Genetic, quantitative and microscopic evidence for fusion of haploid nuclei and growth of somatic calli in cultured $ms10^{35}$ tomato anthers

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Abstract In plant breeding, androgenic doubled haploids represent powerful tools to save time and resources for pure line generation. While in many species efficient protocols are known, in tomato (*Solanum lycopersicum*), the knowledge on the induction of androgenesis is still very scarce, and little is known about the particularities of this highly recalcitrant species. The only known method capable of yielding haploid/doubled haploid tomato plants is anther culture. However, this method has important limitations, including low efficiency of haploid induction and a low proportion of spontaneously doubled haploids. To understand these limitations better, we have analyzed the process of callus formation in anthers of tomato lines carrying the $ms10^{35}$ gene for male-sterility, using light and electron microscopy, flow cytometry and genetic analysis with morphological and molecular markers. Our results demonstrate that haploid, doubled haploid and diploid calli occur in tomato anthers, although at different frequencies. Diploid calli derived either from somatic cells or from the fusion of two genetically different haploid nuclei account for more than 90% of the total of calli produced. Somatic calli are derived from the stubs of connective tissue present in the interlocular septa of anthers. This growth is markedly increased in the $ms10^{35}$ mutants, which explains their higher callogenic rates than standard tomato lines. Together, our results reveal serious drawbacks that explain the low efficiency of anther-derived, doubled haploid production in tomato, and stress the need for alternatives towards doubled haploidy.

Keywords Androgenesis · Anther culture · Doubled haploid · Male sterility · Meiocyte · Nuclear fusion · *Solanum lycopersicum*

Introduction

Androgenesis is defined as a set of biological processes leading to the development of individuals derived from the nuclei of male spores (Seguí-Simarro [2010]). Androgenesis can be a powerful shortcut leading to the production of homozygous, doubled haploid (DH) pure lines in just one in vitro generation. Compared to traditional breeding methods, the considerable savings in terms of costs and time make this method the choice in those species where efficient androgenesis protocols are well established. In species such as tobacco, rapeseed, wheat or barley, it is relatively easy to produce androgenic DHs in some cultivars (Seguí-Simarro

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and Nuez 2008). Unfortunately, this is not the case in tomato.

While tomato is the first vegetable crop worldwide in terms of production and cultivated area (FAO-STAT 2009), little is known about androgenesis in tomato. Since 1971 (Gresshoff and Doy 1972; Sharp and Dougall 1971), nearly all attempts to induce androgenesis in tomato have focused on the in vitro anther culture. A significant number of culture media types, conditions, combinations of nutrients, vitamins, growth factors and supplements have been assessed (reviewed in Bal and Abak 2007). Different laboratories have reported induction of calli (Dao and Shamina 1978; Jaramillo and Summers 1990, 1991; Sharp et al. 1972) or embryo-like multicellular structures (Dao and Shamina 1978; Varghese and Gulshan 1986), and regeneration of roots (Gresshoff and Doy 1972; Gulshan and Sharma 1981; Levenko et al. 1977; Sharp and Dougall 1971) or apical shoots (Ma et al. 1999). Only two laboratories have published on the regeneration of entire plants with a clear haploid origin (Seguí-Simarro and Nuez 2005, 2007; Shtereva et al. 1998; Zagorska et al. 1998, 2004), but also with a high percentage of mixoploid calli and at a low general efficiency of the process. At present, a detailed study about the causes of such a low efficiency and the impact of somatic tissues on the total of calli produced is still lacking.

In general, the most critical parameters in the induction of androgenesis are the genotype and the developmental stage of the microspore (Seguí-Simarro and Nuez 2008). Whereas in nearly all responsive species the inducible stage revolves around the first pollen mitosis (Touraev et al. 2001), in tomato, most studies based on anther culture point to the meiocyte as the inducible stage (Gresshoff and Doy 1972; Seguí-Simarro and Nuez 2005, 2007; Shtereva et al. 1998; Summers et al. 1992; Zamir et al. 1980). The second critical issue is the genotype. Some male-sterile mutant lines of different tomato cultivars have been reported as especially sensitive to the induction of androgenic calli (Seguí-Simarro and Nuez 2005, 2007; Shtereva et al. 1998; Zagorska et al. 1998, 2004; Zamir et al. 1980). Specifically, male-sterile tomato lines carrying the mutant gene $ms10^{35}$ exhibited an increased production of callus masses compared to the fertile lines. It was suggested that somatic tissues of the $ms10^{35}$ mutants' anthers also contribute to the total calli produced, since haploid origin could be demonstrated only for a small percentage of the calli. However, such aspects as to how and which somatic cells are induced to proliferate in these mutants remain largely unknown.

Previous research on androgenic calli from the $ms10^{35}$ mutants also indicated a major undesirable feature of this system, the presence of heterozygous calli. In addition to the proliferation of somatic (diploid) tissues of the anther walls, it is currently believed that heterozygous individuals may also originate from fusion of two different haploid meiotic cells, prior to their release as individual microspores (Seguí-Simarro and Nuez 2007). However, no genetic evidence for such fusions has ever been presented.

From all of these studies it is evident that tomato is extremely recalcitrant to androgenesis, and additional efforts have to be devoted to understand the specifics of the process in this species as a prerequisite step towards the design of an efficient haploid induction protocol. In this work, we provide evidence for the occurrence of nuclear fusions within meiocytes, and study to what extent such fusions of haploid nuclei and the presence of callus of somatic origin may be a drawback in this system. We used flow cytometry, molecular and morphological markers to evaluate the putative gametophytic/sporophytic origin of calli and regenerants. To reconstruct the process of haploid nuclear fusion, we used electron microscopy. Combining light and electron microscopy we reconstructed the process of microsporogenesis in the $ms10^{35}/ms10^{35}$ male-sterile mutant of the 'Resaplus' cultivar as well as in the fertile genotype for comparison. The putative origin of callus and regenerated plants have been addressed through the morphological and molecular characterization of the callus and regenerants produced by the in vitro culture of anthers of the $ms10^{35}/+$ hybrid between 'Resaplus' (the fertile line) and $ms10^{35}/ms10^{35}$ (the male-sterile mutant). The $ms10^{35}$ gene is linked to the aa gene for the absence of anthocyanin in the vegetative parts, with a recombination frequency ranging from 1.7 to 4.1% (Durand 1981; Philouze 1974). The $ms10^{35}$ gene is also linked to the wo gene for strong pubescence in leaves and stems with a recombination frequency of 1.1% (Durand 1981). Thus, we used the presence or absence of the $ms10^{35}$, aa and wo phenotypes as morphological markers in a segregating progeny derived from donor plants heterozygous for the three genes.

Our results shed light on the remarkable impact of somatic cell proliferation in the production of calli from specific cell types of the locular tissues of anthers, as well as on the fusion of genetically different haploid nuclei within the meiocyte. Together, these results explain why it is so difficult to obtain doubled haploids in tomato via anther culture. These results are also relevant for a better understanding of androgenesis induction in tomato, but also for future designs to improve its efficiency.

Materials and methods

Plant material

Tomato donor plants of the cv. 'Resaplus' (purple, moderately pubescent, male fertile) and a male sterile mutant of 'Resaplus' carrying the $ms10^{35}$ gene (green and hairy, recessive for the wo and aa genes) were obtained from the COMAV germplasm collection and used as anther donor plants. Hybrids between these two genotypes (and therefore heterozygous for the three linked genes) were produced and used as anther donors. These three genotypes will be abbreviated as $+/+$, $ms10^{35}/ms10^{35}$, and $ms10^{35}/+$, respectively. Plants were grown in the greenhouses of the COMAV Institute, at the Universidad Politécnica de Valencia, at 18°C under natural light during the months of September to February, for three consecutive years.

In vitro anther culture and plant regeneration

Buds ranging in length from 4 to 5 mm were collected from flowering plants and their anthers were dissected and plated as described in Seguí-Simarro and Nuez (2007). As a control, one out of the six anthers in a single bud was live squashed on a microscopic slide, stained with DAPI and observed under the microscope. Only anthers containing meiocytes before tetrad cellularization (between metaphase I and telophase II) were inoculated in plates with induction medium. Induction medium consisted on Murashige and Skoog (MS) basal medium + vitamins (Murashige and Skoog 1962), pH 5.7, supplemented with 2.5 g l^{-1} Phytagel, 20 g l^{-1} sucrose, 1 mg l^{-1} 2ip and 2 mg l^{-1} IAA). Dishes were kept in a growth cabinet at 25°C, in darkness for 1 month,

and then under a 16/8 photoperiod. Anthers and developing calli were transferred to fresh medium on a monthly basis, discarding anthers either necrotic or not responding to the induction treatment. Green or partially green, proliferating calli were transferred to regeneration medium (4.4 g l^{-1} MS medium + vitamins pH 5.7, 2.5 g l^{-1} Phytagel, 20 g l^{-1} sucrose and 0.25 mg l^{-1} zeatin riboside). Developing shoots were excised from the callus and transferred to glass tubes or magenta pots containing regeneration medium. Non-rooting, developed shoots were transferred to rooting medium (2.2 g l^{-1} MS + vitamins, 2.5 g l^{-1} Phytagel and 10 g l^{-1} sucrose, pH 5.7). Complete, rooted plantlets were transferred to pots with soil and acclimated for 1 week under greenhouse conditions.

Flow cytometry

Small pieces of cultured young calli and leaves from regenerated plants were processed using the CyStain UV Precise P kit (Partec GmbH, Münster, Germany). Additionally, young leaf samples from donor plants were analyzed and used as standards for 2C DNA content. Samples were chopped at 4°C with a razor blade in 400 μl of nuclear extraction buffer (NEB). After 1 min incubation in NEB, 1.6 ml of DAPI-based staining buffer was added and incubated for 2 min. Extracted nuclei were filtered through 30 μm CellTricks filters (Partec GmbH, Münster, Germany) and immediately analyzed in a Partec PA-I Ploidy Analyzer.

Genetic analysis with microsatellite molecular markers

To determine the origin of the regenerants, donor plants of the $ms10^{35}/+$ genotype were screened using microsatellite markers (SSR). Young leaf tissue was sampled from 5 different donor plants, and genomic DNA was isolated from 50 mg of tissue using the modified CTAB (hexadecyl trimethylammonium bromide) method described in Ferriol et al. (2003). Each plant was analyzed using the following 21 SSR markers known to be polymorphic in other tomato cultivars: LE21085, LEATPACAb, LEMDDNb, LEWIPIG, LPHFS24, LEGAST1, LELEUZIP, LE20592, LECAB9, LEGTOM5, LEATPACAa and LEILV1B selected from Smulders et al. (1997); TMS9 and TMS33 selected from Areshchenkova and

Ganal ([1999](#)) and SSR287, SSR63, SSR344, SSR70, SSR20, SSR111 and SSR248 selected from http://www.sgn.cornell.edu/). The forward primers were labeled with different fluorescent dyes and six loci were simultaneously detected using an ABI PRISM 310 Genetic Analyzer. Among the 21 markers tested, 15 did not reveal any polymorphism in the donor plants, and two (TMS9 and SSR20) appeared polymorphic for some individuals and monomorphic for others. Only four markers (TMS33, SSR63, SSR356 and SSR248) were clearly polymorphic for all donor plants tested. These markers were used to verify the genetic status of regenerants.

Light and electron microscopy

We processed samples of anthers of the $ms10^{35}/+$ genotype soon after anther culture initiation and of the $ms10^{35}/ms10^{35}$ and $+/+$ (Resaplus) genotypes at different stages during microsporogenesis. For light microscopy, samples were processed as in Seguí-Simarro and Nuez ([2005](#)). Thin (2 μm) sections were produced with a Leica UC6 ultratome and observed under bright field and phase contrast in a Nikon Eclipse E1000 microscope. For electron microscopy, samples were fixed in the Karnovsky's fixative, post fixed in 2% OsO4, dehydrated in ethanol series, embedded and polymerized in Epon resin according to Seguí-Simarro and Nuez ([2007](#)). Ultrathin (~80 nm) sections were produced with a Leica UC6 ultratome, mounted onto formvar and carbon-coated copper grids, counterstained with uranyl acetate and lead citrate, and observed in a Philips CM10 transmission electron microscope operating at 100 kV.

Results

Callus production and plant regeneration from anther cultures of the $ms10^{35}/ms10^{35}$, $ms10^{35}/+$ and $+/+$ genotypes

A total of 1,842 anthers (744 from $ms10^{35}/ms10^{35}$ donor plants, 720 from $ms10^{35}/+$ plants and 378 from $+/+$ plants) were excised at stages corresponding to meiotic metaphase I through telophase II (Seguí-Simarro and Nuez [2005](#)). Upon culture, callus masses were observed to emerge out of the locules of swollen anthers (Fig. 1a). A total of 965 calli were obtained

from the three genotypes. As long as they were maintained in darkness on the induction medium, most calli proliferated quickly and considerably increased in size. Upon exposure to light, green regions appeared at the callus surface (Fig. 1b). In viable calli transferred to regeneration medium, shoots were observed to grow from the green regions (Fig. 1c) and rapidly regenerated aerial organs (Fig. 1d). Upon excision, they soon rooted and became plantlets (Fig. 1e). A total of 93 plants were regenerated: 62 from the $ms10^{35}/ms10^{35}$ donors, 21 from the $ms10^{35}/+$ donors and 10 from the $+/+$ donors. After acclimatization, plantlets were transferred to a greenhouse. In a few cases these plants had reduced habitus and leaf size (Fig. 1f). However, most of the plants (Fig. 1g) were comparable to the plants used as donors (Fig. 1h). Flow cytometric and molecular marker analyses indicated that among regenerants were haploids (Fig. 1f) and doubled haploids (Fig. 1g).

There were important differences in the regeneration efficiency among the three genotypes used, expressed as a number of calli and regenerated plans per anther. The $ms10^{35}/ms10^{35}$ donor plants produced significantly more calli and regenerants per anther than the $ms10^{35}/+$ and the $+/+$ plants (Fig. 2). This implies that homozygosity for the $ms10^{35}$ allele increased both callus production and plant regeneration. Heterozygosity for this allele did not have any positive effect as the efficiency was not different from homozygotes null.

Flow cytometric, morphological and molecular analysis

Once the effect of the $ms10^{35}$ allele was established, we attempted to determine the origin of the anther-derived calli and regenerants. For this, we focused our study on the $ms10^{35}/+$ genotype, heterozygous for the morphological markers listed above. Additional anther cultures from $ms10^{35}/+$ hybrid donors were performed and a total of 66 different viable calli and 83 regenerated plants were produced. Some calli did not respond to organogenesis, whereas others gave rise to more than one regenerant. Despite the fact that regenerants originating from the same callus may be clones, we decided to study the entire set of plants obtained, for the following reasons: (1) frequent mixoploidy in anther-derived calli is well-documented (Seguí-Simarro and Nuez [2005](#), [2007](#);

Fig. 1 Anther culture and plant regeneration in tomato. **a** Anther with a young callus emerging from the anther locule. **b** Callus with shoot initials at its surface. **c** Regenerating shoot over the surface of the callus. **d** Developing shoots and leaves over old, necrosing calli. **e** In vitro, regenerated and rooted tomato plantlet. **f–g** Tomato plants regenerated from anther cultures, acclimated and grown at the greenhouse. **f** Haploid regenerant. Note the reduced habitus **g** DH regenerant, **h** normal tomato $ms10^{35}/+$ plants, used as donors of anthers. *Bars* in a: 1 mm
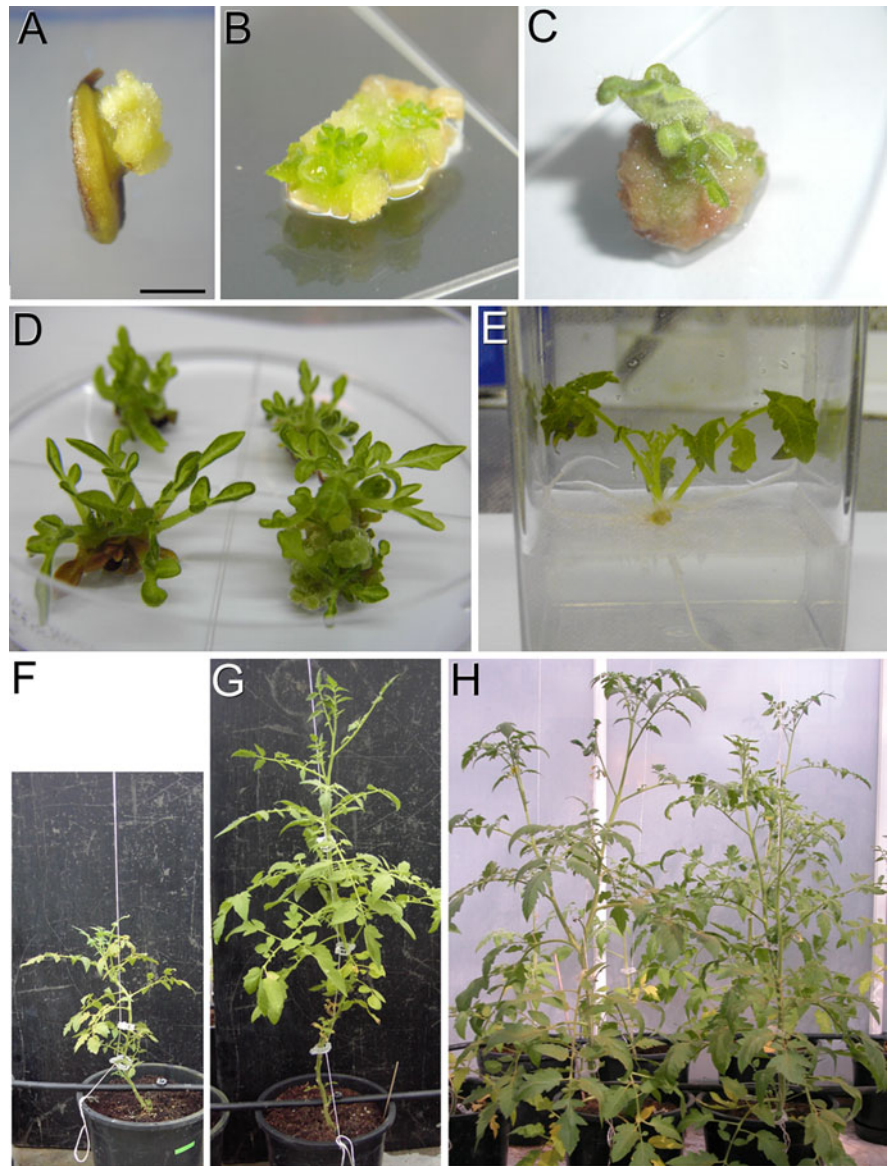
Shtereva et al. 1998; Zagorska et al. 1998, 2004) and it implies the possibility of genetically different individuals originating from different regions of the same mixoploid callus; (2) all plants regenerated from a single callus may provide information about the regenerative capacity of the different tissues involved in the callus and plant production.

The ploidy levels of the $ms10^{35}/+$ calli and regenerants were analyzed by flow cytometry. Leaves from the donor plants presented a G1 DNA peak at channel ∼100, which was set as the 2C standard for diploid cells, together with a small peak at channel ∼200, characteristic of diploid G2 cells. Based on this, 58 of the 66 calli were mixoploid. Among them, 24 presented a 2C + 4C DNA content (Table 1) and 34 presented a 1C + 2C DNA content (Table 1). Seven calli had 2C DNA content, and only one was clearly identified as haploid (1C DNA content). This callus necrosed and died before showing any sign of organogenesis on its surface.

Among the 83 regenerated plants that were analyzed by flow cytometry, no mixoploidy was observed (Table 1). From the 24 2C + 4C calli, 28 plants were obtained of which 23 had a 2C DNA
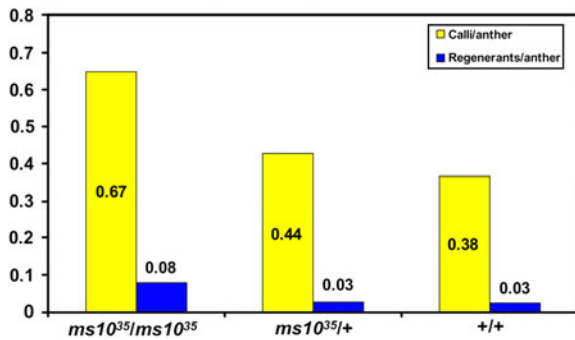
Fig. 2 Analysis of the number of calli (*light bars*) and regenerated plants (*dark bars*) derived from anther cultures of the $ms10^{35}/ms10^{35}$, $ms10^{35}/+$ and $+/+$ genotypes. In order to be able to compare between different experiments, measurements are given as number of calli or regenerants per excised and cultured anther
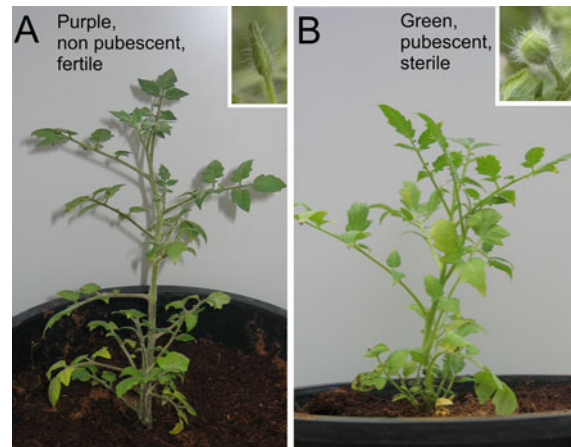

Fig. 3 Phenotypic analysis of regenerants from $ms10^{35}/+$ plants. **a** purple, moderately pubescent, fertile regenerant. **b** Green, strongly pubescent and sterile regenerant. *Insets* in **a** and **b** show a detail of a typical bud of each phenotype

Table 1 Flow cytometric analysis of callus and regenerants derived from anther cultures of the $ms10^{35}/+$ genotype

Calli		Regenerants		
Ploidy	Number	Ploidy	Number	Origin (SSR)
2C + 4C	24	2C	23	Somatic
		4C	5	Somatic
2C	7	2C	21	Somatic
C + 2C	34	C	3	Androgenic
		2C	31	See Table 2
C	1	–	–	–
Total	66	Total	83	

content and five a 4C content. From the seven identified 2C calli, 21 2C plants were obtained, with an average of three plants per callus. From the 34 C + 2C calli, three 1C plants and 31 2C plants were obtained.

The 83 regenerated plants were screened for the three morphological markers present. All 28 plants derived from the 2C + 4C calli and the 21 plants derived from the 2C calli (see Table 1) had the same phenotype: purple stems, moderate pubescence (Fig. 3a) and normal microspores and pollen (data not shown). The phenotypical uniformity of these 49 plants and an absence of haploid cells in their original calli are indicative of somatic origin.

Among 34 plants regenerated from the C + 2C calli (Table 1) there were:

– three haploids (1C as revealed by flow cytometry) that were green, highly pubescent and male

sterile. As expected, they had a reduced plant stature (Fig. 1f) typical of haploids.

– Among the remaining 31 diploid (2C) plants, three were green, highly pubescent and sterile (Fig. 3b), and 28 were purple, moderately pubescent and fertile (Fig. 3a).

Segregation for morphological markers among the 31 diploid plants derived from mixoploid C + 2C calli raised concerns about their origin. Thus, the analysis based on morphological markers was combined with an analysis by microsatellite (SSR) markers. Based on the four polymorphic SSR (Table 2), the following genotypes were observed:

– three individuals were homozygous for all four SSR markers. Since they were also recessive for morphoplogical markers (green, highly pubescent and sterile), we considered them doubled haploids, therefore, originating from haploid nuclei.

– 20 individuals were heterozygous for all four SSR markers. They were also purple, moderately pubescent and fertile, i.e., non-recessive for the *aa, wo* and $ms10^{35}$ genes. Thus, we attributed them a somatic origin.

– eight individuals were heterozygous, three being heterozygous for two SSR markers and five being heterozygous for one SSR marker. In addition, seven out of the eight plants were purple, moderately pubescent and fertile and one was green, strongly pubescent and sterile. The heterozygosis for some

Table 2 Molecular marker (SSR) analysis of *ms10³⁵*/+ callus-derived regenerants

	Number of individuals	Individuals homozygous for 4 SSR	Individuals heterozygous for			
			4 SSR	3 SSR	2 SSR	1 SSR
Regenerants from donor 1	8	–	7	–	–	1
Regenerants from donor 2	8	1	6	–	–	1
Regenerants from donor 3	7	1	4	–	1	1
Regenerants from donor 4	8	1	3	–	2	2
Total regenerants	31	3	20	–	3	5
Total regenerants (%)	100	9.7	64.5	–	9.7	16.1
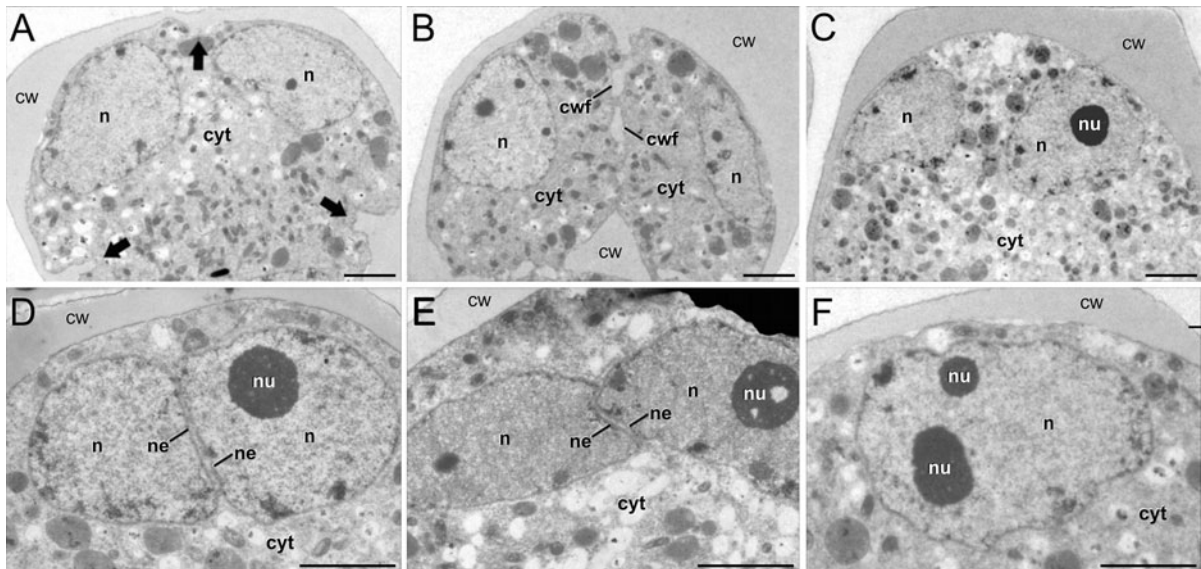
Fig. 4 Electron microscopical reconstruction of the process of meiocytic nuclear fusion. **a** Normally-looking meiocyte at the onset of post-meiotic cytokinesis, as revealed by the appearance of new cell wall protrusions (*arrows*) at defined regions of the original cell wall (*cw*). **b** Incomplete walling of an induced meiocyte, allowing for the appearance of cytoplasmic bridges between cell wall fragments (*cwf*). **c** Coexistence of two nuclei (*n*) in the common cytoplasm (*cyt*) of a meiocyte with absent cell walls. **d** Two nuclei of a non-walled meiocyte with their nuclear envelopes (*ne*) in close apposition. **e** Two nuclei of a non-walled meiocyte with their envelopes in physical contact. **f** Single, enlarged nucleus presumably coming from the fusion of two haploid nuclei, as revealed by the presence of two nucleoli (*nu*). *Bars* 2 μm

markers observed in these these individuals excludes both a somatic and haploid origin. Thus, we analyzed the locular contents of anthers induced to androgenesis in search for a possible explanation for these genetic combinations.

Electron microscopic reconstruction of the first stages of meiocytic induction

We used transmission electron microscopy to analyze the locules of plated anthers few hours after the culture initiation (Fig. 4). By the end of meiosis II, in normally-looking meiocytes the first signs of the post-meiotic cytokinesis were already evident (Fig. 4a). However, as cytokinesis proceeded, clear signs of deviations from the typical syncytial-type pattern of post-meiotic cytokinesis (Seguí-Simarro et al. 2008) were observed in some meiocytes. As seen in Fig. 4b, some cell walls failed to separate the corresponding two neighboring haploid nuclei, giving rise to cytoplasmic bridges between adjacent cells. Other meiocytes showed a total absence of cell walls separating adjacent nuclei (Fig. 4c). The conspicuous presence of a nucleolus was indicative of well formed nuclei, presumably at the G1 stage of the cell cycle, when cell walls are supposed to be completed in

intact meiocytes. In meiocytes with defective or absent cell walls, nuclei coexisting in the same cytoplasm were observed at a very close distance, having their respective envelopes closely apposed (Fig. 4d) or even in tight physical contact (Fig. 4e). Such close contact was highly indicative of subsequent nuclear fusion. Indeed, some of the defective meiocytes showed enlarged nuclei with more than one visible nucleolus (Fig. 4f). Normal meiotic haploid nuclei should exhibit only one nucleolus after nuclear reconstitution (Fig. 4c–e), since in *Solanum* only one nucleolar organizing region (NOR) is present, located at chromosome 2 (Ivanova et al. 2000). Thus, the presence of two nucleoli was an indication of possible of nuclear fusion.

Light and electron microscopic reconstruction of microsporogenesis in $+/+$ and $ms10^{35}/ms10^{35}$ genotypes

In view of the high percentage of somatic regenerants, we next analyzed the development of meiocytes and microspores in both fertile $(+/+)$ and male sterile $(ms10^{35}/ms10^{35})$ lines by means of light and transmission electron microscopy. In the fertile genotype, meiocytes occupied most of the anther locule, surrounded by a well developed tapetum with normally-looking cells and no signs of degeneration (Fig. 5a). Meiocytes at this stage were actively dividing, either at late stages of meiosis I or at early stages of meiosis II (Fig. 6a), the sensitive timeframe for in vitro induction of callus proliferation (Seguí-Simarro and Nuez 2005). Immediately after the inducible timeframe, anthers presented a wider anther locule, surrounded by a thinner tapetal layer with signs of cell death (Fig. 5b). In the locule, most of the cell types were tetrads, with only few young microspores or cellularizing meiocytes present. All tetrads had a normal architecture (Fig. 6b), with four independent cells separated by thick cell walls. At the stage when young microspores are released from the tetrad, microspores appeared well formed (Fig. 6c). At this stage, the anther locule was surrounded by a thin layer of degraded tapetum. The anther wall
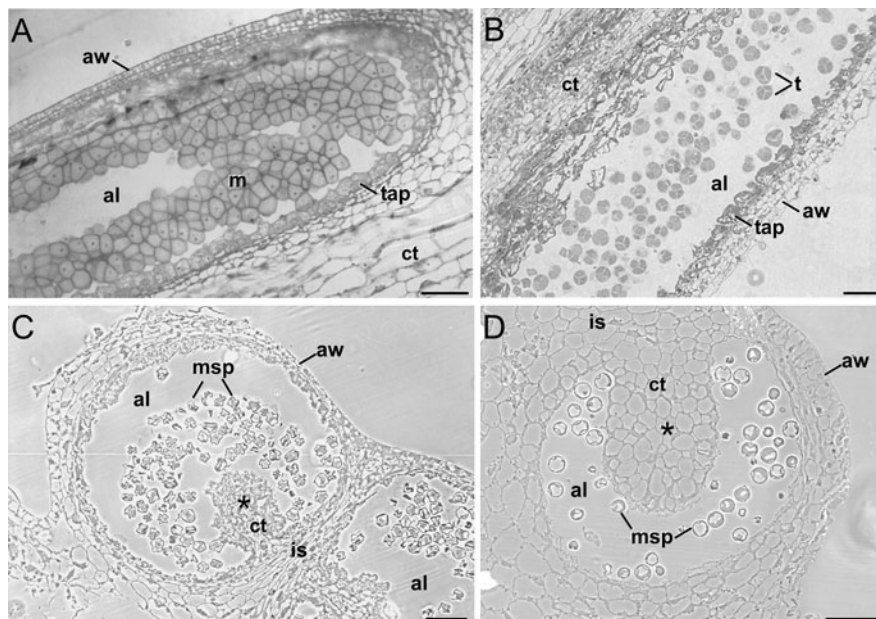

Fig. 5 Light microscopical analysis of meiocyte and microspore-carrying anthers of the $+/+$ donor plants. **a** Longitudinal section of an anther carrying early meiocytes (*m*), at a stage before meiosis II. **b** Longitudinal section of an anther carrying completely walled tetrads (*t*), prior to microspore release. **c** Transversal section of an anther carrying young microspores (*msp*), just released from the tetrad. Note the emergence of a tissue stub (*asterisk*) from the connective tissue (*ct*) of the interlocular septum (*is*) between both adjacent anther locules (*al*). **d** Transversal section of an anther carrying vacuolated microspores (*msp*). Note the growth of the stub (*asterisk*) originated from the layers of connective tissue (*ct*) of the interlocular septum (*is*). *aw* anther wall, *tap* tapetum. *Bars* 50 μm

began to thin along the dehiscence line (Fig. 5c). Interestingly, in transversal sections of all anthers analyzed at this stage, a tissue stub (asterisk in Fig. 5c) could be observed to emerge from the connective tissue separating both pollen sacs of the same theca, partially invading the anther locule. In anthers containing late, vacuolate microspores (Fig. 6d), the stubs appeared swollen, occupying a significant part of the pollen sac (Fig. 5d). Light microscopical sections demonstrated that these stubs had a tissular architecture identical to that of the adjacent connective tissues, being both clearly connected.

In plants of the male-sterile, $ms10^{35}/ms10^{35}$ genotype, anthers at the inducible interval (Fig. 7a) carried 100% of actively dividing meiocytes, as in the fertile genotype (compare Figs. 6a and 8a). However, the next stage in anther development deviated from the fertile line. In anthers of the size equivalent to that of Fig. 5b, the locular width was dramatically reduced

(Fig. 7b). Signs of tapetal degradation were more evident than in the fertile counterpart. Most importantly, the locular structures, presumably tetrads, showed clear signs of death, as revealed by their intense dark staining and the irregular, collapsed shapes (Fig. 7b). Figure 8b shows a tetrad corresponding to an anther of this size, where one of the three cells included in the section (arrow) presented an intensely stained cytoplasm, abundant vacuolation and a general collapse, all signs of cell death. Anthers of a size equivalent to that of Fig. 5c were characterized by the presence of degenerating or completely degenerated tetrads (Fig. 8c) within a collapsed anther locule (Fig. 7c). A callus-like stub linked to the connective tissue, similar to that of fertile anthers but significantly larger, invaded most of the locular volume. Further developmental stages had a similar architecture, but the growing callus-like stub occupied nearly all of the locular space, displacing the tetrad remnants to a thin, peripheral layer (Fig. 7d). No other cell types of

Fig. 6 Transmission electron microscopical analysis of meiocytes and microspores of the +/+ donor plants. a Meiocyte at metaphase II, where two metaphasic plates of aligned chromosomes (chr) are observed at both sides of the equatorial organellar band (ob). b Tetrad just before microspore release, still enclosed within the callosic wall (cw). Note the presence of tapetal debris (td) in the pollen sac. c Young microspore released from the tetrad, with a centrally located nucleus, slight signs of vacuolation, a polygonal shape derived from their previous position within the tetrad, and a sculptured exine coat (ex) at the outermost surface. d Late, vacuolate microspore showing its typical off-centered nucleus, slightly rounded shape and massive vacuole. cyt cytoplasm, n nucleus, v vacuole. Bars 5 μm
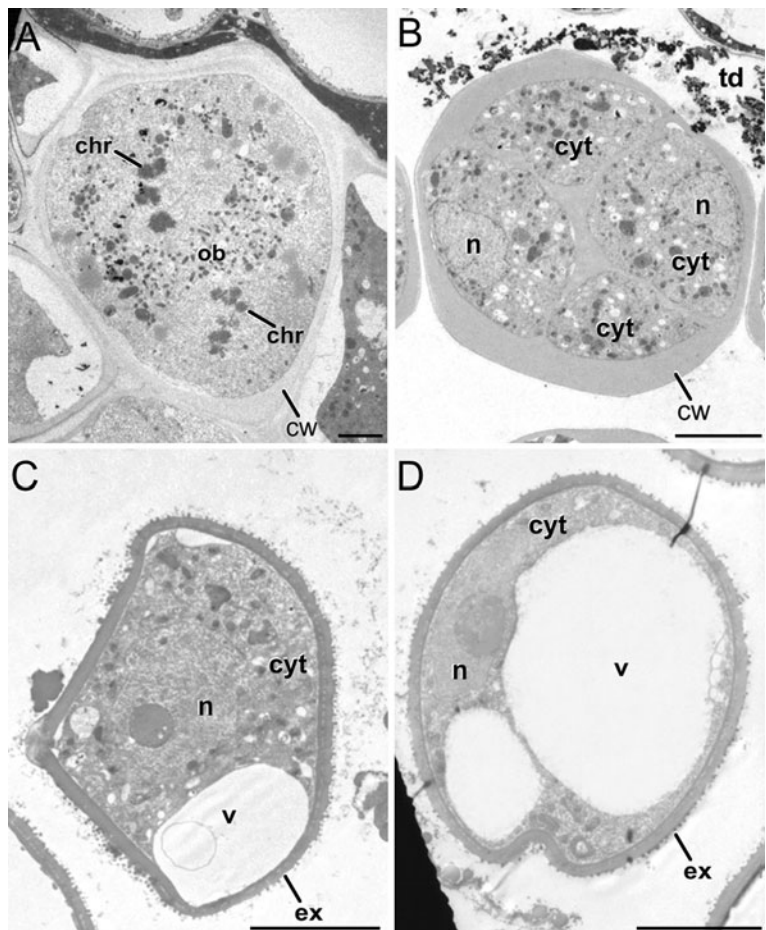
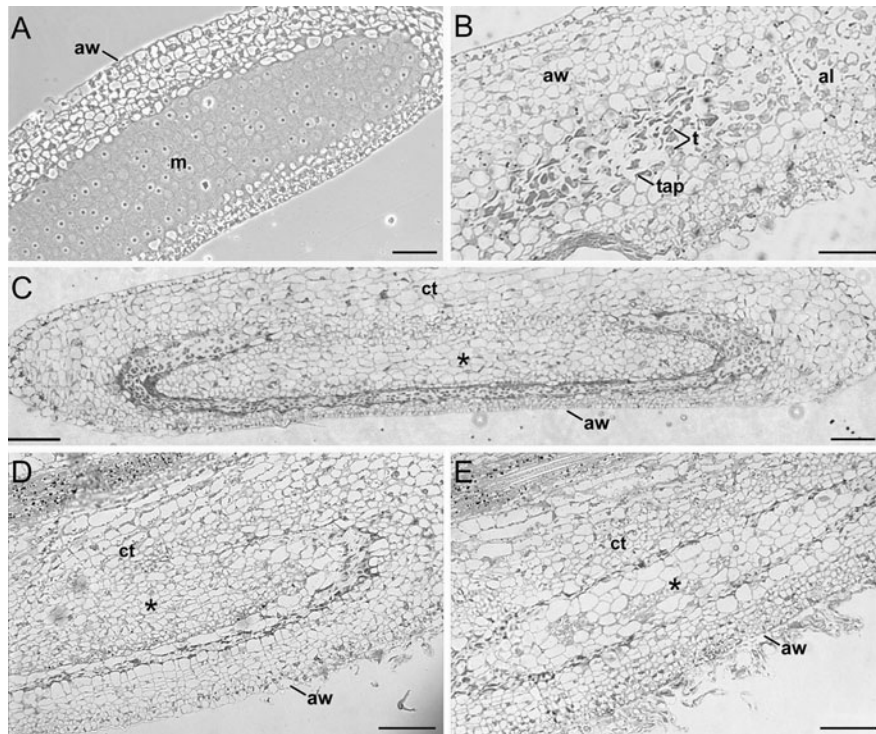**Fig. 7** Light microscopical analysis of meiocyte-carrying anthers of the *ms10^{35}/ms10^{35}* donor plants. **a** Longitudinal section of an anther carrying early meiocytes (*m*), just entering meiosis and still at the uninucleate stage. **b** Longitudinal section of an anther at a stage equivalent to that of Fig. 5b (tetrads). These anthers carry degenerated and/or dying tetrads (*t*) within the anther locule (*al*). **c** Longitudinal section of an anther at a stage equivalent to that of Fig. 5c (young microspores). The locular volume is dramatically reduced by the emergence of a callus-like protrusion (*asterisk*) from the connective layers of the anther (*ct*). Within the locule, degenerating tetrads are present. **d** Longitudinal section of an anther at a stage equivalent to that of Fig. 5d (vacuolate microspores). Nearly all of the locular volume is occupied by the callus (*asterisk*) connected to the connective tissue (*ct*), with only a narrow band of cell remnants present at the periphery of the callus. **e** Longitudinal section of an anther at a stage equivalent to that of Fig. 5d, where a callus (*asterisk*) with no evident link with the connective tissue (*ct*) occupies the locular volume. *aw* anther wall, *tap* tapetum. *Bars* 50 µm

gametophytic nature (microspores or pollen) were observed within the locular space. Occasionally, the link of the callus-like stub with the connective tissue was not evident through a series of serial sections covering all of the anther locule depth (Fig. 7e). This could likely be indicative of the presence of a meiocyte-derived callus, as previously demonstrated (Seguí-Simarro and Nuez 2007).

Discussion

In *ms10^{35}* mutants, meiosis is delayed and the inducible stage is extended in time

In this work we have shown that homozygous *ms10^{35}* mutant gene promotes the degradation of meiotic cells still enclosed within the tetrad, and eventually the collapse of the entire tetrad (Fig. 8). Together with an enhanced rate of callus production, *ms10^{35}* mutants has delayed meiosis, as revealed by the different locular contents of male-sterile anthers equivalent in size to those of the fertile line (compare Figs. 5 and 7). This suggests that the enhanced rate of meiocyte-derived callus production is due to an extension of the inductive window. In other words, the mutation allows for more meiocytes at the optimal stages to be induced. However, this may not be a general feature of all tomato male sterile mutants with disrupted meiosis. As pointed out by Zamir et al. (1980), other meiosis-affecting, male sterile mutants such as the *ms12*, *ms5* and *ms33* fail to show a callogenic response, perhaps due to the presence of additional defects in the tapetal development (Rick 1948).
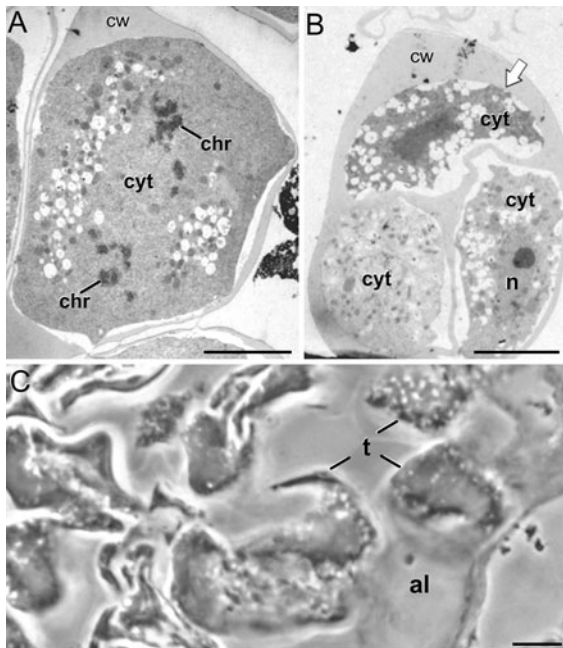
Fig. 8 Transmission electron microscopical analysis of meiocytes of the *ms10³⁵/ms10³⁵* donor plants. **a** Meiocyte at late anaphase I, where each haploid set of chromosomes (*chr*) are reaching the cell poles. **b** Tetrad coming from an anther at a developmental stage equivalent to that of Fig. 6b. In one of the three visible cells (*arrow*), clear signs of cell death can be observed. The other two cells do not show such a dramatic degeneration, but signs of incipient death such as massive vacuolation and detachment of the plasma membrane from the callosic wall (*cw*) can be seen. **c** Tetrad (*t*) remnants from an anther at a developmental stage equivalent to that of Fig. 6c and beyond. Tetrads are dead and collapsed. Not a single viable cell is observed. *al* anther locule, *cyt* cytoplasm, *n* nucleus. *Bars* 5 μm

Three different callus types are produced, but only one is interesting for breeding purposes

Based on the ploidy data, the morphological characterization and the molecular analysis, we conclude that from our 83 anther-derived regenerants, only three (3.6%) were haploid and three were doubled haploid. For the DHs, we cannot entirely exclude a theoretical (although unlikely in practice) possibility that some of these three plants are not in fact true true DHs. The fact that some other individuals were homozygous for all but one of the seven markers establishes a precariously thin line between what is considered a doubled haploid and what is not. This reduced theoretical possibility could further be reduced by making use of more SSR markers, but

this was not feasible in the current study. In conclusion, the total number of haploid-derived regenerants here was six, or 7.3% of all regenerated plants.

Eight (9.6%) diploid regenerants, all of them derived from mixoploid calli, possed degrees of heterozygosity not observed in the donor plants. This excludes both haploid and somatic origins of these plants. It could be reasonably argued that these calli may originate from a mixture of haploid and somatic tissue, forming a chimeric tissue from which haploids, DHs and somatic diploids can arise. The presence of diploid regenerants polymorphic for some of the genetic markers used may also be explained by regeneration from the products of the first meiotic division. A nucleus of a binucleated meiotic intermediate has a haploid number of chromosomes, but with sister chromatids attached. As a consequence of crossing over, some of these sisters may be non-identical. This attractive hypothesis would explain the observed heterozygosity for a very low number of markers (such as one marker out of all tested), as a direct consequence of crossing over, with no need for nuclear fusion. While this may explain a part of the observed results, we are inclined to believe that most mixoploid, partially heterozygous calli and their corresponding regenerants come from a process of nuclear fusion within the meiocyte, based on the following rationale. We have shown (Fig. 4) that after induction, some meiocytes present nuclear profiles highly indicative of the occurrence of nuclear fusions between some of the haploid nuclei of the induced meiocytes. This fusion would be favored by the absence of, or incomplete, cell walls that fail to separate haploid nuclei. Although fusing membrane intermediates are difficult to identify in electron micrographs due the instantaneous nature of the process of membrane fusion, the observation of nuclei closely apposed or even in physical contact, and the presence of enlarged nuclei with two nucleoli are good indications of nuclear fusion events. It was previously proposed that the last steps of meiosis are disturbed by the culture conditions, starting from serious alterations during post-meiotic cytokinesis, including the formation of incomplete or absent cell walls (Seguí-Simarro and Nuez 2007). This, in turn, would allow for the apposition and eventual fusion of two genetically different, haploid nuclei. According to this, the most reasonable origin for these eight partially heterozygous plants would be a diploid region of the C + 2C

mixoploid callus. Whereas the three DHs mentioned above would originate from a haploid region, the eight partial heterozygotes would come from a diploid region formed by cells coming from the fusion of two genetically different meiocytic nuclei. Presence of these plants provides additional support from the genetic side to the model previously proposed (Seguí-Simarro and Nuez 2007) to explain the low efficiency of androgenic DH production in tomato.

However, fusion of meiotic nuclei is neither the only nor the main drawback for the DH production in tomato. Indeed, based on the same ploidy level and the presence of genetic/DNA markers as in the donor plants, we postulate somatic origin for 69 out of the 83 regenerated plants (83% of the total). Such a rate of recovery of non-haploid regenerants is not a trivial issue. Moreover, for all practical purposes this percentage should be increased by the 9.6% of plants derived from meiocyte nuclear fusions, for a total of 92.7% of undesirable individuals. This means that only one in each 13 regenerants will be haploid or DH, and this explains why it is so difficult to obtain androgenic tomato haploids or DHs in quantities sufficient for breeding purposes.

The increased production of callus in the $ms10^{35}$ male-sterile plants is due to a massive growth of the locular stubs of the connective tissue

From the results shown in Fig. 2, it is clear that the $ms10^{35}/ms10^{35}$ genotype outperforms the other two in terms of callus and plant production. Thus, the control of the $ms10^{35}$ mutation over callus production is clearly recessive. This is in agreement with its effect in male sterility, which in turn constitutes an additional proof of the link between these two phenotypic expressions of the mutation. At issue is, why do these male sterile plants produce more calli and regenerants?

In tomato, normal anther development at the microsporogenic stage is characterized by the growth of a stub from the connective tissue at both sides of the interlocular septum (Fig. 5c,d; Goldberg et al. 1993; Senatore et al. 2009). As the anther matures and dehisces during pollen development, the stubs and the interlocular septum undergo a progressive loss of cell layers, becoming thinner and allowing for the aperture of the anther for pollen dispersal through the line of dehiscence (Senatore et al. 2009). This normal succession of events is disrupted in the $ms10^{35}/ms10^{35}$

genotype, where microspores are not released from the tetrad. In parallel to the meiocyte degeneration within the anther locule, we show overproliferation of the stubs (Fig. 7c, d). Subsequently, the cell mass growing from the stub occupies most of the locular volume, at stages where no stub growth is described in the fertile counterpart. Later stages of anther development are characterized by the total locular invasion of the stub-derived cellular mass. In summary, stubs in the $ms10^{35}/ms10^{35}$ genotype resume a proliferative growth that ends up with the production of an intralocular, callus-like cellular mass. When anthers are excised and cultured in the presence of growth regulators, these masses eventually break the anther walls and emerge, giving rise to somatic calli from which most of the tomato regenerants are obtained.

These observations indicate that (1) the stubs from the interlocular septum may be the origin of the somatic callus and (2) the $ms10^{35}$ mutation is not only defined by the degeneration and death of the meiocytes, but also by the induction of proliferation in the connective tissue of the anther. Thus, when we inoculate $ms10^{35}/ms10^{35}$ anthers for in vitro culture, there is already a callus-like, proliferating mass inside, which makes generation of an extralocular callus easier. In fertile lines, where stubs do not proliferate, the growth of the stubs would only be induced in vitro and upon exposure to growth regulators, and therefore, by the same time in culture, their callus would be smaller and less numerous. Most likely, this would be the main reason why significantly lower amounts of calli and regenerants are obtained from fertile genotypes (Seguí-Simarro and Nuez 2007; Zagorska et al. 1998; Zamir et al. 1980).

Haploid or DH calli may have to compete for resources with the massive somatic callus

As seen hereby, somatic calli are predominant but not the only possible. Soon upon culturing some meiocytes are induced to proliferate, becoming haploid or mixoploid calli (this work; Seguí-Simarro and Nuez 2007). Conceivably, meiocyte-derived calli and calli derived from somatic tissues coexist in some anthers. Both callus types compete for the limited resources available within the anther locule. However, the outcome may be biased towards somatic (diploid) tissues, that are genetically more stable and with more regenerative capacity. As seen in Table 1,

diploid calli coming from somatic tissue yielded three plants per callus, significantly higher than the rest, with different origins or ploidy levels.

The fact that the somatic-type callus is only one, proliferates at a faster rate, and is connected to the rest of the anther, will make it a candidate to succeed, displacing and collapsing the growth of the independent meiocyte-derived calli. Perhaps, the actual rate of induction of meiocyte proliferation is higher than that reflected in the number of haploid or DH calli produced, but competition with the somatic cellular mass may make many meiocyte-derived calli abort at early stages of development. This could be an additional argument explaining low percentage of haploid or DH calli. On the other hand, it is clear that some of them are capable to survive and emerge from the anther locule. Following this line of speculation, since meiocytes are formed in the anther locule prior to the growth of the interlocular stub, it might well be possible that once a meiocyte-derived callus is established, the proliferative growth of the stub is somehow inhibited.

Concluding remarks

In this work, we showed that male sterility in $ms10^{35}$ mutants is due to the collapse and death of the tetrads, prior to the release of the microspores to the anther locule. This allows for an extended window for callus induction. We also demonstrated that proliferation of cells from a protruding appendage of the interlocular septum is the main source of anther-derived calli in our tomato genotypes. The callus-like structure formed may likely account for the frequent presence of calli of somatic origin in cultured $ms10^{35}$ tomato anthers. In addition, we provided ultrastructural and genetic evidence for the appearance of calli and regenerants derived from the fusion of two different haploid meiotic nuclei. As seen, although possible, there are many collateral, non desirable processes that make difficult to induce DHs from tomato meiocytes.

These collateral processes carry important practical implications. First, only one plant for every thirteen regenerated is haploid or DH. Second, time and resources must be spent to identify the haploid or DH plants among the total population. Third, the similar percentage of haploids and DHs (50:50) would make mandatory an additional step of genome doubling for haploid regenerants. In conclusion, the work presented hereby provides a reasonable explanation as to why in vitro anther culture in tomato has been highly unsuccessful if not impossible during four decades, and stresses the need for a search of alternative ways to overcome these limitations. Alternatives such as the culture of isolated meiocytes have not been approached yet, possibly due to the discouraging previous reports about the culture of this extremely delicate cell type (Shivanna and Johri 1985). Other more promising alternative, the culture of isolated microspores, has already been addressed. However, results are still very preliminary (Bal and Abak 2005; Seguí-Simarro and Nuez 2007), and more time and efforts will be needed to refine this method.

References

Areshchenkova T, Ganal MW (1999) Long tomato microsatellites are predominantly associated with centromeric regions. Genome 42:536–544

Bal U, Abak K (2005) Induction of symmetrical nucleus division and multicellular structures from the isolated microspores of *Lycopersicon esculentum* Mill. Biotechnol Biotec Eq 19:35–42

Bal U, Abak K (2007) Haploidy in tomato (*Lycopersicon esculentum* Mill.): a critical review. Euphytica 158:1–9

Dao NT, Shamina ZB (1978) Cultivation of isolated tomato anthers. Sov Plant Physiol 25:120–126

Durand V (1981) Relations entre les gènes marqueurs aa et Wo et le gene de stérilité mâle ms35. In: Philouze J (ed) Genetique et selection de la tomate. Proceedings of the Meetings of the Eucarpia Tomato Working Group, Avignon, France, pp 225–228

FAOSTAT (2009) http://faostat.fao.org

Ferriol M, Pico B, Nuez F (2003) Genetic diversity of a germplasm collection of *Cucurbita pepo* using SRAP and AFLP markers. Theor Appl Genet 107:271–282

Goldberg RB, Beals TP, Sanders PM (1993) Anther development: basic principles and practical applications. Plant Cell 5:1217–1229

Gresshoff PM, Doy CH (1972) Development and differentiation of haploid *Lycopersicon esculentum* (tomato). Planta 107:161–170

Gulshan TMV, Sharma DR (1981) Studies on anther cultures of tomato—*Lycopersicon esculentum* Mill. Biol Plant 23:414–420

Ivanova SV, Dolgodvorova LI, Karlov GI, Kuchkovskaja EV (2000) Morphometric and cytogenetic characteristics of haploid tomato plants. Russ J Genetics 36:41–50

Jaramillo J, Summers WL (1990) Tomato anther callus production—solidifying agent and concentration influence induction of callus. J Am Soc Hortic Sci 115:1047–1050

Jaramillo J, Summers WL (1991) Dark-light treatments influence induction of tomato anther callus. Hortscience 26:915–916

Levenko BA, Kunakh VA, Yurkova GN (1977) Studies on callus tissue from anthers. 1. Tomato. Phytomorphology 27:377–383

Ma YH, Kato K, Masuda M (1999) Efficient callus induction and shoot regeneration by anther culture in male sterile mutants of tomato (*Lycopersicon esculentum* Mill. cv. First). J Jpn Soc Hortic Sci 68:768–773

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–479

Philouze J (1974) Marker genes for Ms-32 and Ms-35 male-sterility genes in tomato. Annales De L Amelioration Des Plantes 24:77–82

Rick CM (1948) Genetics and development of nine male-sterile tomato mutants. Hilgardia 18:599–633

Seguí-Simarro JM (2010) Androgenesis revisited. Bot Rev 76:377–404

Seguí-Simarro JM, Nuez F (2005) Meiotic metaphase I to telophase II is the most responsive stage of microspore development for induction of androgenesis in tomato (*Solanum Lycopersicum*). Acta Physiol Plant 27:675–685

Seguí-Simarro JM, Nuez F (2007) Embryogenesis induction, callogenesis, and plant regeneration by in vitro culture of tomato isolated microspores and whole anthers. J Exp Bot 58:1119–1132

Seguí-Simarro JM, Nuez F (2008) How microspores transform into haploid embryos: changes associated with embryogenesis induction and microspore-derived embryogenesis. Physiol Plant 134:1–12

Seguí-Simarro JM, Otegui MS, Austin JR, Staehelin LA (2008) Plant cytokinesis—insights gained from electron tomography studies. In: Verma DPS, Hong Z (eds) Cell division control in plants. Springer, Berlin/Heidelberg, pp 251–287

Senatore A, Trobacher CP, Greenwood JS (2009) Ricinosomes predict programmed cell death leading to anther dehiscence in tomato. Plant Physiol 149:775–790

Sharp WR, Dougall DK (1971) Haploid plantlets and callus from immature pollen grains of *Nicotiana* and *Lycopersicon*. B Torrey Bot Club 98:219–222

Sharp WR, Raskin RS, Sommer HW (1972) The use of nurse culture in the development of haploid clones in tomato. Planta 104:357–361

Shivanna KR, Johri BM (1985) The angiosperm pollen. Structure and function. Wiley Eastern Limited, New Delhi

Shtereva LA, Zagorska NA, Dimitrov BD, Kruleva MM, Oanh HK (1998) Induced androgenesis in tomato (*Lycopersicon esculentum* Mill). II. Factors affecting induction of androgenesis. Plant Cell Rep 18:312–317

Smulders MJM, Bredemeijer G, RusKortekaas W, Arens P, Vosman B (1997) Use of short microsatellites from database sequences to generate polymorphisms among *Lycopersicon esculentum* cultivars and accessions of other *Lycopersicon* species. Theor Appl Genet 94:264–272

Summers WL, Jaramillo J, Bailey T (1992) Microspore developmental stage and anther length influence the induction of tomato anther callus. Hortscience 27:838–840

Touraev A, Pfosser M, Heberle-Bors E (2001) The microspore: a haploid multipurpose cell. Adv Bot Res 35:53–109

Varghese TM, Gulshan Y (1986) Production of embryoids and calli from isolated microspores of tomato (*Lycopersicon esculentum* Mill.) in liquid media. Biol Plant 28:126–129

Zagorska NA, Shtereva A, Dimitrov BD, Kruleva MM (1998) Induced androgenesis in tomato (*Lycopersicon esculentum* Mill.)—I. Influence of genotype on androgenetic ability. Plant Cell Rep 17:968–973

Zagorska NA, Shtereva LA, Kruleva MM, Sotirova VG, Baralieva DL, Dimitrov BD (2004) Induced androgenesis in tomato (*Lycopersicon esculentum* Mill.). III. Characterization of the regenerants. Plant Cell Rep 22:449–456

Zamir D, Jones RA, Kedar N (1980) Anther culture of male sterile tomato (*Lycopersicon esculentum* Mill.) mutants. Plant Sci Lett 17:353–361