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Histological aspects of microsporogenesis in fertile, cytoplasmic male sterile and restored fertile *Petunia hybrida*

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Summary. A comparative histological study is made of microsporogenesis in fertile, cytoplasmic male sterile and restored fertile *Petunia*. Microsporogenesis in sterile anthers proceeds normally until leptotene. The development of the restored fertile type at 25 °C is normal until the tetrad stage. In both types sporogenesis arrests and the meiocytes, c.q. microspores ultimately degenerate. The first phenomena of deviation are found in the tapetum. The effects of degeneration on cellular structure, vacuolation and cytoplasmic organization of the tapetal and sporogenous cells are variable. The deposition of callose around the meiocytes appears independent of the process of degeneration. The absence of an increase in callase activity possibly explains the remnants of callose found at late stages of development. The failure of callose wall dissolution appears to be the result of metabolic abnormalities in the tapetum and is regarded as an indirect effect of sterility.

Key words: *Petunia* – Cytoplasmic male sterility – Restored fertility – Tapetum – Callose

Introduction

The anatomical and cytological aspects of cytoplasmic male sterility (cms) in approximately 140 species of angiosperms have been reviewed by Laser and Lersten (1972). They concluded that among the different species, the failure of microsporogenesis can occur at almost any time during development and that probably more than one mechanism is involved. Various aspects of cms in *Petunia* have been described earlier. Recently, Izhar et al. (1983) have studied the transfer of cms in *Petunia* via somatic hybridisation; Boeshore et al. (1983) reported the mitochondrial DNA restriction patterns of fertile and cms somatic hybrids and Kool et al. (in preparation) described the physicochemical characteristics of mitochondrial DNA isolated from cultured cell suspensions. These reports were focussed on the elucidation of the molecular basis of cms in *Petunia*. Kool et al. demonstrated that mitochondrial modifications in DNA and its translation products were associated with cms. Obviously I, *Petunia* was chosen for these studies because this genetically well-researched genus belongs to the economically important family of the *Solanaceae*. Since cms is a powerful tool in the production of hybrid seeds to avoid the costly procedure of hand emasculation, it may be of great commercial value to broaden the applicability of cms in this family.

While considerable knowledge is available on the histological aspects of sterility in cms lines of many species, there is little information concerning *Petunia*. According to Van Marrewijk (1968), the development of the anther of cms *Petunia* closely resembles that of the sterile forms of many other species. Izhar and Frankel (1971) suggested that faulty timing of enzymatic digestion of the meiotic callose wall was a primary cause of cms. They recognized three main patterns of cms in *Petunia* related to callase activity in the locule. This faulty timing of enzymatic digestion resulted in a breakdown of microsporogenesis during either meiosis, or tetrad stage, or the young microspore stage. The callase activity is found to be different for various plasma types and is influenced by the presence of fertility restorer genes and environmental conditions (Izhar and Frankel 1976). Estimations of the moment of breakdown are primarly based on the abortion symptoms of the sporogenous cells. The authors did not pay much attention to the development of the tapetum or other anther tissues nor to the process of abortion of the meiocytes or microspores themselves.

The first detectable cytological deviations in many cms species are found to occur in the tapetum (Laser and Lersten 1972). The tapetum seems critical in the arbortive process and

its malfunctioning is often regarded as the direct or indirect cause of cms. The tapetum is found to be an important tissue for many enzymatic processes in the locule; for instance callase appears to originate from it (Mepham and Lane 1969).

The present report compares microsporogenesis in fertile, restored fertile and cms *Petunia*. The emphasis of this study is placed on the development of the tapetum and the process of abortion of the sporogenous cells. Cytological aspects of cms will be discussed in relation to the newly gained insights into the molecular basis of sterility in *Petunia*.

Material and methods

Three types of *Petunia hybrida* (Hook.) Vilm. were used in this study, i.e. male fertile cv. 'Blue Bedder' (BBF), the cms 'Blue Bedder' (BBS) described by Van Marrewijk (1968) and the heterozygous restored fertile F_1-cross of BBS with a restorer line descending from restorer material, developed at the Institute of Plant Breeding, Agricultural University, Wageningen by Ferwerda (1963). BBF and BBS are highly isogenic. The plants were cultivated in a growth chamber under a regime of 16 h light, 8 h dark. BBF and BBS were cultured at 17 °C, the F_1 cross at 25 °C. Dissected anthers were fixed in 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 6.5) for 1 h and subsequently postfixed in 1% osmiumtetroxide in the same buffer for 16 h at room temperature. The material was dehydrated in a graded ethanol series and embedded in Epon 812 through propylene oxide.

To check for the presence of callosic compounds, sections (2 µm) were placed in a solution of 0.05% aniline blue in 0.06% potassium phosphate and examined with a UV-fluorescence microscope. Light micrographs were made with a Nikon Optiphot microscope equiped with Nomarski optics.

Results

Fertile microsporogenesis

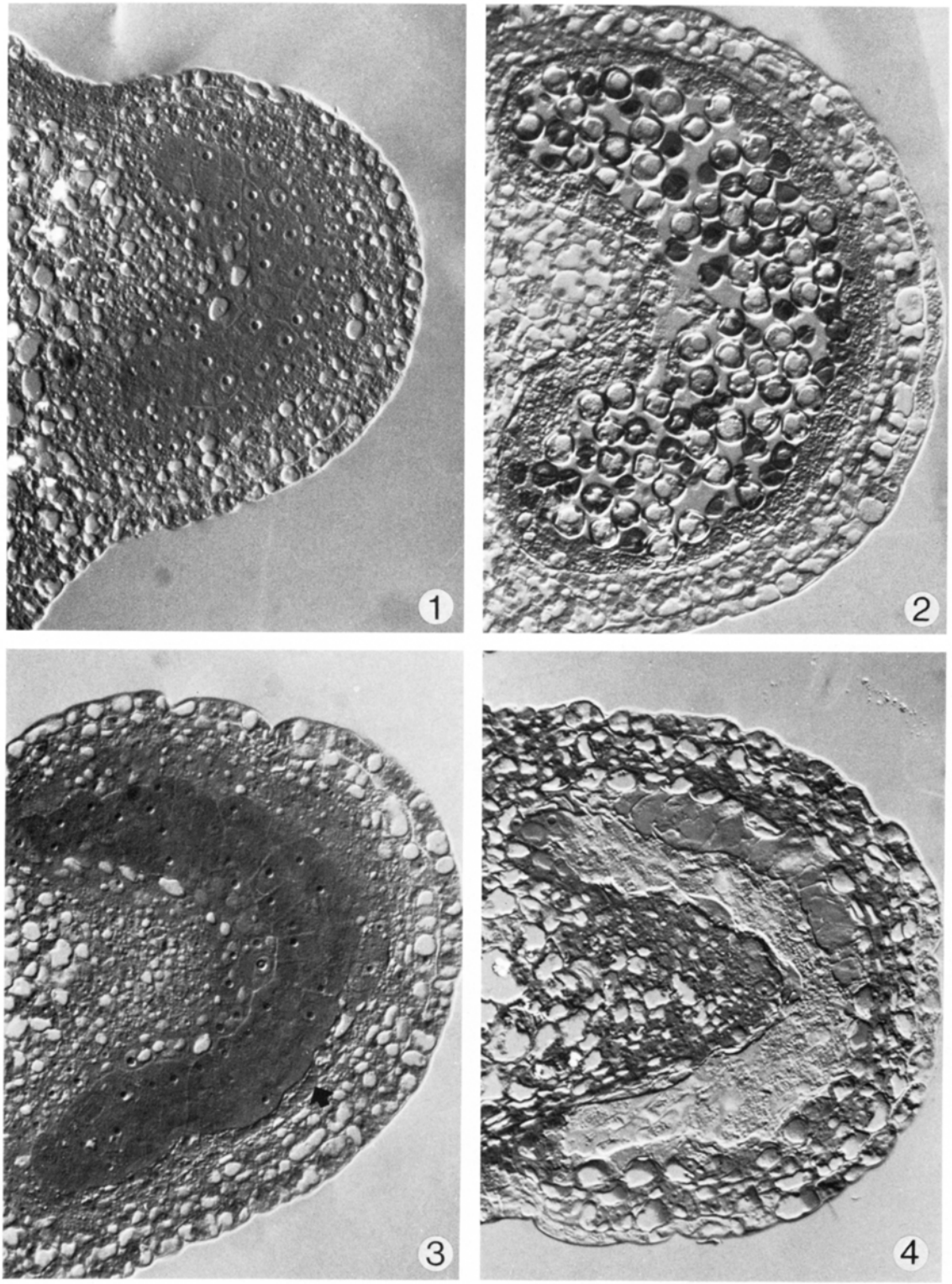
Microsporogenesis in the male fertile type (BBF) generally follows the well-established pattern described for many other species (Bennett 1976). Attention will be focussed on some species specific aspects of *Petunia hybrida*.

Each microsporangium consists of four distinct tissues: epidermis, three parietal layers, a uniserale tapetum surrounding two or three layers of sporogenous cells. The locules appear crescent shaped in cross section. The tapetum is of the secretory type. Before meiosis the tapetal cells of the connective side of each locule (the inner tapetum) become cytologically different from the tapetal cells of the external side (the outer tapetum) (Fig. 1). The inner tapetal cells become larger and more irregularly shaped compared with the outer ones, a dimorphism which has been reported for some other plant families (Gupta and Nanda 1978). During leptotene of the meiocytes, a nuclear division occurs in most of the tapetal cells, though some cells do not undergo karyokinesis and remain uninucleate. During pachytene, callose is deposited around the meiocytes. By the end of the tetrad stage the inner tapetal cells increase in volume and several large vacuoles are formed. The regularly shaped cells of the outer tapetum contain some small vacuoles and the cytoplasm remains dense. During the first pollen mitosis the tapetal cells start to degenerate (Fig. 2). Microsporogenesis in the four locules of one anther does not progress completely simultaneously. Among anthers of one flower the differences are somewhat larger, this variation appears to be the highest at the prophase stages, after meiosis I the development is synchronous.

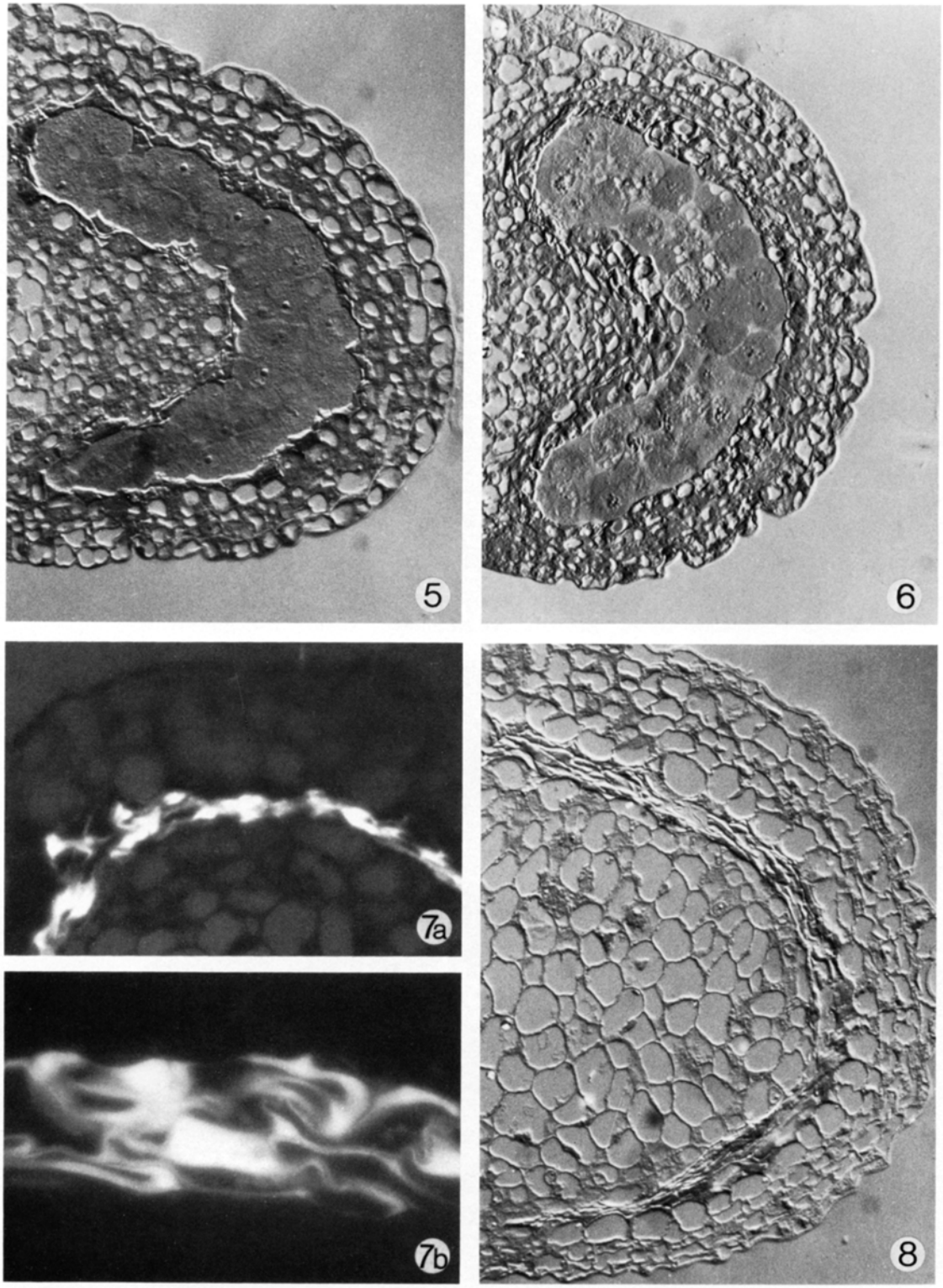
Sterile microsporogenesis

The early developmental stages of the BBS anthers are comparable with those of the fertile type. Cytologically, both lines appear similar during the premeiotic period. Meiosis starts in flower buds of about the same length. At leptotene stage the first signs of aberrant development in the tapetal cells are manifest (Fig. 3). The initial deviation can be observed in the outer tapetum in one locule and in the inner tapetum of another locule in the same anther. During leptotene most cells become reduced in size, have a dense cytoplasm and contain some large vacuoles in comparison with tapetal cells of BBF at the same developmental phase. In general the cells stay uninucleate, a binucleate tapetum cell may be found infrequently. The effect on the structure is variable, although most of the tapetal cells decrease in volume, several may enlarge at first (Fig. 4), to reduce in size at a later stage in the development. At pachytene the cytoplasm of a majority of the tapetal cells is dense with a high affinity for osmiumtetroxide (Fig. 5). Ultimately at anaphase I, abortion of the tapetal cells is distinct; the cells are highly deformed, the nucleus is disrupted and the cytoplasm appears disorganized. The abortion results in a thin tapetal layer, crushed between the sporogenous cells and the parenchymous cells of the parietal layer and the connectivum.

Although tapetal breakdown already begins at leptotene, the first deviations in sporogenous development are generally observed between the leptotene and zygotene stage. Occasionally complete metaphase I or anaphase I configurations are reached (Fig. 6). Like the tapetum layer, the process of degeneration in the sporogenous tissue is rather variable. Irregularly shaped cells are found along with meiocytes of apparently normal dimensions in the same locule. The cytoplasm of most cells becomes dense, showing a high affinity for osmiumtetroxide. The structure of the nucleus disintegrates. The process of degeneration does not proceed completely simultaneously among locules of an anther. Among anthers of the same flower the



Figs. 1–4. 1 Premeiotic stage, BBF. **2** First pollen mitosis, BBF. **3** Leptotene stage, BBS, *arrow* indicates the first signs of aberrant development of the outer tapetal cells. **4** Prophase I, BBS. All figures, ×300



Figs. 5–8. **5** Pachytene stage, BBS. **6** Metaphase I, BBS. **7a** Distorted callose cell walls, aniline blue staining, BBS. **7b** ×400. **8** Meiocyte degeneration completed, BBS. All figures except 7b, ×300

differences are somewhat larger. During pachytene, independent of the stage of breakdown, callose is deposited around the meiocytes. Since most cells are deformed at this phase, the encasing may result in a distorted callose wall (Fig. 7). When meiocyte degeneration is complete, the remnants of callose remain visible until late in floral development (Fig. 8). Changes in the shape of the sporogenous and tapetal cells are followed by an enlargement of the adjacent parietal cells. Though the volume of a sterile anther is smaller than the volume of a fertile one, most of the space originally occupied by sprogenous cells and tapetum is taken up by these parenchymous cells. Neither in the connective, nor the vascular and the epidermal tissues differences are found between the fertile and the sterile type at any stage during development.

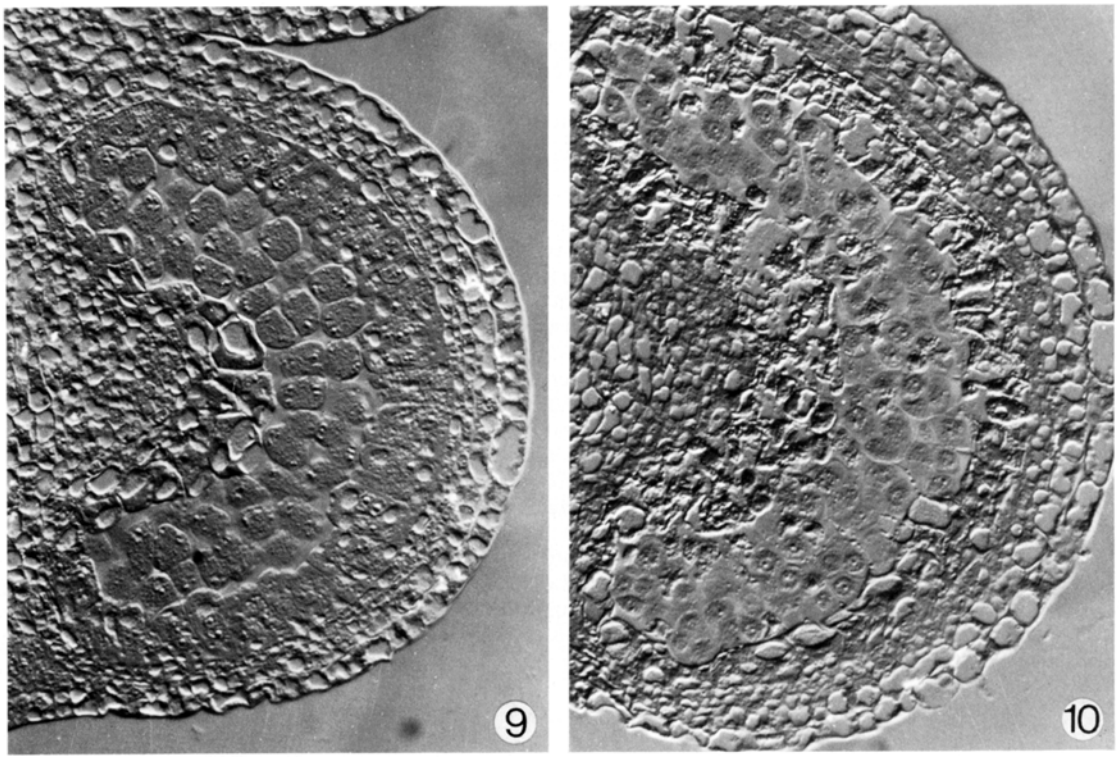
Restored fertile microsporogenesis

Fertility of the F_1 cross between BBS and a restorer line is influenced by temperature (Van Marrewijk 1968). At 17 °C the cross results in a fully fertile F_1, while at 25 °C no microspores are produced. The restorer line itself is fertile at both temperatures, though fertility decreases at 25 °C. Microsporogenesis of the F_1 at 25 °C is comparable with the BBF development until the end of meiosis II (Fig. 9). During the tetrad stage the tapetal cells deform, become vacuolated, and develop a dense cytoplasm (Fig. 10). Development of the sporogenous cells ceases at the late tetrad stage and the microspores collapse inside the callose envelope. Remnants of the callose walls can be found inside the thecae until dehiscence.

Discussion

Microsporogenesis in BBS anthers proceeds normally and is indistinguishable from that in BBF anthers until the first stages of meiosis. The development of the F_1 cross at 25 °C is normal until the tetrad stage. The first phenomena of deviating sporogenesis are found in the tapetum layer in both types. The tapetum is believed to play an important role in microspore development. It appears to serve as a nutritive tissue for microspore formation (Vasil 1967). Aberrant tapetal behaviour has been reported for cms lines in many species (Laser and Lersten 1972).

Malfunctioning of this tissue is often regarded as the direct or indirect cause of cms. Whether cause or effect, early changes in tapetal structure preceeds alterations in sporogenous development in both types of sterility described here. The consequences of these differences



Figs. 9 and 10. **9** End of meiosis II, F_1 cross BBS × restorer line (× 300). **10** Tetrad stage, F_1 cross BBS × restorer line (× 300)

in development on cellular structure, vacuolation and cytoplasmic organization are variable. The same variation in the process of degeneration of the sterile tapetum has been reported for some other plant families, including maize (Lee et al. 1979) and *Sorghum* (Overman and Warmke 1972). In the present study the pattern of breakdown of the sporogenous cells has the same variance as observed in the tapetal cells. Since the different features can be found in the same locule, this variation is likely to be independent of sterility itself.

Timing of breakdown of sporogenesis is not completely simultaneous among the anthers of one flower. Differences are sustained by the asynchronism of the BBF sporogenesis. These differences appear to be correlated with the relative short duration of meiosis in *Petunia;* i.e. about 12 h (Izhar and Frankel 1973).

Izhar and Frankel (1971) found that a premature burst in callase activity results in the too early dissolution of the callose in BBS anthers. Using their methods they could not detect the callose wall after prophase I. The authors suggested that this faulty timing of callase activity results in male sterility. Despite the use of similar material, the present study does not confirm these observations. Independent of the stage of breakdown, callose is deposited around the meiocytes during pachytene. Since the sporogenous cells predominantly deform before the callose wall formation, their encasing results in a distorted callose envelope. Degeneration of the tapetum will influence the enzymatic activity of these cells. Remnants of callose found at late stages of anther development indicate the absence of a normal callase activity. The failure of wall dissolution in the BBS and the F_1 cross appears to be the result of metabolic abnormalities in the tapetum. Malfunctioning is likely to be determined by the premature degeneration of the tapetal cells and is regarded as an indirect effect of sterility. Preliminary results could not confirm an increase in callase activity at any stage of BBS development (Van Marrewijk, personal communication).

Substantial evidence suggests that the cms trait is encoded by the mitochondrial genome. In maize, tobacco, field beans and *Sorghum* mitochondrial modifications associated with cms have been observed (Leaver and Gray 1982).

Investigations of translational products in maize from isolated mitochondria revealed several unique polypeptides. In *Petunia* it was also demonstrated that changes in mitochondrial DNA and its translational products are associated with cms (Kool et al., in preparation). The distinct polypeptide composition, determined by the cms mitochondria influences the development of the microspores directly or indirectly. The effect of the different protein patterns may ultimately result in a stage specific degeneration of tapetal cells. It is interesting to know what polypeptides are connected with cms, how they effect microsporogenesis and in what way they are effected by restorer genes and influenced by environmental conditions. Comparing the molecular insights with histological aspects may provide answers leading to a more complete understanding of these questions.

References

Bennett MD (1976) The cell in sporogenesis and spore development. In: Yeoman MM (ed) Cell division in higher plants. Academic Press, London New York, pp 161–198

Boeshore ML, Lifshitz I, Hanson RM, Izhar S (1983) Novel composition of mitochondrial genomes in *Petunia* somatic hybrids derived from cytoplasmic male sterile and fertile plants. Mol Gen Genet 190:459–476

Gupta SC, Nanda K (1978) Onotgeny and histochemistry of dimorphic tapetum in *Tecoma stans* anthers. Soc Bot Fr Actualités Botaniques 125:129–134

Izhar S, Frankel R (1971) Mechanism of male sterility in *Petunia:* the relationship between pH, callase activity in the anthers, and the breakdown of the microsporogenesis. Theor Appl Genet 41:104–108

Izhar S, Frankel R (1973) Duration of meiosis in *Petunia* anthers in vivo and in floral bud culture. Acta Bot Neerl 22:14–22

Izhar S, Frankel R (1976) Cytoplasmic male sterility in *Petunia.* 1. Comparative study of different plasmatype sources. J Hered 67:43–46

Izhar S, Schlicter M, Swartzberg D (1983) Sorting out of cytoplasmic elements through several meiotic cycles. Mol Gen Genet 190:468–474

Kool AJ, de Haas JM, Mol JNM, van Marrewijk GAM (in preparation) Isolation and physicochemical characterization of mitochondrial DNA from cultured cells of *Petunia hybrida*

Laser KD, Lersten NR (1972) Anatomy and cytology of microsporogenesis in cytoplasmic male sterile angiosperms. Bot Rev 38:425–454

Leaver CJ, Gray MW (1982) Mitochondrial genome organization and expression in higher plants. Annu Rev Plant Physiol 33:373–402

Lee SLJ, Gracen VE, Earle ED (1979) The cytology of pollen abortion in C-cytoplasmic male sterile corn anthers. Am J Bot 66:656–667

Marrewijk GAM van (1968) Cytoplasmic male sterility and restoring fertility in the garden petunia. Doctorate Thesis, Agricultural University, Wageningen, The Netherlands (in Dutch, with English summary)

Mepham RH, Lane GR (1969) Formation and development of the tapetal periplasmodium in *Tradescantia bracteata.* Protoplasma 68:175–192

Overman MA, Warmke HE (1972) Cytoplasmic male sterility in *Sorghum.* 2. Tapetal behavior in fertile and sterile anthers. J Hered 63:226–234

Vasil IK (1967) Physiology and cytology of anther development. Biol Rev Cambridge Philos Soc 42:327–373