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CYTOPLASMIC MALE STERILITY IN PETUNIA HYBRIDA

A structural and histochemical analysis

Proefschrift

ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
in het openbaar te verdedigen
op vrijdag 9 mei 1986
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen

STELLINGEN

1. De conclusie van Van Damme, dat in natuurlijke populaties van Plantago lanceolata, mannelijke steriele planten zes keer zo lang zouden leven als mannelijke fertiele is ongegrond.
   Van Damme JMM (1983) Proefschrift Rijksuniversiteit Groningen

2. Het verloop van de callaseaktiviteit in de helmknop vormt geen juiste basis voor een indeling van cytoplasmatische mannelijke steriliteit bij de petunia.
   Izhar S, Frankel R (1971) Theor Appl Genet 41: 104-108

3. Het verschil in fluorescentiepatroon tussen bloemen van mannelijke fertiele en mannelijke steriele planten kan leiden tot een voorkeur van de bestuiver voor de stuifmeelbevattende bloemen, waardoor de kans op bevruchting van mannelijke steriele planten wordt gereduceerd.
   Nakanishi T (1982) Scientia Horticulturae 18: 57-63
   Kevan PG (1978) In: The pollination of flowers by insects pp 51-78

4. De bestuiving van Orchis galilaea geeft inzicht in de wijze waarop de bloem-insekt relatie in orchideeën is geëvolueerd van het algemene Orchis-type tot de sexuele verlokking zoals die in het genus Ophrys wordt gevonden.
   Bino RJ, Dafni A, Meeuse ADJ (1982) New Phytol 90: 315-319

5. Bij onvoldoende aandacht voor het initieel vochtgehalte van pollen kan de fluorochromatische vitaliteitstoets aanleiding geven tot een onderschatting van het percentage kiemkrachtig stuifmeel.
   Heslop-Harrison J, Heslop-Harrison Y, Shivanna KR (1984) Theor Appl Genet 67: 367-375
   Hoekstra FA (in press) In: Membranes, metabolism and dry organisms

6. Om te kunnen bepalen of DNA dat is toegevoegd aan het kiemmedium van pollen ook werkelijk via de pollenbuis overgebracht kan worden naar de eicel, dient dit DNA voorzien te zijn van genen met selectie-eigenschappen.
   De Wet JMJ, Bergquist RR, Harlan JR, Brink DE, Cohen CE, Newell CA, De Wet AE (1985) In: Experimental manipulation of ovule tissues pp 197-209
   Hess D, Dressler K, Konle S (1985) In: Experimental manipulation of ovule tissues pp 224-239
   Sanford JC, Skubik KA, Reisch BI (1985) Theor Appl Genet 69: 571-574

7. Het schrijven van een wetenschappelijke publikatie wordt aanmerkelijk vergemakkelijkt door gebruik te maken van een personal computer met een goed tekstverwerkingsprogramma.

8. Bij het sluiten van de Hortus Botanicus van de Universiteit van Amsterdam verliest de leus 'Amsterdam heeft het' veel van zijn waarde.

9. Acceptatie van assimilatie betekent nog geen integratie.

10. Dat bloemisten in toenemende mate kruiden als dille en thijm in een boeket snijbloemen vermengen, geeft aan dat veredelaars van bloemgewassen meer aandacht aan de geur van hun produkten moeten geven.

Wageningen, 9 mei 1986. Raoul J. Bino

"The use of each trifling detail of structure is far from a barren search to those who believe in natural selection".

Charles Darwin (1904). The various contrivances by which orchids are fertilised by insects.

Alan Madelon

Voorwoord

Gaarne wil ik iedereen bedanken die, direct of indirect, betrokken is geweest bij de totstandkoming van dit proefschrift. Enkelen zou ik hierbij met name willen noemen.

Prof. dr. J.L. van Went, mijn promotor, Jac, onze gesprekken voerden van het kleinste detail van een EM-foto tot de richting die de Landbouwhogeschool in de verre toekomst hoopt in te gaan. Ongeacht het onderwerp, ik heb altijd met veel plezier met je gediscussieerd en ik dank je voor de kundige begeleiding. Ook mijn co-promotor, dr. ir. G.A.M. van Marrewijk dank ik voor zijn enthousiaste ondersteuning van mijn onderzoek. Bert, hoewel je handschrift niet altijd even duidelijk te lezen was, de kritische kanttekeningen waren niet mis te verstaan. Prof. dr. M.T.M. Willemse ben ik zeer erkentelijk voor zijn belangstelling in het onderwerp en de vele waardevolle opmerkingen tijdens de 'pinus'-werkgroepsbesprekingen. Joke Cobben dank ik voor haar vingervlugge typewerk; Sybout Massalt voor zijn gekleurde kijk op al het zwart-witte fotowerk; Allex Haasdijk en Paul van Snippenburg voor hun mooie figuren; Jan Verburg, Gerrit van Geerenstein en Aart van Ommeren voor de verzorging van de vele petuniaplanten. Peter Vos bedank ik voor de kweek van het uitgangsmateriaal en de verzorging van de planten in het fytotron. Aan het hierbeschreven onderzoek hebben ook studenten meegewerkt: Clementine van Gerven, Anja van der Neut, Simon-Jan de Hoop en Jaap Duijs; zij allen zullen gedeeltes van dit proefschrift als hun werk herkennen. Koos Keijzer dank ik voor de introductie in de vakgroep en zijn geduld om al mijn gesteun, geslurp en geschuivel aan te horen. De, ook voor mijn onderzoek, zo belangrijke biochemische bepalingen werden veelal uitgevoerd door Luc Suurs en Jan Molenveld. De samenwerking en de discussies met Mei Lie Tan, Frits van der Mark, Ad Kool, Linus van der Plas en Folkert Hoekstra heb ik als zeer stimulerend ervaren.

Tenslotte wil ik mijn ouders, Madelon en Ilana bedanken voor hun liefde die mij de rust heeft gegeven om aan dit proefschrift te werken.

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Chapter 1
General Introduction

1.1. Aim and approach

At the beginning of this century, Correns (1904) found in Satureja hortensis a type of male sterility that was transmitted through the egg. Von Wettstein (1924) explained Correns observations by introducing the concept of cytoplasmic inheritance. In the years following, many new cases of this type of sterility were found in various plant species.

Plant breeders frequently use cytoplasmic male sterile plants in their breeding programs. Application of the cytoplasmic male sterility (cms) trait makes the production of hybrid seed possible without the need of emasculation of the maternal line (seed line). Nowadays, cms is the most widely applied tool for the production of hybrid seed. However, in spite of its economic importance, little is known on the initial step and the primary reactions leading to pollen abortion in cms plants. Moreover, there is no comprehensive insight in the molecular base of cms and the relation between the molecular background and the phenotypic effects.

Considerable information on the structural effects of cms is already available for many plant species. In 1972, Laser and Lersten reviewed the observations on the anatomy and cytology of microsporogenesis in cms angiosperms of 140 species of 47 genera from 20 families. The authors, however, were impressed by the 'profusion and imprecision of much of the descriptions of many authors'. Only a few studies give clear descriptions of cytological abnormalities in the development of the sporogenous tissue and paid attention to other anther tissues. In a number of species, more detailed observations were made by using the electron microscope: i.e. Triticum aestivum (De Vries and Ie, 1970), Sorghum bicolor (Overman and Warmke, 1972), Capsicum annuum (Horner and Rogers, 1974), Zea mays (Warmke and Lee, 1977; Lee et al., 1979; Lee et al., 1980; Colhoun and Steer, 1981), Beta vulgaris (Nakashima, 1975), Helianthus annuus (Horner, 1977), and Impatiens walleriana (Van Went, 1981). Most of these studies revealed

that the initial abnormalities in anther development of cms plants are not manifested in the sporogenous cells itself but in the tapetal tissue. The aberrations may vary in timing and expression among different species. Tapetal cells may degenerate too soon, or may become excessively enlarged and invade the locule (cf. Horner and Rogers, 1974). Nevertheless, in the gametophytic type of cms, exemplified in cms-S maize (Lee et al., 1980) and particular cms idiotypes of rice (Edwardson, 1970), aberrant development is only manifested in the microspores, i.e. other anther tissues are unaffected. Among and between cms forms of different species, the stage at which the initial symptoms of abnormal development are observed may vary. First alterations may become apparent from any period between the early meiotic phases until the end of spore maturation. However, for a certain cms plasmatype in a particular genotypic background, the moment of first aberration is rather stable. Obviously, there are different systems conditioning the expression cms in higher plants.

The diversity in expression of the cms trait is impressive. Much of our knowledge on cytosterility is highly fragmentary. Probably, only integrated research will on the long term lead to a comprehensive insight in the relations between the regulation and expression of sterility controlling genes and their effects on anther development. The present thesis represents one part of such an integrated approach. It contains information on the structural and histochemical aspects of cms in petunia and maize. The study is performed in a research group of which the participants cover the field of plant breeding, cytology, molecular biology, physiology, and cell- and tissue culturing. To enable the comparison and integration of the different results, all investigations are done on similar and well defined plant material, grown under the same and controlled conditions. Petunia hybrida was chosen as a model crop for practical, theoretical, economical and scientific reasons. In petunia, a stable system of cms is present and maintainer and restorer lines are available. The vegetative multiplication is convenient and it is possible to raise the plants under standard conditions in a climate room (cf. Van Marrewijk, 1969). Petunia is a member of the economically important family of the Solanaceae and the obtained information may be applicable to other members of the family. Recent research achievements in petunia

plant-cell culture, protoplast manipulation, and molecular genetics provide means to complete the conventional cms plant breeding methods with novel techniques.

Cms appears to be a tissue specific and development associated phenomenon. The value of a microscopical analysis lies in the ability of the observer to examine individual cells in individual anthers. Although microscopy alone may not elucidate the mechanism of cms, it is an essential element in the comprehensive research into the fundamental base of cytoplasmic male sterility.

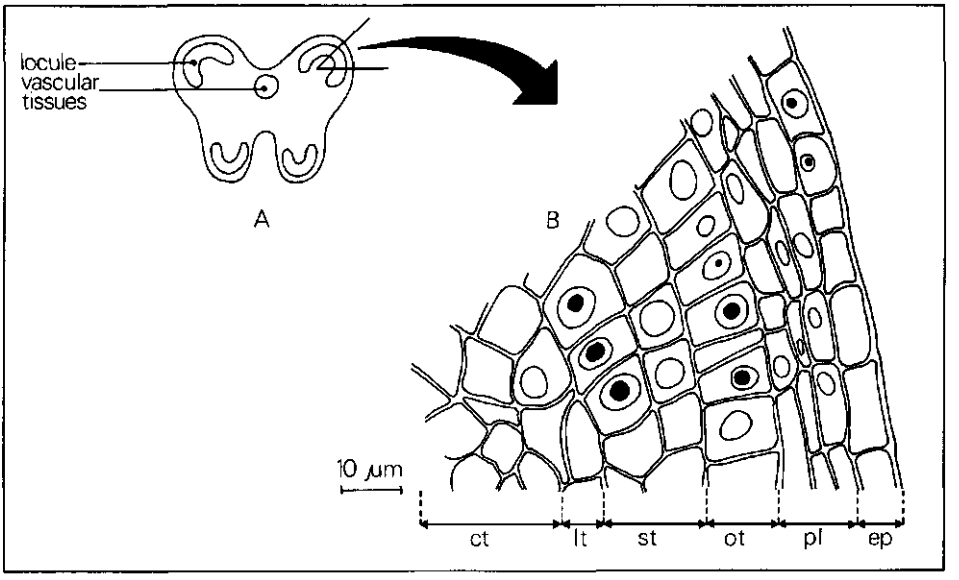


Fig. 1. Anther of *Petunia hybrida* in transverse section at premeiotic stage. **(A)** outline showing connective and four pollen sacs.
(B) pollen sac in greater detail, showing the principal tissues (connective tissue, ct; inner tapetum, it; sporogenous tissue, st; outer tapetum, ot; parietal layers, pl; epidermis, e. Redrawn after Van der Neut, 1984).

1.2. Microsporogenesis and pollen development

The formation of functional pollen grains is the result of a series of finely co-ordinated processes. The outcome of pollen formation depends on the synchronous development of various tissues and on the exact timing of the synthesis and breakdown of diverse products. Several of the physiological, biochemical, cytological, and morphological aspects of these interactions are recently reviewed (cf. Heslop-Harrison, 1972; Mascarenhas, 1975; Bennett, 1976; Shivanna et al., 1979; Knox, 1984; and Bhandari, 1984).

Pollen grain formation results from two subsequent processes, sporogenesis and pollen development. Sporogenesis is the period between the formation of the archespore and the production of spores at the end of meiosis. Pollen development begins after sporogenesis and includes the whole pollen grain development until functional male gametophytes are produced. At the early stages of sporogenesis, the principal tissues of an anther constitute: the epidermis, the parietal layers, the outer and inner tapetal layers, the parenchymous cells of the connective tissue, and the sporogenous cells (Fig.1).

During microsporogenesis and pollen development the various anther tissues interact intensively. Especially, the tapetal tissue is considered to be of considerable physiological significance (Maheshwari, 1950). Pacini et al. (1985) distinguished as many as ten important functions for this tissue, mostly associated with: nourishment of the sporogenous tissue, production and release of callase (the enzyme which depolymerizes the callose envelope of the tetrads), and formation of pollen wall components. All these functions are essential for normal pollen grain development. Malfunctioning of the tapetal tissue is often regarded as a cause for male sterility. However, also other anther tissues may influence the outcome of pollen formation. For instance, several important functions are fulfilled by the cells of the parietal layers. The layers are storage centers for starch and other reserves which are mobilized during the later development of the pollen (Heslop-Harrison, 1972). During pollen maturation, one or more parietal cell layers differentiate to form the endothecium. At dehiscence, the endothecium cells produce the outward bending of the locule wall and the exposure of the pollen grains (Keijzer, 1985). In fact, the formation of functional pollen depends on the

synchronous development of several sporophytic tissues. Success of pollen function, however, is not guaranteed with the formation of the pollen grain. The pollen has to be transferred to a compatible and receptive pistil. On the stigmatic surface, the grain has to form a pollen tube which enters the style and finds its way to the embryo sac. Only by the specific interaction between the male gametophyte and the female sporophyte and gametophyte, a successful fusion of the gametes may be accomplished (cf. Van Went and Willemse, 1984).

Conclusively, the formation of functional pollen grains involves a closely ordered sequence of changes in which fundamental genetical events are associated with elaborate processes of cell differentiation.

1.3. Male sterility

Normal pollen grain development may disarrange in many ways, ultimately resulting in male sterility. The disarrangement of normal pollen development may arise from causes ranging from the total suppression of anther growth to the failure of male gamete nuclei to fuse with the female gamete nuclei at the very conclusion of fertilization. Male sterility may be determined by:

1) environmental conditions,
2) application of chemicals,
3) nuclear genes,
4) cytoplasmic genes, or
5) a combination of the different agents.

Various environmental stress conditions disturb the normal process of pollen grain formation. For instance, in wheat, high temperature (30 ^{o}C for 3 days) and water deficit may result in male sterility (Saini et al., 1984). In rice cv., a period of low temperature (12 ^{o}C for 4 days) during meiotic stages of the sporogenous cells induces partial male sterility (Nishiyama, 1984). In maize, pollen fertility is modified by the light regime, i.e. short days experienced during the initiation of the apical inflorescence reduce the number of florets and depress the fertility of those that are produced (Moss and Heslop-Harrison, 1968). Also in the grass Dichanthium aristatum, an increase in number of short days may

result in partial male sterility (Knox, 1962). Additionally, in a number of species, various mineral deficiencies inhibit microspore development (Lohnis, 1940; Agarwala et al., 1979; and Dell, 1981).

The cytological effects of environmentally induced male sterility are only described in a few studies. In maize, sterility associates with characteristic changes and precocious degeneration of the tapetal tissue (Moss and Heslop-Harrison, 1968). In wheat, abnormal microsporogenesis initially involves either premature tapetal degeneration or loss of contact between the tapetum and the microspores (Saini et al., 1984). In rice, low temperature induces hypertrophy of tapetal cells, usually accompanied by the increase in the number of cytoplasmic organelles (Nishiyama, 1984). Also in Dichanthium aristatum, the first aspects of aberrant development are observed in the tapetal tissue (Knox, 1962).

Many different chemical agents are known to induce male sterility in several crop plants. A list of the most common gametocides and crops with a positive response is given by Van Marrewijk (1979). Most gametocides, however, do not only influence pollen development but also cause necrosis and yellowing of leaves and may adversely affect inflorescence emergence, floral morphology and female fertility. Hence, the wide scale use of gametocides has seriously been impeded. Only in wheat, a chemical agent is known to be used commercially (Jensen, 1985). The compound causes the inhibition of sporopollenin deposition, ultimately resulting in male sterility.

Male sterility determined by nuclear genes is widespread in flowering plants. Gottschalk and Kaul (1974) could distinguish four different groups of genes controlling the fertility of higher plants. Two of the gene groups are directly correlated with the meiotic behaviour, causing the failure of homologous chromosomes to pair or the prevention of chiasma formation. Both groups act principally in a similar way on micro- and macrosporogenesis. The third group of genes is not related to the meiotic system but induces a misdifferentiation of the sex organs due to abnormalities in the development of the growing points destined for flower formation. The fourth group, the male sterility genes, becomes only effective during microsporogenesis and does not influence

macrosporogenesis. Most of the genes belonging to this group cause the breakdown of normal pollen grain development when present in the recessive state. Gottschalk and Kaul (1974) carefully analyzed 99 male sterility genes of 48 species belonging to 12 families. The majority of the male sterility genes influence the final stages of sporogenesis between interphase II and pollen formation. However, some genes also affect the early and middle stages of the first meiotic prophase. From comparative developmental and biochemical studies, Gottschalk and Kaul (1974) concluded, that the non-formation of functional pollen grains is generally preceded by abnormalities in tapetal development. The authors assumed that 'the primary action of the male sterility genes is not directed to the pollen mother cells but to the tapetum'.

Cytoplasmically inherited male sterility may frequently arise in the progeny of interspecific and intergeneric crosses (Edwardson, 1970). Additionally, cms has been found in natural populations of different species (e.g. in Plantago lanceolata, Van Damme and Van Delden, 1982), and after mutagenic treatment (Erichsen and Ross, 1963). A cms plant normally transmits its male sterility expression to the whole progeny. This correlates with the assumption that, in angiosperms, the cytoplasm of the offspring is largely inherited trough the maternal line. The male gamete generally contributes only a small amount of cytoplasm to the zygote. Nevertheless, in many cases of cms, the paternal genotype may totally or partially 'restore' the male fertility expression of the progeny of a cms maternal seed line. Apparently, the nuclear background influences the phenotypic expression of the cytoplasmic genes. Monogenic and polygenic restorer systems are known, and recessive and dominant sterility modifiers have been identified (cf. Hanson and Conde, 1985). Hence, the sterility expression is dependent upon the presence of sterilizing cytoplasm and on the absence of restorer genes in the nuclear background of the plant. Additionally, pollen grain formation in anthers of plants with restorer genes in sterilizing cytoplasm is very susceptible to environmental conditions. Especially, temperature conditions highly affect male fertility (Van Marrewijk, 1969). Conclusively, cms is a manifestation of an aberration in the nuclear-cytoplasmic-physiological interactions. Some of the molecular aspects of these interactions will be discussed in the

next chapter.

1.4. The molecular base of cytoplasmic male sterility

The cytoplasm of higher plants has long been recognized as a source of extranuclear genetic information. Notwithstanding that, the existence of unique DNA in both mitochondria and plastids was not demonstrated until the early 1960s. After the assessment that cytoplasmic genes play an important role in plant development, productivity, and susceptibility to diseases, there has been an increased interest in the information content and function of plant mitochondrial DNA and plastid DNA. Several lines of evidence indicate that the genetic determinants responsible for cms are carried by the mitochondrial genome:

1) Fragments of mitochondrial DNA from fertile-type and sterile-type cytoplasms, cleaved with restriction endonucleases and fractioned by agarose gel electrophoresis, are readily distinguishable irrespective of the endonuclease employed. In contrast to the marked heterogenity in mitochondrial DNA, the restriction patterns of chloroplast DNA from comparable cytoplasms are in most cases almost identical. Restriction endonuclease fragment analysis has been carried out for cms forms of many different species: e.g. *Zea mays* (Levings and Pring, 1976), *Nicotiana tabacum* (Belliard *et al.*, 1979), *Petunia hybrida* (Kool *et al.*, 1982), *Sorghum bicolor* (Dixon and Leaver, 1982), *Beta vulgaris* (Powling, 1982), *Vicia faba* (Boutry and Briquet, 1982), *Brassica* spec. (Pelletier *et al.*, 1983), and *Daucus carota* (De Bonte *et al.*, 1984). However, the analyses of the mitochondrial genomes of cms and fertile lines generally reveal so many points of divergence, that in most cases the genomic features relevant to cms are masked (Hanson and Conde, 1985). Hence, the effects of the differences in mitochondrial DNA restriction patterns on phenotypic expression are not established in all species.

Mitochondrial component	Mitochondrial gene product in		
	Maize	Yeast	Man
Cytochrome c oxidase			
Subunit I	+	+	+
Subunit II	+	+	+
Subunit III	+	+	+
Ubiquinol cytochrome c reductase			
Apocytochrome b	+	+	+
ATPase complex			
Subunit 6	+	+	+
Subunit 9	+?	+	-
Subunit 8	?	+	+
F1 subunit α	+	-	-
Large ribosomal subunit			
rRNA	+	+	+
Small ribosomal subunit			
rRNA	+	+	+
Ribosome-assoc. protein	?	+	-?
RNA processing enzymes			
Intron 2 COB maturase	?	+	-
Intron 4 COB maturase	?	+	-
tRNAs	?	25	22
URFs	?	≥8	8

Table 1. An inventory of mitochondrial genes (from Borst et al., 1984).

2) A more elaborate evidence for the mitochondrial involvement in cms is the comparison of mitochondrial translation products from fertile and sterile type cytoplasms.

Mitochondrial proteins are assembled by a complex interaction between two protein synthesizing systems. On the one hand, the nucleo-cytoplasmic system coding for the major components of the mitochondrial matrix and the outer and inner mitochondrial membranes. On the other hand, the mitochondrial system coding for most mitochondrial RNAs and some mitochondrial proteins (Tedeschi, 1976). Quantitatively the role of the mitochondrial system is minor: it has been estimated that only 5 to 15% of the total mitochondrial proteins are coded for by the mitochondrial genome (Schatz et al., 1972). Nevertheless, an active mitochondrial translation system is essential for a cell. In maize, the system codes for three of the seven subunits of cytochrome c oxidase, the apoprotein of the seven subunits of the cytochrome bc complex, four of the nine subunits of the oligomycin sensitive ATPase complex, and subunits of the mitochondrial ribosomal RNAs (Table 1). Hence, the major mitochondrial complexes compose of a combination of mitochondrially and cytoplasmically synthesized polypeptides. Most of the complexes are an integral part of the inner mitochondrial membrane and are responsible for key steps in the process of oxidative phosphorylation and the generation of ATP (Leaver and Forde, 1980).

The most successful approach to establish the identity of the mitochondrial translation system in plant cells has been the analysis of proteins synthesized by isolated mitochondria (Leaver and Gray, 1982). Sufficient quantities of mitochondria, uncontaminated with plastids, can be isolated from different plant tissues as leaves, tubers, etiolated shoots, and suspension cultured cells (Boutry et al., 1984). Active protein synthesis of the isolated mitochondria is dependent upon the isolation of intact organelles and a suitable incubation medium (Leaver and Gray, 1982). The mitochondrial translation products are radioactively labelled, solubilized and separated by SDS-polyacrylamide gel electrophoresis, and the labelled polypeptides are detected by autoradiography (Forde et al., 1979). Using these techniques, several authors could distinguish the translational products of mitochondria isolated from fertile- and sterile-type plants. For instance, Dixon and Leaver (1982) revealed a variation in mitochondrial translation products of fertile and cms plasmatypes of Sorghum. Mitochondria, isolated from etiolated shoots of 24 different cms plasma sources, show in three cases a

polypeptide composition different from that of the fertile-type cytoplasm. In one case, the mitochondria of the cms plasmatype lack a 38-kdalton polypeptide, which is characteristic of all other cytoplasm sources examined, and produce a new 42-kdalton component. Both polypeptides immunoprecipitate with an antiserum raised against yeast cytochrome c oxidase subunit I, and the 42-kdalton polypeptide probably represents an altered form of this enzyme. The rate of synthesis of the aberrant subunit I is about one tenth that of the normal form as detected by labelling mitochondrial translation products in vitro (Dixon and Leaver, 1982). However, whether these differences have effect on enzyme activity in vivo is not established yet. Additionally, two other sources of cms Sorghum synthesize aberrant polypeptide compositions (Dixon and Leaver, 1982). A variation in mitochondrial translation products is also found in Zea mays (Forde and Leaver, 1980), Vicia faba (Boutry and Briquet, 1982), Beta vulgaris (Powling and Ellis, 1983), Nicotiana tabacum (Boutry et al., 1984), Triticum aestivum (Boutry et al., 1984), and Petunia hybrida (Nivison and Hanson, 1984). In most of these studies, however, the functional identification of the polypeptides requires further investigation.

3) A third line of evidence indicating the involvement of mitochondrial DNA, has been revealed by the analysis of the mitochondrial genome of somatic hybrid plants. Several laboratories have synthesized cytosterile and male fertile somatic hybrid plants following the fusion of protoplasts from lines with cms and male fertile plasmatype. The mitochondrial DNA and plastid DNA restriction patterns of the somatic hybrid plants are examined to identify any fragments which segregate exclusively with the fertility expression. In fusion combinations of Nicotiana tabacum (Belliard et al., 1978), Brassica spec. (Pelletier et al., 1983), and Petunia hybrida (Hanson et al., 1985) restriction patterns of the mitochondrial genome are found to segregate together with the male sterile phenotype. In petunia, Boeshore et al. (1985) characterized the mitochondrial DNA arrangement of 17 stable sterile somatic hybrids and 24 fertile somatic hybrids. The authors subjected the cms-associated DNA region and a homologous mitochondrial DNA region from the fertile counterpart to sequence and transcription analysis. Besides an unidentified part, the cms-associated

region is found to contain imperfect reading frames encoding for ATPase subunit 9 and cytochrome c oxidase subunit II (Hanson, personal communication). However, more details on the expression of the genes will be necessary to conclude whether or not these deviations in mitochondrial DNA arrangements are functionally associated with cms

4) Further evidence suggesting the mitochondrial involvement with cms is more specifically revealed for T-cytoplasm of maize. In 1970 the southern corn leaf blight disease swept the USA and caused enormous losses in corn production (Ullstrup, 1972). The disease is caused by the pathogen Drechslera maydis, which has been found to display a preferential virulence on cms maize lines with T-cytoplasm but has little effect on normal fertile and other male-sterile types (Laughnan and Gabay-Laughnan, 1983). A very low level of the host-specific toxin purified from the pathogen is highly active against mitochondria of the cms-T maize line. The toxin elicits a wide range of effects, including uncoupling of oxidative phosphorylation, stimulation of succinate and NADH respiration, inhibition of malate respiration, increased mitochondrial swelling, loss of mitochondrial matrix density, and unfolding of the mitochondrial inner membrane (Gregory et al., 1980). The pathogen only mildly affects mitochondria isolated from plants with a similar nuclear genotype but with cms-C, cms-S, or normal fertile type cytoplasm (Laughnan and Gabay-Laughnan, 1983). The phenotypic reversion of cms-T maize to male fertility is coupled with disease resistance (Dixon et al., 1982). These observations support the idea that male sterility and T-toxin sensitivity are the result of a similar mutation in the mitochondrial genome (Laughnan and Gabay-Laughnan, 1983).

A different line of evidence implicating mitochondrial involvement with sterility in cms-T maize comes from comparative cytological studies of microsporogenesis in anthers of fertile and sterile plants. Warmke and Lee (1977) found that a loss of internal mitochondrial structure initially indicated abnormal anther development. The early breakdown of mitochondria has been observed only in the tapetum and middle layers, and occurs before tapetal irregularities become manifest with the light microscope. However, in contrast to Warmke and Lee (1977), Colhoun and Steer (1981) reported

that degeneration of mitochondria does not necessarily precede microspore abortion in cms-T maize. The authors described the presence of apparently intact mitochondria in tapetal cells from anthers at various stages of microspore abortion.

Besides differences in the mitochondrial genome, some authors described aberrations in plastid DNA as related with the cms trait. Chen and Meyer (1979), and Jigeng and Yi-nong (1983), found a small difference in the plastid DNA composition of cms and fertile forms of Zea mays, Triticum aestivum, Brassica napus, and Nicotiana tabacum. However, only in Brassica the aberrations in nucleotide sequence generate some alterations in restriction fragment patterns (Jigeng and Yi-nong, 1983). Ahokas (1978) suggested that cms in Hordeum vulgare is the result of a mutation in the plastid genome. The leaves of cms Hordeum exhibit a different proportion of xanthophyll and chlorophyll b as compared with leaves of fertile-type plants (Ahokas, 1978). Other lines of research identified virus or virus-like particles as being associated with cms (recently reviewed by Grill et al., 1983). In the cytoplasm of cms Vicia faba 447, Edwardson et al. (1976) observed cytoplasmic spherical bodies. These bodies are never found in fertile-type or restored fertile-type plants (Grill and Garger, 1981). Subsequent studies revealed that the spherical bodies contain a large double-stranded RNA normally characteristic for a virus-like agent (Grill et al., 1983). A virus-like entity as the agent for cms would be sustained by the possibility of graft transmission of the trait. Some years ago, a number of authors reported the feasibility of the direct transfer of cms in various species. However, Van Marrewijk (1970) critically analyzed several of these experiments and concluded that the reported cases were not conclusive and are to be ascribed to causes other than sterility transmission.

1.5. Cytoplasmic male sterility in Petunia hybrida

Different aspects of cms in Petunia hybrida are described by various authors. According to Izhar and Frankel (1976), a single plasmagene is responsible for cms in petunia. This assumption is supported by the observation that different fertility restorer genes similarilly affect

fertility expression of cms plasma sources. The interaction between the plasmatype, the restoration of fertility and the influence of external conditions is reported by Van Marrewijk (1969), Izhar and Frankel (1976), and Izhar (1975, 1977, and 1978). The fertility expression of restored fertile plants is specifically affected by temperature conditions. In experiments with homozygous and heterozygous clones at different temperatures, the formation of functional pollen grains followed an optimum curve. The position of the optimum and the width of the fertility range depended on the restorer genotype and the nuclear background of the plant (Van Marrewijk, 1969).

Frankel et al. (1969) suggested that faulty timing of enzymatic digestion of the meiotic callose wall is a primary cause of cms in petunia. Premature or retarded callase activity resulted in the disarrangement of microsporogenesis during either meiosis, or tetrad stage, or the young microspore stage. The callase activity is found to be influenced by the presence of fertility restorer genes and by environmental conditions (Izhar and Frankel, 1971). In contrast to Frankel et al. (1969), Van Marrewijk and Suurs (1985) did not observe a faulty timed increase in callase activity. During all stages of microsporogenesis, the authors found a strongly reduced enzyme activity in anthers of cms plants as compared with the activity observed in fertile-type anthers.

In 1971 Frankel proposed a model explaining cms in petunia. According to Frankel, nuclear male sterility was induced in an originally fertile scion grafted on a cms stock (Frankel, 1962). Apparently, in this exceptional case, the male sterility trait transferred from its cytoplasmic origin to some place in the nuclear genome. This unique system allowed the study of genetic factors controlling male sterility in petunia. Frankel (1971) suggested that the non-formation of functional pollen is correlated with the presence of hypothetical sterility elements in the cytoplasm of the cells and the formation of functional pollen with the presence of fertility elements. In cms petunia, the sterility elements are in their 'autonomous' state while in genic male sterile plants the elements are in their 'integrated' state. Subsequent studies revealed that the 'autonomous' state and the 'integrated' state may coexist in cells of

the same plant (Evenor and Izhar, 1984). Fusion of cells with fertile and sterile plasmatype resulted in cells with both fertility and sterility elements in the same cytoplasm (Izhar and Tabib, 1980). The sterility and fertility elements do not show simple dominance-recessive relations. In most cases, the heteroplasmic cybrid is fertile, and only a threshold of sterility elements may result in sterility expression (Izhar et al., 1983). In subsequent generations the heteroplasmic cybrid plant may segregate in homoplasmic male fertile and homoplasmic male sterile plants. Apparently, both the sterility and the fertility elements sort out after several meiotic or mitotic cycles (Izhar et al., 1983). Izhar (1984) assumed that the sterility elements are normally located inside the mitochondria but occasionally may move to other cell organelles. Recent molecular biological studies demonstrated the possibility of a gene transfer between different intracellular compartments in the evolutionary past of some organisms (Yaffe and Schatz, 1984). During phylogenesis, DNA sequences are transferred from chloroplasts into mitochondria, and from mitochondria into the nucleus (Börner, 1984). Additionally, several genetic elements that transpose from one site in the genome to another are well established in Petunia hybrida (cf. Doodeman, 1984 and Gerats, 1985).

Whether gene transfer or some other mechanism plays a role in cytoplasmic male sterility awaits further evidence on the molecular base of the trait. Probably, the progression of our knowledge on the relations between structure, function, and regulation of genes involved in cms and their phenotypic effects will lead to a more comprehensive understanding of cms. Research at the histological and cytological level is in this sense essential and is the subject of the present thesis.

Chapter 2
Histological Aspects of Microsporogenesis in Fertile, Cytoplasmic Male Sterile and Restored Fertile Petunia hybrida

Summary

A comparative histological study is made of microsporogenesis in fertile, cytoplasmic male sterile and restored fertile Petunia hybrida. 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 the callosic cell wall 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 hybrida - 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 may occur at almost any time during development and that probably more than one mechanism is involved. Various aspects of cms in Petunia hybrida have been described earlier (cf. Van Marrewijk, 1968). Recently, Izhar et al. (1983) demonstrated 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. (1985) 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. (1985) demonstrated that mitochondrial modifications in DNA and its translation products are associated with cms. Obviously, petunia is 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 a faulty timing of the enzymatic digestion of the meiocytic callose cell wall is a primary cause of cms in petunia. The authors recognized three main patterns of cms as related to the callase activity in the locule. The 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 variable for the different plasma sources and is influenced by the presence of fertility restorer genes and environmental conditions (Izhar and Frankel, 1976). Estimations of the moment of breakdown are primarily 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 hybrida.

Material and methods

Three types of Petunia hybrida (Hook.) Vilm. were used in this study, i.e., the 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 (2μm) 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

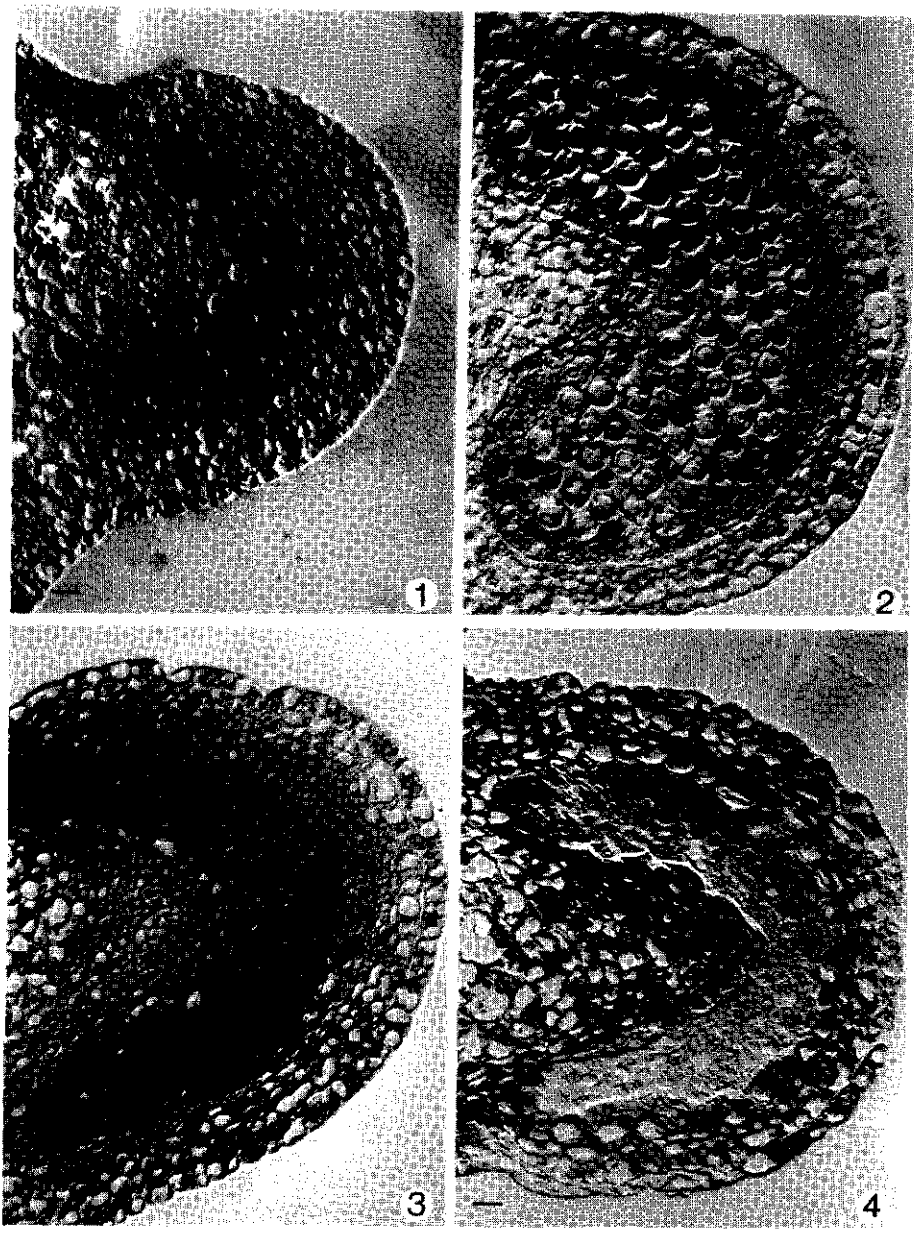
Microsporogenesis in the male fertile type (BBF) generally follows the well-established pattern described for many other species (Bennett, 1976). Attention will be focussed on some species specific aspects of Petunia hybrida.

Each microsporangium consists of four distinct tissues: epidermis, three parietal layers, a uniseral tapetum surrounding two or three layers

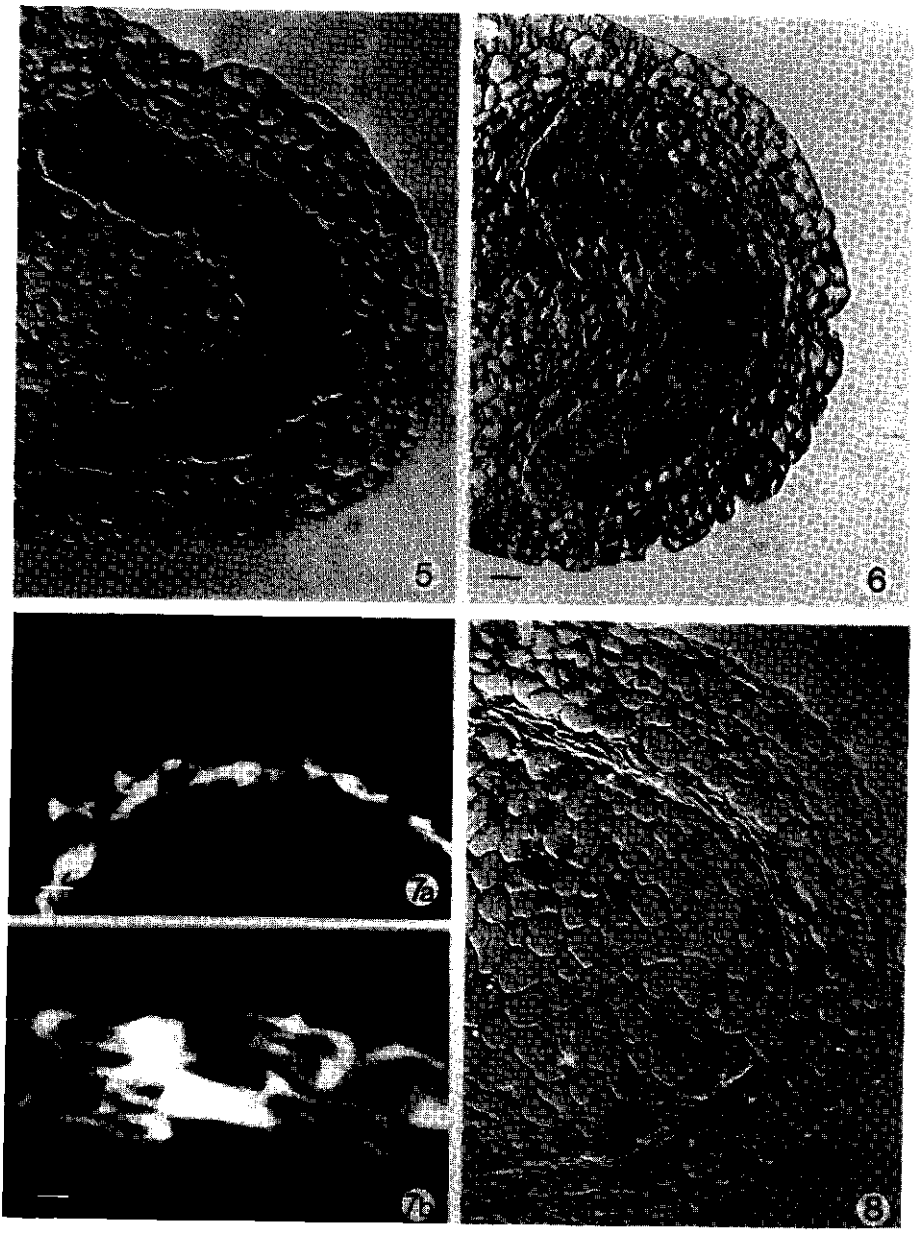
of sporogenous cells. The locules appear crescent shaped in cross section. The tapetum is of the secretory type. Before meiosis, the tapetal cells at the connective side of each locule (the inner tapetum) become cytologically different from the tapetal cells at the external side (the outer tapetum) (Fig. 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,



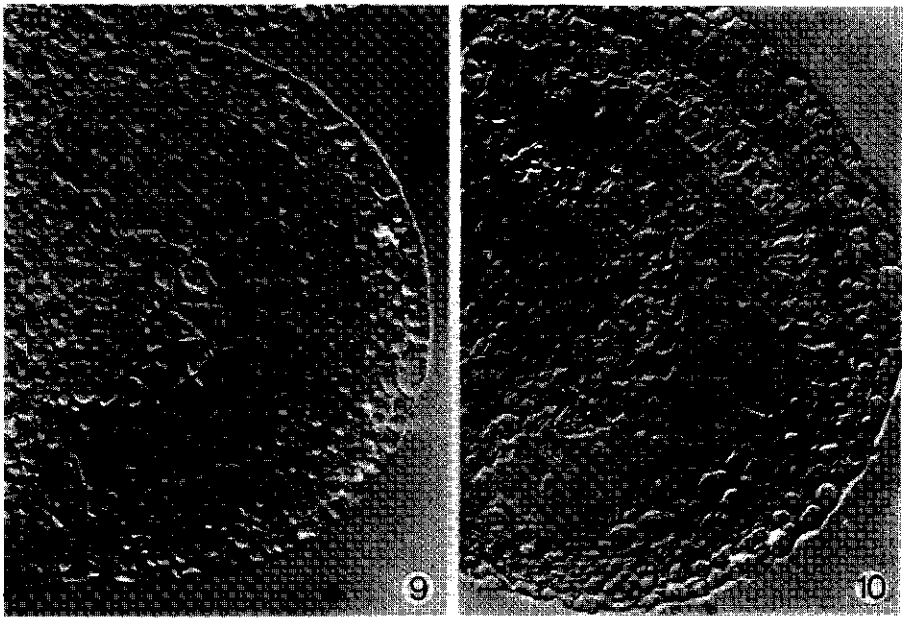
Figs. 1–4. 1. Premeiotic stage, BBF. 2. First pollen mitosis, BBF. 3. Leptotene stage, BBS, arrow indicates the first signs of aberrant development of the outer tapetal cells. 4. Prophase I, BBS. All figures bar, 20 μm.



Figs. 5–8. 5. Pachytene stage, BBS. **6.** Metaphase I, BBS. ***7a*** Distorted callose cell walls, aniline blue staining, BBS. **7b.** Bar, 10 µm. **8.** Meiocyte degeneration completed, BBS. All figures, except 7b, bar, 20 µm.

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 becomes distorted. The process of degeneration does not proceed completely simultaneously among locules of an anther. Among anthers of the same flower the differences are somewhat larger. Independent of the stage of breakdown, callose is deposited around the meiocytes during pachytene. 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 sporogenous cells and tapetum is taken up by these parenchymous cells. Neither in the connective, nor in the vascular and the epidermal tissues are differences found between BBF and BBS anthers at any stage of development.



Figs. 9 and 10. 9. End of meiosis II, F_1 cross BBS x restorer line. **10.** Tetrad stage, F_1 cross BBS x restorer line (Bar, 20 µm).

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 cell 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 tapetal layer in both types. The tapetum is believed to play an important role in pollen grain 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 the tapetal tissue is often regarded as the direct or indirect cause of cms. Whether cause or effect, early changes in tapetal structure precede alterations in sporogenous development in both types of sterility described here. The consequences of these differences 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 Zea mays (Lee et al., 1979) and Sorghum bicolor (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 hybrida; 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. The authors could not detect the callose wall after prophase I. Izhar and Frankel (1971) suggested that the faulty timing of callase activity is the direct cause of male sterility in petunia. Despite the use of similar plant 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. In BBS anthers, tapetal breakdown begins at prophase stage of the meiocytes. Probably, the enzymatic activity of the tapetal cells is influenced by the process of degeneration. Remnants of callose found at late stages of anther development may indicate the absence of a normal callase activity. Conclusively, the failure of callose wall dissolution in the BBS and the F_1-cross appears to be the effect 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. Also a biochemical analysis could not confirm the results of Izhar and Frankel (1971). Van Marrewijk and Suurs (1985) carefully analyzing enzyme activity in BBS anthers, did not observe a faulty timed increase in the callase activity of BBS anthers. During all stages of microsporogenesis, the authors found a strongly reduced callase activity as compared with the level observed in fertile-type anthers.

Substantial evidence suggests that the cms trait is coded for 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 from isolated mitochondria in maize, 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., 1985). Apparently, 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.

Chapter 3
Ultrastructural Aspects of Cytoplasmic Male Sterility in Petunia hybrida

Summary

Anther development of isogenic male fertile and cytoplasmic male sterile types of Petunia hybrida cv. Blue Bedder is studied by electron microscopy. First deviation in sporogenesis of the sterile type, is observed during leptotene stage of the meiocytes. Initial aberration is represented by the presence of large vacuoles in the cytoplasm of the tapetal cells. These vacuoles reveal the first aspects of degeneration; no other ultrastructural differences are observed. Vacuolation is accompanied by the condensation of cytoplasmic organelles. The tapetal cells become distorted and ultrastructural aberrations in mitochondria do occur. The mitochondria elongate and contain several tubular cristae.

Substantial evidence suggests, that cytoplasmic male sterility in petunia is encoded by the mitochondrial genome (Boeshore et al., 1983). However, before degeneration becomes manifest, no consistent ultrastructural differences in mitochondrial organization are observed.

Abortion of the tapetum and the sporogenous tissue in cytoplasmic male sterile plants, generally follows a corresponding pattern. Ultimately, the cells are highly distorted, the nucleus is disrupted and the cytoplasm disorganized. Mitochondria and plastids degenerate and many lipid droplets are present.

Key words: Cytoplasmic male sterility - Mitochondria - Petunia hybrida - Tapetum - Ultrastructure - Vacuoles.

Introduction

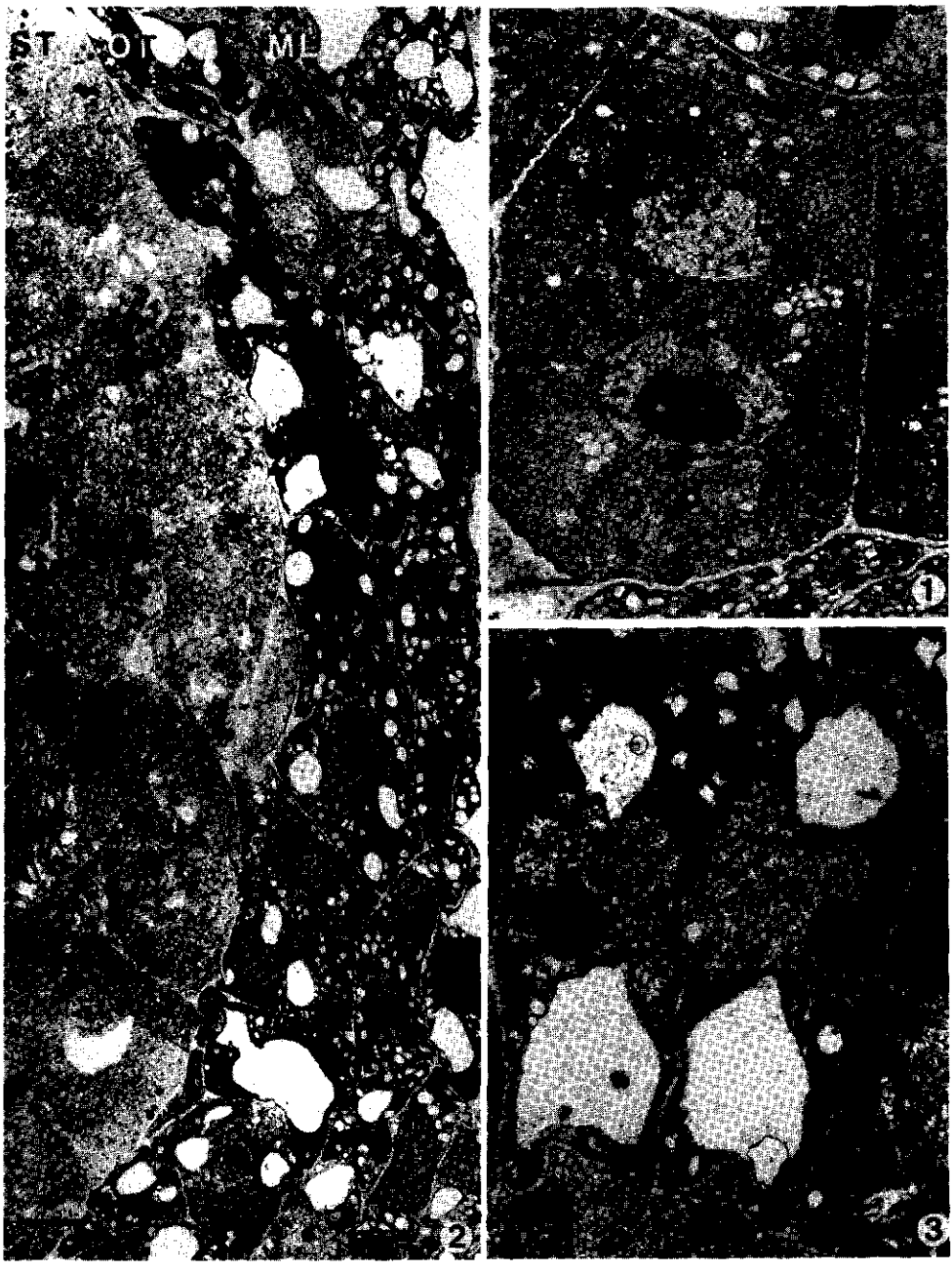
Cytoplasmic male sterility (cms) is used in various crops for hybrid seed production to exclude self-fertilization. Considerable knowledge is available about histological aspects of sterility in cms lines of many

species (Laser and Lersten, 1972). In only a few cases a comparison is made of microsporogenesis in fertile and cms lines at the electron microscopical level. Substantial evidence suggests, that the cms trait in several species is encoded by the mitochondrial genome (Leaver and Gray, 1982). Hence, in most studies special attention is paid to changes in mitochondrial ultrastructure. Warmke and Lee (1977) found that a loss of internal mitochondrial structure initially indicated abnormality in sterile anthers of cms-T maize. The early breakdown of mitochondria has been observed only in the tapetum and middle layers, and occurs before tapetal irregularities become manifest with the light microscope. In contrast to Warmke and Lee (1977), Colhoun and Steer (1981) reported that degeneration of mitochondria does not necessarily precede microspore abortion in cms-T maize. Colhoun and Steer (1981), described the presence of apparently intact mitochondria in tapetal cells from anthers at various stages of microspore abortion. Several authors reported that ultrastructural and histological deviations are coincident. According to Horner (1977), the enlargement of tapetal cells in cms sunflower is concomitant with a disarrangement of cytoplasmic organelles. Overman and Warmke (1972), observed that cytoplasmic disorganization accompanies degeneration of the tapetal layer in the anthers of male sterile Sorghum bicolor.

Fig. 1. BBF outer tapetal cell, after karyokinesis. The cytoplasm contains numerous organelles including several small vacuoles (V). Bar, 2 µm.

Fig. 2. Section of a BBS loculus at early prophase stage. Successive stages in the process of degeneration may be followed in the outer tapetal layer. The cells become highly vacuolated, the cytoplasm heavily stained and the structure largely deformed. ST; sporogenous tissue, OT; outer tapetum, ML; middle layer, E; epidermis. Bar, 5 µm.

Fig. 3. BBS outer tapetal cells at leptotene stage of the meiocytes. Initial deviation from fertile development is represented by the presence of large vacuoles invading the cytoplasm of tapetal cells. Bar, 2 µm.


ST
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1
2
3

In cms wheat, De Vries and Ie (1970), found no cytoplasmic differences which ocurred consistently before the start of degeneration is evident. In cms-C maize lines (Lee et al., 1980), neither the mitochondria, nor the plastids undergo specific changes prior to the general sequence of events that characterizes tapetal or microspore abortion. In cms pepper, an ultrastructural study could not reveal any disruption of organelles, though histological evidence showed various aspects of degeneration (Horner and Rogers, 1974). The authors reported vacuolation and enlarging of tapetal cells, without any disorganization of cytoplasmic organelles. First ultrastructural and histological features of sterile microsporogenesis are generally found in the tapetal layer. The tapetum plays an important role during microsporogenesis (Echlin, 1971). Its malfunctioning has often been interpreted as a primary cause for the initiation of abortion of sporogenous tissue. Also in cms Petunia hybrida cv. Blue Bedder, the first phenomena of deviation are observed in the tapetum (Bino, 1985a). During leptotene of the meiocytes, most tapetal cells become reduced in size, have a dense cytoplasm and contain some large vacuoles in comparison with cells of the fertile type. In the present study these light microscopical observations are extended to the electron microscopical level. Especially the first ultrastructural differences between fertile and cms microsporogenesis are reported.

Figs. 4-9. Tapetal cells at leptotene stage of the meiocytes.

Fig. 4. BBS inner tapetal cell. The vacuole contains an amount of loose osmiophilic material. Bar, 2 µm.

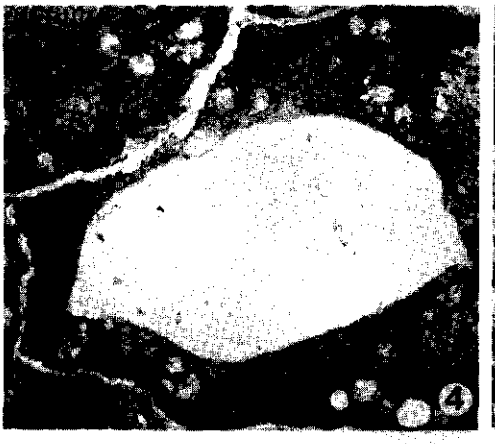
Fig. 5. BBS outer tapetal cell. A membrane divides the vacuole in a translucent area and a part containing some osmophilic matter. Bar, 1 µm.

Fig. 6. BBS outer tapetal cell. Mitochondria (M) are oval, contain some plate-like cristae and a lightly stained matrix. Bar, 1 µm.

Fig. 7. BBF outer tapetal cell. Note that mitochondria (M) show corresponding characteristics as observed in sterile development (Fig. 6). Bar, 1 µm.

Fig. 8. BBS outer tapetal cell. Plastids (P) are irregularly shaped, they have a densely stained matrix and contain some membranous configurations. Bar, 1 µm.

Fig. 9. BBF outer tapetal cell. Plastids (P) show similar features as observed in sterile development (Fig. 8). Bar, 1 µm.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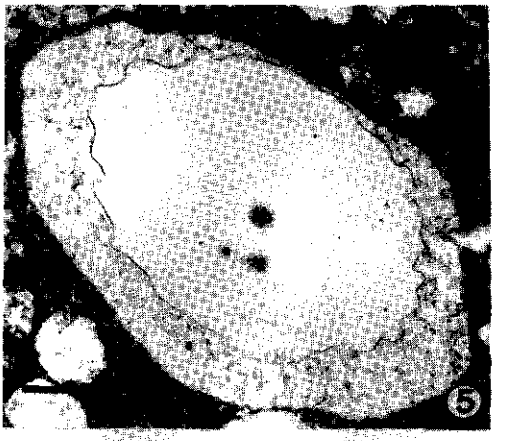
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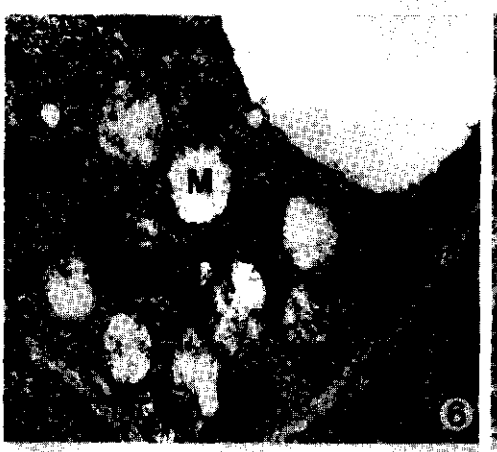
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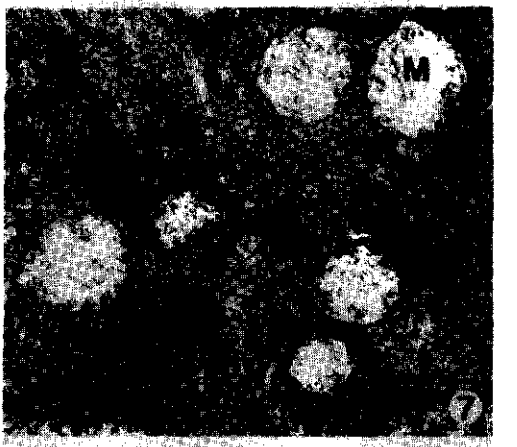
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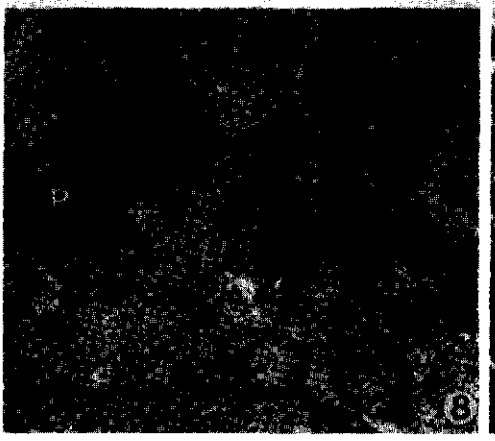
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Material and methods

Two isogenic types of Petunia hybrida (Hook.) Vilm. were used in this study, i.e. male fertile cv. Blue Bedder (BBF), and cms Blue Bedder (BBS), described by Van Marrewijk (1969). The plants were cultivated in a growth chamber under a regime of 16 h light, 8 h dark at 17°C. Dissected anthers were dehydrated and embedded as reported previously (Bino, 1985a). Thin sections, cut with a LKB ultratome III, were stained with uranyl acetate and lead citrate and examined with a Philips 301 electron microscope at 60 kV.

Results

Tapetum

Before meiosis, tapetal cells at the connective side of each locule (the inner tapetum) become cytologically different from tapetal cells at the external side (the outer tapetum). The inner tapetal cells are larger and more irregularly shaped compared with the outer ones (Bino, 1985a). During the leptotene stage of the meiocytes, BBF development shows in most cases the typical karyokinesis of inner and outer tapetal cells. The cytoplasm of the cells is dense, it contains numerous organelles and some small vacuoles (Fig. 1).

Fig. 10. BBS outer tapetal cells at leptotene stage of the meiocytes. Infrequently, karyokinesis is followed by formation of a periclinal cell wall between the two nuclei (note that second nucleus is out of section plain). Bar, 2 µm.

Fig. 11. Detail from Fig. 10, the irregular wall (arrows) is thin and comprises some gaps. Bar, 1.4 µm.

Fig. 12. BBS outer tapetal cell at prophase stage of the meiocytes. The vacuolic system occupies a large part of the cell. The cytoplasm has become heavily stained. Bar, 2 µm.

Fig. 13. Higher magnification of a part out of same tapetal layer as cell in Fig. 12. Mitochondria (M), plastids (P) and ribosomes (numerous small dark dots in cytoplasmic background) are condensated. Bar, 1 µm.


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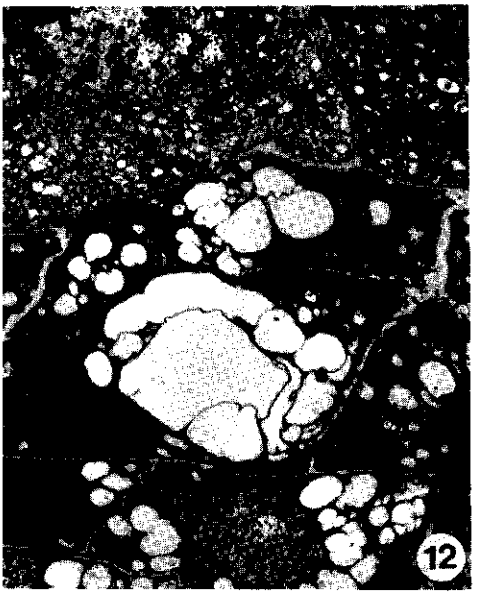
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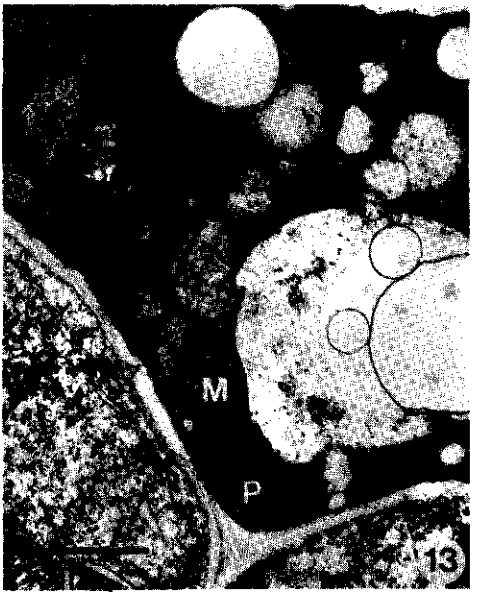
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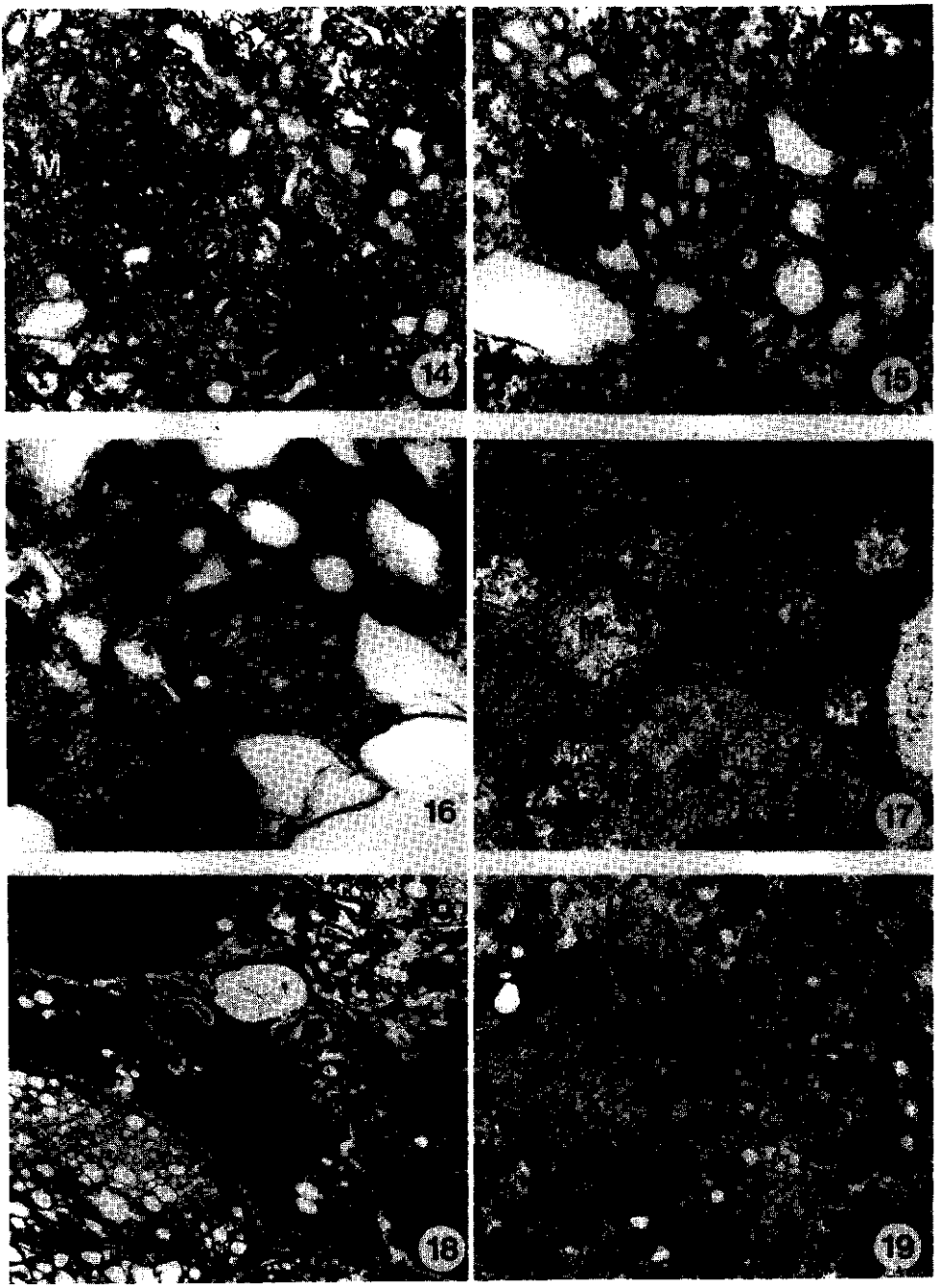
During leptotene, first aspects of deviating development of BBS anthers are observed. Successive stages in the process of degeneration may be followed in the same tapetal layer (Fig. 2). Cells with some small vacuoles, adjoin highly vacuolated cells. The vacuolic system may occupy a large part of the volume of the cell (Fig. 3). Similar features are found in outer and inner tapetal cells. Most vacuoles are located adjacent to the sporogenous tissue. Several contain membranous inclusions and an amount of loose osmiophilic material (Fig. 4). Membranes may restrict the osmiophilic matter to a distinct part of the vacuole and consequently divide the organelle in different areas (Fig. 5). At leptotene stage, cytoplasmic organelles in tapetal cells of BBF and BBS anthers show corresponding characteristics. Mitochondria of the cells are oval, contain some plate-like cristae and a lightly stained matrix (Figs. 6 and 7). Plastids are irregularly shaped, they have a densely stained matrix and contain some membranous configurations (Figs. 8 and 9). Vacuolation of tapetal cells of the BBS type, may take place before karyokinesis, ultimately resulting in degeneration of the uninucleate cell.

Figs. 14, 15, and 16. Mitochondria of BBS outer tapetal cell at late prophase stage of the meiocytes. The mitochondria (M) are elongated-oval (Fig. 14). At cross-section the tube like cristae become evident (Fig. 15). The organelles may contain several of these cristae (Fig. 16). Fig. 14: Bar, 1 µm; Fig. 15: Bar, 0.5 µm; Fig. 16: Bar, 0.5 µm.

Fig. 17. BBF outer tapetal cell at late prophase stage of the meiocytes. The mitochondria (M) are ellipsoid to oval and contain some plate-like cristae that are arranged perpendicularly to the envelope of the organelles. Note ultrastructural differences with mitochondria of sterile development (Figs. 14, 15, and 16). Bar, 1 µm.

Fig. 18. BBS outer tapetal cells at late stage of degeneration. The cells are highly distorted, the nucleus is disrupted and the cytoplasm disorganized. Bar, 2 µm.

Fig. 19. Higher magnification of a part out of same tapetal layer as cells in Fig. 18. Cytoplasmic organelles are mostly lysated and many lipid droplets are present. Bar, 1 µm.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Occasionally, vacuolation proceeds karyokinesis, resulting in degeneration of the binucleate tapetum cell. In approximately 5% of the outer tapetal cells, a cell wall is formed following karyokinesis. In these cells, cytokinesis has taken place and a periclinal wall is clearly present (Fig. 10). The irregular wall is thin and comprises some gaps (Fig. 11). Wall development precedes, or is simultaneous with vacuolation of the cells. Formation of a cell wall after karyokinesis is never observed in the inner tapetal cells. The cytoplasm of vacuolated cells heavily condenses (Fig. 12). Apparently, the increased volume of the vacuolic system results in condensation of ribosomes and other organelles (Fig. 13). Subsequently in the course of degeneration, the tapetal cells largely deform. The volume of most cells is considerable less then that of cells of the fertile type, at a corresponding period of anther development. At the end of prophase stage of the meiocytes, first ultrastructural differences in mitochondria of tapetal cells are evident.

Fig. 20. BBF meiotic cell at premeiosis. Plastids (P) are irregularly formed, they have a densely stained matrix and a moderately developed lamellar system. Bar, 1 µm.

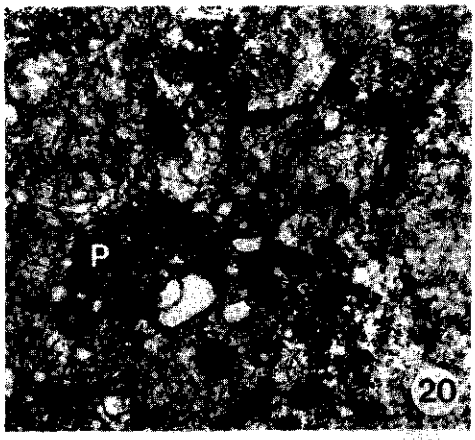
Fig. 21. BBF meiotic cell at premeiosis. Mitochondria (M) are spherical or ellipsoid, they have a lightly stained matrix and a small number of plate-like cristae. Bar, 1 µm.

Fig. 22. BBS meiotic cell at prophase stage. The first features of degeneration are represented by vacuoles invading the cytoplasm of the cell. Bar, 2 µm.

Fig. 23. Detail from Fig. 22. Though first phases of degeneration are represented, mitochondria (M) do not show ultrastructural abnormalities. The organelles are spherical, they have a lightly stained matrix and a small number of plate-like cristae, comparable with fertile development (Fig. 21). Bar, 0.15 µm.

Fig. 24. BBS meiotic cell at end of prophase stage. A callosic cell wall (arrows) is synthesized. Note that outer tapetal cells (OT) are already degenerated. Bar, 4 µm.

Fig. 25. Higher magnification of a part out of same meiotic tissue as Fig. 24. Mitochondria (M) enlarge and develop tubular cristae. Bar, 1 µm.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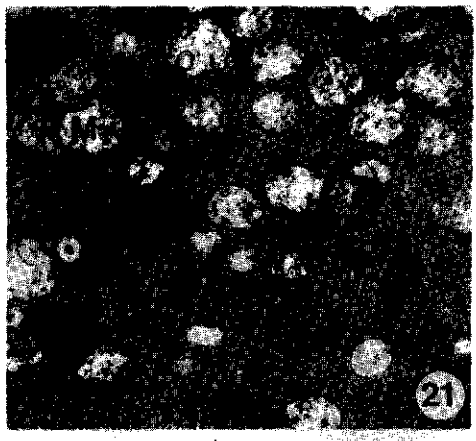
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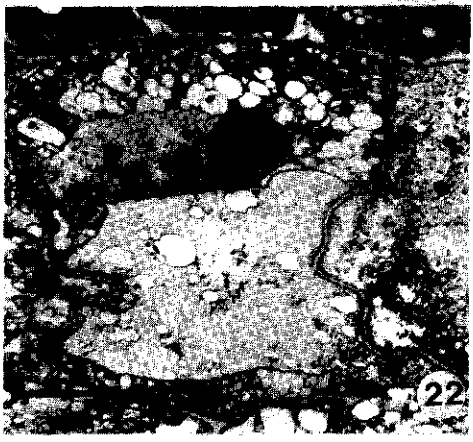
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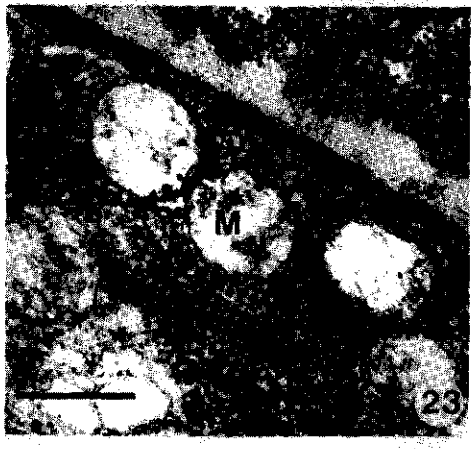
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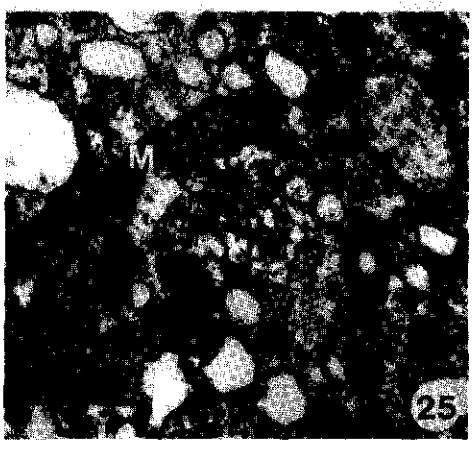
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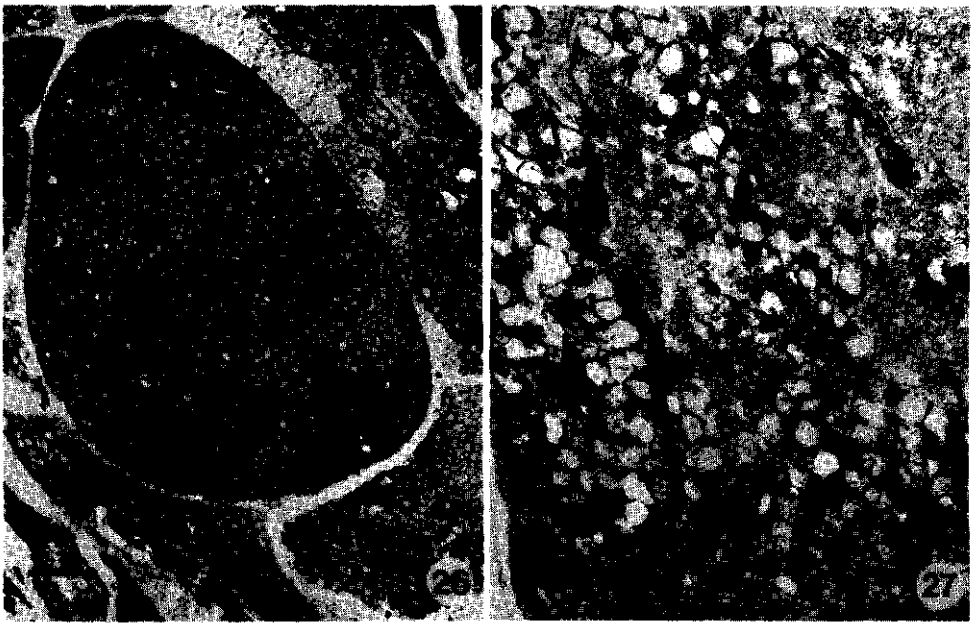


Fig. 26. BBS meiotic cell at late stage of degeneration. The cytoplasm is highly disorganized. Bar, 2 µm.

Fig. 27. Detail from Fig. 26. The mitochondria and plastids disintegrate, leaving a darkly stained mass of cytoplasm, with many lipid droplets. Bar, 1 µm.

Mitochondria from tapetal cells of BBS are elongated and contain several tubular cristae (Figs. 14, 15, and 16). Mitochondria from the fertile type, at a similar phase of meiocyte development, are ellipsoid to oval and contain some plate-like cristae, that are arranged perpendicularly to the envelope of the organelles (Fig. 17). Continuation of degeneration is rather variable, as already described in a previous report (Bino, 1985a). Ultimately at anaphase I of the meiocytes, degeneration of tapetal cells is distinct. The cells are highly distorted, the nucleus is disrupted and the cytoplasm disorganized (Fig. 18). Plastids and mitochondria are mostly lysated and many lipid droplets are present (Fig. 19).

Sporogenous tissue

Observations on ultrastructural development of meiocytes during meiosis have been reviewed by Heslop-Harrison (1971). Throughout the course of meiosis, major changes do occur in both cytoplasm and organelles. Also in petunia these alterations are found. During premeiosis, dump-bell shaped mitochondria and plastids are found. These profiles are suggestive of division stages of the organelles. However, most plastids are irregulary formed, they have a densely stained matrix and a moderately developed lamellar system (Fig. 20). Mitochondria are spherical or ellipsoid, they have a lightly stained matrix and a small number of plate-like cristae (Fig. 21). In pachytene, division ceases and the organelles reach maximum structural simplification. Plastids are ellipsoidal, with a compact matrix and without lamellae. Mitochondria are spherical with a lightly stained matrix and contain rarely some small cristae. During prophase, massive cytoplasmic channels are developed between neighbouring cells. Formation of these channels precedes synthesis of the callosic cell wall, which begins in pachytene.

Aberration in BBS development may begin at any period during prophase. Till this initial commence of deviation, 'sterile' and 'fertile' sporogenesis are indistinguishable. Abortion of meiocytes generally follows a similar pattern as that what is found in tapetal tissue. The first phases of degeneration are represented by large vacuoles invading the cytoplasm of the cells (Fig. 22). Though the process of degeneration has started, mitochondria do not demonstrate ultrastructural abnormalities (Fig. 23). Also in sterile development a callosic cell wall is synthesized (Fig. 24). The mitochondria of these cells enlarge and develop tubular cristae (Fig. 25). Ultimately, the cytoplasm of degenerated cells is disorganized (Fig. 26). The mitochondria and plastids disintegrate, leaving a darkly stained mass of cytoplasm, with many lipid droplets (Fig. 27).

Discussion

In cms Petunia hybrida cv. Blue Bedder, first deviation in sterile development is noticed during leptotene stage of the meiocytes. Initial aberration in sporogenesis of the BBS type, is generally represented by formation of large vacuoles in the cytoplasm of inner and outer tapetal

cells. These vacuoles manifest the very first aspects of degeneration; no other ultrastructural differences are observed at this phase. In various species a similar situation is reported. Several light and electron microscopical studies revealed, that vacuolation of tapetal cells frequently precedes pollen sterility. E.g. Lee et al. (1979), described the existence of two different pathways of pollen abortion in cms-C maize, both starting with the vacuolation of tapetal cells. In cms pepper, tapetal cells vacuolate before any other cytoplasmic alteration is evident (Horner and Rogers, 1974). Overman and Warmke (1972) found in cms Sorghum bicolor, that vacuolation of tapetal cells accompanies cytoplasmic disorganization. In addition, various genic male sterile species share similar characteristics. For example, vacuolation of tapetal cells is described in genic male sterile pigeon pea (Dundas et al., 1981), male sterile soybean (Graybosch et al., 1984) and ms 10 mutant of maize (Chen et al., 1979). Vacuoles have many funtions in the cell. The organelles immobilize toxic products, transport and accumulate cell substances and play a prominent role in autophagic processes (Matile and Wiemken, 1976). Formation of vacuoles in tapetal cells of sterile plants may manifest disturbances in one of these functions. In petunia, several vacuoles contain osmiophilic material and membranous configurations. These inclusions may represent accumulation of substances inside the vacuole. Alternatively, partially cytoplasmic enclosement is correlated with vacuole formation (Marty et al., 1980). In this regard, the study of Amelunxen and Heize (1984), which describes vacuole development is perhaps significant. The authors demonstrate vacuolic inclusions as a result of disarrangement of groundplasm followed by fusion of smaller vacuoles.

In Petunia hybrida, modifications in the ultrastructure of mitochondria occur only after the process of degeneration has started. The mitochondria elongate and contain several tubular cristae. Alterations in mitochondrial ultrastructure may be associated with changes in the energy requirements of the cell (Smith and Ord, 1983). However, a tissue specific localization of cytochrome c oxidase could not establish any change in enzyme activity. At different stages of development, a similar cytochrome oxidase activity is demonstrated in cristae and at membranes of mitochondria of BBF and BBS anthers (Bino et al., 1985).

BBS outer tapetal cells, following mitosis, infrequently develop a

thin wall between the nuclei. A similar situation is observed in a genic male sterile mutant of maize, in which a periclinal wall is formed after karyokinesis (Greyson et al., 1980). In both species, formation of the wall is probably evoked by developmental disorganization of the tapetum cell.

Resemblances in ultrastructural aspects of sterility, between cytoplasmic and genic male sterile species are impressive. Possibly, these similarities are a consequence of a corresponding process. Many recent observations suggest that mitochondria interact intensively with other cellular structures, and may even be able to transfer DNA to the nucleus (Yaffe and Schatz, 1984). Whether these phenomena play a role in sterility, may be a question for future research. Combining the cytological and molecular observations, hopefully will give a better insight into the process of cytoplasmic male sterility.

Acknowledgments

I wish to express my gratitude to Prof. dr. J.L. van Went, Dr. G.A.M. van Marrewijk and Prof. dr. M.T.M. Willemse for valuable suggestions and comments on the manuscript. Thanks are due to Mr. S. Massalt for help in the photographic work and to Mrs. J. Cobben-Molenaar and Mrs. G.G. van de Hoef-van Espelo for secretarial assistance. I also like to thank Mr. A.B. Haasdijk and Mr. P.A. van Snippenburg for their help in preparing the plates.

Chapter 4
Characterization of Cytoplasmic Male Sterility in Petunia hybrida. Localization, composition and activity of esterases

Summary

Anthers of male fertile, cytosterile and restored male fertile clones of Petunia hybrida are compared for esterase activity and composition in subsequent stages of microsporogenesis. Three methods are applied (I) ultra-thin layer isoelectric focussing on polyacryl amide gels, (II) quantitative spectrophotometrical assay, (III) histochemical determination of total esterase activity associated to the azo-coupling method (Pearse, 1972).

In male fertile and restorer idiotypes the isozyme patterns are quite similar. Both the number and intensity of bands increase gradually till the tetrad stage. In contrast, esterase activity in cytosterile anthers remains at a low level and hardly any new bands show up during the later stages. This unvariable, low activity level in anthers of cms plants is also found in the spectrophotometric assay. Histochemical determinations reveal that in male fertile anthers, esterase activity is concentrated in the outer tapetal layer at late prophase and that it accumulates there till the early microspore stage. In male sterile anthers, esterase accumulation in the tapetal cells stops at the moment that tapetal breakdown becomes visible. This suggests that differences in esterase activity and composition are an effect rather than a cause of the failing pollen formation.

Key words: Petunia hybrida - Cytoplasmic male sterility - Esterase activity - Esterase isoenzymes - Histochemical analysis.

General introduction

As yet cytoplasmic male sterility (cms) is the most widely adopted tool for hybrid seed production. Male sterility is necessary to prevent the maternal line (seed line) from selfing or sibbing where hand emasculation is not feasible. Nevertheless, its application is limited by a number of severe drawbacks: (a) the non-availability of the cms characteristic in many crops and their wild relatives; (b) the necessity of a time-consuming series of backcrossings to introduce cms in breeding stocks; (c) the need for fertility restorer genes in seed or fruit producing hybrids; (d) the complexity of seed production and maintenance of parental lines. Breeders are therefore looking for systems of artificial prevention of selfing which can be applied on call. In connection with this, the main attention has been directed at developing gamete killing chemicals (gametocides) such as maleic hydrazide, gibberellins, Ethrel (2-chloroethane phosphonic acid) and sodium 2,3-dichloroisobutyrate. Wide scale use of gametocides has hitherto been impeded by serious shortcomings, of which female sterility and unreliable activity are the most notorious. Only in one single case (wheat) have hybrids that were produced on the basis of gametocides, been released or have been put on trial (Jensen, 1985).

An alternative way to tackle the above problem is to try to imitate male sterility induction as it occurs in nature in heritable male sterile plants. This approach requires knowledge of the primary step and the primary causative factor of the disarrangement in microsporogenesis leading to the non-formation of viable pollen grains. Such knowledge could be obtained by a careful inventarization of all cytological, anatomical, histological and biochemical abnormalities in male sterile plants and comparing them with highly nuclear isogenic male fertile counterparts during the successive stages of microspore development. By assembling the points of deviation as pieces of a jigsaw puzzle, one may obtain insight in their relation and in the sequence in which they occur.

Many cases of male sterility, both genic and cytoplasmic, were described earlier and the observed phenomena were summarized by several authors, most recently by Laser and Lersten (1972) and by Frankel and Galun (1977). However, the most obvious shortcoming of nearly all these studies is that they are highly fragmentary. The majority of the authors deal only with light microscopically detectable differences and, even in

that respect, only registrate the macro-deviations, which must be considered as resultant rather than causative factors of defective sporogenetic processes. Biochemical observations are generally restricted to easily detectable indicators as the total protein content or free amino-acid composition of anthers or other plant parts. Only a few studies concentrate on processes that are supposed to have direct impact on pollen formation (e.g. Frankel et al., 1969; Izhar and Frankel, 1971).

Another serious shortcoming of most investigations is that they are conducted on inadequately defined plant material - in several cases not even the variety is referred to, but merely the species name - grown under uncontrolled conditions. This makes comparison of the results obtained within the same crop very hazardous or even impossible.

The above considerations have led us to set up a project for the purpose of characterizing comprehensively cytoplasmic male sterility in a model crop. On the strength of the following points we have chosen for petunia:

a) the presence of a stable system of cytoplasmic male sterility and the availability of maintainer and restorer lines;
b) the convenience of vegetative multiplication, so that all experiments can be done with genetically identical material;
c) the possibility of raising the crop under standard conditions in a climate room.

This report comprises a biochemical and histochemical study of the activity and the isozym composition of esterases.

The first and third author are primarily responsible for the biochemical part and the second author for the histochemical aspect.

BIOCHEMICAL DETERMINATION OF ESTERASE COMPOSITION AND ACTIVITY

Introduction

Esterases are rather unspecific enzymes occurring in all plant parts. Esterase isozyme patterns are used as an aid in identifying crop cultivars (Bassiri, 1976; Payne and Kaszykovski, 1978). They also have proven to be a valuable tool for distinguishing one sterilizing plasm from the other

(Tripathi et al., 1982; Karim et al., 1984). Some esterases appear to play a role in the production of the outer pollen wall. In Brassica oleracea, esterases are synthesized in the tapetal cells and transferred to the exine cavities when the tapetal cells degenerate at early pollen maturation (Knox et al., 1973; Vithanage and Knox, 1976). Since in petunia cytosterility results in an untimely degeneration of the tapetum (Bino, 1985a), it is investigated in what way such is reflected in the esterase activity.

Material and methods

Plant material

Esterase analyses were conducted on a number of well-defined idiotypes of Petunia hybrida derived from the material described by Van Marrewijk (1969), namely:

(I) cytoplasmic male sterile clones of cvs. Blue Bedder (BBS1), Rosy Morn (RMS1) and Snowball (SBS1);

(II) nuclear isogenic male fertile counterparts of those cytosterile idiotypes (BBF1, RMF1, SBF1);

(III) clones derived from highly inbred homozygous restorer lines (R1-2, R2-1, R2-2, R3-2);

(IV) clones derived from F1 crosses between cytosterile and restored fertile plants (BBS1 x R2-1, RMS1 x R1-2, SBS1 x R3-1).

Growing conditions

All clones were grown in a climate room at 17°C, a relative humidity of 70% and a light intensity of 60 Watt/m^2 for 16 h/day. A few clones (BBS1, BBF1, R2-2) were also raised at 13°, 21° and 25°C to study the effect of temperature on esterase compostion and activity. Determinations were carried out on anthers of 8 developmental stages ranging from premeiosis to dividing microspore. Flower buds were collected early in the morning and stored in petri-dishes on ice before analysis. Determination of stage was done by cytological observation in one of the anthers or by an empirically established relationship between flower bud size and anther development (Table 1). The latter method was mainly applied in cytosterile

idiotypes which display abnormal microspore development.

Enzyme extraction

Anther samples of about 10 mg were homogenized in H_2O (2 μl/mg anther tissue). After 10 minutes centrifugation (10,000 x g), 20 μl supernatant was dialyzed against 2 l H_2O for 90 minutes. Following dialysis, esterase composition and relative activity of isozymes was determined by means of ultrathin layer isoelectric focussing. Another part of the supernatant was directly used for a quantitative assay of esterase activity.

Isoelectric focussing

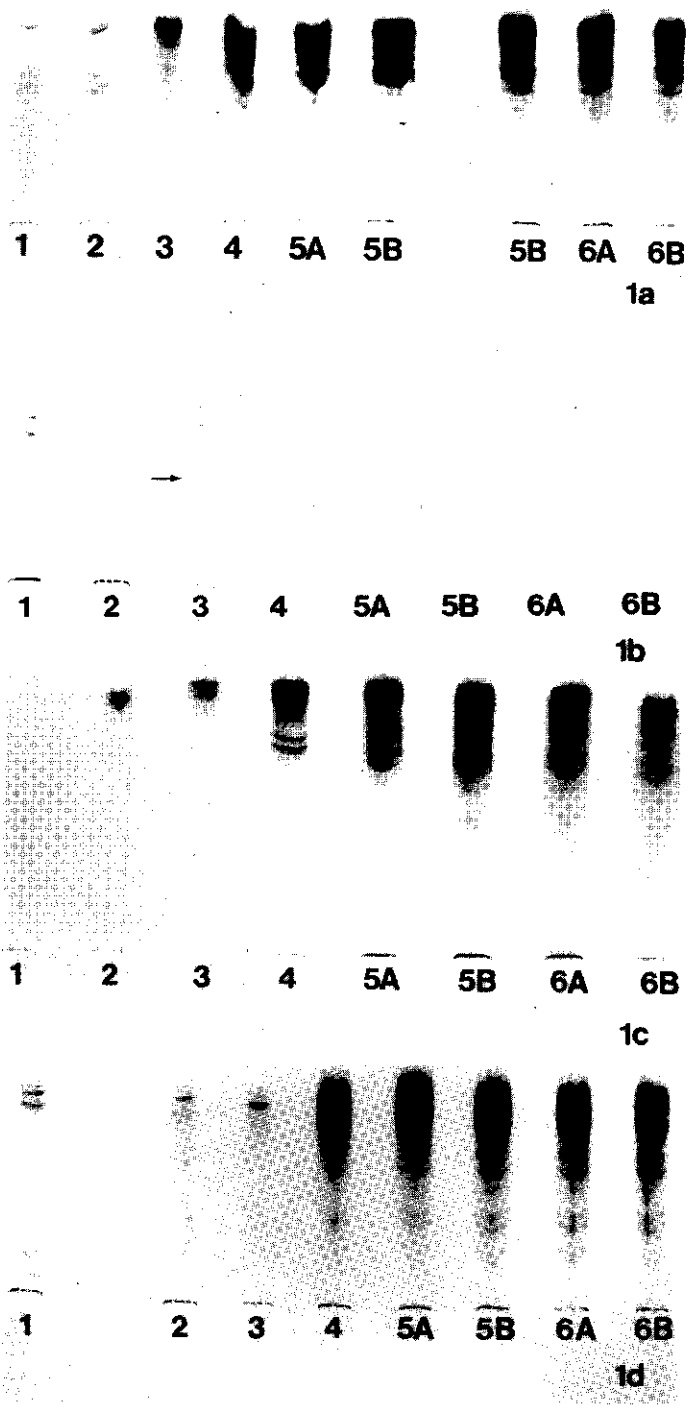
Polyacrylamide gels (5% T, 3% C) including 3% w/v 'Servalyt T', pH 4-6 ampholine carrier, 10% v/v glycerol and 0.2 ml 1.5% ammonium persulphate were prepared on polyester films according to the 'flap' technique of Radola (1980). Ultrathin layer isoelectric focussing was done at a temperature of 4^{o}C in a LKB Ultraphor flat bed container connected to a power supply (Pharmacia ECPS 3000/150 with Volthour integrator VH-1) at a constant input of 3 Watt.

Isozyme staining

Esterase bands were made visible by incubating the gels in 150 ml 0.15 M phosphate buffer, pH 7.2, containing 150 mg Fast Blue RR salt and 60 mg 1-naphtyl acetate dissolved in 5 ml aceton, and rinsing them for 5 minutes in a 15:4:1 mixture of H_2O-methanol-acetic acid. After air drying the staining intensity was measured with an ultrascan LKB Laser Densitometer.

Esterase activity determination

After the reaction was started by adding enzyme extract in an appropriate dilution, linear extinction changes at 620 nm were measured in a spectrophotometer (Beckman-M24) during 10 minutes at 22^{o}C in a cuvette containing 0.05 M phosphate buffer pH 8.0 and $2.5.10^{-4}$ M indophenylacetate (IPA; 0.8% in ethanol). Controls were run by incubating enzyme free indophenylacetate and IPA supplemented with tissue extract inactivated by heat treatment.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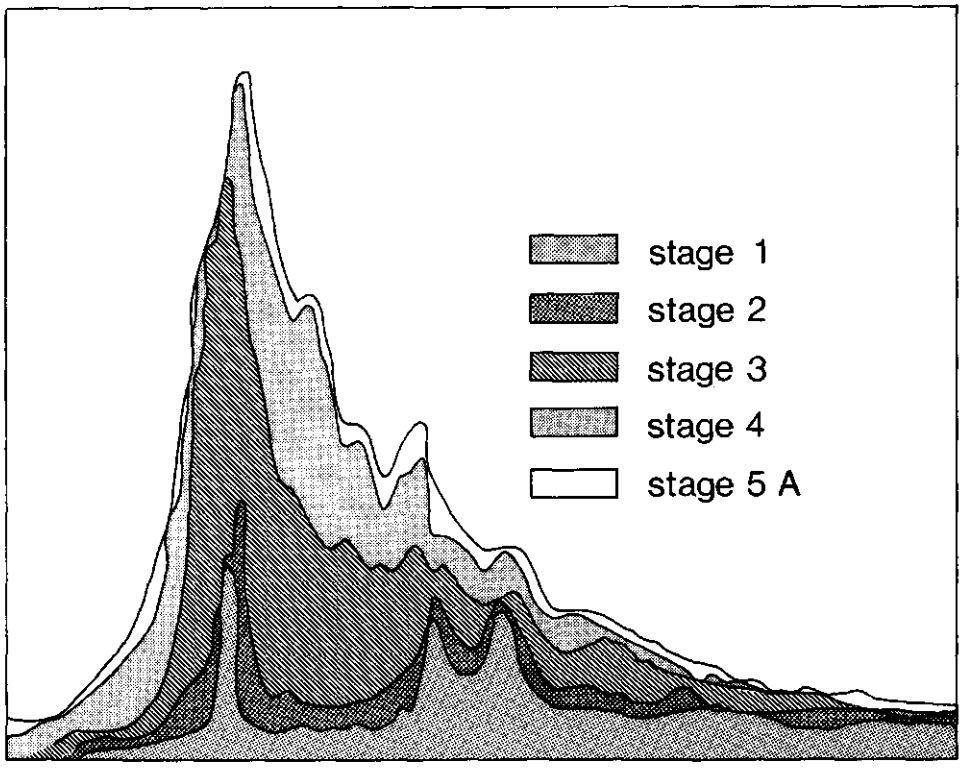


Fig. 2. Densitogram of esterase activity at subsequent stages of microsporogenesis in the male fertile *Petunia hybrida* clone BBF1. For stage code see Table 1.

Fig. 1. Esterase isoenzyme patterns of different idiotypes of *Petunia hybrida*. **a.** male fertile clone BBF1. **b.** cytoplasmic male sterile clone BBS1, at stage 3, only one new band shows up (arrow). **c.** restored male fertile clone R2-2. **d.** F_1-clone BBS1 x R2-1.
Lane numbers refer to stages of microsporogenesis mentioned in Table 1.

Table 1. The relationship between flower bud size and stage of microsporogenesis in *Petunia hybrida* cv. Blue Bedder.

Code	Stage	Flower bud size (mm)
1	Premeiosis	≤ 1.4
2	Prophase	1.5 - 1.8
3	1st Meiotic Division	1.9 - 2.4
4	2nd Meiotic Division	2.5 - 3.0
5A	Early tetrad	3.1 - 3.5
5B	Late tetrad	3.6 - 4.0
6A	Microspore	4.1 - 5.0
6B	Young pollen	5.1 - 6.0

Results

Apart from trifle intensity differences, the male fertile idiotypes BBF1, RMF1 and SBF1 show quite similar isoenzyme patterns. In all cases the number and intensity of bands gradually increase till the tetrad stage (Figs. 1a and 2). Restorer idiotypes have exactly the same patterns (Fig. 1c). Also among the cytosterile idiotypes, BBS1, RMS1 and SBS1, we observed a nearly identical isozyme composition and activity spectrum. In contrast to the situation in male fertile idiotypes, both the number and intensity of bands remain at a unvariably low level throughout the developmental stages. Compared to the premeiotic situation only one new band shows up at early meiosis (Fig. 1b; arrow). The results of the electrophoretic activity determination are corroborated by the results of the spectrophotometric assessment of esterase activity (Table 2). Anthers of F_1 crosses between cytosterile and restorer lines have isoenzyme patterns and activities almost identical to those of the restorer parent (Fig. 1d). Effects of temperature differences are not traced. The zymograms of the idiotypes grown in different temperatures do not exhibit any notable variation.

Table 2. Esterase activity ± SE in a cytoplasmic male sterile idiotype of petunia (BBS1) and its nuclear-isogenic male fertile counterpart (BBF1) expressed as extinction changes per time unit (ΔE). Averages of 5 measurements.

Stage	BBF1		BBS1	
	$\Delta E\ min^{-1}$ anther	$\Delta E\ min^{-1}$ mg anther	$\Delta E\ min^{-1}$ anther	$\Delta E\ min^{-1}$ mg anther
Premeiosis	0.01 ± 0.00	0.06 ± 0.01	0.01 ± 0.00	0.06 ± 0.01
Meiosis	0.31 ± 0.09	0.52 ± 0.11	0.02 ± 0.01	0.06 ± 0.01
Microspore	1.90 ± 0.39	1.99 ± 0.21	0.04 ± 0.01	0.08 ± 0.01

HISTOCHEMICAL DETERMINATION OF ESTERASE ACTIVITY AND DISTRIBUTION

Introduction

A disadvantage of biochemical analyses is that they generally require large samples. The mean of such a sample represents a great number of individual values and completely masks the variation between single anthers or flower buds. Another disadvantage of biochemical analyses is that they are mostly done on entire organs and not on separate tissue layers. Differences in activity of a particular tissue can thus remain unnoticed, because such tissue has no significant effect on the total activity or because the other layers increase their activity to compensate for the deficiency of the particular tissue.

Especially in the study of cytoplasmic male sterility these disadvantages may play a role. Cms appears to be a tissue specific and development associated phenomenon. The first ultrastructural and histological deviations are generally found in the tapetum. Initial aberration in microsporogenesis occurs at a well defined developmental stage. This stage varies among different cms sources, both within and between species, but is rather stable for a particular cms plasma-type (Laser and Lersten, 1972).

This part of our study focusses on the localization of esterase activity in the various cell layers and tissues of the developing anther.

Material and methods

All histochemical investigations were performed on clones of the cytosterile cv. Blue Bedder (BBS1) and its male fertile counterpart (BBF1).

Dissected anthers were sectioned directly after flower bud collection. The sections (≤ 20 μm) were placed in an 0.2 M phosphate buffer, pH 6.8. Total activity of non-specific esterases was determined according to the azo-coupling method (Pearse, 1972). Incubation was performed in 1-naphtyl acetate (1 mM), coupled to hexazotized pararosanilin (1 mM) at pH 6.8 for 30 min at room temperature. The reaction was stopped by rinsing the slides in phosphate buffer. Control sections were made by omitting the substrate in the reaction mixture and by pre-incubating sections for 20 min in phosphate buffer at 80°C. Inhibition was tested by pre-incubating the sections for 10 min in a 0.1 mM eserine solution, and placing them in the incubation medium supplemented with the inhibitor. Eserine is known to inhibit cholinesterase activity (Pearse, 1972).

For electron microscopical studies the method of Holt and Hicks (1966) was used. The dissected anthers were fixed in 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 6.8) for 1 h. Subsequently, the sections were placed in the incubation medium, rinsed and postfixed in 1% osmiumtetroxide for 16 h at room temperature. The slides were dehydrated, embedded and sectioned as reported previously (Bino, 1985a). Thin sections were examined with a Philips 301 electron microscope at 60 kV.

Results

The male fertile idiotype BBF1

Microsporogenesis in the male fertile idiotype (BBF1) mainly follows the well established pattern described for many other species (Bennett, 1976). Some species specific aspects of Petunia hybrida were reported previously (Bino, 1985a). At the premeiotic stage, the esterase reaction product is diffusely distributed (Fig. 3a). All cells colour faintly red. At the end of the prophase, esterase activity is mainly located in the outer tapetal cells of the locule (Fig. 3b). In the inner tapetal cells

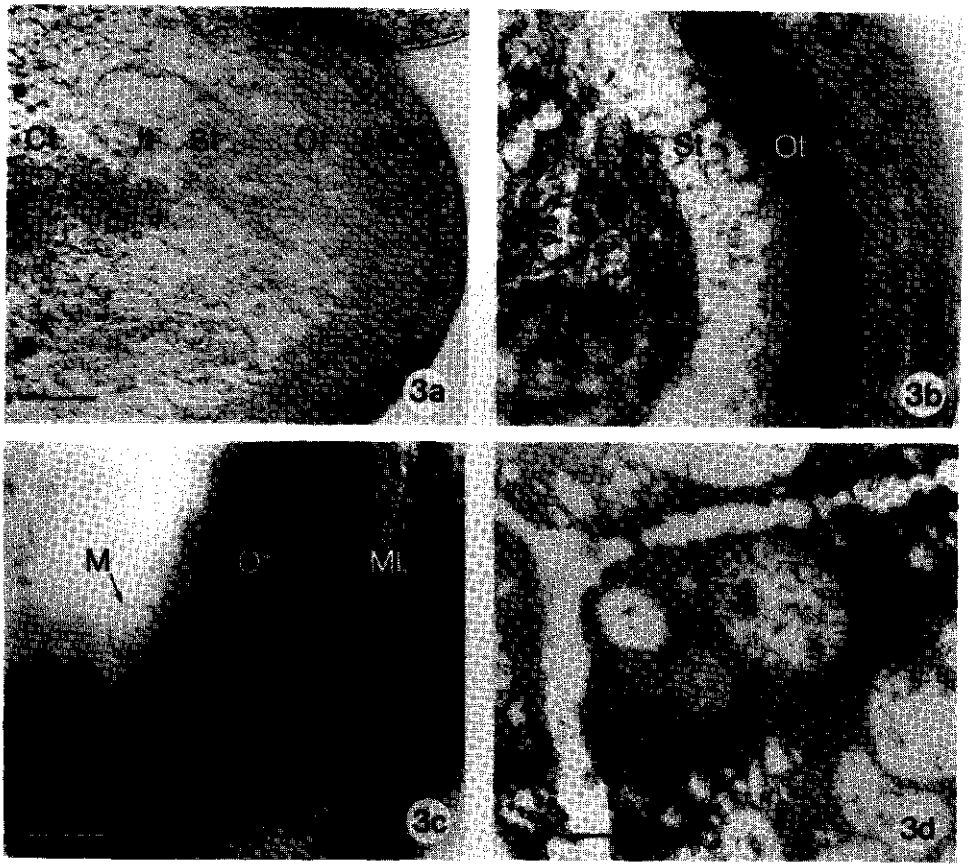
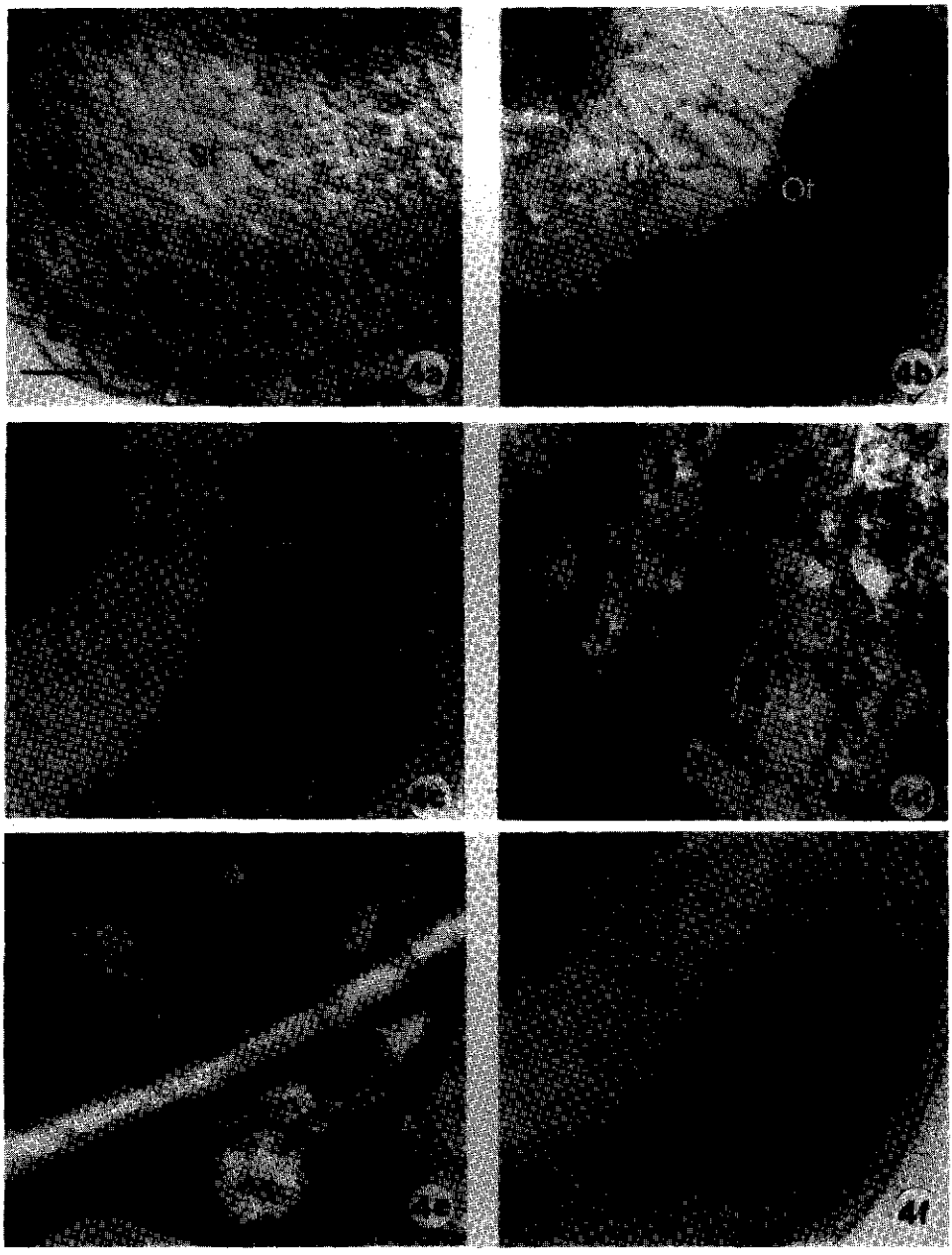


Fig. 3a. Cross section of a BBF1 anther at premeiosis. Esterase reaction product is diffusely distributed in cells of the epidermis (E), middle layer (ML), outer tapetum (Ot), sporogenous tissue (St), inner tapetum (It) and connective tissue (Ct). Bar, 20 µm.

b. BBF1 anther at the end of first meiotic prophase. Esterase activity is mainly located in the outer tapetal (Ot) cells of the locule. The degree of esterase activity correlates with the intensity of the shade. Bar, 20 µm.

c. BBF1 anther at microspore stage. Deposition of esterase reaction product accumulates in the cells of the outer tapetum (Ot). Some reaction product is present at the exine of the young microspore (M, arrow). Bar, 20 µm.

d. Tapetal cell of BBF1 anther at the end of first meiotic prophase. Esterase activity is manifest as an osmiophilic precipitate at the enzyme site (arrows). Esterase activity appears associated with the plasmalemma and concentrates at the plasmodesmatal regions. Bar, 1 µm.


Ot
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and the parenchymatic cells of the middle layer and the connectivum some diffusely distributed reaction product can be observed. At this stage, the sporogenous cells which become enveloped by callose, do not show any deposition of reaction product. At the early microspore stage, esterase activity continues to accumulate in the outer tapetal tissue (Fig. 3c), the cells colouring intensely red. Some weak activity is observed in the parenchymatic cells and the inner tapetum. Deposition of reaction product also occurs at the developing exine of the young microspores, staining the cells pale-yellow.

At the electron microscopical level, esterase activity is manifested as an osmiophilic precipitate at the enzyme site. Esterase activity appears associated with the plasmalemma and concentrates at the plasmodesmatal regions (Fig. 3d). From the end of prophase onward, the outer tapetal cells show a dense deposition of esterase reaction product. Slight esterase activity is noticable in the parenchymatic, sporogenous and inner tapetal tissues.

Fig. 4a. Cross section of a BBS1 anther at premeiosis. Esterase reaction product is diffusely distributed. Bar, 20 µm. For abbreviations, see caption to Fig. 3a.
b. BBS1 anther at early first meiotic prophase. Note that esterase reaction product concentrates in the outer tapetal cells (Ot). Bar, 20 µm.
c. BBS1 anther at the end of first meiotic prophase. At this stage, tapetal breakdown has started; the tapetal cells become disorganized and reduced in size. Bar, 20 µm.
d. BBS1 anther at postmeiotic stage. Cells of the tapetal- and sporogenous tissue are crushed between parenchymatic cells of the middle layer (ML) and the connectivum. Esterase activity is still visible in the deformed outer tapetal (Ot) cells. Bar, 10 µm.
e. Tapetal cells of BBS1 anther at first meiotic prophase. Subcellular distribution of esterase appears associated with the plasmalemma (arrows). L = lipid droplet. Bar, 0.5 µm.
f. BBS1 anther at early first meiotic prophase. Control section, incubated in absence of substrate. Note that there is no deposition of reaction product. Bar, 20 µm.

The cytoplasmic male sterile idiotype BBS1

The location of esterase enzyme activity in early developmental stages in anthers of male sterile plants does not differ from the position in male fertile counterparts. During premeiosis, esterase reaction product is diffusely distributed in the locule (Fig. 4a) and at early prophase, it concentrates in the outer tapetal cells (Fig. 4b). Some reaction product is also observed in the parenchymatic cells of the middle layer and the connectivum. No hydrolysis of esterase substrates is established in the callose enveloped meiocytes. During late prophase, esterase activity accumulates in the outer tapetal tissue (Fig. 4c), the inner tapetum showing only slight activity. Some diffusely distributed reaction product is noticed in the parenchymatic cells. At this stage, tapetal breakdown has started; the tapetal cells become disorganized and reduced in size. During subsequent stages of degeneration, the tapetal and sporogenous cells are crushed between parenchymatic cells of the middle layer and the connectivum. Esterase activity is still observed in the deformed tapetal cells (Fig. 4d). Remnants of meiocytes do not show any deposition of esterase reaction product. Like in fertile development, subcellular distribution of esterase appears associated with the plasmalemma (Fig. 4e).

Control experiments

Similar results are obtained in BBF and BBS anthers. Control sections incubated in absence of substrate do not show any deposition of reaction product (Fig. 4f). No esterase activity is observed in pre-incubated sections at 80^{o}C. Esterase activity is not inhibited by a supplement of eserine in the reaction mixture.

Discussion

In male fertile petunia, a sharp increase in esterase activity is observed during microsporogenesis. This increase appears to parallel the esterase accumulation in the outer tapetal layer from early meiosis onward. Contrarily, in cytosterile idiotypes an accumulation of esterase activity does not take place. This is probably connected with the deviations in the development of the tapetum as observed by Bino (1985b).

At the early meiotic stages, the cytoplasm of BBS tapetal cells disorganizes and the cells deform in a large degree. The volume of the tapetal cells largely reduces as compared to the BBF tapetum. Nevertheless, from premeiosis to the microspore stage, the total esterase activity in BBS anthers slightly increases (Table 2). This might result from the growth of the parenchymatic cells of the middle layer and the connectivum, which continue to show some esterase activity. Besides, some esterase activity remains in the deformed and disorganized tapetal cells.

The specific role of the tapetal layer with respect to esterase activity was also found in earlier studies. According to Knox et al. (1973), esterases are synthesized in the tapetal cells. In Brassica oleracea, esterase activity accumulates in the tapetum until the tapetal cells degenerate. At this stage, pollen wall esterase activity increases, reflecting the transfer from tapetal cells to the exine cavities (Vithanage and Knox, 1976). This phenomenon was also observed in the present study.

During the development of BBF anthers, the pattern of esterase isozymes changes. Abbott et al. (1984), obtained similar results in fertile maize plants. The authors described the appearance of new esterases at subsequent stages of microsporogenesis. Synthesis of esterase isozymes may be correlated with a defined developmental stage. For instance, in barley, Ahokas (1976) distinguished a 'yellow staining period' specifically associated with exine development of the microspores. The esterases responsible for the yellow staining of the sporogenous cells are probably synthesized in the tapetum (Ahokas, 1976). During meiosis in anthers of male sterile petunia plants, the number of esterase isozymes remains stable. Apparently, developmental disorganization of the tapetum results in disturbances in the de novo synthesis of esterases.

Höhler and Börner (1980) compared esterase isozyme patterns of male fertile and cytosterile wheat cv. Iljicewka. The three major esterases typical for wheat are present in a comparable intensity. However, a characterizing feature of the male sterile anthers is the lack of two isoenzyme bands. The authors explained the difference in isoenzyme pattern by the assumption that the sporopollenin hydrolyzing esterase isoenzyme, as described by Ahokas (1976), is absent in anthers of cms plants. Unfortunately, they did not report at what stage of development the

observed differences in esterase isozym pattern occur.

According to Gahan and McLean (1969), esterases in root tips of Vicia faba are initially synthesized on the ribosomes of the rough endoplasmic reticulum and secondarily transported to the cell walls. Depending on the incubation time, the osmiophilic material is precipitated in the different organelles. After 20 min of incubation, the end product appears associated with the plasmalemma. In anther tissues of cms and fertile petunia, a similar subcellular localization is observed, i.e. after 30 min of incubation, the reaction product is precipitated at the plasmalemma.

In conclusion, our results imply that changes in total esterase activity and esterase isozyme pattern are initiated by the degeneration process of BBS tapetal cells. The results strongly suggest that differences in esterase activity and esterase composition are an effect rather than a cause of the failing pollen formation.

Acknowledgments

The authors wish to thank J. W. Molenveld and P. Vos for their technical assistance.

Chapter 5
Characterization of Cytoplasmic Male Sterility in Petunia hybrida. Localization and activity of cytochrome c oxidase

Summary

Anthers of male fertile, cytoplasmic male sterile (cms), and restored male fertile Petunia hybrida, are analyzed for cytochrome c oxidase (cox) activity in subsequent stages of microsporogenesis, and compared with anthers of male fertile, cms-S and cms-C Zea mays. The cox activity is determined in anther extracts and cytochemically. In petunia anthers, the first differences in cox activity occur from meiosis onward. However, at these stages, the initial symptoms of degeneration are already apparent. It is suggested that the decline in enzyme activity of the cms petunia anthers is the result rather than the cause of the non-formation of functional pollen.

In maize anthers, the cox activity of sterile-type anthers is reduced in comparison with fertile-type anthers from premeiosis onward. There are also consistent cytochemical differences in the mitochondrial organization of cox activity between pollen of cms-S and male fertile maize anthers. In fertile-type mitochondria, the DAB reaction product indicating cox activity is localized in the cristae and within the space between the outer and inner limiting membranes of the organelles. In mitochondria of pollen of cms-S maize, cox activity is only observed between the outer and inner membranes of the mitochondria. The biochemical and cytochemical differences are observed at stages of development at which no structural signs of degeneration are apparent. The results suggest that cms in maize correlates with deviations in cytochrome c oxidase activity.

Key words: Petunia hybrida - Zea mays - Cytoplasmic male sterility - Biochemical analysis - Cytochemical analysis - Cytochrome c oxidase

Abbreviations: cox = cytochrome c oxidase
DAB = 3,3'-diaminobenzidine

Introduction

The role of the mitochondrial genome in plant cells is limited to the synthesis of 10-12 polypeptides (Leaver and Forde, 1980). The majority of these mitochondrial encoded polypeptides are components of three enzyme complexes, including the three largest of the seven subunits of cytochrome c oxidase, the apoprotein of the seven subunits of the cytochrome bc complex, and two to four of the nine subunits of the oligomycin-sensitive ATPase complex (Borst et al., 1984). Most of these complexes are an integral part af the inner mitochondrial membrane and are responsible for key steps in the process of oxidative phosphorylation and the generation of ATP (Leaver and Forde, 1980).

Several studies report that the genetic determinants responsible for cytoplasmic male sterility (cms) are carried by the mitochondrial DNA (see review by Hanson and Conde, 1985). Possibly, in cms plants, an aberrant synthesis of one of the mitochondrial encoded proteins initiates the abnormalities in tapetal and sporogenous development which ultimately result in male sterility. In several studies, male sterility is associated with alterations in the cox complex. For instance, mitochondria of a cms type of Sorghum bicolor, synthesize a cox subunit I, 4000 daltons larger than the form found in fertile plasmatypes (Dixon and Leaver, 1982). In Zea mays, differences occur in the number of cox isozymes in anthers of male fertile and male sterile plants (Watson et al., 1977). In addition, the cox activity of male sterile-type maize anthers is significantly lower than that observed in anthers of male fertile plants (Watson et al., 1977; Ohmasa et al., 1976). A general reduction of the activity of mitochondrial redox processes in anther extracts has been reported for cms types of wheat (Borisenko and Dmitrieva, 1975), rye (Dmitrieva, 1971), rice (Dai et al., 1978), and lucerne (Fursov and Fisenko, 1975).

In cms Petunia hybrida, Kool et al. (1985) demonstrated that the cms plasmatype is correlated with changes in mitochondrial DNA composition and its translational products. The cms-associated DNA arrangement and a homologous mitochondrial DNA region from a male fertile line are subjected to sequence and transcription analysis (Boeshore et al., 1985). The authors identified and mapped the point where the mitochondrial genomes diverge from each other, but could not conclusively elucidate whether or not the observed deviations in mitochondrial DNA are functionally

associated with the abnormalities in pollen development.

In the present study, we attempt to establish whether the alterations in mitochondrial DNA do have implications for the cox activity in anthers of cms petunia. Cytochrome c oxidase is determined in two ways: biochemically and cytochemically. The biochemical analysis enables us to determine the enzyme activity in anthers at different stages of development. The cytochemical analysis provides information about the subcellular localization of cox. Differences in enzyme activity are correlated with differences in the ultrastructural organization of mitochondria in the various anther tissues. The results obtained with the petunia material are compared with observations on the localization and activity of cox in anthers of cms and male fertile maize types.

Material and methods

Plant material

Analyses of the total cox activity were conducted on genetically well-defined plant material of Petunia hybrida and Zea mays. The petunia material included:

(I) cytoplasmic male sterile clones of cvs. Blue Bedder (BBS1) and Rosy Morn (RMS1);

(II) nuclear isogenic male fertile counterparts of those cytosterile idiotypes (BBF1, RMF1);

(III) clones from highly inbred homozygous restorer lines (R2-1, R2-2).

All petunia plants were clones derived from the material originally described by Van Marrewijk (1969).

The maize material included:

(I) cytosterile lines W182 BN-H and W182 BN-RB (classified by Sisco et al. (1982) as respectively cms-S and cms-C type of male sterility)

(II) male fertile line W182 BN-N

Seeds of the maize lines were supplied by Veredelingsbedrijf Zelder bv, Ottersum, The Netherlands.

The cytochemical determination was only performed on the BBF1, BBS1, W182 BN-N, and W182 BN-H material.

Growing conditions

The petunia clones were grown in a climate room at 17 °C day and night, a relative humidity of 70 % and a light intensity of 60 Watt/m^2 for 16 h/day. The maize plants were grown in a glasshouse at 18-22 °C.

Biochemical methods

Preparation of anther suspension

From each petunia flower bud, four anthers were dissected and ground in a micocentrifuge tube with a slightly fitting plastic rod in 75 μl potassium phosphate buffer (0.1 M, pH 7.2) at 4 °C. The remaining anther of each flower bud was used to determine the stage of development. Stage determination was done by a cytological observation or by an empirically established relationship between flower bud size and anther development (Van Marrewijk et al., 1986).

For the preparation of maize anther suspensions, two anthers of the same tassel were dissected and ground in 50 μl potassium phosphate buffer (0.1M, pH 7.2) and another anther was used to determine the stage of development.

Preparation of reduced cytochrome c

Fourty mg cytochrome c (horse heart ferrocytochrome c; Boehringer, Mannheim) was dissolved in 5 ml H_2O and reduced by adding a small quantity of sodium dithionite ($Na_2S_2O_4$). The surplus of sodium dithionite was removed by passing the reduced cytochrome c solution through a PD-10 Sephadex G-25M standard column (Pharmacia) using H_2O as the solvent. The degree of cytochrome c reduction was verified according to Smith (1961), and was higher than 80%. The solution was stored at 4 °C in a closed bottle.

Spectrophotometrical determination of cytochrome c oxidase activity

Cytochrome c oxidase was assayed by observing the decrease in absorption at 550 nm resulting from the oxidation of reduced cytochrome c. We adjusted the methods of Hoekstra and Van Roekel (1983) for our plant material. Twenty μl of the anther suspension was pipetted into a quartz cuvette containing 1.3 ml potassium phosphate buffer (0.1 M, pH 7.2), and

80 µl 1% Triton X-100. The reaction was initiated by adding 100 µl of the reduced cytochrome c solution, and subsequent mixing. After 3.5 min the remaining cytochrome c was oxidized by adding a droplet of a saturated solution of potassium ferricyanide. The absorption blank contained 1.4 ml potassium phosphate buffer (0.1 M, pH7.2) and 80 µl 1% Triton X-100. The reduced cytochrome c was not oxidized in a control reaction medium without the anther suspension. Cytochrome c oxidase activity was effectively inhibited by adding 100 µl KCN (0.01 M) to the reaction mixture or by heating the anther suspension for 5 min at 60 ^{o}C. Activities were calculated as the first order rate constant K (min $^{-1}$).

Determination of total soluble protein

Of each anther suspension, the total protein content was determined in a Marius Proti-analyzer (Marius Instruments, Utrecht) according to Bradford (1976). The Proti-analyzer is a bichromatic colorimeter able to measure very low protein concentrations. After staining with a Coomassie Brilliant Blue reagent, the analyzer measures the protein samples simultaneously at 465 and 590 nm.

Cytochemical methods

Incubation procedure

For the localization of cox activity we used the methods originally developed by Seligman et al. (1968). The authors used 3,3'-diaminobenzidine (DAB) for the visualization of an oxidase system in the compartment between the inner and outer membranes and in the intracristate spaces of mitochondria. The mechanism of mitochondrial DAB oxidation depends upon cytochrome c acting as the electron acceptor. The next step is the reoxidation of cytochrome c by cytochrome oxidase. The oxidized DAB forms a nondroplet, amorphous reaction product at the enzyme site. The precipitate is osmiophilic and insoluble in the dehydration and embedding procedures used for electron microscopical studies.

Specimen preparation

Anthers were sectioned directly after flower bud collection. The

sections (≤60 μm) were placed in 0.05 M phosphate buffer, pH 7.2. The unfixed tissue slices were incubated for 1 h in the dark at room temperature in phosphate buffer (0.05 M, pH 7.2) containing 2.5 mM DAB (BDH Chemicals Ltd) and 1% (v/v) catalase (Sigma Chemical Company). After incubation, the segments were rinsed in three changes of the phosphate buffer.

Control treatments

Different control experiments were performed:

1) KCN was added to the standard incubation medium in a final concentration of 0.01 M.

2) Before incubation, the sections were heated at 60 °C for 15 min in the phosphate buffer.

3) Before incubation, the sections were prefixed for 15 min in the phosphate buffer containing 2.5 % (v/v) glutaraldehyde.

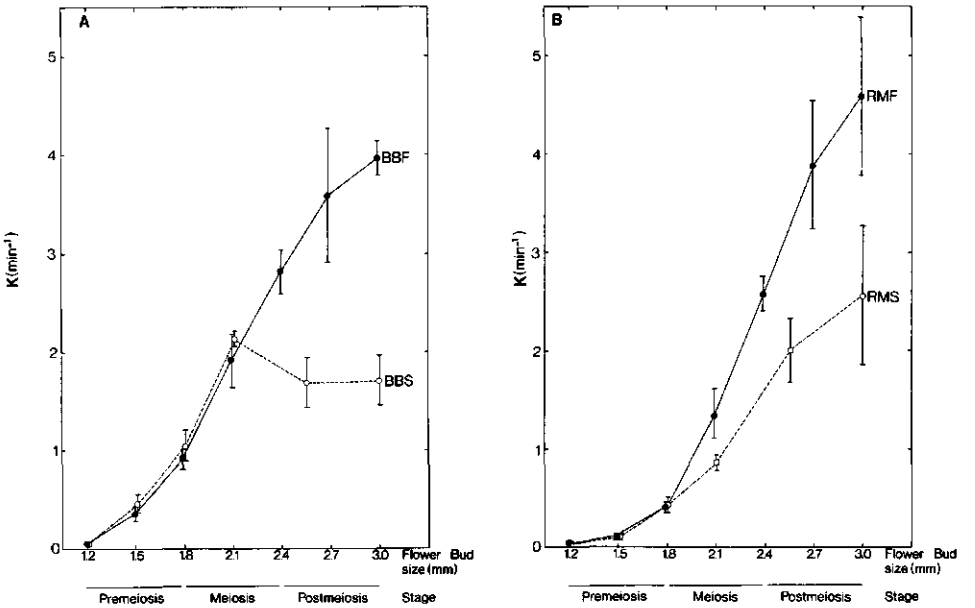
4) The sections were placed in the standard incubation medium supplemented with 0.5 % antimycin A (Boehringer Mannheim)

Electron microscopy

After rinsing, the tissues were fixed in 0.05 M phosphate buffer, pH 7.2, with 2.5 % (v/v) glutaraldehyde. The sections were washed thoroughly in phosphate buffer. The material was then fixed for 16 h in 1 % osmiumtetroxide in phosphate buffer, dehydrated in an ethanol series and embedded in Epon 812 through propylene oxide. Thin sections were examined with a Philips 301 electron microscope at 60 kV without counterstaining.

Morphometric procedures

Anthers were incubated in the standard DAB medium. Cells selected for morphometric analysis were photographed at 20,000 x. The micrographs were examined with a MOP-30 image analyzer (Kontron Messergeräte) on 20-30 randomly selected cells per anther tissue by measuring 3-4 radially sectioned mitochondria of each cell. The total length of DAB reacting membranes per mitochondrial section was determined. Additionally, we measured the surface area of each mitochondrial section. The results were expressed as the mean ± standard deviation. The Student's t-test was used to evaluate the significance level of differences in means.



Figs. 1A and B. Cytochrome c oxidase activity (K) in four anthers of one flower, at subsequent stages of petunia anther development. Each value represent the average ± SE of 3-7 separate determinations.

1A. Cox activity in BBF 1 and BBS1 anthers.

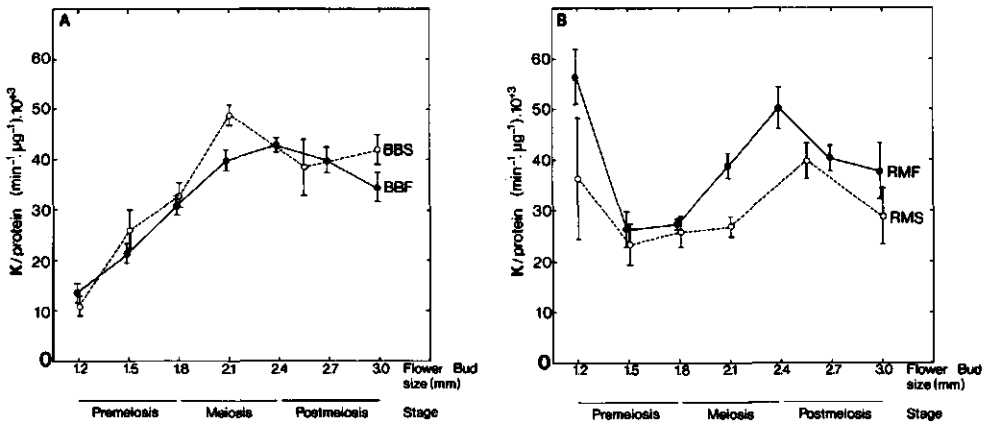
1B. Cox activity in RMF 1 and RMS1 anthers.

Results

Cytochrome oxidase activity in petunia anthers

The activity of cytochrome c oxidase in anther extracts at subsequent stages of flower bud development are presented in Fig. 1. At premeiotic stage, there is no difference between the total cox activity in anthers of the petunia cultivars BBF1 and BBS1 (Fig. 1A), and between RMF1 and RMS1 (Fig. 1B). At postmeiosis, the enzyme activity of both sterile-type cultivars is reduced compared to the activity in fertile-type anthers. Reduction in activity of the male sterile type starts earlier during development in the Rosy Morn cultivar than in the Blue Bedder. The cox

activity is also expressed on the basis of the total soluble protein content (Fig. 2 A and B). At premeiotic and postmeiotic stages, the values of both idiotypes are not significantly different. At premeiosis, the values of the Rosy Morn anthers show considerable variation (Fig. 2B). However, during meiosis, the activity of RMF1 anthers is significantly higher than the activity observed in RMS1 anthers (Fig. 2B). In contrast, at the same stage of development the activity in BBF1 anthers tended to be reduced compared to the activity of BBS1 anthers (Fig. 2A). Cytochrome c oxidase activities of the restorer lines R2-1 and R2-2, are similar to those of the male fertile idiotypes (data not shown).



Figs. 2A and B. Cytochrome c oxidase activity in four anthers of one flower, expressed on the basis of the total soluble protein content (K/protein content) at subsequent stages of petunia anther development. Each value represents the average $\pm$ SE of 3-7 separate determinations.

2A. BBF1 and BBS1 anthers.

2B. RMF1 and RMS1 anthers.

Cytochrome c oxidase activity in maize anthers

The cox activities of maize anthers are shown in Table 1. From premeiosis onward, the male fertile line shows a considerable increase in cox activity. In contrast, the cox activity in anthers of the male sterile lines is very low at all stages of development.

Table 1. Cytochrome c oxidase activity (min^{-1}) at subsequent stages of development in anthers of different maize lines. Each value represents the activity of two anthers.

	Plasmatype		
Stage	N-plasm	Cms-S	Cms-C
Premeiosis	0.19	0.00	0.02
Meiosis	6.61	0.01	0.01
Postmeiosis	8.47	0.02	0.01

Localization of cytochrome c oxidase in anthers of male fertile and cytoplasmic male sterile Petunia hybrida

At the premeiotic stage, the DAB reaction product is predominantly deposited in the intracristate spaces of the mitochondria of BBF1 (Fig. 3) and BBS1 (Fig. 4) tapetal cells. In some mitochondria an unevenly distributed reaction product is observed between the outer and inner limiting membranes. All mitochondria of a tapetal cell are stained to a similar degree, and all cells show a positive reaction. Plastids, vacuoles, endoplasmic reticulum, nuclear envelope and other cytomembranes do not show any activity. Formation of the reaction product is entirely abolished by cyanide (Fig. 5) and by short prefixation in glutaraldehyde (Fig. 6). Addition of antimycin A causes no change in the deposition of reaction product (Fig. 7). Heat pretreatment at 60 °C for 15 min disturbs the normal cellular structure of tapetal cells. Organelles become swollen and disrupted. The mitochondria, however, still exhibit a patchy

distributed formation of DAB reaction product at the membranes (Fig. 8).

The total length of the DAB reacting membranes per mitochondrial section is similar for mitochondria from tapetal cells of BBF1 and BBS1 anthers (Table 2). Also the mitochondrial surface area is not significantly different for tapetal cells of both petunia types. Furthermore, there is no difference in length of the DAB reacting membranes and in relative volume of mitochondria from sporogenous cells of BBF1 and BBS1 anthers. However, mitochondria of tapetal cells of both petunia types exhibit a somewhat greater surface area and have a longer total length of reacting membranes than the mitochondria of sporogenous cells (Table 2).

Table 2. Total length of DAB reacting membranes per mitochondrial section and the surface area of mitochondria from tapetal and sporogenous tissues of BBF1 and BBS1 anthers at premeiotic stage. The readings are expressed as mean $\pm$ standard deviation of n mitochondria.

	Length (μm)	Surface area (μm^2)	n
Tapetal tissue			
BBF1	2.00 ± 0.96	0.29 ± 0.16	59
BBS1	1.97 ± 0.83	0.26 ± 0.12	61
Sporogenous tissue			
BBF1	1.67 ± 0.60	0.20 ± 0.10	63
BBS1	1.82 ± 0.70	0.22 ± 0.12	75

Localization of cytochrome c oxidase in anthers of male fertile and S-cytoplasmic male sterile Zea mays

At the mononucleate pollen stage, the DAB reaction product is deposited both in the intracristate spaces and in the matrix of mitochondria of pollen of male fertile maize anthers (Figs. 9 and 10). The organelles have many small cristae, are ellipsoidal and have a heavily stained matrix (Fig. 10). In some mitochondria an unevenly distributed reaction product is observed between the outer and inner limiting membranes. In pollen of cms-S anthers, however, a totally different

distribution of the DAB reaction product is observed, i.e. the osmiophilic precipitate is only localized between the inner and outer membranes of the mitochondria (Figs. 11 and 12). There is no reaction product in the matrix or at the cristae of the mitochondria. The organelles are spherical, have a lightly stained matrix and no DAB reacting cristae (Fig. 12). In male fertile anthers, formation of the DAB reaction product is completely inhibited by cyanide (Fig. 13).

The length of the DAB reacting membranes per mitochondrial section in pollen of N anthers is significantly higher than in mitochondria of S anthers (Table 3). The mitochondrial surface area of both maize types is not significantly different (Table 3).

Table 3. Total length of DAB reacting membranes per mitochondrial section and the surface area of mitochondria from pollen of N-type and S-type maize anthers at the mononucleate pollen stage. The readings are expressed as mean ± standard deviation of n mitochondria.

	Length (µm)	Surface area (µm²)	n
Normal plasmatype	6.26 ± 1.56	0.24 ± 0.08	34
Cms-S plasmatype	1.32 ± 0.34	0.29 ± 0.08	23

Discussion

Biochemical implications

The initial signs of deviating development of cms-type petunia anthers are apparent at the first stages of meiosis (Bino, 1985a). The first abnormalities are represented by the presence of large vacuoles in the cytoplasm of the tapetal cells (Bino, 1985b). Until early meiosis, microsporogenesis in cms petunia anthers proceeds normally and is indistinguishable from that in male fertile-type anthers. Correspondingly, during these early developmental stages, the biochemical characteristics of cox activity in male fertile and cms anthers are similar. The first differences in the total cox activity of Blue Bedder and Rosy Morn anther extracts occur at the meiotic and postmeiotic stages. The decrease in activity of cms-type anthers, corresponds with the decline in the total soluble protein content. Apparently, the differences in cox activity are a consequence of the proceeding process of degeneration of the tapetal and sporogenous tissues.

Figs. 3-8. Distribution of DAB reaction product in mitochondria (M) of petunia BBF1 and BBS1 tapetal cells at premeiotic stage of the meiocytes. All figures, Bar, 0.5 µm.

Fig. 3. BBF1 tapetal cell. Note that there is no deposition of reaction product at the membranes of the nucleus (N) and the plastids (P).

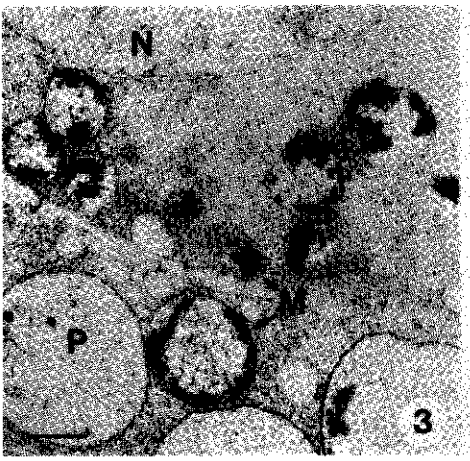
Fig. 4. BBS1 tapetal cell. As in male-fertile development (Fig. 3), the DAB reaction product is predominantly located in the intracristate spaces.

Fig. 5. BBF1 tapetal cell. Control section, formation of DAB reaction product is inhibited by the addition of KCN to the standard incubation medium. Mitochondria (M), Lipid droplet (L).

Fig. 6. BBF1 tapetal cell. Control section, incubated after short prefixation in glutaraldehyde. Note that there is no deposition of reaction product at the membranes of the mitochondria (M).

Fig. 7. BBS1 tapetal cell. The section was placed in the standard medium supplemented with antimycin A.

Fig. 8. BBF1 tapetal cell. Heat pretreatment disturbs the normal cellular structure. Mitochondria (M) exhibit a patchy distributed formation of DAB reaction product at the membranes.


N
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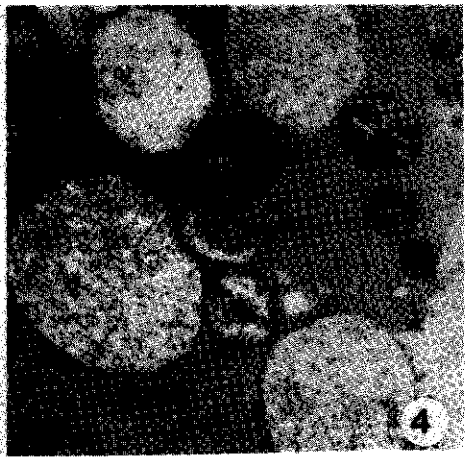
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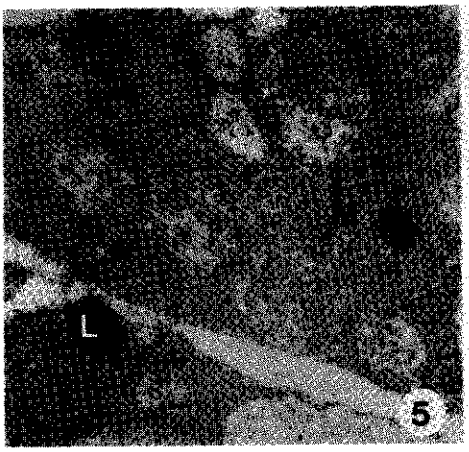
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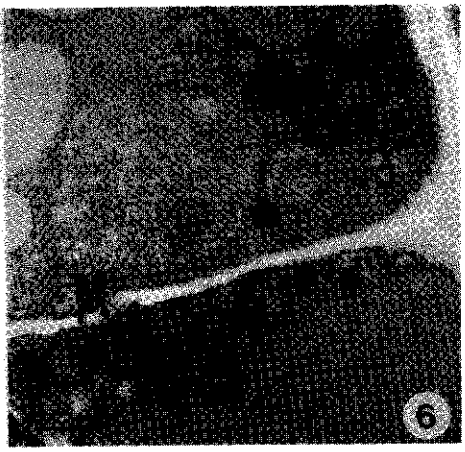
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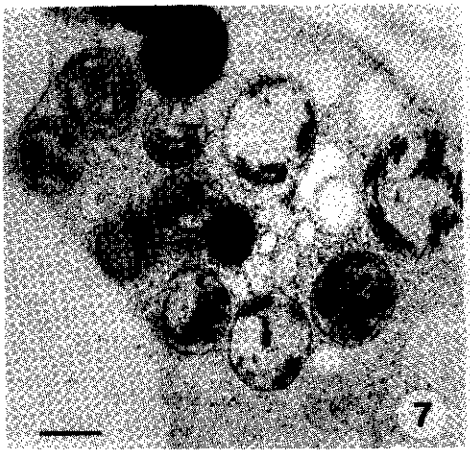
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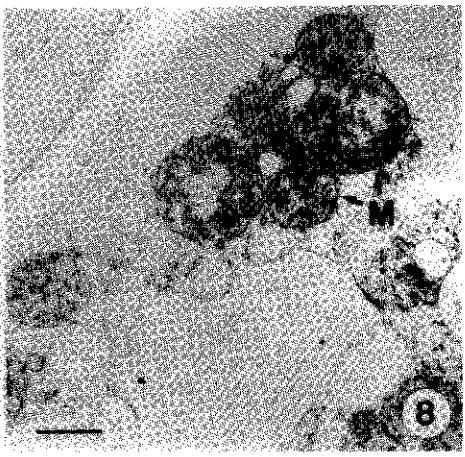
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In cms-S maize, Lee et al. (1980) and Colhoun and Steer (1981), revealed that the first signs of degeneration become apparent at the binucleate stage of pollen development. Just before anthesis, the pollen grains abruptly disintegrate while the other anther tissues remain unaffected. In cms-C anthers, the first symptoms of degeneration are observed in the tapetum at the tetrad stage of the sporogenous cells (Lee et al., 1979). The present study demonstrates that cox activity of cms-S and cms-C anthers is reduced in comparison with male fertile anthers from premeiosis onward. Hence, in both cms maize types, changes in cox activity occur before any visible signs of the degeneration of the anther tissues has become apparent. These results are in accordance with the observations of Watson et al. (1977) and Ohmasa et al. (1976) for some other cms maize genotypes. In all cms maize systems, the cox activity differs from premeiosis onward. Nevertheless, the phase at which the first symptoms of degeneration become evident varies from the early meiotic stages to the binucleate pollen stage. Apparently, some other factor determines the stage at which the consequences of the aberrant cox activity become structurally manifest.

Figs. 9-13. Distribution of DAB reaction product in mitochondria (M) of pollen of male fertile and cms-S Zea mays at the mononucleate pollen stage. All figures, except Fig. 12, Bar, 0.5 µm.

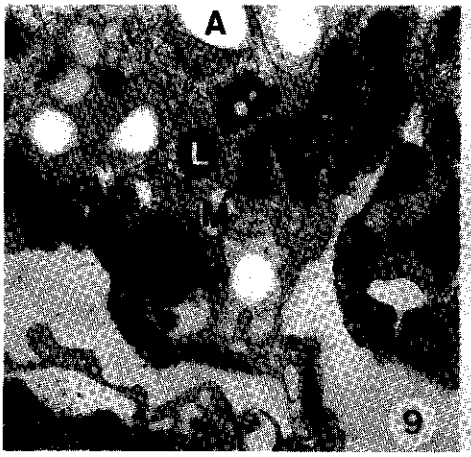
Fig. 9. Part of pollen grain of male fertile maize type. Lipid droplet (L), amyloplast (A).

Fig. 10. Higher magnification of a sector out of same anther tissue as Fig. 9. Note that DAB reaction product is both deposited in the intracristate spaces and in the matrix of the mitochondria.

Fig. 11. Part of pollen grain of cms-S maize type.

Fig. 12. Higher magnification of a sector out of same anther tissue as Fig. 11. DAB reaction product is deposited between the outer and inner limiting membranes, there is no reaction product in the matrix of the mitochondria. Bar, 0.25 µm.

Fig. 13. Detail of pollen grain of male fertile maize type. Control section, formation of DAB reaction product is inhibited by the addition of KCN to the standard medium.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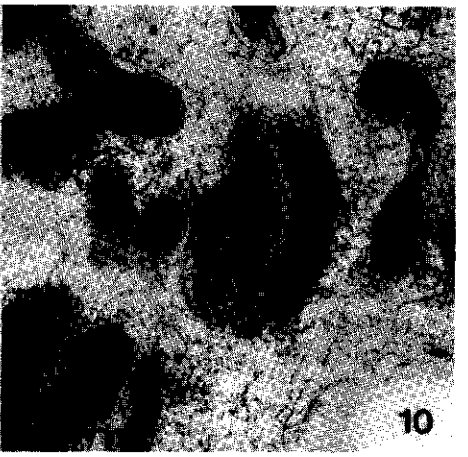
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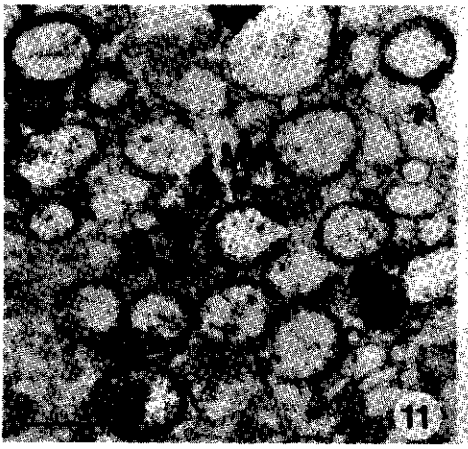
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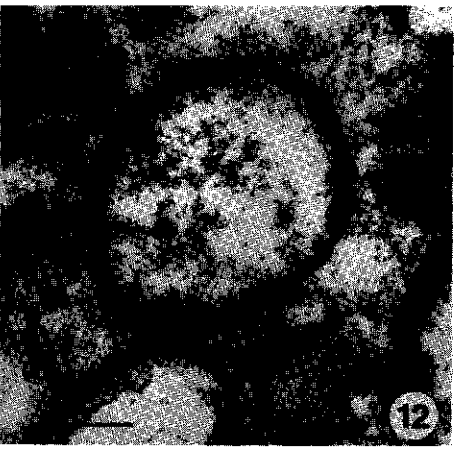
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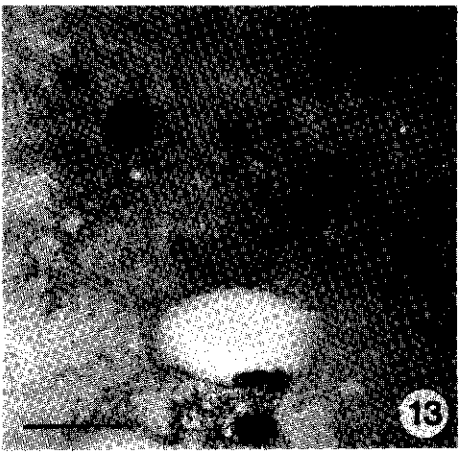
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Cytochemical implications

In maize and petunia, the cox activity is determined cytochemically at the stage of development just preceding the moment at which the initial signs of structural degeneration are evident. In both cms and male fertile petunia anthers, the DAB reaction product is deposited predominantly in the intracristate spaces of the mitochondria. Apparently, there is no change in cox activity between male fertile and cms petunia anthers at the early developmental stages. The situation in maize is totally different. In maize, the DAB precipitate is differently localized in mitochondria of N and cms-S anthers. In N-type mitochondria, an excess of the reaction product is found in the intracristate spaces and the mitochondrial matrix in pollen at the mononucleate stage. In cms-S mitochondria, oxidation of DAB is limited to the space between the inner and outer mitochondrial membranes. Correspondingly, the cms-type mitochondria exhibit a significantly smaller amount of the DAB reaction product as found in mitochondrial sections of fertile-type organelles. Since most of the DAB reaction product is deposited in the matrix of the mitochondria, the change in the length of the DAB reacting membranes (Table 3) is proportional to the biochemically established difference in the total cytochrome c oxidase activity of cms and male fertile maize anthers (Table 1). Apparently, the DAB reaction in mitochondria of the principal anther tissues of petunia and maize, affords a dependable criterion for indicating cox activity. In conlusion, in cms maize anthers, there are biochemical and cytochemical differences in cox activity at stages before the initial effects of pollen disintegration are manifest.

Specificity of the DAB reaction

Precipitation of DAB at the outer surface of the inner mitochondrial membranes is considered to be specifically due to the activity of cox (Seligman et al., 1968). This specificity is established in several ways. For instance, cox is sensitive to KCN and glutaraldehyde (Seligman et al., 1973). Correspondingly, the formation of the DAB reaction product is inhibited by short prefixation in these compounds. Other cytochromes may possibly participate in the oxidation of DAB. However, antimycin A which blocks the electron transport between cytochrome b and c, does not affect DAB precipitation (see Cammer and Moore, 1973). Nonetheless, the

specificity for cox is not completely certain. A critical point is, that in contradiction to the heat sensitivity of cox, we did not observe inhibition of the DAB oxidation by high temperatures. Öpik (1975) found this heat resistance also to occur in the mitochondria of rice coleoptiles and suggested the participation of other autooxidazible, heat stable cytochromes in the reaction. Nevertheless, Litwin (1979), who carefully analyzed the histochemistry and cytochemistry of DAB, concluded that DAB localizes cytochrome c and demonstrates cox.

Molecular aspects

Cms-S plants are characterized by having in their mitochondria abundant quantities of two plasmid-like DNAs called S-1 and S-2 (Pring et al., 1977). Recently, S-1 and S-2 have recieved much attention, because of the possible involvement with the cms-S sterility mechanism (Laughnan and Gabay-Laughnan, 1983). Additionally, Pring and Levings (1978) already showed that mitochondrial DNAs isolated from vegetative cells of normal and cms-S maize strains possess distinctive endonuclease restriction characteristics. Nevertheless, the exact nature of the mitochondrial encoded products which are responsible for the non-formation of functional pollen is still not elucidated. The present report reveals consistent differences in the mitochondrial cox activity in the pollen of cms-S maize anthers. The results suggest that the aberrant mitochondrial DNA composition is functionally associated with deviations in the cox complex. Such differences in the mitochondrial enzyme activity are, however, not found in vegetative parts of the plants (Ohmasa et al., 1976; Watson et al., 1977). Apparently, the defects in the mitochondrial genome are only expressed in specific anther tissues at certain moments of development.

The results obtained in the present investigation indicate that differences in cox activity are apparently not involved in the non-formation of functional pollen in cms petunia. Currently, it is still uncertain which mitochondrial encoded protein is responsible for the developmental abnormalities in the cms petunia anthers. Deviations in mitochondrial DNA arrangement are found in organelles purified from suspension cultured cells (Kool et al., 1985; Boeshore et al., 1985). Certainly, a cms-specific variation in gene expression may be expressed in these vegetative cells, as well as in reproductive tissues. But as in

maize, it may well be that the essential tissues to examine are the reproductive structures undergoing the developmental alterations. Moreover, the variation in the mitochondrial DNA sequence is not necessarily functionally associated with the male sterility trait. It is possible that the change in the DNA arrangement is expressed in the suspension cultured cells but not in the reproductive tissues. Hence, a better understanding of cms may only be obtained by comparing the mitochondrial translation products in the specific anther tissues of cms and male fertile plants at various stages of development.

Acknowledgment

The authors wish to thank dr. F. A. Hoekstra for his valuable suggestions during the work and for critically reading the manuscript.

Chapter 6
Energy Metabolism in Petunia hybrida Anthers: A Comparison Between Fertile and Cytoplasmic Male Sterile Development

Introduction

Cytoplasmic male sterility (cms) in petunia is associated with aberrations in mitochondrial DNA (Kool et al., 1985). Cms-specific differences in mitochondrial DNA arrangement are revealed in mitochondria purified from vegetative cells (Kool et al., 1985). However, the structural effects of cms-plasmatype only become manifest during anther development (Bino, 1985a). The mitochondrial genome encodes for a number of proteins, which are involved in energy-generating processes (Dillon, 1981). Hence, defects in mitochondrial DNA may affect the energy supply of cells containing cms-plasmatype.

Many metabolic reactions are depending on the energy status of the cell. An index of the energy status is the adenylate energy charge: AEC= [(ATP)+(ATP+ADP)]/2(ATP+ADP+AMP). According to Atkinson (1968), AEC ratio modulates activity of various metabolic sequences related to energy utilization and regeneration. The AEC can have values ranging from 0 (all AMP) to 1 (all ATP), but in normally metabolizing cells and tissues the AEC value is usually higher than 0.8 (Pradet, 1982). In the present study, the energetic balance of anthers of fertile and cms petunia is determined at different stages of flower bud development.

Material and methods

Plant material

Two idiotypes of Petunia hybrida are used in this study, i.e. the male fertile cv. "Blue Bedder" (BBF), and the cms "Blue Bedder" (BBS) described by Van Marrewijk (1969). BBF and BBS are highly isogenic. The plants are cultivated in a growth chamber under a regime of 16 h light, 8 h dark at 17 °C.

Extraction methods

In order to determine quantitatively the amounts of adenine nucleotide mono-, di- and triphosphates, we used perchloric acid to inactivate hydrolytic enzyme activity. A problem with the PCA extraction method is, that the activity of phosphatases (which hydrolyze ATP and ADP into AMP) may partly be restored when the extract is neutralized (Pradet, 1982). As a consequence, adenylate ratios (ATP/ADP, ATP/AMP or AEC) may be low compared to results obtained with other extraction methods, such as formic acid dissolved in ethanol or trichloroacetic acid dissolved in diethylether (Pradet, 1982). However, when these methods were applied, quantitative recoveries of ATP, ADP and AMP were not as satisfactory as those obtained with the perchloric acid extraction (Ikuma and Tetley, 1976). Hence, for studies whith small amounts of plant material, perchloric acid turned out to be more suitable. According to Ikuma and Tetley (1976), ATP hydrolyzing activity in solanaceous plants is optimal at pH 5, whereas no activity was detected below pH 3 and above pH 9. To circumvent the hydrolysis of ATP and ADP, we followed the extraction procedure as proposed by Ikuma and Tetley (1976), and maintained the pH of the tissue extract below 3 throughout the extraction procedure to adjust the pH to 8.5 prior to the quantitative assay.

Plant material for extraction was prepared in the following manner. The length of a dissected flower bud was measured. One anther was used to determine the stage of development. The other four anthers were directly frozen in liquid N_2, whereafter they were pulverized in 65 μl ice-cold 1 N $HClO_4$ (final pH below 3). After 10 min at 4 ^{o}C, the mixture was centrifuged at 13,000 g for 3 min; 40 μl of the supernatant was pipetted into a test tube containing 240 μl buffer (0.06 M Tricine/$MgSO_4$, with 1% (w/v) $KHCO_3$, pH 7.6). The pH of the extract was directly adjusted to 8.5 with 1 N KOH, and the $KClO_4$ was pelleted in the cold (2,500 g, 1 min).

Determination of adenine nucleotide levels

For adenine nucleotide phosphates determination, we used the methods described by Hoekstra (1979) and adapted them for small amounts of plant material. (ATP + ADP), and (ATP + ADP + AMP) were determined after enzymatic conversion of ADP and AMP into ATP. For (ATP + ADP) determination, 40 μl of the extract was diluted in buffer (40 μl 0.06 M

Tricine/$MgSO_4$, pH 7.6), containing 40 µl 0.125% (w/v) hydrated phosphoenolpyruvate (Sigma) and 2.75% (v/v) pyruvate kinase (EC 2.7.1.40) (Sigma). For (ATP + ADP + AMP) determination, 1.6% (v/v) myokinase (EC 2.7.4.3) (Boehringer) was added to the above mentioned reaction mixture. The extracts were incubated for 30 min at 35 °C. The resulting ATP was determined by the luciferin - luciferase assay, using a luminometer 1250 (LKB - Wallac). Twenty µl of a concentrated ice-cold firefly lantern extract (Boehringer), was injected into a small vial containing 20 µl of the ATP extract and 0.3 ml 0.02 M Tricine/$MgSO_4$ buffer (pH 7.6). Exactly 10 s after injection, bioluminoscence was measured for 6 s at 18 °C. Samples with and without an internal standard were alternately counted. In each extract, the ATP, (ATP + ADP), and (ATP + ADP + AMP) were assayed 3 times. The amounts of ADP and AMP were determined by difference.

Respiration measurements

O_2 consumption of anthers was measured polarographically with a Clark-type O_2 electrode. Eight to 15 anthers were inserted in a reaction chamber containing 1 ml 0.1 M mannitol and 0.5 mM $CaSO_4$ at 24 °C. O_2 consumption was measured for 15 min and respiration rates were calculated according to Hoekstra (1979).

Results and Discussion

Effect of plasmatype on anther fresh weight

First structural aspects of abnormal anther development in BBS plants become apparent at leptotene stage of prophase I. Initial aberration is represented by the presence of large vacuoles in the cytoplasm of tapetal cells (Bino, 1985b). At meiosis I, sporogenesis arrests and meiocytes and tapetal cells degenerate. During premeiosis, however, BBF and BBS development is indistinguishable (Bino, 1985a). Table 1 indicates that at similar flower bud length anther fresh weights of fertile and sterile plants are similar until meiosis. As degeneration progresses, anther fresh weight of sterile plants decreases in comparison to fertile-type anthers. The relation between stage of development and flower bud length is in accordance with the data obtained by Van Marrewijk and Suurs (1985).

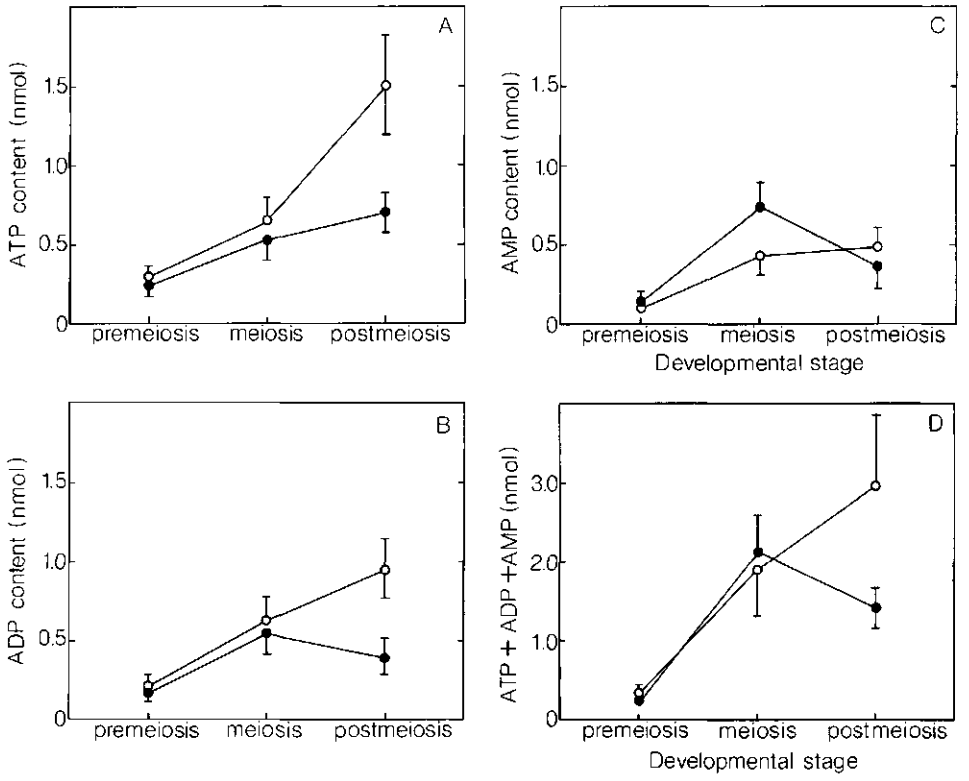
Table 1. Effect of plasmatype on anther fresh weight at different stages of development (values are means $\pm$ SE, of 500 to 1,000 anthers). The data are representative of a number of experiments.

	Developmental stage	Bud length (mm)	Anther fresh weight (mg)
BBF	premeiosis	1.0 $\pm$ 1.7	0.15 $\pm$ 0.02
	meiosis	1.8 $\pm$ 2.8	0.56 $\pm$ 0.04
	postmeiosis: tetrads and microscopes	2.9 $\pm$ 4.5	1.08 $\pm$ 0.07
BBS	premeiosis	1.0 $\pm$ 1.7	0.15 $\pm$ 0.03
	meiosis: first aspects of degeneration	1.8 $\pm$ 2.8	0.42 $\pm$ 0.05
	postmeiosis: sporogenous and tapetal tissues degenerated	2.9 $\pm$ 4.5	0.54 $\pm$ 0.06

Adenine nucleotide levels in BBF and BBS anthers

ATP and ADP contents in BBF anthers increase from premeiosis to postmeiosis (Fig. 1 A and B). At premeiosis and meiosis, ATP and ADP levels in BBF and BBS anthers are similar. During postmeiosis, ATP and ADP contents in BBS anthers are significantly lower than the amounts found in fertile-type anthers. In contrast, the AMP contents in BBS anthers exhibit a significant increase during meiosis (Fig. 1 C). This increase is coincident with degeneration of sporogenous and tapetal tissues. When the process of abortion is completed, the AMP contents in BBS anthers decline sharply. Total amounts of adenine nucleotides in BBF and BBS anthers are similar at premeiosis and meiosis (Fig. 1 D). At postmeiosis, (ATP + ADP + AMP) levels in BBS anthers decrease significantly in comparison to fertile-type anthers. The change in total adenine contents is comparable with the observed differences between fresh weights of BBF and BBS anthers.

Dissimilarities in adenylate contents between BBF and BBS anthers are associated with degeneration of sporogenous and tapetal tissues. At premeiosis, ATP, ADP and AMP levels are similar.



Figs. 1 A–D. Adenine nucleotide phosphates contents in BBF (-0-) and BBS (-●-) anthers at different stages of development. Each point represents the mean (± SE) of 7 to 11 separate extractions of four anthers of one flower bud. The fifth anther is used to determine the stage of development. **A**, ATP contents. **B**, ADP contents. **C**, AMP contents. **D**, (ATP + ADP + AMP) contents.

AEC ratios of BBF and BBS anthers

To ascertain the effectiveness of the extraction method, we determined adenylate levels in leaf, and in combined leaf and anther extracts. AEC ratio obtained in petunia leaf tissue is high (AEC = 0.83 ± 0.05; n = 2). Combined leaf and anther extracts did not show any evidence for an anther specific increase in phosphatase activity. Table 2 indicates that AEC ratios of fertile- and sterile-type anthers are similar during premeiosis and postmeiosis. At the meiotic interval, AEC values of BBF and BBS anthers decrease significantly. The drop in AEC ratio of BBS anthers exceeds the decrease in BBF anthers ($P \leq 0.025$). The difference in AEC ratios of fertile- and sterile-type anthers is correlated with the increase in AMP contents of BBS anthers at meiosis (Fig. 1 C).

At all stages of development, AEC values of BBF and BBS anthers are low in comparison to the ratios obtained in petunia leaves and other normally metabolizing cells or tissues (Pradet, 1982). According to Atkinson (1968), low AEC ratio may exhibit a disparity between energy-generating and energy-utilizing systems. Metabolic activity of anther tissues is reported by Porter *et al.* (1983), who found that the amounts of mRNA in *Lilium* meiocytes varied considerably as meiosis progressed. Williams and Heslop-Harrison (1979) observed a similar variation in the synthetic activity of *Lilium* and *Rhoeo* tapetal cells during meiosis. Possibly, the low AEC ratios of petunia anthers reflect the special metabolic state of the tapetal and sporogenous tissues.

Table 2. AEC values of fertile and sterile plants at different stages of development. Values represent the mean ± SE of n extractions.

Stage	BBF	n	BBS	n
premeiosis	0.64 ± 0.04	11	0.61 ± 0.03	11
meiosis	0.56 ± 0.04	7	0.46 ± 0.02	9
postmeiosis	0.69 ± 0.04	10	0.67 ± 0.04	8

Respiratority rate of BBF and BBS anthers

O_2 consumption of BBF anthers increases during sporogenesis (Table 3). At premeiosis and meiosis, respiration rates of BBS and BBF anthers are similar. However, at postmeiotic stages, O_2 consumption of BBS anthers is significantly reduced.

Table 3. Effect of plasmatype on O_2 consumption (pmol/min/anther) at different stages of development. Values represent the mean $\pm$ SE of n separate measurements.

Stage	BBF	n	BBS	n
premeiosis	50.3 $\pm$ 9.7	3	51.8 $\pm$ 4.7	2
meiosis	81.4 $\pm$ 4.7	4	74.6 $\pm$ 8.7	3
postmeiosis	91.7 $\pm$ 10.8	5	62.3 $\pm$ 1.6	2

Conclusions

Before the structural aspects of degeneration in BBS anthers become apparent, there is no difference in energy metabolism between fertile- and sterile-type anthers. At premeiosis, adenylate contents, AEC ratios, respiration rates and fresh weights are similar in BBF and BBS anthers. At meiosis, however, AMP levels of BBS anthers increase and the corresponding AEC values decline. During this stage, sporogenous and tapetal tissues degenerate. Apparently, the process of degeneration disturbs the equilibrium in AMP contents. At postmeiosis, sporogenous and tapetal tissues are aborted and the metabolic activity of sterile anthers is reduced.

Acknowledgments

We thank Mr. P.A. Van Snippenburg for drawing the figures. We are grateful to Prof. dr. M.T.M. Willemse and Dr. F.A. Hoekstra for critically reading the manuscript and many helpful discussions. RJB thanks the "Fonds Landbouw Export Bureau 1916/1918" for grant No. 95(B).

Chapter 7
General Discussion

In Petunia hybrida, the genetic determinants responsible for cms are presumably carried by the mitochondrial DNA. As discussed in Chapter 1, the role of the mitochondrial genome in plant cells appears limited to the synthesis of 10-12 polypeptides. The majority of these mitochondrial encoded polypeptides are components of complexes which are responsible for key steps in the process of oxidative phosphorylation and the synthesis of ATP (Leaver and Forde, 1980). Hence, an active mitochondrial translation system is essential for energy generating processes. In sterile somatic hybrids of petunia, Boeshore et al. (1985) identified an aberrant mitochondrial DNA arrangement segregating with the cms plasmatype. However, the authors did not establish whether the deviation in the mitochondrial DNA composition was functionally associated with the non-formation of viable pollen. Moreover, the exact nature of the product, encoded by the aberrant part of the mitochondrial DNA, is still not elucidated. Nevertheless, it may be postulated that the aberrant mitochondrial genome of cms plants codes for components of one of the protein complexes which are involved in the synthesis of energy-rich products. Correspondingly, these deviations may adversely affect the general energy status of cms cells. In cms petunia, the initial symptoms of aberrant development are found in the tapetum. There are at least two explanations for this tissue specific character of cms. The first possibility is, that the aberrant mitochondrial gene is expressed only in the tapetal cells at a certain stage of development. An alternative explanation is, that the mitochondrial deficiency is expressed in all cells of a cms plant but becomes critical only in the tapetal tissue. In the present chapter, both possibilities will be discussed in greater detail.

The first symptoms of aberrant development in cms Petunia hybrida cv. Blue Bedder are found in the tapetal tissue (Chapter 2). The initial

deviation is observed at the leptotene stage of the meiocytes. In isogenic male fertile plants, this stage is characterized by the enlargement of the tapetal volume and the occurence of a nuclear division in most of the tapetal cells. D'Amato (1984) correlated the multiplication of the genome in the reproductive tissues of a plant with the augmentation of the transcriptional and translational machinery of the cells. Also in the tapetal tissues of an anther, karyokinesis is associated with a vigorous RNA and protein synthesis (Shivanna et al., 1979). Apparently, in anthers of cms petunia, the normal development is impaired at the moment at which the tapetal function requires a considerable rise of the metabolic activity of the cells. Possibly, in anthers of cms petunia, the mitochondrial energy production is insufficient to meet the energetic demands for the normal functioning of the tapetal cells.

The first aberrations in cms petunia plants are, however, not always found at the start of the prophase stage of the meiocytes. Depending on the plasmagene, the initial deviations may become apparent from any period between the early meiotic phases until the end of spore maturation (Izhar, 1977). The variation may correspond with the assumption that there are several stages during anther development which impose a stress situation on the energy production of tapetal cells. Indications for the stage specific augmentation in cellular activity are found by some seperate lines of evidence. For instance, Williams and Heslop-Harrison (1979) have found a massive protein synthesis in the tapeta of Rhoeo spathacea and Lilium longiflorum during the diplotene and the tetrad stage of the sporogenous cells. Raghavan (1981) observed a transient RNA accumulation in the tapetum of Hyoscyamus niger from the end of the tetrad stage until the degeneration of the tapetal tissue. Furthermore, morphometric analysis in Zea mays, revealed a 40-fold increase in the number of mitochondria per tapetum cell between the diplotene and the end of tetrad stage (Lee and Warmke, 1979). Additionally, at the meiotic stages, the adenylate energy charge values of cms and male fertile petunia anthers are low in comparison to the ratios obtained in leaves and other normally metabolizing cells or tissues (Chapter 6). The adenylate energy charge is an index of the energy status of a cell, and the low ratios possibly reflect the special metabolic state of the petunia anther tissues. Finally, Maheshwari and Prakash (1965) have found a high

respiratory rate in anthers of Agave americana between early meiosis and the tetrad stage. From premeiosis onward, a similar increase in oxygen consumption is observed in anthers of Lilium longiflorum (Erickson, 1947), Trillium erectum (Stern and Kirk, 1948), and Petunia hybrida (Chapter 6). The latter observations, however, do not indicate which tissues of the anther contribute to the respiratory rate and at what relative levels. Nevertheless, Heslop-Harrison (1972) already noted that: 'In view of the undoubted metabolic activity of the tapetum, it is entirely possible that the respiration of this tissue is always the dominant factor when observations are made on the intact organ'. The stage specific differences in metabolic activity of tapetal tissues correlate with the observation that the tapetal functions are staggered in time (Pacini et al., 1985). For instance, the production and release of callase is limited to the tetrad stage, the formation of exine precursors is restricted to the free microspore stage, and the synthesis of locular fluid is confined to the stages between the start of meiosis and the first haploid mitosis (Pacini et al., 1985). Additionally, in petunia, esterase activity is found to accumulate in the outer tapetal layer from late prophase till the early microspore stage (Chapter 4).

Abnormalities in tapetal development, ultimately resulting in male sterility, are determined by the nuclear background, the environmental conditions, and the plasmatype of the plant. All these factors may influence the metabolic state of the tapetal cells. Effects of nuclear genes are found in genic male sterile plants. In these plants, the initial symptoms of abnormal development are generally observed in the tapetal tissue (Gottschalk and Kaul, 1974). For instance, in genic male sterile soybean, vacuolation of the tapetal cells at prophase stage of the meiocytes is the earliest sign of abnormality (Graybosch et al., 1984). Bennett (1976) suggested that the genetic background plays a major role in the initiation, the duration, and the synchronization of meiosis. Although it is not clear till what extent these factors influence tapetal functioning, it may be presumed that they also affect the metabolic state of the tapetal cells and that deviations may induce abnormalities in the tapetal development.

In various plant species, nutritional deprivation, temperature stress, water stress, and an inadequate light regime may adversely affect

the outcome of pollen formation (see Chapter 1). In most cases these environmental stress conditions initially induce abnormalities in the tapetal cell development. Furthermore, the application of auxins, ethylene and abscisic acid sometimes induces male sterility (Heslop-Harrison, 1972). The cytological effects of hormonal induced male sterility are described in Triticum aestivum (Saini et al., 1984). The authors found that the exogenous application of abscisic acid initially resulted in the degeneration of the tapetal tissue. Possibly, it may be stated that conditions that adversely affect the energy status of a plant or individual flower may cause pollen sterility. Since the tapetal tissue is most vulnerable for the perturbations in the energetic balance, the unfavorable circumstances initially induce aberrations in the tapetal development. Other plant parts are much more tolerant for the stress conditions. For instance, in Triticum aestivum, Saini et al. (1984) showed that a short episode of water deficit causes male sterility whereas there is no effect on female fertility. Additionally, in rice, anther development is severely depressed by environmental stress conditions while female reproductive tissues are hardly affected (Nishiyama, 1984).

The influence of the cytoplasm on tapetal development is exemplified in plants with cms plasmatype. In these plants, tapetal malfunctioning is possibly correlated with defects in the mitochondrial synthesis of adenine nucleotide phosphates. The total synthesis of energy-rich products in plant cells, however, is not only provided by the mitochondria but also by the chloroplasts. In fact, in the light the mitochondrial oxidative phosphorylation is possibly suppressed and the production of ATP by chloroplasts considerably exceeds that by mitochondria (Hampp et al., 1984). Only in the dark, the ATP requirements in the cytosol are primarily met by the mitochondrial oxidative phosphorylation (Stitt et al., 1982). At the early stages of flower bud development, the reproductive organs are covered by several leaflet structures. Hence, at these stages, the anther tissues are readily concealed from the light. Correspondingly, with the exception of the parietal wall layers, the anther tissues generally do not contain any active chloroplasts. As demonstrated in Chapter 3, the tapetal cells of petunia include numerous plastids. The organelles, however, have only a moderately developed lamellar organization. Conclusively, it may be assumed that the energy production in tapetal

cells is mainly regulated by the mitochondria. Likely, there is considerable transfer of energy-rich products towards the specific anther tissues. However, not much is known on the interchange of such products between the tapetal and other anther tissues at the early stages of development. Reznickova and Willemse (1980), suggested that, at later stages of development, the parietal wall layers of Lilium function as the main source of lipids, carbohydrates, and starch for the developing microspores.

It may be questioned whether the development of other plant tissues is evenly disturbed by the mitochondrial defects. Several authors identified pleiotropic effects of the cms plasmatype in cms forms of various species. For instance, Panayotov (1980) associated the non-formation of functional pollen in Triticum aestivum with detrimental effects on general aspects of plant development. The author found that culm length, ear length, number of spikelets per spike, and flag leaf length were adversely influenced by the cms plasmatype. In Daucus carota the flowers of cms plants, may differ in appearance, produce little nectar and aroma, and therefore, compete poorly for the attention of pollinators in comparison with flowers of male fertile plants (Erickson et al., 1982). Probably, the best described effects of cms on morphological characters other than pollen development, are reported by Duvick (1965) in his review on cms in Zea mays. Plants of the cms-T type of maize, are significantly shorter and have a reduced number of leaves as compared with male fertile plants. Nevertheless, the grain yield of cms plants is sometimes higher than their fertile counterparts. Duvick (1958) explained these differences by the sparing effect of pollen sterility on the total energy status of the plant. Correspondingly, in natural populations of Plantago lanceolata, differences were found in seed production and weight per seed in favor of the male sterile type (Van Damme, 1983). Moreover, in one particular experiment the male sterile plants tended to have a somewhat higher survival rate than the male fertile counterpart. Notwithstanding that, the tendency was only found in one of two plots while the comparison of the survival rates is severely impeded by the unknown effects of the environmental conditions. Hence, in cms forms of most species, the plasmatype appears only to influence the pollen grain formation. Likewise, external stress conditions may selectively influence pollen formation

without inducing effects on other aspects of plant development.

It may be postulated that the differences in mitochondrial energy synthesis between cms and male fertile plants only become apparent in cells without chloroplasts at specific moments of energetic stress. This hypothesis is rather difficult to prove because of the complex assessment of the *in planta* mitochondrial ATP production in specific tissues at certain stages of development. Van Der Plas and Otto (1985) however, simulated the anther situation *in vitro* by the cultivation of isolated vegetative cells under external stress conditions. The authors found a variation in the growth characteristics of batch cultured cells of male fertile and cms *Petunia hybrida* cv. Rosy Morn, and preliminary concluded that the cms-type cells grow less economically and 'spill' a part of the ATP produced. Additional differences between the cellular energy metabolism of cms and male fertile plants are observed in isolated mitochondria from vegetative tissues of different origins. For instance, in a cms form of *Vicia faba*, Boutry and Briquet (1982) found a smaller capacity of the respiratory chain in mitochondria isolated from seedling tissues. Moreover, the capacity of the cyanide resistent alternative electron transport pathway is higher in isolated mitochondria of cms *Vicia faba* and cms *Oryza sativa* as compared with the male fertile counterparts (Briquet, personal communication). A similar observation is made in batch cultured cells of petunia (Van Der Plas and Otto, 1985). The higher capacity of the alternative pathway in cms mitochondria represents a less efficient generation of ATP. These results are, however, in contrast to Van Marrewijk (personal communication), who found no difference in the percentage of cyanide resistent electron transport in anther suspensions of cms and male fertile petunia. Additionally, as discussed in Chapter 5, aberrations in cytochrome c oxidase activity and other mitochondrial redox processes are sometimes correlated with cms.

In conclusion, there are some indications that the differences between cms and male fertile plants are integral present in all mitochondria of a cms plant but only become critical in the tapetal tissues at moments of energetic stress. Nevertheless, it cannot be ruled out that there are mitochondrial encoded polypeptides which are translated in specific cells of the anther. In fact, the variation in polypeptide composition between cms and male fertile plants may, for instance, only

become apparent in the tapetal tissue. Additionally, it may well be that a mitochondrial function which is satisfactory for vegetative growth becomes defective during anther development due to improper interaction with a nuclear gene that is expressed only in the male reproductive tissues.

Obviously, consistent differences between cms and male fertile plants may only be detected by an appropriate technique visualizing the cms specific deviations in the mitochondrial encoded polypeptides. Recently, Van Lammeren et al. (1985) used immunocytochemical methods for the quantitative determination of tubulin in the pollen of Gasteria verrucosa. After the raising of antibodies against mitochondrial encoded polypeptides, similar methods could be applied for the determination of relative amounts of cms-specific polypeptides in individual anther tissues of cms and male fertile petunia at different stages of development. Presumably, this combination of biochemical and cytological methods will help to bring us nearer to the unraveling of the functional base of cytoplasmic male sterility.

Summary

This thesis presents an analysis of the structural and histochemical aspects of cytoplasmic male sterility (cms) in Petunia hybrida. In petunia and in other crops, cms is the most commonly used tool for hybrid seed production. Application of the trait makes hybrid seed production possible without the need of emasculation of the maternal line. However, in spite of its economic importance, little is known on the primary causative factor and the initial step of pollen abortion in cms plants. Insights in the initial effects of cms may lead to a more comprehensive understanding of the regulation and expression of male sterility controlling genes, and, additionally, may possibly provide strategies for the introduction or induction of male sterility in crops in which cms systems are not available.

In the first Chapter, some molecular aspects of cms are evaluated. Several lines of evidence indicate that the genetic determinants responsible for cms are carried by the mitochondrial genome. The mitochondrial involvement is found in a variety of plant species, including Petunia hybrida. Most of the mitochondrial encoded polypeptides are components of complexes which are responsible for key steps in the process of oxidative phosphorylation and the generation of ATP. Correspondingly, mitochondria isolated from tissues of cms plants, may code for an aberrant polypeptide composition of components of one of these complexes. Nevertheless, more information on the expression of mitochondrial genes in different anther tissues at various stages of development is necessary before we can conclude whether or not the deviations in mitochondrial DNA are functionally associated with the non-formation of viable pollen.

The initial abnormalities in anther development of cms plants are generally found in the tapetal tissue. Also in the cms form of Petunia hybrida cv. Blue Bedder (BBS), the first symptoms of deviation are found

in the tapetum (Chapter 2). Light microscopical analysis shows, that in BBS anthers, the tapetal breakdown begins at the prophase stage of the meiocytes. At the preceding stages of development, microsporogenesis in BBS anthers is normal and indistinguishable from the development in the male fertile counterpart (BBF). At the ultrastructural level, the initial aberration of BBS anthers is represented by the presence of large vacuoles in the cytoplasm of the tapetal cells (Chapter 3). At the leptotene stage of the meiocytes, these vacuoles are the first symptoms of degeneration. At later stages, the tapetal and sporogenous cells are highly distorted, the nucleus is disrupted and the cytoplasm disorganized. Mitochondria and plastids degenerate and many lipid droplets are present.

Chapter 4 describes the way in which the biochemical and histochemical aspects of an enzyme system are influenced by the degeneration of the tapetal and sporogenous tissues. The Chapter gives information on the isoenzyme pattern, the activity, and the localization of esterases in anther tissues of cms and male fertile petunia cultivars. Esterases are rather unspecific, nuclear encoded enzymes occuring in all plant parts. The biochemical data show that, from the early meiosis onward, esterase activity in cms-type anthers remains at a low level and hardly any new isoenzyme bands show up as compared to the situation in the male fertile counterpart. The histochemical determinations reveal, that in male fertile-type anthers, esterase activity is concentrated in the outer tapetal layer at late prophase and that it accumulates there till the early microspore stage. In anthers of cms plants, esterase accumulation in the tapetal cells ceases at the moment that tapetal breakdown becomes evident. These results suggest that the differences in total esterase activity and esterase isoenzyme patterns are an effect rather than a cause of the failing pollen formation.

In cms forms of different species, aberrations in cytochrome c oxidase activity and other mitochondrial redox processes are associated with the cms plasmatype. A biochemical determination of the cytochrome c oxidase activity in anthers of Petunia hybrida and Zea mays is given in Chapter 5. The biochemical analysis is combined with a cytochemical localization of enzyme activity in mitochondria of sporogenous and tapetal tissues in both species. The data show that in anthers of different cms maize strains, the cytochrome c oxidase activity is reduced in comparison

with the level found in male fertile-type anthers. Additionally, there are consistent cytochemical differences in the mitochondrial organization of cytochrome c oxidase activity between pollen of cms-S and male fertile maize plants. The aberrations in enzyme activity are observed at stages of development at which the structural aspects of degeneration are not yet evident. In fact, the deviation in cytochrome c oxidase may represent the initial symptom of male sterility in this maize type. Contrarily, in petunia, the first detectable differences in the mitochondrial enzyme activity occur only after the initial effects of tapetal degeneretion are apparent. Hence, in petunia, the decline in cytochrome c oxidase activity is the result rather than the cause of the proceeding process of degeneration.

In Chapter 7 it is postulated that the cms specific deviations in the mitochondrial genome induce alterations in protein complexes which are essential for energy generating processes. Possibly, these aberrations adversely affect the energy status of cms cells. However, BBF and BBS plants possess similar growth characteristics, and, apparently, the viability of plants with cms plasmatype, is not diminished by the mitochondrial defects. In fact, abnormalities in the development of cms plants are only observed in particular anther tissues. These results may suggest that the aberrations in the mitochondrial genome are only expressed in the tapetal or sporogenous tissues at certain moments of development. However, this assumption is inconsistent with the fact that deviations in mitochondrial products are sometimes found in organelles isolated from vegetative parts of the plant. An alternative explanation for the tissue specific character of cms is, that the degeneration of the anther tissues is initiated by the specific metabolism of the cells. The adenylate energy charge ratios of petunia anther tissues is discussed in Chapter 6. As compared with petunia leaf tissue, the results give evidences for the particular metabolic state of the tapetal and sporogenous tissues. Examples of the metabolic activity in anthers of other plant species are evaluated in Chapter 7. Furthermore, the structural analyses as presented in the second and the third Chapter of this thesis, reveal that the cms petunia anther development is distorted at the moment at which there is a considerable rise in the metabolic activity of the tapetal cells of the male fertile counterpart. Possibly,

during moments of energetic stress, the mitochondrial synthesis of energy-rich products in tapetal cells of cms petunia is insufficient to meet the energetic demands for the normal functioning of the cells at that stage. Hence, as a result of defects in the mitochondrial genome, the tapetal, and consequently, the sporogenous tissues degenerate.

Samenvatting

In dit proefschrift worden de resultaten gepresenteerd van een analyse van een aantal structurele en histochemische aspecten van cytoplasmatische mannelijke steriliteit (cms) in Petunia hybrida. Cytoplasmatische mannelijke steriliteit is een algemeen toegepast hulpmiddel bij de produktie van zaaizaad van hybride rassen. Toepassing van de cms eigenschap maakt het tijdrovende en kostbare emasculeren van de moederplanten overbodig. Ondanks het economisch belang van cms zijn er toch nog slechts weinig gegevens bekend over de primaire reacties in de helmknoppen die uiteindelijk leiden tot het niet vormen van functioneel stuifmeel. Meer kennis omtrent de eerste afwijkingen vergeleken met de ontwikkeling van de normale mannelijke fertiele plant, kan ons inzicht in de regulatie en expressie van steriliteit inducerende genen verbeteren. Bovendien kan deze kennis methoden opleveren om cms te introduceren of te induceren in gewassen die deze eigenschap nog niet bezitten.

In het eerste hoofdstuk wordt een aantal moleculaire aspecten van cms belicht. In het algemeen neemt men aan dat de cms eigenschap op het mitochondriële DNA is gelokaliseerd. De samenhang tussen veranderingen in het mitochondriële genoom en cms is voor een aantal plantesoorten, waaronder Petunia hybrida, aannemelijk gemaakt. De meeste van de mitochondrieel gecodeerde eiwitten vormen onderdelen van complexen die verantwoordelijk zijn voor belangrijke stappen in het proces van de oxydatieve fosforylering en de synthese van ATP. In sommige gevallen is een correlatie gevonden tussen het afwijkende DNA van 'cms' mitochondriën en het eiwitpatroon van een van deze complexen, Echter, om te bepalen of de gevonden afwijkingen ook daadwerkelijk betrokken zijn bij het niet ontstaan van functioneel pollen is er meer informatie nodig omtrent de expressie van de mitochondriële genen tijdens de ontwikkeling van de verschillende helmknopweefsels.

De eerste afwijkingen in de ontwikkeling van de anthere van een cms plant blijken algemeen in het tapetum gevonden te worden. Ook in de cms vorm van Petunia hybrida cv. Blue Bedder (BBS), treden de primaire symptomen van de abnormale ontwikkeling in het tapetum op (Hoofdstuk 2). De lichtmicroscopische onderzoekingen geven aan, dat in BBS antheren de ontsporing van het tapetum tijdens het profase stadium van de meiocyten begint. Tijdens de daaraan voorafgaande stadia, verloopt de ontwikkeling in de BBS antheren normaal en verschilt niet met de ontwikkeling zoals die in de helmknoppen van de mannelijke fertiele tegenhanger (BBF) wordt gevonden. Op ultrastructureel niveau worden de eerste afwijkingen in de BBS antheren zichtbaar door de aanwezigheid van grote vacuoles in het cytoplasma van de tapetumcellen (Hoofdstuk 3). Gedurende het leptoteen stadium van de meiocyten zijn deze vacuoles de enige aanwijzing van een abnormale ontwikkeling. Tijdens latere stadia vervormen de tapetale en sporogene cellen, degenereert de kern en raakt de organisatie van het cytoplasma verstoord. Mitochondriën en plastiden degenereren en er ontstaan veel vetdruppels in het cytoplasma.

In hoofdstuk 4 wordt beschreven hoe biochemische en histochemische aspecten van een enzymsysteem mogelijk worden beïnvloed door de degeneratie van het tapetale en sporogene weefsel. In het hoofdstuk wordt een beschrijving gegeven van het isoenzympatroon, de aktiviteit en de lokalisatie van esterases in anthere weefsels van cms en mannelijke fertiele petunia cultivars. Esterases zijn kern-gecodeerde enzymen die in de meeste delen van een plant kunnen worden aangetroffen. Biochemische onderzoekingen geven aan dat vanaf het begin van de meiose, de esteraseaktiviteit in antheren van het cms type laag blijft en dat er weinig nieuwe isoenzymen worden gevormd in vergelijking met de situatie in de mannelijke fertiele tegenhanger. Uit histochemische bepalingen komt naar voren dat in antheren van de mannelijke fertiele planten de esteraseaktiviteit geconcentreerd is in de buitenste tapetumlaag. De esteraseconcentratie doet zich voor vanaf de late profase tot aan het vroege microspore stadium. In antheren van cms planten stopt de accumulatie van esterases in het tapetum op het moment dat de degeneratie van dit weefsel zichtbaar wordt. Deze resultaten suggereren dat de veranderingen in de totale esteraseaktiviteit en het isoenzympatroon eerder effect dan oorzaak zijn van de verstoring in de pollenontwikkeling.

In cms vormen van verschillende plantesoorten is een samenhang gevonden tussen het cms plasmatype en afwijkingen in de cytochroom c oxydase aktiviteit en andere mitochondriële redox processen. Een biochemische bepaling van de cytochroom c oxydase aktiviteit in antheren van Petunia hybrida en Zea mays wordt gegeven in hoofdstuk 5. In beide soorten wordt de biochemische analyse gecombineerd met een cytochemische lokalisatie van de enzymaktiviteit in de mitochondriën van de sporogene en tapetale weefsels. De gegevens tonen aan, dat in de antheren van enkele cms maisrassen de cytochroom c oxydase aktiviteit gereduceerd is in vergelijking met het niveau dat gevonden wordt in antheren van het fertiele type. Bovendien geven de cytochemische bepalingen aan dat er verschillen zijn in de mitochondriële organisatie van cytochroom c oxydase tussen het pollen van cms-S en mannelijke fertiele maisplanten. De afwijkingen worden gevonden op het moment dat er nog geen aanwijzingen zijn dat de degeneratie van het sporogene weefsel is begonnen. De verschillen in cytochroom c oxydase vormen de eerste symptomen van mannelijke steriliteit in dit maistype. Daartegenover staat, dat in petunia de eerste verschillen in cytochroom c oxydase aktiviteit pas optreden als de degeneratie van het tapetum reeds is ingezet. Dus in petunia lijkt de afname van de cytochroom c oxydase aktiviteit eerder een resultaat van het voortschrijdende proces van degeneratie dan de oorzaak ervan.

In hoofdstuk 7 wordt gepostuleerd dat de cms-specifieke veranderingen in het mitochondriële genoom afwijkingen opwekken in eiwitcomplexen, die essentieel zijn voor de energie-leverende processen. Het is mogelijk, dat de veranderingen een nadelig effect hebben op de energiestatus van de cms plant. Echter, een plant met het cms plasmatype lijkt even goed te groeien als een mannelijke fertiele tegenhanger en klaarblijkelijk wordt de energiehuishouding niet in zo'n mate verstoord dat de levensvatbaarheid van de plant afneemt. In feite komen de verschillen alleen in bepaalde anthereweefsels op een specifiek moment in de ontwikkeling tot uiting. Dit zou er op kunnen wijzen, dat de mitochondriële genen, die verantwoordelijk zijn voor de afwijkingen, alleen in deze weefsels tot expressie komen. Een probleem van de weefselspecifieke expressie is, dat dit moeilijk in overeenstemming te brengen is met het feit dat de afwijkingen in de mitochondriële produkten soms ook gevonden worden in geïsoleerde

organellen uit vegetatieve delen van de cms plant. Een alternatieve verklaring is, dat de degeneratie van de tapetale en sporogene weefsels samenhangt met het specifieke metabolisme van de betrokken cellen. De energiehuishouding in de anthereweefsels van petunia wordt besproken in hoofdstuk 6. De resultaten geven inderdaad aanwijzingen dat de antheren een bijzondere stofwisseling bezitten, die hoger is dan wat bijvoorbeeld in bladweefsel wordt gevonden. Voorbeelden van een verhoogde metabolische aktiviteit in de antheren van andere plantesoorten worden besproken in hoofdstuk 7. Bovendien geeft de structurele analyse, zoals die is gepresenteerd in het tweede en derde hoofdstuk van dit proefschrift, een aanwijzing dat de ontwikkeling in de anthere van een cms petuniaplant van het normale verloop gaat afwijken op het moment dat er in de mannelijke fertiele ontwikkeling een duidelijke stijging is in de metabolische aktiviteit van de tapetumcellen. Het is mogelijk dat de mitochondriële synthese van energie-rijke produkten in de tapetumcellen van cms petunia onvoldoende is op momenten van een verhoogde energie behoefte. De normale werking van het tapetum wordt dan verstoord en als een gevolg daarvan degenereren de tapetale en sporogene weefsels.

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Curriculum Vitae

Raoul John Bino werd op 23 november 1957 geboren te Amsterdam. In 1976 deed hij eindexamen atheneum-B. In datzelfde jaar begon hij met de studie biologie aan de Universiteit van Amsterdam. In 1982 werd het doctoraalexamen afgelegd met als hoofdvak genetica en bijvakken plantenfysiologie en bijzondere plantkunde. Van 1 april 1983 tot en met 31 maart 1986 werd een promotieonderzoek verricht bij de vakgroepen Plantencytologie en -morfologie en Plantenveredeling van de Landbouwhogeschool te Wageningen, hetgeen resulteerde in dit proefschrift. Vanaf 1 april 1986 is hij als bloembioloog in dienst getreden van het Instituut voor de Veredeling van Tuinbouwgewassen.