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Structural studies of microsporogenesis in fertile and male-sterile onions (*Allium cepa* L.) containing the cms-S cytoplasm

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Summary. The structure of anther tissues has been studied during microsporogenesis in male-sterile and -fertile onions. Three types of abnormal tapetal behaviour have been observed within the single line II/3ms containing the cms-S cytoplasm: type 1, the premature breakdown of the tapetum at the tetrad stage, type 2, the hypertrophy of the tapetum after the diad stage followed by its premature autolysis and, type 3, in which the tapetum remains in good condition but for an abnormally long period of time. Tapetal autolysis proceeds in the same manner in both male-steriles and -fertiles with only the stage at which it occurs differing between the types of plants. Mitochondria were prominent in the tapetal tissue of all onion types throughout all stages of microsporogenesis and were still visible during the last stages of tapetal autolysis. In a detailed study of type 2 behaviour, no differences in mitochondrial volumes were found until the tapetum hypertrophied.

Key words: *Allium cepa* – Cytoplasmic male sterility – Microsporogenesis – Transmission electron microscopy

1 Introduction

During microsporogenesis there is a differentiation of the cells of the anther into various tissue types, and a coordination in the development and functioning of these tissues must occur for successful microsporogenesis. The most important association appears to lie between the developing microspores and the tapetum, as it is through this tissue that microspores must derive all of their nutrition. Therefore, any perturbations within the tapetum may affect their development. Cytological studies on male-sterile plants from a number of species have suggested that there is a lack of synchronisation between the developmental processes that occur in anther tissues during microsporogenesis. This lack of co-ordination may lead to the production of inviable pollen.

Onions are an important world-wide crop with 25×10^6 tons produced in 1987 (Anon 1987). Increasingly, hybrid varieties are being grown following the discovery of a cytoplasmic male-sterility system by Jones and his co-workers in the United States (Jones and Emsweller 1937; Monosmith 1926) that facilitates large-scale hybridisation. This source of male-sterility has been shown to be under the genetic control of a cytoplasmic factor S and a recessive nuclear gene *ms* (Jones and Clarke 1943). In onions, cms caused by the cms-S cytoplasm has been correlated with restriction fragment length polymorphisms of both the mitochondrial and chloroplastal genomes (Holford et al. 1988; De Courcel et al. 1989). In a number of other species it has been possible to define a relationship between the organisation of the mitochondrial genome and the cms-conferring cytoplasm (Leaver and Grey 1982; Leaver et al. 1988). For two species, *Zea mays* and *Petunia hybrida,* defined mitochondrial genes have been strongly implicated in the breakdown of normal pollen production (Dewey et al. 1986; Dewey et al. 1987; Wise et al. 1987; Young and Hansen 1987).

The work described here was performed using two inbred onion lines which were near-isogenic except for the presence of the normal fertile cytoplasm (in line II/3) or the cms-S cytoplasm (in line II/3ms). In order to determine the stage at which pollen production was aborted in line II/3ms, the development of the tapetum and sporogenous tissues was followed at both the cellular and sub-cellular levels. Particular attention was focussed upon the number and state of mitochondria within

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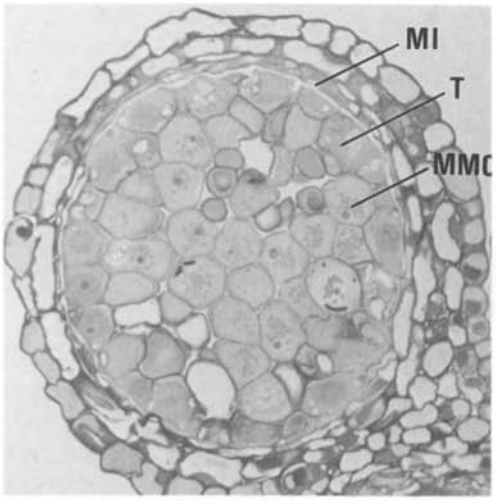
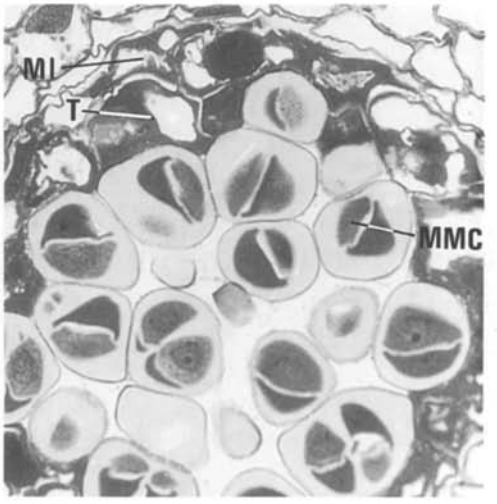
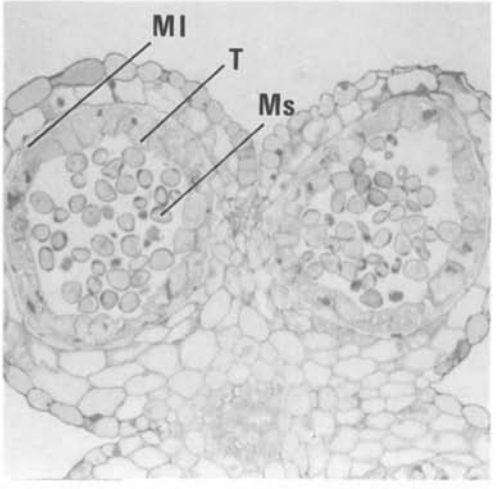
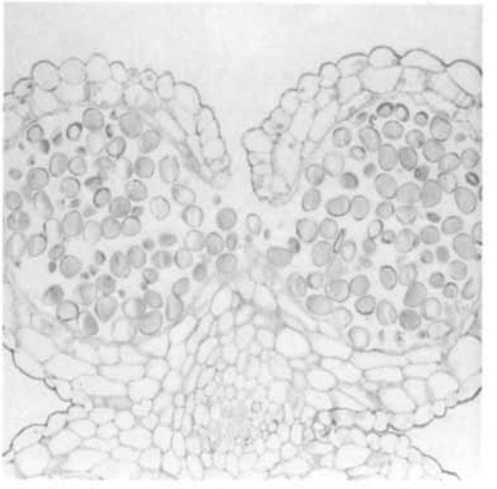
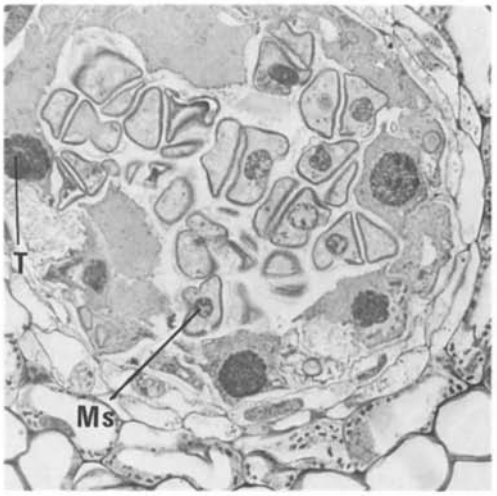










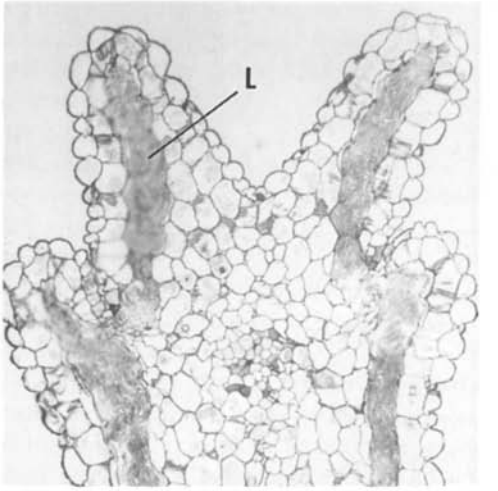


Fig. 1. Early meiosis in the anther from a male-sterile onion flower. *Ml* Middle layer, *MMC* microspore mother cell, *T* tapetum. Approx. ×315

Fig. 2. Formation of tetrads within the callose capsule of a male-sterile onion exhibiting type 1 behaviour. Approx. ×490

Fig. 3. A male-fertile anther with microspores undergoing mitotic divisions. *Ms* Microspore. Approx. ×150

Fig. 4. Anthesis in a male-fertile anther. Approx. ×150

Fig. 5. Anther from a male-sterile plant showing type 1 behaviour. Approx. ×450

Fig. 6. Anther from a male-sterile plant showing the failure of dehiscence and the compressed walls of empty pollen grains. *L* Locule with shrivelled pollen grains. Approx. ×130

tapetal cells in order to discover whether structural characteristics of these organelles differed in male-fertile and male-sterile lines.

2 Methods

Fertile and male-sterile onion material of the lines II/3 (N cytoplasm) and II/3ms (cms-S cytoplasm) were obtained from the Institute of Horticultural Research (UK) during the flowering season. Anthers were removed from the florets and squash preparations made to determine the approximate stage of microsporogenesis. The remaining anthers were given a primary fixation in Karnovski's fixative (Karnovski 1965) overnight followed by secondary fixation in 1% (w/v) osmium tetroxide. The samples were block-stained in 1% (w/v) tannic acid, washed in 1% (w/v) Na_2SO_4 and then dehydrated through an ethanol series. The samples were infiltrated with L. R. White resin for 3 h in 50% resin: 50% alcohol, followed by five changes of 100% resin in a 48- to 72-h period. Gold sections were taken with an L.K.B. ultramicrotome and placed onto formvar- and carbon-coated grids. These were stained with 1% (w/v) uranyl acetate and Reynolds' lead citrate (Reynolds 1963), before examination using a Philips 301 electron microscope.

For light-microscopy 2- to 3-μm sections were taken from blocks prepared for electron-microscopy and placed on slides coated with chrome alum/gelatin-subbing solution (Pappas 1971). After drying, the sections were treated with saturated sodium ethoxide for 20 s, washed and then stained of 30 s with 0.15% (w/v) toluidene blue in 0.1 *M* phosphate buffer, pH 6.5.

The average areas occupied by tapetal tissue and the mitochondria within this tissue were measured from light- and electron-micrographs of transverse sections of the central portion of male-fertile and male-sterile anthers. Acetate sheets, with points at 5 or 10 mm centres and arranged in a square pattern, were used as overlays to the micrographs, and the number of points falling within the area of interests were recorded. These data were then used to calculate the actual areas, and hence the relative volumes occupied by a particular tissue or organelle (Weibel 1969; Weibel and Bolander 1973).

3 Results

Differentiation into the anther tissue types occurred normally in both male-fertile and -sterile onion lines (Fig. 1). The microspore mother cells passed through their meiotic divisions normally (Fig. 2), resulting in the formation of diads and tetrads within callose capsules. Callose wall breakdown occurred without problem to liberate the young microspores. In the anthers of male-fertile onions the tapetum remained as a discrete ring of cells surrounding plump microspores (Fig. 3). These completed their mitotic division, the tapetum degenerated and the anther dehisced (Fig. 4).

At the light-microscopy level three types of abnormal developmental behaviour were observed in male-sterile material. In type 1 behaviour, the cells of the tapetum remained as a discrete ring of cells until the stage at which the microspores in equivalent male-fertile anthers were proceeding through their mitotic division. The cytoplasm of the tapetal cells contained within male-sterile material, however, appeared to be disorganised. Within the locule, the microspores started to shrivel, became deformed, and lost their cell contents (Fig. 5). After the onset of the shrivelling process, the cells of the tapetum lost their integrity and the cell contents spilled out into the anther locule. Finally, the anther dehydrated, the locule cavity shrank, compressing the remains of the pollen grains (Fig. 6), and the anther failed to dehisce.

A second category of abnormal behaviour, type 2, was observed. During the formation of tetrads, the cells of the tapetum hypertrophied almost filling the locule cavity (Fig. 7). Some swelling of tapetal cells was seen at the diad stage, and this may represent the onset of hypertrophy (Fig. 8). The tapetal cells retained their cell walls until after the release of microspores from the callose capsule. After this stage, the tapetum degenerated releasing its cell contents into the locule cavity (Fig. 9). The microspores then shrivelled, the locule cavities compressed and again there was no anther dehiscence.

A third form of tapetal behaviour, type 3, was observed in one male-sterile anther. The microspores within this anther were at the uni-nucleate stage and were shrivelled and deformed (Fig. 10). The tapetum, however, showed no hypertrophy, thus resembling type 1 behaviour, but there was no sign of the cytoplasmic disorganisation. Abnormal behaviour types 1, 2 and 3 were seen in the same line, II/3ms, in the material prepared for microscopy in 1988. In the following year only type 2 behaviour was observed.

At the electron-microscopy level the cytoplasm of male-fertile microspore mother cells (MMC's) was found to be relatively electron-lucent with little endoplasmic reticulum and many mitochondria and plastids during the early meiotic stages (Fig. 11). The mitochondria within the MMC's at this stage had distinct finger-like cristae and contained ribosomes (Fig. 12). By the time the chromosomes within the MMC's were fully condensed, the mitochondria were reduced in size, their structure simplified with cristae being few or absent (Fig. 13), and they remained in this state during the first meiotic division. After this stage, the mitochondria regained their former size and structure (Fig. 14). Plastids followed a similar reduction in size during the meiotic divisions and were large and electron-opaque before the microspores were released from the callose capsule.

The mitochondria and chloroplasts of microspores from male-sterile plants showing either type 1 or 2 behaviour underwent a similar de- and re-differentiation (Figs. 15 and 16). After the release of the microspores from their callose capsules, cristae could be seen within the mitochondria similar to those in fertile grains at a similar stage. It was at this point that abnormalities appeared in the microspores. The cytoplasm started to shrivel and shrink away from the microspore wall. Many

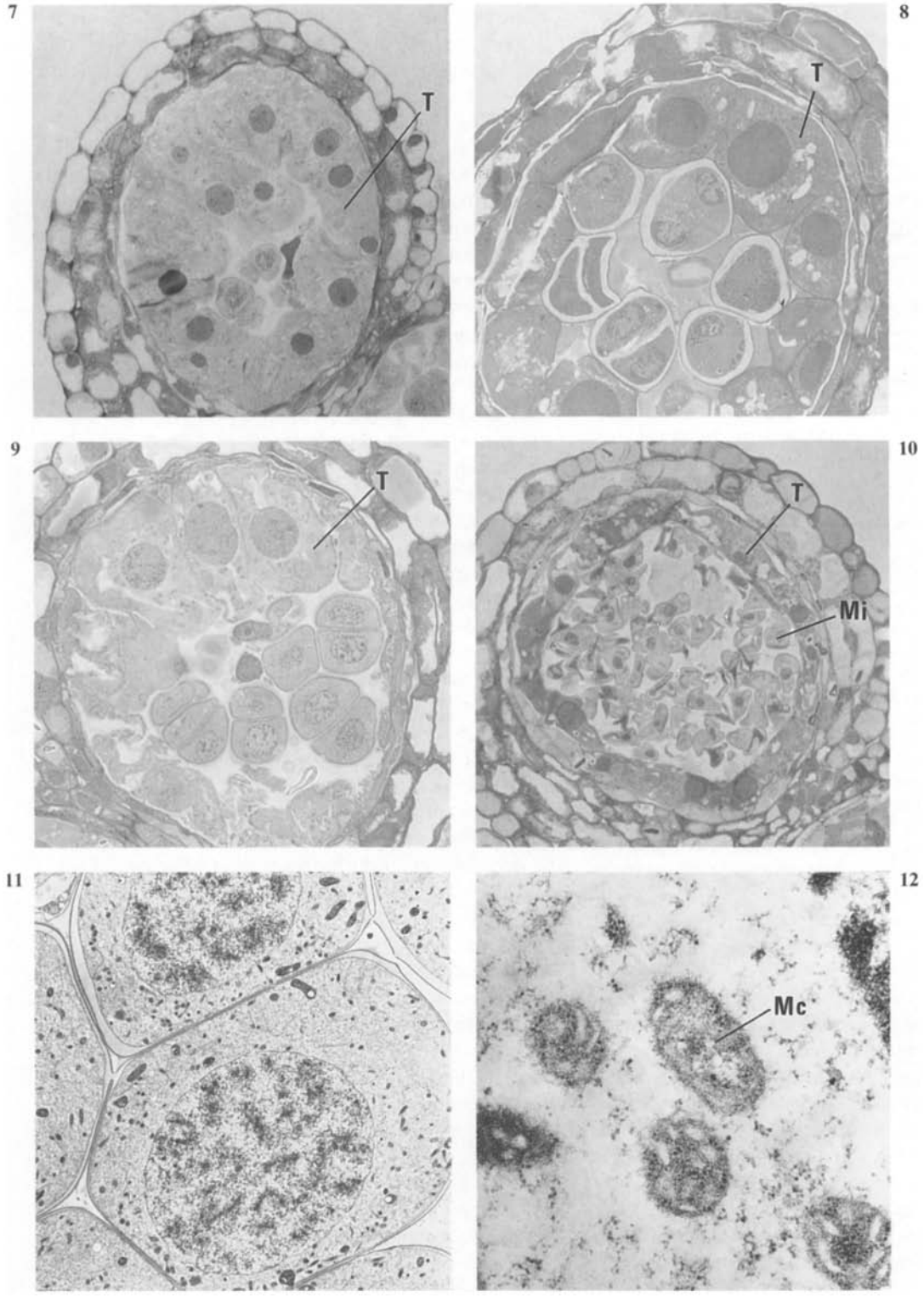


Fig. 7. Anther from a male-sterile plant showing type 2 behaviour after the release of the tetrads from their callose capsule. Approx. × 200

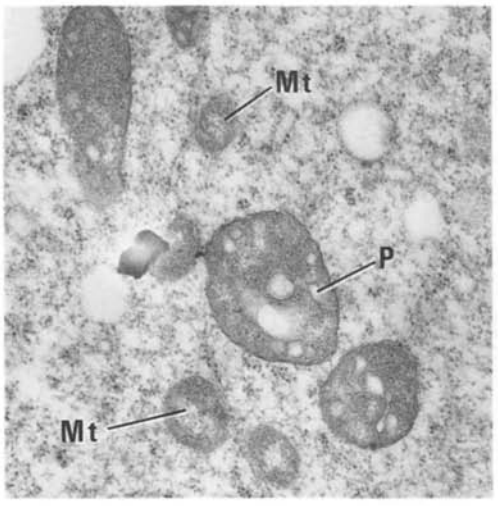
Fig. 8. Diad formation in an anther from a male-sterile plant showing type 2 behaviour. Approx. × 500

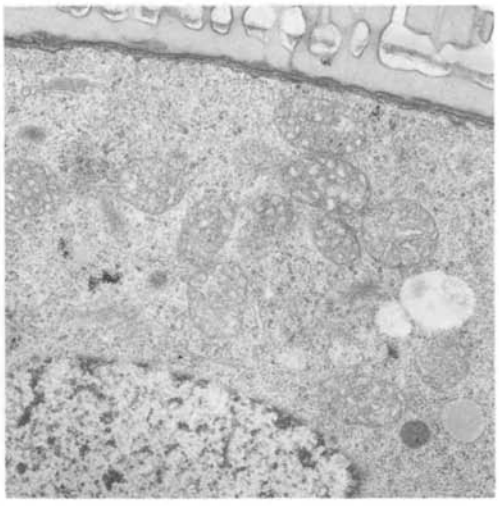
Fig. 9. Degeneration of the tapetum in a male-sterile onion anther showing type 2 behaviour. Approx. × 540

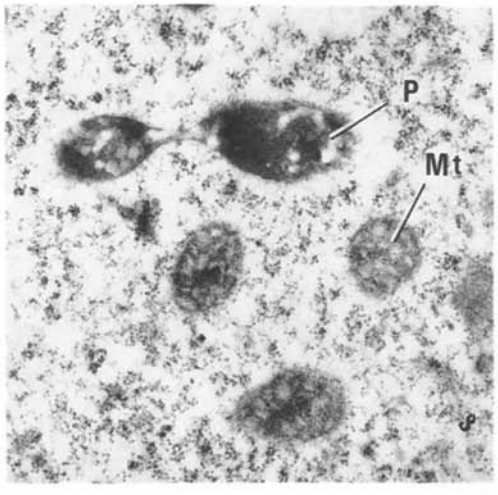
Fig. 10. Male-sterile onion anther showing type 3 behaviour. *Mi* Microspore. Approx. × 220

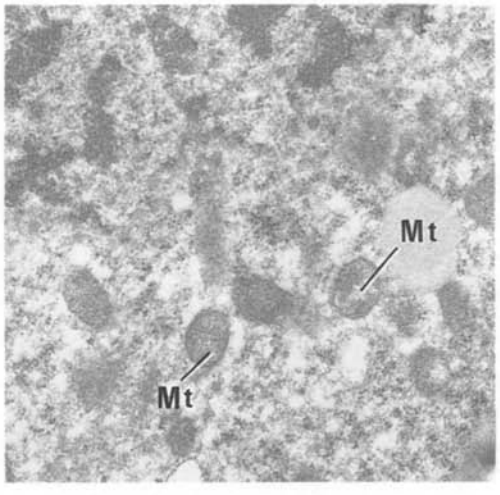
Fig. 11. Early meiotic stage in microspore mother cells of a male-fertile onion anther. Approx. × 3,000

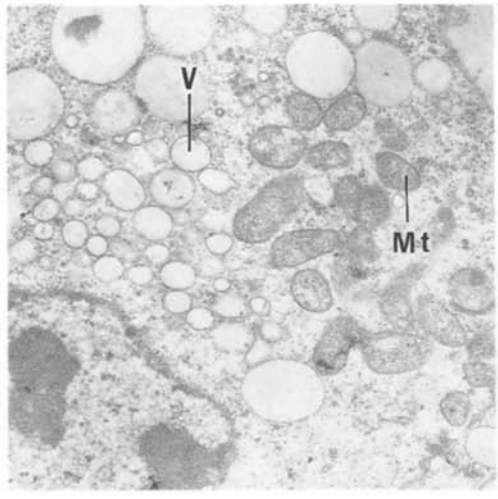
Fig. 12. Mitochondria in a pre-meiotic, male-fertile anther. *Mt* mitochondrion. Approx. × 38,000











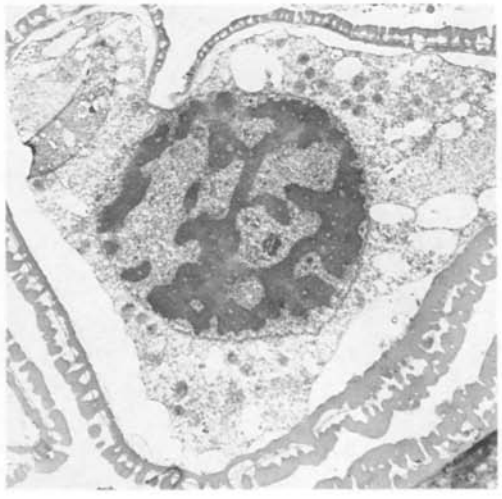


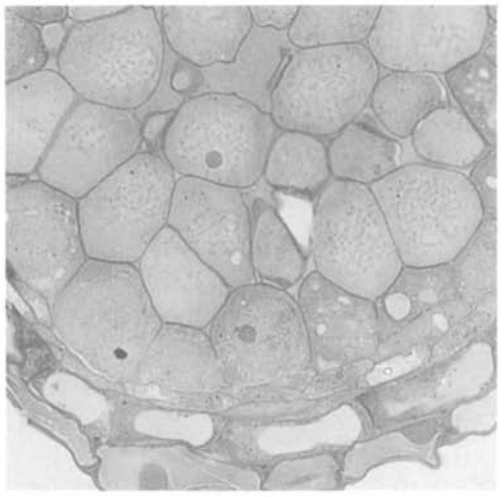
Fig. 13. Cytoplasm in a male-fertile anther during meosis. *Mt* Mitochondrion, *P* plastid. Approx. × 28,000

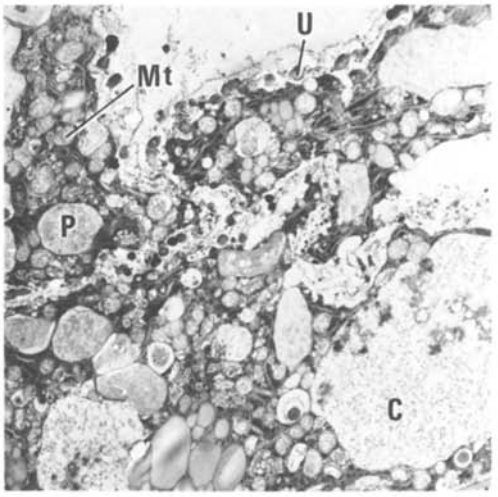
Fig. 14. Microspore from a male-fertile plant near anthesis. Approx. × 16,000

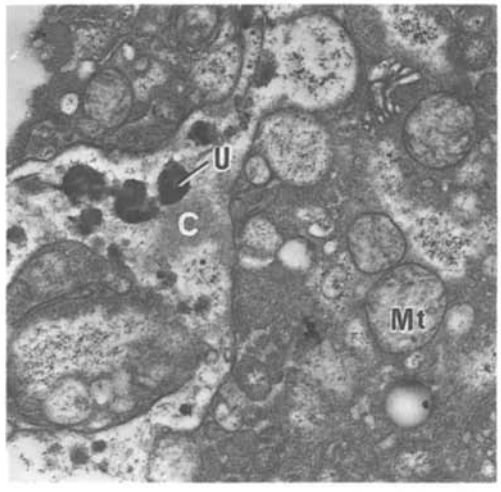
Fig. 15. Organelles within the cytoplasm of a premeiotic, male-sterile anther. Approx. × 28,000

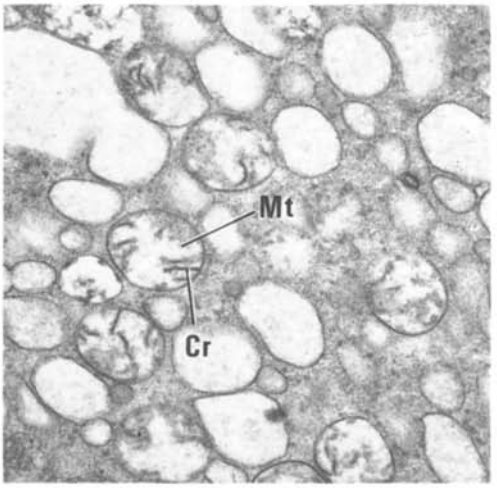
Fig. 16. Mitochondria in the cytoplasm of a male-sterile anther during meiosis. Approx. × 27,000

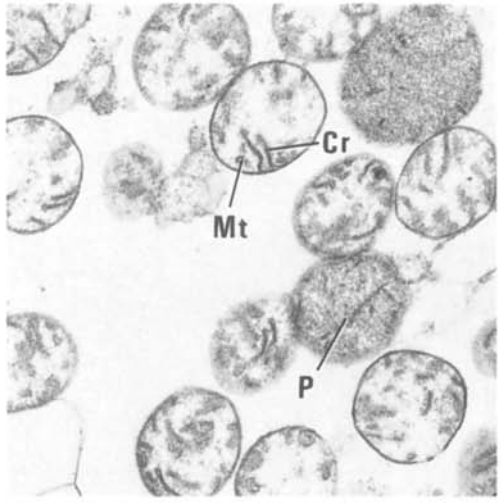
Figs. 17 and 18. Breakdown of the cytoplasm within pollen grains from male-sterile anthers. *V* vesicle. Approx. × 20,000 (Fig. 17) and × 6,300 (Fig. 18)











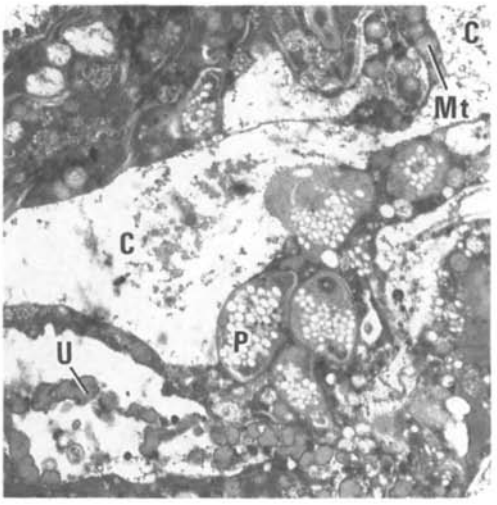
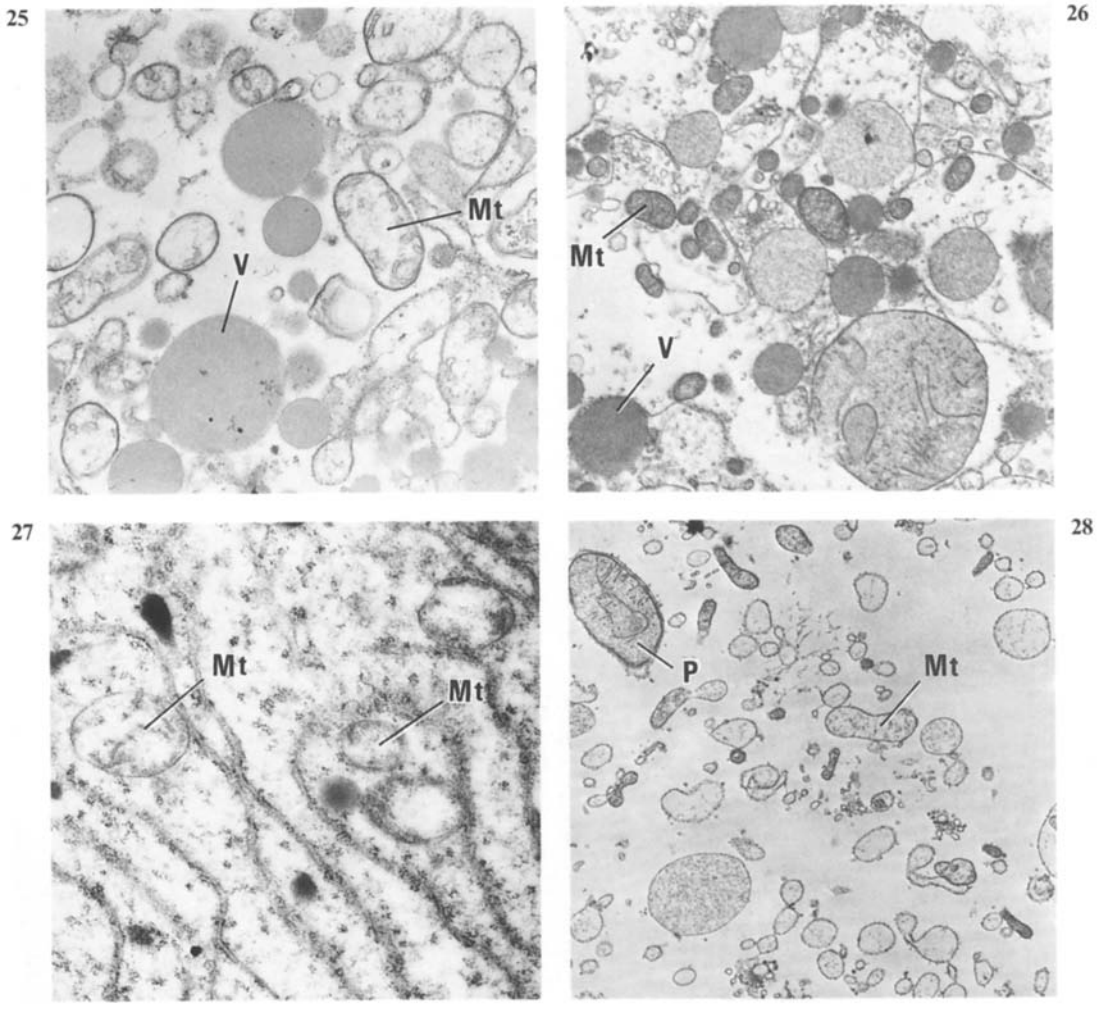


Fig. 19. Male-fertile onion anther during prophase. Approx. × 500

Figs. 20 and 21. Tapetal cytoplasm of male-fertile anthers after tetrad formation. *C* Cytoplasmic channel, *U* Ubisch body. Approx. × 16,000 (Fig. 10) and × 22,000 (Fig. 21).

Figs. 22 and 23. Remains of tapetum in male-fertile anthers during the mitotic divisions of the microspores. *Cr* Crista. Approx. × 25,000 (Fig. 22) and × 28,000 (Fig. 23)

Fig. 24. Tapetum of a male-sterile anther showing type 1 behaviour during the tetrad stage. Approx. × 18,000



Figs. 25 and 26. Final stages of tapetal autolysis in male-sterile anthers showing type 1 behaviour. Approx. ×22,000 (Fig. 25) and ×14,000 (Fig. 26)

Fig. 27. Autoloysis of tapetal tissue during tetrad formation of male-sterile anther showing type 2 behaviour. Approx. ×25,000

Fig. 28. Final stages of tapetal autolysis in a male-sterile anther showing type 2 behaviour. Approx. ×18,300

membrane-bound vesicles become apparent within the cytoplasm, some containing small amounts of fibrillar material (Fig. 17). Next, all membranes progressively lost their integrity (Fig. 18) until the microspore contents disappeared completely (Fig. 6).

Tapetal differentiation in male-fertiles is complete before the microspores pass through their meiotic divisions. The cells of this tissue were characterised by a densely staining cytoplasm and prominent nuclei and nucleoli (Fig. 19). The electron-opacity of the cytoplasm was largely due to the very high numbers of ribosomes. Large amounts of endoplasmic reticulum were also observed, some forming channels through the cytoplasm (Figs. 20 and 21). The cell vacuole was progressively lost and had completely disappeared by the tetrad stage. Also at this time, the inner tangential tapetal wall disappeared to leave partially surrounded protoplasts. At this point vesicles containing a lipid-like substance appeared, as did Ubisch bodies in cytoplasmic channels. Around the time of the mitotic division the tapetum rapidly autolysed (Fig. 22) to leave a collection of vesicles and mitochondria with a few plastids (Fig. 23). The tapetal cell membrane and associated Ubisch bodies were the last vestiges of the tapetum left at the time of dehiscence.

The tapetal tissue of male-steriles showing type 1 behaviour followed the same pattern of development seen in male-fertiles up to the tetrad stage (Fig. 24). During this stage the tapetum started to autolyse in the same manner seen in fertiles and, at the stage at which fertile microspores in fertile anthers were going through their

mitotic division, the autolysis was almost complete. This degeneration resulted in the formation of a collection of vesicles and mitochondria (Figs. 25 and 26), which were then progressively lost.

In anthers showing type 2 behaviour, most cells at the diad stage had the typical densely-staining cytoplasm found in the tapetal cells of male-fertile plants at this time. In the other cells, where hypertrophy was occurring, the cytoplasm was much less electron-opaque and the autolysis of the cell contents had started to occur. Most of the ribosomes had disappeared, as had much of the endoplasmic reticulum. Hypertrophy and autolysis proceeded throughout the formation of tetrads (Fig. 27). When the microspores were released from their callose capsule the hypertrophied cells collapsed spilling their contents into the anther locules. The final stages of autolysis followed the same pattern as that seen with type 1 behaviour, again forming a collection of vesicles and mitochondria (Fig. 28).

The state of the internal structure of the mitochondria, during the autolysis of the tapetal tissue, was dependent on the type of material studied. In male-fertile tapetal tissue at this time, cristae were well formed (Figs. 22 and 23). However, in male-sterile plants the internal structure of the mitochondria was somewhat simplified, especially in plants exhibiting type 2 behaviour (Figs. 25 and 28). Vesicles containing lipid-like material were also seen during this time in male-sterile plants with type 1 behaviour (Figs. 25 and 26); these were not seen to the same extent in male-fertiles (Figs. 22 and 23) or in type 2 male-steriles (Fig. 28).

The volumes occupied by tapetal tissue, and by mitochondria within this tissue, were measured in the central portion of male-fertile and -sterile onion anthers showing type 2 behaviour. The area of tapetal tissue remained constant up to the formation of diads and was the same in male-fertiles and -steriles (Fig. 29). The hypertrophy of the tapetal tissue in the anthers of male-sterile plants was most evident at this stage as it occupied nearly twice the volume of the same tissue in fertile plants. The volume of the tapetal tissue occupied by mitochondria, expressed as a percentage, increased during the first meiotic division and then fell up to, and after, the second division (Fig. 30). Up to the formation of diads, the proportion of mitochondrial volume was similar in male-fertile and -sterile anthers (Fig. 30). After this stage the fall in the proportion of tapetal tissue occupied by mitochondria, as the cells hypertrophied, was greater in male-sterile plants. The actual mitochondrial volume in male-fertile and -sterile tapetal tissue during microsporogenesis is given in Fig. 31. The volume of mitochondria increased during the first meiotic division and then remained more or less constant. The mitochondrial volume was similar in male-sterile and -fertile tapetal tissue at each developmental stage. The hypertrophy of the tapetal tissue, seen

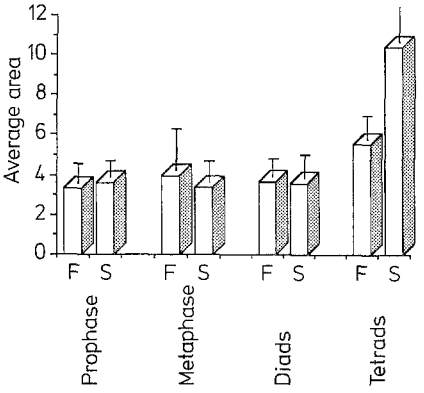


Fig. 29. Average area of tapetal tissue (in $\mu m^2 \times 10^4$) in anthers at different stages of microsporogenesis in male-fertile (*F*) and -sterile (*S*) onions (error bars = std. error of mean)

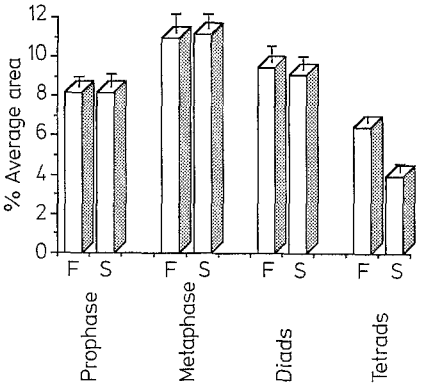


Fig. 30. Average area of tapetal tissue (in percentage) occupied by mitochondria at different stages of microsporogenesis in male-fertile (*F*) and -sterile (*S*) (error bars = std. error of mean)

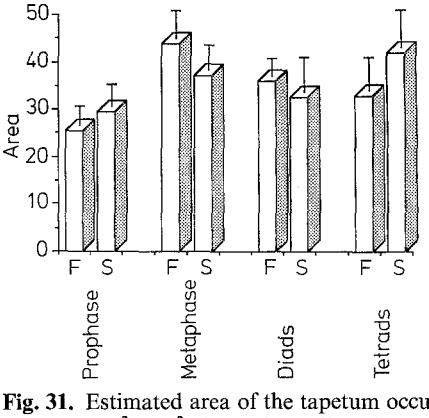


Fig. 31. Estimated area of the tapetum occupied by mytochondria (in $\mu m^2 \times 10^2$) in male-fertile (*F*) and -sterile (*S*) onion anthers at different stages of microprogenesis (error bars = std. error of mean)

at the tetrad stage in male-sterile anthers, was accompanied by a decrease in the percentage of the tapetum occupied by mitochondria. As a result of these two processes there was not a large difference between the area occupied by mitochondria in male-fertile or -sterile tapetal tissue at the tetrad stage.

4 Discussion

In all of the onion anthers studied, abnormalities in the microspores of male-sterile onions were never observed before the stage at which the microspores of male-fertile plants were going through their mitotic division. Tapetal behaviour was found to be variable. Type 1 behaviour, where tapetal degeneration is precocious, has previously been reported by Tatebe (1952) in a Japanese line of onion derived from the American variety 'Yellow Globe Danvers'. No hypertrophy of the tapetal tissue was observed, and the microspores degenerated after the autolysis of the tapetal tissue was under way. Type 2 behaviour, with hypertrophy of tapetal tissue followed by its collapse, has been recorded in the American cultivars 'Italian Red 13–53' and 'Scott County Globe' (Monosmith 1926; Peterson and Foskett 1953), the New Zealand variety 'Pukekohe Longkeeper' (Yen 1959) and the Indian 'Maharashtra' onion (Patil et al. 1973). The third type of behaviour has been observed in the cultivar 'Zittauer Gelbe' (Kobabe 1958; Virnich 1967) and also in the Polish line 'Kasticka' (Jirik and Novak 1969). The present article is the first report of several different types of abnormal tapetal behaviour in a single line (II/3ms) of onion.

Onion is not alone in having different forms of abnormal tapetal behaviour in male-sterile plants with the same nuclear and cytoplasmic constitution. Male-sterile maize plant containing the cms-C cytoplasm show two forms tapetal behaviour. In the first, the tapetum development was similar to that of fertiles up to the tetrad stage apart from the appearance of numerous small vesicles and irregular Ubisch body deposition; both the tapetum and microspores degenerated after microspore release. Anthers showing the second form of behaviour had tapetal cells that were highly vacuolate at the tetrad stage, the inner and radial wall remained intact and the tetrads showed signs of degeneration (Lee et al. 1979). The authors suggested that the same mechanism is responsible for the two types of behaviour and that the difference is due to the timing of its action.

The lesion causing cms in the onion cms-S cytoplasm must be capable of exhibiting differential expression in order to cause the range of abnormal behaviour observed in this study. This expression must be independent of the nuclear genotype as all behavioural types were seen in the line II/3ms, which is highly inbred. The expression of the cms-S lesion is most likely influenced by the environment as all forms of abnormal behaviour were seen in the 1988 season, whilst only type 2 behaviour occurred in the following year.

In both types 1 and 2 behaviour tapetal degeneration occurred before any abnormalities were observed in the microspores. In type 3 behaviour microspore degeneration appeared to be independent of tapetal behaviour, but occurred at the same stage in development as in the other two types. This may suggest that the lesion has independent effects on the tapetum and the microspores. It has been suggested that male-sterility in cms-S onions results from the lack of the appropriate nutrients being passed from the tapetum to the developing microspore due to its premature autolysis (Saini and Davis 1969). If this is the case then another mechanism must be sought to explain the degeneration of the microspores in anthers showing type 3 behaviour. Virnich (1967) suggested that onion microspores in anthers exhibiting type 3 behaviour were, in some way, unable to absorb nutrients found in the anther loculus. It seems more likely that the pool of nutrients made accessible to the microspores of male-fertile plants, due to the autolysis of the tapetum, is not available in type 3 anthers. The cause of male-sterility in anthers with the cms-S cytoplasm could be explained as resulting from a lack of correct nutrition for the microspore either before the mitotic division (types 1 and 2) or during and after this stage (type 3). It seems unlikely, however, that the lesion could be expressed at two different developmental stages.

Where the histological development of the anther tissue has been studied, 49% of species exhibiting gene-cytoplasmic male-sterility have shown abnormal tapetal behaviour and a breakdown in the microspores after their liberation from the tetrads (Kaul 1988). Male-sterile plants of both *Allium fistulosum* and *Allium schoenoprasum,* and an inter-specific cross between. *A. cepa* and *A. pskemense,* also show tapetal hypertrophy and microspore breakdown after their release from their callose capsule (Nishi and Hiraoka 1958; Singh and Kobabe 1969; Saini and Davis 1969). Interspecific crosses between *A. cepa* and *A. ascalonicum, A. drobovii* and *A. galanthum* and also between *A. roylei* and *A. drobovii* all resulted in male-sterile plants (Little et al. 1944; Saini and Davis 1969). Although the microspores in the plants degenerated after release from the callose capsule, no abnormalities in the tapetum were observed. Microspore breakdown after their release from the callose capsule is, then, a common feature of male-sterile alliums, but tapetal abnormalities are not a prerequisite for this process. This again may suggest a separate, pleiotropic effect of the gene(s) causing male-sterility on both the microspores and tapetal tissue.

In view of the links between cms and the mitochondrion, particular attention has been paid to the be-

haviour of the mitochondrial population. No difference in the relative mitochondrial total volumes within the tapetal tissue were observed at any stage nor were structural differences found. Indeed the mitochondrion was the last organelle that was recognisable during tapetal autolysis in both fertiles and steriles and was found in large numbers.

To date, the only cms-related ultrastructural abnormalities reported in mitochondria have been found in male-sterile maize anthers containing the cms-T cytoplasm. The first observed aberration in these anthers occurred at the tetrad stage in the mitochondria of the tapetum. Although their outer membrane remained intact, the cristae became disorganised and the matrix was light and without structure (Warmke and Lee 1977). Although differences in the structure of tapetal mitochondria in cms-T maize plants could be shown, no differences could be found in mitochondrial volume or numbers per cell (Warmke and Lee 1978). A study of the *ogu* cms line of *Brassica napus* (Polowick and Sawhney 1990) showed differences in vacuole formation, sporopollenin deposition and the distribution and structure of mitochondria compared to fertile material. However, variability in these characteristics between anthers or even adjacent locules precluded them from being the primary cause of sterility. In barley containing the *msm1* cytoplasm, male-sterility was attributed to the uncontrolled production and secretion of sporopollenin, and the tapetal plastids showed an abnormal behaviour pattern (Ahokas 1978). In other plants, organelle behaviour is similar in fertiles and steriles. Liu et al. (1987) showed that the tapetal cytoplasm of male-sterile *Petunia* was similar to that of male-fertiles with no abnormalities in the nuclei, plastids, mitochondria or ribosomes. However, a difference could be shown between the amounts of endoplasmic reticulum, especially during the meiotic prophase. Ultrastructural studies have also been made on male-sterile wheat (De Vries and Ie 1970), pepper (Horner and Rogers 1974), sorghum (Laser 1972), soybean (Albertson and Palmer 1979) and maize containing the cms-C cytoplasm (Lee et al. 1979). In none of these studies were disruptions in organelle behaviour or structure detected; however, De Vries and Ie (1970) did find a reduction in the overall number of organelles in their male-sterile material.

Male-sterility in cms-S onions cannot, then, be linked to the abnormal behaviour of any organelle either in the tapetum or microspore. It would appear from these results that there is a mistiming of normal tapetal behaviour that leads to its premature autolysis in type 1 and 2 plants or possibly to its retention in type 3 plants. None of the lesions in the plant species studied, except cms-T maize, result in gross changes in mitochondrial structure until other aberrations in anther ultrastructure are observed. Proteins, shown to be correlated with cms, are either found in the inner mitochondrial membrane (Dewey et al. 1987; Wise et al. 1987) or associated with it (Nivison and Hanson 1989). There is an apparent large requirement for energy during microsporogenesis, and these proteins may interfere with energy production. This is likely to have a wide pleiotropic effect resulting in the different types of tapetal behaviour seen in plants exhibiting cms such as those observed in onion.

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