somatic hybridization between the wild species *S. commersonii* and the cultivated *S. tuberosum* resulted in male-sterile hybrids, except for one fully male-fertile hybrid. Although a number of features seem to be common between the *S. tuberosum* fusion partner and the sterile somatic hybrids, it is important to note some differences regarding the occurrence of male sterility. The sterile hybrids exhibited a "pollen-less" phenotype, with rare pollen grains which were abnormal in shape and exine sculpture.

The *tuberosum* fusion partner SVP11 showed the formation of pollen grains, although pollen stainability was only 5%. Previously, pollen stainability values of 10–15% in SVP11 were reported in other studies (Mattheij et al. 1992; Pijnacker et al. 1992; Cardi T., unpublished results). The high incidence of sterile pollen grains in SVP11 has not been explained in previous investigations, and it is likely that, as postulated in other *S. tuberosum* dihaploids (Ross et al. 1964; Carroll and Low 1976; Nilmalgoda 1994), the occurrence of homozygous deleterious recessive genes, as a consequence of dihaploidy, could affect male fertility. Based on microsporogenesis data obtained in the present study, asynapsis and degeneration of PMCs before cytokinesis can be considered as causes of male sterility in SVP11, although meiotic restitution mechanisms restore fertility in large-sized 2n pollen at a low frequency. However, the frequency of pollen abortion is much higher than meiotic abnormalities, and possibly other deleterious mechanisms might be expressed at the haploid stage causing abortion.

Although it cannot be excluded that the meiotic defects of SVP11 are, at least partly, transferred to somatic hybrids, the predominant meiotic abnormality, i.e. the breakdown of meiosis-II, observed in the sterile hybrids seems independent from SVP11. The male-sterile sexual hybrids between *S. commersonii* and different *S. tuberosum* dihaploids also showed meiotic arrest at even earlier stages (Novy and Hanneman 1991). Moreover, when SVP11 was used as a fusion partner in combination with *S. phureja* and *S. circaeifolium*, somatic hybrids resulted in either male-fertile or male-sterile plants, respectively (Mattheij et al. 1992; Pijnacker et al. 1992). Other somatic hybrids between *S. commersonii* and a different *S. tuberosum* dihaploid also resulted in male-sterile plants (Carotenuto and Bastia 1995). The male fertility of sexual hybrids between 4*x S. commersonii* (2EBN) and 2*x S. tuberosum* (2EBN) depended on the direction of the cross: no pollen production was found when *S. commersonii* was used as a male parent (Novy and Hanneman 1991), whereas hybrids with 4*x commersonii* as female parent exhibited stainable pollen ranging from 5% to 74% (Carputo et al. 1995).

Thus, based on these considerations it can be suggested that the male sterility of both sexual and somatic hybrids is caused by the interaction between *tuberosum* sensitive cytoplasm and nuclear genes of

Fig. 4a–h Transverse sections through anthers from the male-fertile somatic hybrid SH9A **(a, c, e, g)** and the male-sterile somatic hybrid SH9B **(b, d, f, h)**. PMCs at the meiosis-I stage **(a, b)** and inner tapetal cells (*it*) showing signs of degeneration in SH9B. The outer tapetal cells (*ot*) are not aberrant. At the end of meiosis-II the inner tapetum has completely degenerated in the sterile hybrid, and remnants of the outer tapetal cells are visible **(d)**, whereas tapetal cells start to vacuolize in the fertile hybrid **(c)**. Anther locules at anthesis are filled with pollen grains in the fertile hybrid **(e)** and completely empty in the sterile hybrid **(f)**. A callose wall is present in PMCs of both male-fertile **(g)** and sterile somatic hybrids **(h)** at the end of meiosis-II. (The bars represent 40 μm in Figs. **a**, **b**, **c**; 30 μm in Figs. **d**, **e**, **f**; and 10 μm in Figs. **g**, **h**)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S. commersonii, as commonly found in other interspecific crosses in *Solanum* (Hanneman and Peloquin 1981; Iwanaga et al. 1991). The cytoplasmic involvement in the male sterility of somatic hybrids obtained in the present study is also supported by the fact that the fertility phenotype of the fertile hybrid SH9A and the sterile hybrid SH9B was inherited maternally, since no segregation was observed in progenies (Cardi et al. 1993c).

The pattern of male sterility in the somatic hybrids in this study was complex, arising through mechanisms expressed at both sporophytic and gametophytic levels. Various abnormalities occurred first in the tapetum and later during meiosis-II and cytokinesis. The pattern of PMC degeneration in these hybrids has some features analogous to the "sporad" form of genic-cytoplasmic male sterility observed in different diploid species of *Solanum* characterized by non-dehiscent anthers (Grun and Aubertin 1966). Similar to most CMS genotypes, the first cytological abnormality detected in the present somatic hybrids involves the tapetum; but additionally in this material both tapetum and PMCs are equally affected, and the development of both tissues is defective. The male-fertility phenotype of the hybrids could be dependent on rearrangements of the mitochondrial genome, as observed in CMS genotypes (Dewey et al. 1986; Young and Hanson 1987; Johns et al. 1992). However, it is not clear whether tapetal breakdown and the meiosis-II and cytokinesis defects are related to each other. The male fertility of SH9A, occurring with the restoration of normal development in tapetum and PMCs, seems to indicate a common pathway. Fertile SH9A and sterile SH9B were regenerated from the same callus, but their mtDNA was rearranged differently prior to regeneration (Cardi et al. 1993c). Similarly, *S. tuberosum* (+) *S. brevidens* somatic hybrids, regenerated from the same callus, did not possess the same mitochondrial genome (Kemble et al. 1986).

Because of the formation of multivalent configurations, gene exchange between *S. commersonii* and *S. tuberosum* is supposed to occur in somatic hybrids. The number of quadrivalents per cell, however, is lower in the tetraploid somatic hybrids than in autotetraploid *S. commersonii* as well as in allotetraploids produced from intra- and inter-series hybrids between diploid species (Beamish et al. 1957; Dvorak 1983; Matsubayashi 1983). The low number of multivalents suggests the existence of differentiation between the genomes of *S. commersonii* and *S. tuberosum*, although they are reported to share a common genome designated as A. According to Matsubayashi (1991) the genome formula for *S. commersonii* is AA and for *S. tuberosum* is AAA^tA^t. The superscript "t" indicates minor chromosome differentiation, which can be interpreted as structural (Matsubayashi 1991) or "non-structural due to the evolution of non-coding sequences" (Dvorak 1983), and is responsible for reduced homoeologous pairing. Heteromorphic bivalents also occur in somatic hybrids, and therefore pairing is not preferential, at least in certain chromosomes.

Multivalent chromosome configurations have also been reported in other interspecific and intergeneric somatic hybrids involving *S. tuberosum* (Ehlenfeldt and Helgeson 1987; Mattheij et al. 1992; Pijnacker et al. 1992; de Jong et al. 1993; Wolters et al. 1994). In *S. tuberosum* (+) *S. brevidens* somatic hybrids, intergenomic recombination was confirmed by molecular marker analysis (Williams et al. 1990).

Backcrosses between the somatic hybrids and 4*x* potato varieties have been performed recently (Cardi et al. 1996). Complete fertility of SH9A and female fertility of other somatic hybrids, together with intergenomic recombination, can offer potential perspectives for the introgression of useful traits from *S. commersonii* into *S. tuberosum*.

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