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DNA synthesis and cytoplasmic differentiation in tapetal cells of normal and cytoplasmically male sterile lines of *Petunia hybrida*

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Summary. A new method is described by which tapetal cells may be isolated from anthers of cytoplasmic male sterile (CMS) and fertile lines of *Petunia hybrida*. Using a combination of stereometry and Feulgen densitometry it has been possible to chart DNA synthesis and nuclear division with great precision within these cells. Results are presented which show CMS lines both to synthesize far less DNA than the fertiles and also to undergo less cell division. These differences in DNA kinetics and cytokinesis are obvious long before any differences between sterile and fertile lines may be detected in the meiocyte mass. In addition to these difference in nuclear behaviour, the tapetal cells of CMS lines also synthesize far lower levels of rough endoplasmic reticulum than do their fertile counterparts. Again, this difference is evident at a very early stage of anther development when all other cell components, including mitochondria and plastids, appear normal. These data are discussed in terms of the very special differentiation undergone by tapetal cells in angiosperms, and the conclusions drawn are considered in the perspective of current hypotheses proposed to explain the operation of CMS.

Key words: Cytoplasmic male sterility – DNA synthesis – Mitochondria – Pollen development – Tapetum

Introduction

The monolayer of tapetal cells investing the developing pollen mother cells in angiosperm anthers has long been held to play an important role in nutrition (Goebel 1905). The content of these cells, principally banks of rough endoplasmic reticulum (RER), mitochondria, dictyosomes and, frequently, more than one nucleus, indicates them to be very active physiologically (Vasil 1967). Clearly, all materials assimilated by the pollen mother cells must first pass through the tapetum but studies are few concerning the manner by which compounds may be modified during this transfer (Dickinson 1968).

Any single event has yet to be identified as the primary cause of any of the several types of cytoplasmic male sterility (CMS) observed in angiosperms, but the first detectable divergences from normal development frequently involve changes in the tapetum (Overman and Warmke 1972; Chuahan and Singh 1966; Nanda and Gupta 1974; Lee et al. 1979). Most recently, Bino (1985a, b) has also reported that the first signs of deviation from normal development in CMS anthers of *Petunia* appear in the tapetal tissue. Differences in the pH of the locular fluid and in the activity of a 1,3 glucosidase, both of which are presumably controlled by the tapetal tissue, have also been recorded in CMS lines of *Petunia hybrida* by Izhar and Frankel (1971) and Frankel et al. (1979). Although the inheritance of CM dictates that it cannot be regulated by the tapetal nuclear genome, nuclear events in this tissue can be quite dramatic (Heslop-Harrison and Moss 1968). Since this unusual nuclear differentiation is a feature solely of male development its study may assist in the search for a molecular basis for CMS.

In recent years, evidence has accumulated that CMS is accompanied, almost without exception, by lesions in mitochondrial DNA (Hanson and Conde 1984). In some cases, aberrant polypeptides can be shown to be synthesized in vitro by mitochondria from CMS lines, and there is now little doubt that CMS is regulated through the mitochondrial genome. Not surprisingly, investigations into the mitochondrial DNA of CMS *Petunia* have revealed a number of lesions in the mol-

ecule (Boeshore et al. 1985). It remains true, however, that since CMS only becomes evident during male sex cell development, and cannot be detected elsewhere in the plant body, there must be another 'component' of the CMS response, one which is specific to male development.

Materials and methods

Plants of *Petunia hybrida* var. Rosy Morn were grown from seed (kindly supplied by Dr. S. Izhar, Volcani Centre, Bet Dagan, Israel) under glass with supplementary illumination. The fertile and CMS lines employed in the study were isogenic, except in the possession of nuclear restorer gene by the fertile. Under a mean day temperature of 25 °C, both lines normally flowered within 10 weeks. Tapetal cells were isolated from developing buds of these plants using the method set out below.

Following staging of excised anthers by the preparation of temporary squashes stained with lacto-proprionic orcein, appropriate anthers were excised and fixed immediately in standard formalin-acetic alcohol solution, pre-cooled in ice. The low temperature during fixation induces the tapetum to detach from the anther middle layer. Following two washes in distilled water to remove the fixative each of 30 min duration, anthers were placed on a slide and carefully bisected longitudinally using a sharp razor blade. Anther halves were then incubated at 30 °C in an enzyme solution containing 5 mg/ml Macerozyme and 5 mg/ml Cellulysin in 0.05 M phosphate buffer at pH 5.8; 5 min of this digestion proved adequate to loosen the tapetal cells from their walls.

The anthers were then simply transferred to a drop of distilled water on a clean slide, covered with a cover slip and gently pressed to release the tapetal cells. The preparations were finally frozen to −20° for 5 min, the cover slips prised off, and the material allowed to dry at room temperature.

Nuclei were then stained by means of the Feulgen reaction (McLeish and Sunderland 1961; Moss 1966) using a hydrolysis period of 12 min in 1 normal HCl at 60 °C. DNA levels were measured with a Vickers M 85 microdensitometer (Bennett and Smith 1976) employing a scanning wavelength of 565 nm, and a × 100 objective lens with oil immersion optics. Using these methods it was possible not only to estimate the quantity of DNA contained within individual tapetal nuclei as a function of the haploid (1 C) levels found in the young microspores, but also to count the nuclei contained within each tapetal cell.

For the measurement of the endoplasmic reticulum (ER) in tapetal cells, anthers of *Petunia hybrida* were fixed for electron microscopy as described in Dickinson and Bell (1976a), sections cut of this material, and cells photographed at a constant magnification of × 12,000. Using these micrographs, the length of sectioned ER present in a large number of tapetal cells was measured and then divided by the area studied to give a factor representative of the amount of ER present in a constant area. These figures were then plotted against developmental stage. Although the result is clear-cut when analysed statistically (see Fig. 2), there are a number of errors implicit in this type of analysis, and these results should thus be regarded only as indications of the ER content of each type of cell.

Results and discussion

Despite the proposition by Goebel in 1905 that the tapetum acted as a nurse tissue to the developing pollen grains, physiological evidence to support this contention has been sparse. Tapetal materials have been shown to move on to the surface of the pollen grains in a number of investigations (e.g. Heslop-Harrison 1968; Dickinson and Lewis 1973; Reznickova and Dickinson 1982) but it has proved difficult to demonstrate that material imported to the tapetum is modified and then passed on to the sporocyte mass. Most of the evidence of this type has been circumstantial, and has emerged from simple studies on the levels of proteins and other reserves contained in the pollen, loculus, and the meiocytes (Dickinson and Bell 1976a, b). Labelling experiments do show, however, that precursors provided to the tapetal cells are retained for periods which differ depending upon the stages of development of the anther (Dickinson 1968). In this type of experiment it is quite impossible to determine the nature of the polypeptides which may be synthesized in the tapetum and passed on to the developing pollen grains. Nuclear events within the tapetal cells have proved very difficult to study for, depending upon the particular species, tapetal nuclei can divide up to 3 times by endomitosis. The C value of these nuclei has been studied by Heslop-Harrison and Moss (1968), and been found to vary between 1 and 6 C. Tapetal cells are unusual in that they possess a relatively short life, during which time their activities are restricted to the phasic synthesis of a small number of compounds (Dickinson and Bell 1976a, b). For this reason, there is the remote possibility that this rapid tapetal endomitosis may not involve the faithful replication of all nuclear DNA – but rather the amplification of sequences required for particular syntheses. While gene amplification in tapetal nuclei might explain some forms of CMS, in that in lines requiring complementation between nuclear and mitochondrial genomes to compensate for lesions in mitochondrial DNA, the genes involved in the complementation might not be amplified, it still remains unlikely. Male sterility more probably results from mitochondria being unable to meet either the very specific synthetic requirements, or the very high energy needs of the tapetal cells undergoing these elevated levels of DNA synthesis. Any investigation into any of these possibilities should clearly commence with the study of nuclear number in individual tapetal cells during maturation of the anther, and measurement of the DNA contained within them.

In tapetal cells of fertile *Petunia* lines, the number of nuclei rises during meiosis to an average of 2.1 as the pollen mother cells undergo meiosis (see Fig. 1). Very little endomitosis occurs in subsequent stages. In CMS lines, however, little nuclear division takes place at all, resulting in an average nuclear number of about 1.75, a figure which remains unaltered until the tapetum degenerates during the tetrad stage. Cebrat and Zadecka (1978) have suggested that in *Secale cereale* the fun-

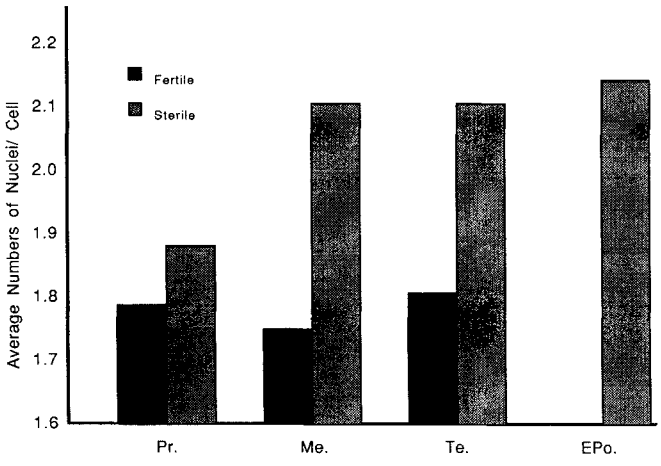

Fig. 1. The average numbers of nuclei contained in the tapetal cells of male sterile and fertile *Petunia hybrida* lines throughout microsporogenesis. The stages depicted are PR: prophase, ME: metaphase I, TE: telophase I and Epo: early pollen. Since the tapetum has completely degenerated by the early pollen stage in male sterile lines, figures have not provided for this stage. These results represent means from the study of an average of 165 cells for each developmental stage for both male fertile and sterile lines.

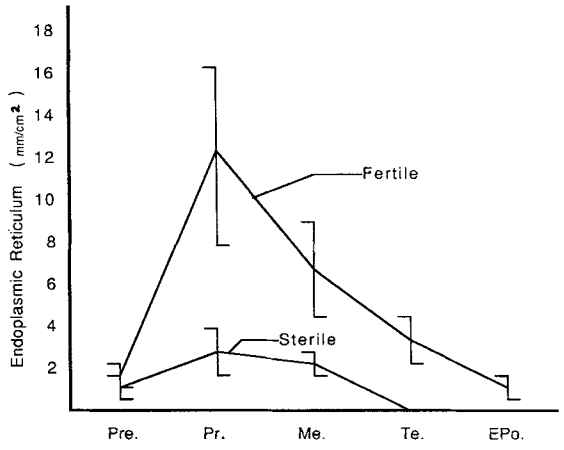

Fig. 2. Changes in the amount of endoplasmic reticulum contained in tapetal cells during meiosis in fertile and CMS anthers of *Petunia hybrida.* Levels of endoplasmic reticulum are expressed in measurements taken directly from micrographs. *Vertical bars* denote standard errors, and the developmental stages shown are those depicted in Fig. 1 except for premeiosis

damental cause of abnormal microsporogenesis in CMS lines is the aberrant function of the tapetum, and particularly its failure to undergo nuclear division. Whilst these authors did not provide clear evidence in support of this contention, it was proposed that the absence of a second nucleus reduced the area of nuclear envelope available for the production of rough ER, thus affecting the ability of the cells to synthesize protein.

Our preliminary studies on the area of ER available in CMS and fertile lines (see Figs. 2 and 3a, b) strongly support this contention. The stereological technique employed here, albeit somewhat imprecise, suggests that tapetal cells from fertile lines contain vastly more ER than do those of male sterile. Further, these differences are at their greatest during early prophase, a point when virtually no signs of abortion may be detected in the male sterile anther. Apart from this difference in ER quantity, the cytoplasm of tapetal cells from these different lines is remarkably similar, containing normal nuclei, apparently healthy mitochondria and plastids, and large numbers of ribosomes (see Fig. 3a, b).

No sensible conclusions may be drawn with regard to the significance of nuclear number within tapetal cells unless the amount of DNA contained within them is known. From Fig. 4 it is clear that tapetal nuclei of CMS lines cease in their ability to synthesize DNA during early meiotic states in the pollen mother cells (PMCs), while nuclei of fertile plants continue DNA synthesis well into the early pollen stages. In a similar investigation into DNA levels in tapetal cells Heslop-Harrison and Moss (1968), using an autoradiographic method, also showed a rise in DNA levels within normal anthers.

Throughout the later stages of meiosis and early part of microsporogenesis in the PMCs, the tapetal cells of CMS lines would thus appear to contain quantities of DNA significantly lower than normal.

In Fig. 5, the proportion of nuclei containing DNA at different C levels is shown for both fertile and CMS lines throughout meiosis. As might be expected, tapetal nuclei from fertile cells show a distribution between 2–4C during meiotic prophase in the PMCs, advancing to higher levels as development proceeds. By the time that early pollen is being formed, the range of C values observed extends to 7 and 8C in the largest nuclei. A completely different pattern is exhibited by CMS lines. Here, tapetal cells at PMC meiosis contain nuclei possessing levels of DNA as low as 1C, which increase only to 2C during the later stages of meiotic prophase. By the time the tapetal cells degenerate between the tetrad and early pollen stages a spread of DNA levels is observed ranging only between 2 and 4C. These results dramatically confirm those data presented in Fig. 4, and indicate that tapetal DNA synthesis is seriously affected in CMS lines.

A number of important points emerge from this study. Firstly, and most significantly, both nuclear division and DNA synthesis is seriously inhibited in the tapetum of CMS plants. It is difficult to determine exactly when this inhibition commences, but it seems to be operating early in meiotic prophase. Although small

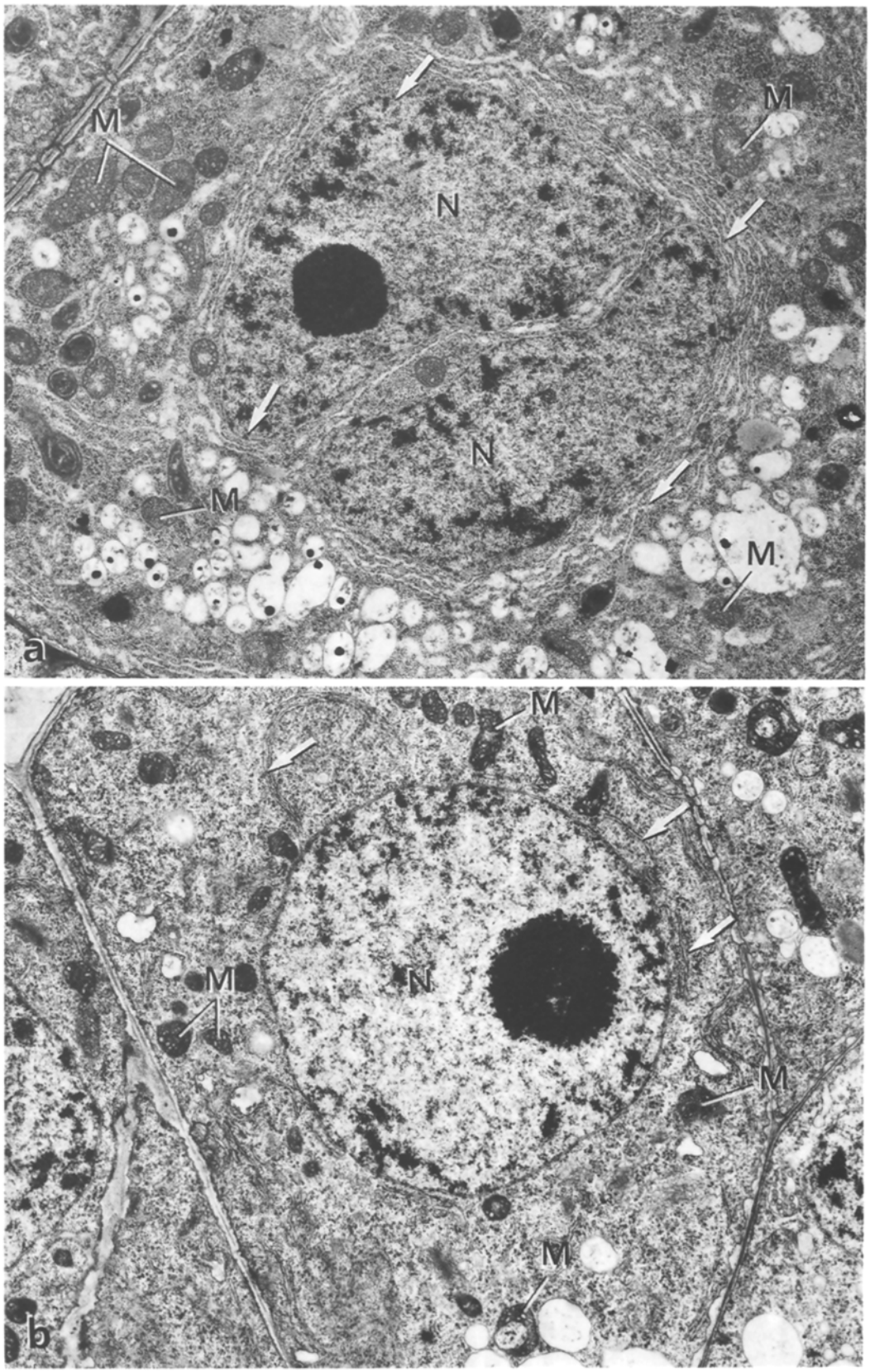

Fig. 3. **a** Tapetal cell contained in a fertile anther of *Petunia hybrida* at the stage when meiocytes are in mid-prophase. Note the presence of two nuclei (N), copious endoplasmic reticulum (*arrows*) and a number of mitochondria (M). × 12,000. **b** Material as in **a** but from a CMS line. The nucleus (N) and mitochondria (M) remain visible, but far lower levels of endoplasmic reticulum (*arrows*) exist in these cells. × 10,000

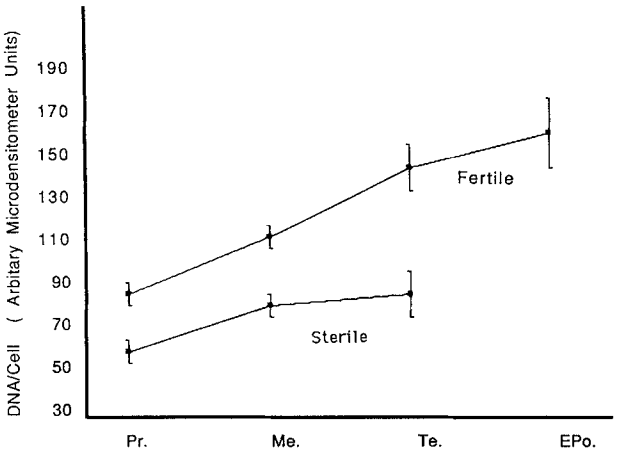

Fig. 4. The amount of DNA (expressed in arbitrary microdensitometer units) contained in the tapetal cells of male sterile and fertile lines at different stages of development. These results represent the means of measurements of over 300 cells at each stage of development for both lines. The developmental stages studied are those shown in Fig. 1. *Vertical bars* delineate standard errors

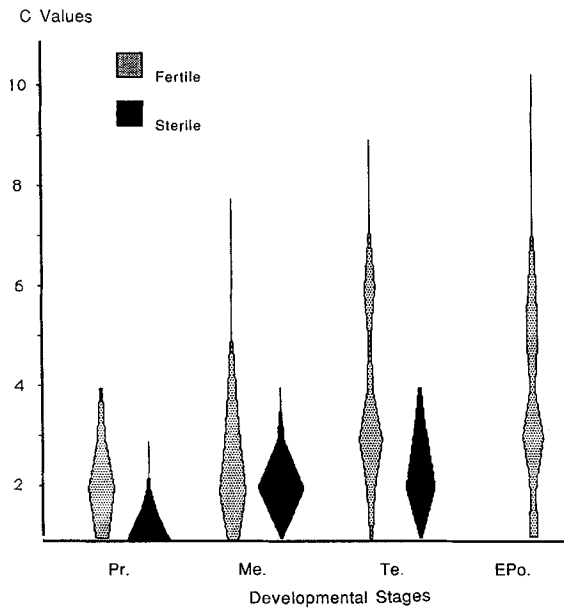

Fig. 5. The distribution of DNA quantitiy, as represented by C value, in tapetal nuclei of fertile and male sterile in *Petunia hybrida* at stages of development shown in Fig. 1 and 2. The width of each histogram at any point indicates the percentage of the total proportion of nuclei at that particular C value; a width of demonstrates that 20% of that nuclear population were at a particular C value. C values were not calculated directly, but were deduced by assuming that the microdensitometer units generated by individual members of a tetrad of microspores represented 1C, and those from an undivided meiocyte 4C. These results represent means from the study of ca. 200 cells for each developmental stage in both fertile and CMS lines

cytoplasmic differences are detectable within the meiocytes at this stage, it is within the tapetal cells that the first evidence of sterility is seen (Bino 1985a, b). A number of authors have poined out that developments within tapetal cells parallel to a very large extent those taking place in the meiocytes (Lersten 1972; Dickinson and Bell 1976b; Horner 1977) and it is therefore reasonable to assume that the pattern of gene expression in these two tissues is similar, with the significant exception of sequences controlling meiosis and, at a later stage, some aspects of pollen wall synthesis (Sheldon and Dickinson 1983). In this connection it it interesting to note that while cell division is relatively unaffected within the meiocyte mass of male steriles, results from the work reported here suggest that it is inhibited at an earlier stage in the tapetal cells.

This evidence, and the previous data of Heslop-Harrison and Moss for *Zea* (1968) all reinforce the view that DNA synthesis within normal tapetal cells possesses a number of important and unusual features. Firstly, it seems to proceed unchecked throughout PMC meiosis and microsporogenesis; secondly it is only accompanied by nuclear, rather than cell division and thirdly, it continues until some nuclei possess DNA levels of up to 8C. If much of this DNA synthesis were to involve the amplification of sequences coding for materials secreted by the tapetum, a reasonable explanation would be provided for these unusual kinetics. Since sequences involved in supporting nuclear-mitochondrial genomic complementation would be unlikely to be amplified, the regulation of mitochondrial activity could seriously be impaired in plants possessing lesions in mitochondrial DNA, with a resulting decrease in the input of energy to these very active cells. Such a scheme would account for some of the phenomenology of CMS in *Petunia* and perhaps, in other species. More persuasive evidence of gene amplification would obviously come from detailed analysis of tapetal DNA – work which is currently in progress. In the meantime, it still remains more probable that these differences in DNA synthesis, nuclear division and ER production result from an incapacity of the mitochondrial population to produce ATP in the unique environment of the anther. Reasons for this mitochondrial insufficiency might include changes in metabolic pathways, the synthesis of materials specific to pollen development, or even a requirement for high levels of ATP not encountered elsewhere in the plant life cycle.

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