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STUDIES ON THE REPRODUCTIVE BIOLOGY OF MALE-STERILE MUTANTS OF SOYBEAN (GLYCINE MAX (L.) MERR.)

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Studies on the reproductive biology of male-sterile mutants of soybean (*Glycine max* (L.) Merr.)

by

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GENERAL INTRODUCTION

Male sterility is a condition in plants in which hermaphroditic or monoecious individuals lose the ability to differentiate functional male gametophytes, while retaining the capacity of seed production. Male sterility may be structural or nonstructural. The former includes situations where differentiation of stamens or sporogenous tissue is suppressed, as well as mutants in which functional pollen is formed but fails to effect fertilization due to structural abnormalities of the flower (Johns et al., 1981). Nonstructural sterility occurs when microsporogenesis and/or microgametogenesis are prevented. Male-sterile mutants also may be classified as incomplete or complete. Incomplete male sterility occurs when individuals with male-sterile genotypes revert to fertile, or partially fertile, phenotypes. Such responses are usually temperature-dependent (i.e. Rick and Boynton, 1967; Stelly and Palmer, 1980a). In complete male sterility, reversion to fertility does not occur.

Male-sterile mutants also may be classified by their mode of inheritance. The traits may be biparentally (nuclear male sterility or NMS) or uniparentally (cytoplasmic male sterility or CMS) inherited. CMS is rarely completely independent of the nuclear genome; in many cases nuclear genotypes or loci occur that are capable of restoring

fertility. Such systems have been designated genecytoplasmic (nuclear-cytoplasmic) (Jain, 1959).

NMS often arises spontaneously in natural or cultivated populations of plants, although many NMS mutants have been induced through mutagenesis (Gottschalk and Kaul, 1974). Most cases of NMS are due to recessive Mendelian loci (Gottschalk and Kaul, 1974) but digenic recessive (Weaver, 1968; Ross, 1969; Abdalla and Hermsen, 1972) and dominant (Weaver and Ashley, 1971) mutants have been reported. CMS usually arises through interspecific or interracial hybridizations (Duvick, 1965; Edwardson, 1970; Virmani and Edwards, 1983) though cases of spontaneous origin are known (Edwardson, 1970).

The anatomical and developmental features of male-sterile mutants are diverse. Johns et al. (1981) reported that structural sterility was due to either the prevention of meiosis, or the prevention of pollination. Meiosis was omitted either through the absence, abortion, deformation or transformation (into other floral parts) of stamens. In many structural steriles, pollination was prevented due to various floral abnormalities (i.e. abnormal filaments, aberrant style lengths, or indehiscent anthers) that inhibit the ability of pollen grains to reach the stigma. In nonstructural sterility, abortion of reproductive cells has been noted at every stage of anther ontogeny. For both NMS and CMS, the

tetrad and young microspore stages have been cited most often as the time of action of the mutants (Laser and Lersten, 1972; Gottschalk and Kaul, 1974). Abnormalities of the tapetal layer have been correlated with male sterility in the majority of reports (Heslop-Harrison, 1971).

The observations that abnormalities of the germline cells most frequently appear at the tetrad/young microspore stages, and the correlation of tapetal abnormalities with male sterility, may be related. Most anthers are first differentiated as masses of meristematic cells (Buchen and Sievers, 1981). Sporogenous and tapetal cells then follow separate developmental pathways during meiosis. Sporogenous cells differentiate into microspore mother cells (MMCs) that become isolated from the rest of the anther via the secretion of callose. At the completion of meiosis, callose dissolves. Tapetal cells accumulate cellular attributes that indicate their function is largely secretory in nature. Maximum tapetal differentiation generally coincides with the release of microspores from callose. Roles assigned to the tapetum include: secretion of enzymes involved in callose dissolution (Stieglitz, 1977), production of sporopollenin precursors for use in pollen wall synthesis (Echlin, 1971; Horner and Pearson, 1978), the production of various substance (i.e. tryphines and "pollenkits") that coat mature pollen (Dickinson, 1973; Pacini and Casadoro, 1981; Stevens and

Murray, 1981); and the release of material for the engorgement of pollen (Christensen and Horner, 1974; Reznickova and Dickinson, 1982). Thus, most of the tapetal functions are postmeiotic. In male-sterile systems, aberrant tapetal development may lead to male sterility through the inability of the tapetal cells to fulfill functions that typically begin at the tetrad/young microspore stages.

The molecular basis for male sterility is poorly understood, especially in NMS. Studies have demonstrated differences in the free amino acid content of fertile and sterile anthers in both NMS and CMS in Petunia (Izhar and Frankel, 1973). Alam and Sandal (1969) found qualitative differences for both total protein and specific enzymes (peroxidase and cytochrome oxidase) when anthers of male-fertile and male-sterile lines of Sorghum vulgare were compared. It is not clear from such studies whether amino acid and protein differences represent the cause, or merely another effect, of male sterility.

Much of plant development is under the control of growth regulators (hormones) (Moore, 1979). Recent studies have demonstrated potential involvement of plant hormones in the induction of male sterility. Ahokas (1982) demonstrated a correlation between reduction in cytokinin translocation from root to shoot and CMS in Hordeum. Certain genotypes were capable of partially restoring fertility. In such cases,

fertile spikelets were found in the basalmost regions of the inflorescence, indicating that cytokinin translocation was partially restored as well. Saini and Aspinall (1982) were able to induce male sterility in Triticum by the application of absciscic acid to flowering spikes. The concentration of absciscic acid that was necessary to elicit this response was similar to that induced by moisture stress, which also resulted in male sterility. Colhoun and Steer (1983) found that the cytological effects of gametocides mimiced those of male-sterile mutants. The compounds used were thought to act as plant growth regulators.

Dell (1981) has discovered a link between mineral status of plants and male sterility. He was able to induce male sterility in several species by subjecting plants to growth media deficient in copper. Copper ions may be necessary as cofactors to enzymes involved in pollen differentiation.

Studies with CMS indicated several possible causes of sterility, depending on the species in question. Grill and Garger (1981) have demonstrated that CMS in Vicia faba resulted from the presence of a seed transmissible RNA virus. In Nicotiana, CMS may be induced through the substition of a nucleus from one species into the cytoplasm of another. Gerstel et al. (1978) and Burns and Gerstel (1981) found that fertility was restored when chromosomes from the cytoplasmic donor carrying an NOR (nucleolar organizing region) were

added to the nuclear genome of the sterile line. Cells of restored plants demonstrated amphiplasty (the NORs of the nuclear donor were suppressed by NORs of the cytoplasmic parent). Thus, Burns and Gerstel (1981) proposed that male fertility required the interaction of NOR's and cytoplasm from the same species.

Male sterility in Nicotiana also may be linked to variation in chloroplast (cp) DNA. Frankel et al. (1979) analyzed cp-DNAs from male sterile lines produced by substituting the nucleus of N. tobaccum into the cytoplasms of six other species. Four of the six CMS lines were indistinguishable from their maternal parents. Two, however, showed cp-DNA organization that varied when compared to that of their maternal parents. These changes may have interfered with the successful cooperation between nuclear and organellar genomes.

Male sterile cytoplasms of maize (Zea) have been placed in three groups(C, S, and T), based on their response to nuclear restorer genes (Duvick, 1965). Male sterile cytoplasms also have been distinguished from normal (N) cytoplasm, and from each other, by DNA fragment patterns generated by restriction endonuclease digestion (Pring and Levings, 1978). Hybridization studies with comigrating fragments indicated that extensive divergence of sequence organization distinguished cms-T from N cytoplasm (Spruill et al., 1981).

Mitochondrial plasmid-like DNAs were linked to CMS in S cytoplasm (Levings et al., 1980). Reversions to fertility in cms-S occurred through insertion of these plasmid-like molecules into the mitochondrial genome.

The CMS cytoplasms of maize also were characterized by the production of unique mitochondrial translation products (Forde et al., 1980). In cms-T, restoration of fertility led to the suppression of synthesis of a unique 13,000 MW polypeptide not found in N cytoplasm (Forde and Leaver, 1980). No changes in translation products were associated with fertility restoration in cms-S and cms-C. Plants with cms-T are susceptible to a toxin produced by Helminthosporium maydis. The toxin acts on the mitochondrial membrane, perhaps affecting an intrinsic protein (Levings, 1983). Umbeck and Gengenbach (1983) recovered plants that had reverted to both male-fertility and pathotoxin resistance after being regenerated from tissue cultures derived from male-sterile cms-T plants. Five of six reverted lines tested showed mitochondrial DNA organizations that differed from the parental line. Thus, both pathotoxin sensitivity and male-sterility in cms-T lines may be due to the same mitochondrially encoded function.

Thus, there is substantial evidence linking CMS in maize to mitochondria. These organelles are uniparentally inherited (Conde et al., 1979), thus satisfying the requirement that

the cause of CMS be maternally inherited. The first study on anther development in cms-T lines reported premature degeneration of tapetal mitochondria as the earliest indication of sterility (Warmke and Lee, 1977), but Colhoun and Steer (1981) detected this pattern in only a minority of cells examined. Mitochndrial degeneration was not observed in cms-S and cms-C (Lee et al., 1979; 1980).

In sorghum (Sorghum bicolor), mitochondria also have been suggested as the determinants of CMS. Pring et al. (1982a) detected plasmid-like DNAs in the mitochondria of male-sterile lines. These molecules possessed homology to those associated with cms-S of maize. Pring et al. (1982b) were able to recognize seven groups of male-sterile cytoplasms based on mitochondrial DNA organization. Three of these groups also were characterized by unique cp-DNAs. Dixon and Leaver (1982) found differences in mitochondrial-encoded proteins associated with male steriltiy. For instance, male fertile combinations of Milo nucleus/Milo cytoplasm and Kafir nucleus/Kafir cytoplasm showed identical mitochondrial translation products, with the exception of the presence of a 65,000 MW protein unique to the Milo line. In the male-sterile combination of Kafir nucleus/Milo cytoplasm, the production of this protein increased dramatically. Unique translation products were found in other male-sterile cytoplasms as well.

CMS, then, may arise as a result of incompatibilities between the nucleus and the cytoplasm of certain hybrid combinations. CMS usually arises through the introduction of a nuclear genome into a foreign cytoplasm (from either a different species or race). Nuclear and cytoplasmic genomes may represent coevolved systems. Removing a nucleus from the cytoplasm it is adapted to leads to the generation of abnormalities and male sterility. Restoration of fertility is possible when a locus or chromosome compatible with the foreign cytoplasm is introduced into the nuclear genome of the male-sterile line.

Several NMS, but no CMS, mutants have been assigned gene symbols in soybean (Brim and Young, 1971; Palmer et al., 1980; Stelly and Palmer, 1980b; Johns and Palmer, 1982; Buss, 1983; Graybosch et al., 1984). Most are nonstructural, however, one structural sterile has been reported (Johns and Palmer, 1982). All are inherited as monogenic recessive characters, except the structural sterile (fs1fs2), which is a digenic trait. No documented heterozygous effects have been reported. That is, individuals heterozygous or homozygous dominant at a male-sterile locus show identical (male-fertile) phenotypes.

Several male-sterile mutants have been reported but not assigned gene symbols (Caviness et al., 1970; Chaudhari and Davis, 1977; Patil and Singh, 1976). Little is known about

these mutants other than their being monogenic recessive characters.

In soybean, male sterility may be either partial or complete. Partial male-sterile mutants are capable of occasional pollen production. Phenotypic expression of such mutants is temperature sensitive. Complete male-sterile mutants are incapable of generating pollen, regardless of the environmental conditions.

Many attempts have been made to establish linkage relationships between the sterility loci and marker loci or specific chromosomes. These have been summarized by Graybosch and Palmer (1984), Palmer (1984) and Yee and Palmer (1984). To date, only ms1 has been placed in a linkage group (Palmer and Kaul, 1983). It resides in linkage group 8, along with the flower color loci w1 and wm, the synaptic mutant st5 and the breakpoint of a translocation from a Plant Introduction of Glycine soja. Sadanaga and Grindeland (1984) have determined that linkage group 8 occurs on the satellite chromosome of soybean. This is the only morphologically distinct chromosome in the karyotype. Keaschall et al. (1981) reported tentative linkage between ms2, lf1 and pd1, but the results have not been confirmed.

Observations on the developmental reproductive biology of many of the sterility mutants have been published. The floral structure mutant (fs1fs2) was studied by Johns and

Palmer (1982). All flowers produced were cleistogamous. In some cases, styles protruded above the closed perianth. An expanded receptacle led to abnormal perianth development, which suppressed filament elongation. Pollen was produced, but anthers dehisced at the base of the pistil, rather than in the normal position adjacent to the stigma. Thus, self-pollination was absent or infrequent. Ovule development was also impaired. Embryo sac ontogeny was determined to be normal. However, the integuments failed to mature properly. Abnormal micropyles, associated with aberrant positioning of ovules within the ovary, effectively prevented pollen tubes from reaching their destinations. Seed production was rare, even when controlled pollinations were attempted.

The nonstructural male-sterile mutants of soybean exert their effects by interrupting pollen development, generally at a mutant-specific stage. In order to adequately discuss the phenotypic characteristics of male-sterile plants, a description of anther development in male-fertile plants is warranted. Anther ontogeny in male-fertile plants has been discussed by Carlson (1973), and Albertsen and Palmer (1979).

Anthers are initiated as individual primordial masses of meristematic cells. Within each, five tissues arise. These are: sporogenous, tapetal, parietal (2), endothecial and epidermal layers. The latter three constitute the anther wall. Each undergoes tissue-specific developmental changes

during the course of anther maturation. Based on the events in the sporogenous cells and their immediate descendents, anther development may be divided into the following stages: sporogenous, meiotic, tetrad, free microspore and pollen. Each shall be discussed in turn.

Sporogenous stage: The innermost layer of primordial cells enlarges and may now be recognized as sporogenous cells. Cytoplasm of all cells is uniformly dense, and organelles are similar in number and distribution throughout.

Meiotic stage: The sporogenous cells mature into microspore mother cells (MMCs). Callose is secreted within the primary cell walls of the MMCs. Within a given microsporangium, the first meiotic division is highly synchronous; the second division is less so. During this stage, tapetal cells accumulate numerous organelles. The inner tangential wall of the tapetal cells begins to dissolve. Cells of the anther wall become vacuolate and accumulate starch grains in their plastids.

Tetrad stage: Subsequent to telophase II, MMCs are divided into tetrads of microspores by simultaneous cytokinesis that proceeds in a centripetal fashion. The initial cleavage furrows are filled with callose. Microspore wall formation begins with the deposition of the primexine. Within the primexine, the tectum, columellae and pedium (foot layer) of the ektexine are deposited. The tapetal cells are

characterized by extensive profiles of endoplasmic reticulum, numerous Golgi bodies and mitochondria, and the absence of an inner tangential cell wall. The parietal layers become crushed at this time, and the epidermis begins to degenerate.

Microspore stage: The sheath of callose surrounding the tetrads dissolves, releasing the microspores into the locule. At this time, the endexine of the microspore wall is deposited. Microspores become vacuolate as reserves are expended in wall formation. The tapetal layer still appears to be functioning as a secretory tissue, although vacuoles become more common in the tapetal cytoplasm. The radial cell walls of the endothecial layer expand.

Pollen stage: Microspore mitosis gives rise to a two-celled pollen grain. Pollen wall formation is completed with the production of the intine. The mature pollen wall thus consists of the ektexine (tectum, columellae and pedium), endexine and the intine. The lattermost appears cellulosic in nature; the remainder of the pollen wall is largely composed of sporopollenin. Three colpi or grooves are visible on the surface of each pollen grain. The tapetal layer collapses, although it is still bound by a plasmalemma. Tapetal cytoplasms accumulate lipid bodies and the plastids are rich in lipid-like osmiophilic bodies. The endothecial layer forms thickenings of secondary wall material along the expanded radial walls. Partitions between adjacent locules

break down and anthers dehisce along the stomia. At anthesis, all anthers are situated adjacent to the stigma. The close proximity of pollen to the stigma at dehiscence, as well as the lack of any self-incompatibility system, ensures autogamy.

Mutations at the msl locus have arisen independently in four different populations (Palmer et al., 1978). Albertsen and Palmer (1979) determined that male sterility was due to the failure of postmeiotic cytokinesis. A quadrinucleate cell (referred to as a coenocytic microspore or CM) was formed. Degeneration of these cells was not immediate, rather they showed a tendency to mimic the development of normal pollen. A pollen-like wall was produced and food reserves in the form of starch and lipid accumulated. Nuclear fusion, mitotic-like divisions and the formation of structures resembling pollen tubes occurred. CMs were frequently partitioned internally through the deposition of intine-like walls. Although CMs possessed many characteristics of normal pollen, their participation in fertilization was not documented.

The msl mutant also influences female reproduction, but it never conditions complete female sterility. Kenworthy et al. (1973) observed twin seedlings among progeny of male-sterile plants. Most twins were diploid:diploid, but one diploid:haploid and three diploid:triploid pairs were found.

This haploid, and all reported in subsequent studies, was shown to be of maternal origin. Beversdorf and Bingham (1977) noted polyembryony at a frequency of 2.3% in progeny of male-sterile individuals. Most polyembryonic seeds contained twins, but triplets, a quadruplet and a quintuplet also were found. Chromosome numbers of seedlings ranged from 2Ø (n) to 8Ø (4n). As previously noted, mutations at the msl locus have arisen in four different genetic backgrounds. Chen et al. (1984) determined that the frequency of polyembryony and polyploidy varied as a function of the source population. They also found monoembryonic polyploids to be common. Such polyploids were easily isolated through the screening of "abnormal" seedlings.

Cutter and Bingham (1977) attempted to determine the cytological basis for polyploidy and polyembryony associated with msl. Supernumerary nuclei in the vicinity of the egg apparatus were common. They believed that the fusion of these nuclei and/or their differentiation into multiple egg cells was responsible for the observed abnormalities. Only 28% of the ovules examined contained a normal embryo sac. Kennell (1984) also has examined this phenomenon. Wall formation after meiosis was partly or completely absent; this impaired subsequent megagametophyte development. Increased ploidy levels were attributed to the fusion of supernumerary nuclei early in megagametogenesis. Multiple egg cells,

synergid cells and central cells were also observed. Kennell showed that the frequency of these abnormalities varied as a function of the source population.

Seed set on haploids was studied by Sorrells and Bingham (1979). In n X 2n crosses, most progeny were diploid (2n=40) although four trisomics, one triploid, nine tetraploid and one plant with 70 chromosomes were found. Thus, haploids were capable of generating "unreduced" haploid eggs. Crane et al. (1982) found a low level of bivalent formation (0-5 per cell) during meiosis in haploid plants. All haploids studied produced CMs, some of which contained aneuploid nuclei. Binucleate CMs with 20 chromosomes per nucleus, and uninucleate CMs with 40 chromosome nuclei were noted. Variation in gametic chromosome number may have been due to the formation of restitution nuclei. Whether such nuclei formed as a consequence of irregular meiosis associated with haploidy, or as a result of the ms1 allele being present in a hemizygous condition, was not discussed. Both factors may have contributed.

Graybosch et al. (1984) demonstrated that the ms2 mutant renders plants male-sterile by preventing microspore development subsequent to the tetrad stage. The microspores aborted while still enclosed in callose. Tapetal dysfunction was associated with male sterility. During the meiotic stages abnormally large vacuoles were evident in the tapetal

cells. By the tetrad stage, approximately 90% of the cellular volume was in the form of vacuoles.

The ms2 locus also may influence female reproduction. Sadanaga and Grindeland (1981) reported spontaneous trisomy and polyploidy in progeny of male-sterile plants. Buss and Autio (1980) noted twin seedlings and polyploids. In both cases, the frequency of the observed abnormalities was far less than that which occurs with the ms1 mutant.

The ms3 mutant was first analyzed by Palmer et al. (1980). Abortion at the young microspore stage was cited as the cause of sterility. Excessive tapetal vacuolation was evident at the time of, but not before, microspore degeneration. Buntman (1983) and Buntman and Horner (1983) also studied the ms3 mutant. They noted that the microspore walls were abnormal and that those of adjacent cells often fused. The release of microspores from the callose sheath was delayed. In and about tapetal cells, a refractive material suspected to be sporopollenin or its precursors was seen to accumulate. Nakashima et al. (1984) showed that this material possessed primuline-induced and auto-fluorescent properties similar to those of sporopollenin.

Buntman and Horner (1983) determined that the earliest sign of abnormality in the ms3 system was the presence of aberrant mitochondria in the tapetal cells. When tapetal cells of male-fertile and male-sterile plants were compared

at the meiotic stage, the mitochondria of male-sterile plants were larger in size than those of normal tapetal cells. Buntman (1983) wrote "...all ms3 tapetal cells, by the dyad stage, possess the swollen mitochondria...". At the tetrad stage, tapetal cells were characterized as being disorganized and in states of degradation. In contrast to the observations of Palmer et al. (1980) tapetal cells were said to degenerate prior to the microspores. Mitochondria of tapetal cells at the tetrad stage were variable in appearance, some being degenerate, others normal.

H. T. Horner, Department of Botany, Iowa State University (personal communication) is presently conducting a more extensive study of the ms3 mutant. He has found that the mitochondria of both meiotic and tapetal cells condense dramatically between the sporogenous stage and the meiotic stage. In sterile anthers, however, this response is delayed. During and after meiosis, tapetal mitochondria appear normal. Tapetal abnormalities do not appear until the late tetrad stage, just before the microspores degenerate.

Delannay and Palmer (1982) have noted similarities between the expression of the ms4 mutant and that of ms1. Postmeiotic cytokinesis often failed, resulting in a CM. Subsequent development of these cells was variable. A pollen-like wall usually was deposited. In some locules, CMs enlarged, became vacuolate and degenerated. Excessive

vacuolation of the tapetal layer occurred in such cases. In other locules, CMs engorged with starch and lipids, and persisted to maturity as giant pollen grains. Fusion of some or all of the nuclei was noted. In many CMs, a delayed cytokinesis occurred. These divisions often were irregular in distribution or incomplete. However, at times, tetrads of normal appearing pollen were formed. Some of these may have functioned in fertilization. The frequency of male-sterile progeny from open pollinated male-sterile plants often exceeded the maximum expected frequency of 50%.

The ms5 mutant has not been the subject of any detailed cytological investigation. Buss (1983) did note that mature anthers were dark and shrunken, and pollen grains were small and shrivelled.

Stelly and Palmer (1980b) determined that phenotypic expression of the msp mutant was temperature dependent. When grown under a warm daytime temperature regime (35C) male-sterile plants had near-normal seed set. However, at cooler temperatures (29C or 24C), plants were partially or completely male sterile. Stelly (1979) employed genetic markers to demonstrate that the seed produced by male-sterile plants was of sexual and not of apomictic origin. Stelly (1979) and Stelly and Palmer (1982) determined that the effect of the msp locus on anther development also was variable. Infrequently, anthers were reduced in size or feminized.

Many anthers produced normal pollen. However, in others, abortion of reproductive cells occurred at any time from the sporogenous stage to the pollen stage. Degeneration was most common during prophase I and the tetrad stage. Excessive vacuolation of tapetal cells generally preceded degeneration of reproductive cells.

The "Arkansas" male-sterile mutant (Caviness et al., 1970) also was characterized by a temperature-dependent phenotype. The response differs from that of msp. Daytime temperatures of 35C lead to complete male sterility. When daytime temperatures were maintained at either 21C or 29C, plants were partially male fertile. Seed set ranged from approximately 10% to 50% that of fertile controls grown in the same environment (Caviness and Fagala, 1973).

Format of the Dissertation

Only 13 years have elapsed since the first male-sterile mutant was described in soybean (Brim and Young, 1971). From the preceding discussion one may discern that a wealth of knowledge on sterility has been obtained. The general goal of the present study is to enhance further current understanding of the reproductive biology of male-sterility in soybean. Five specific problems were examined. The results of each study are presented in separate sections to follow. These are:

Section I. Phenotypic expression of a male-sterile mutant (ms2) of soybean (Glycine max (L.) Merr.).

Section II. Aneuploids from a male-sterile mutant (ms2) of soybean (Glycine max (L.) Merr.).

Section III. Observations on the reproductive biology of a male-sterile mutant (ms4) of soybean (Glycine max (L.) Merr.).

Section IV. Partial fertility in a "male-sterile" mutant of soybean (Glycine max (L.) Merr.).

Section V. Analysis of a male-sterile mutant in 'Wabash' soybean.

All work presented in sections I, III, IV, and V is my own. In section II, some of the chromosome counts were obtained by Dr. K. Sadanaga, Department of Genetics, Iowa State University.

SECTION I: PHENOTYPIC EXPRESSION OF A MALE-STERILE MUTANT (ms2) OF SOYBEAN (GLYCINE MAX (L.) MERR.)

Abstract

The effects of a nuclear male-sterile mutant (ms2) of soybean (Glycine max (L.) Merr.) on anther development were analyzed by means of light- and electron-microscopy. The structure of microspore mother cells (MMCs) in male-sterile plants was identical to that of male-fertile plants. Meiosis was completed, and tetrads of microspores formed. Microspores degenerated after the deposition of primexine and probacullae. The sheath of callose surrounding microspores did not dissolve. No structural abnormalities of the microspores were detected before the onset of degeneration. The tapetal and anther wall layers were characterized by aberrant development. Tapetal abnormalities included premature vacuolation, a persistent inner tangential cell wall, failure to differentiate normal concentrations of endoplasmic reticulum and dictyosomes, disruption of plastids and premature degeneration. Malfunction of the tapetal layer preceded, and may have induced, microspore degeneration. Gross anther morphology was not influenced until advanced stages of development.

Introduction

Male sterility is a condition in plants in which male gametophytic function is prevented, while female reproduction remains unimpaired. Based on inheritance patterns, two types of male sterility are recognized: nuclear male sterility (NMS) and cytoplasmic male sterility (CMS). In many cases of CMS, nuclear genes that restore fertility have been found. Such systems are referred to as nuclear-cytoplasmic male-sterile mutants. NMS mutants often occur spontaneously (Gottschalk and Kaul, 1974) while CMS generally arises through interspecific or interracial hybridization (Duvick, 1965; Edwardson, 1970; Virmani and Edwards, 1983). In NMS, sterility factors are simply inherited Mendelian loci. Alleles are usually recessive, though dominant mutants have been reported (Weaver and Ashley, 1971). CMS has a variety of causes depending on the species in question. Alterations of mitochondrial genomes (Leaver and Gray, 1982), viral-like agents (Grill and Garger, 1981) and incompatibilities between donor NOR chromosomes and recipient cytoplasms in interspecific crosses (Bürns and Gerstel, 1981) are some examples.

Male sterility provides for a greater degree of outcrossing in species that lack mechanisms of self-incompatibility. This function has been exploited in the

production of hybrid seed in several crop species (Pearson, 1981). CMS has seen wider commercial application, largely because of the ease in obtaining male-sterile populations for use as female parents in crosses. True breeding lines of male-sterile plants can not be produced with NMS mutants. In many cultivated plants, i.e. soybean (Glycine max (L.) Merr.), CMS has not been reported. Several NMS mutants of soybean are known (Graybosch and Palmer, 1984). Though male-sterile soybeans have been used to generate limited quantities of hybrid seed for estimations of heterosis (Nelson and Bernard, 1984), and have served as source populations for recurrent selection programs (Burton and Brim, 1981), no attempt has been made to generate commercial quantities of F1 hybrid seed via male-sterility in soybean.

Research emphasis on male-sterile mutants of soybean has focused on their effects on the processes of microsporogenesis and microgametogenesis (Albertsen and Palmer, 1979; Palmer et al., 1980; Delannay and Palmer, 1982; Stelly and Palmer, 1982; Buntman and Horner, 1983). These studies are important in two respects. The effect of mutants on the reproductive biology of higher plants provide useful information on aspects vital to successful reproduction in normal individuals. For instance, the crucial role of the tapetum in pollen differentiation is evidenced by the frequent correlation of tapetal malfunction with male-

sterility (Laser and Lersten, 1972; Gottschalk and Kaul, 1974). Secondly, means of inducing male-sterility by use of pollen-specific gametocides are available (Colhoun and Steer, 1983) and are currently being evaluated for use in hybrid seed production (Virmani and Edwards, 1983). The restoration of fertility to male-sterile plants for one generation would allow the production of large populations of male-sterile progeny, but no successful attempts at fertility restoration have been reported. Observations on the effects of male-sterile mutants on pollen differentiation may be useful in attempts to design and implement gametocides and fertility restorers.

As part of a continual program of evaluating NMS mutants of soybean, observations on the reproductive biology of the ms2 mutant were obtained. This mutant arose spontaneously in a breeding population at Eldorado, Illinois, in 1971 (Graybosch et al., 1984). Female fertility was nearly as high in male-sterile plants as in male-fertile plants. There may be a slight pleiotropic effect of the ms2 locus on female reproduction as low frequencies of spontaneous trisomics, twin seedlings, and polyploids have been found among progeny of male-sterile plants (Buss and Autio, 1980; Sadanaga and Grindeland, 1981).

Male sterility in the ms2 system is complete; reversion of male-sterile plants to a fertile phenotype has not been

observed. Observations with the light microscope showed that male sterility resulted from the abortion of microspores at the tetrad stage of development (Graybosch et al., 1984). Abnormalities of the tapetal layer were evident before the occurrence of microspore abortion. Presented herein are additional microscopic observations on anther development in ms2 male-sterile plants.

Materials and Methods

Plants used were obtained from a green-seeded (d1d1d2d2) line, segregating at the ms2 locus, developed by Sadanaga and Grindeland (1981). Reproductive buds of various sizes were collected from male-fertile (Ms2 _) and male-sterile (ms2ms2) individuals. Buds were dissected and anthers removed and fixed in a mixture of 2.0% paraformaldehyde/ 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.25, for 2-4 hours. Anthers subsequently were placed in the same fixative, less paraformaldehyde, for an additional 16-20 hours. Following three buffer washes, anthers were postfixed in 1.0% OsO_4 in 0.1 M phosphate buffer, washed again, dehydrated in a graded acetone series, and embedded in Spurr's.

Specimen blocks were sectioned on a Sorvall Mt-5000 ultramicrotome. For light microscopic observations, one micrometer "thick" sections were stained with methylene-blue

and safranin-O (Warmke and Lee, 1976). Specimens were observed and photographed on a Leitz Dialux-20 microscope. "Thin" sections (60-100nm) were stained in aqueous solutions of uranyl acetate and lead citrate for one hour per solution. Ultrastructural observations were obtained through use of a Hitachi HU-11C transmission electron microscope operated at 50kV.

Stamen development in male-fertile and male-sterile plants also was studied. Buds of various sizes were fixed under vacuum in the mixture cited above. Dehydration was in a graded ethanol series. Specimens were then gradually infiltrated with Freon-TF and critical point dried (CPD) in a home-made critical point dryer. Stamens were exposed by either freeze-fracturing buds in liquid nitrogen before the introduction of Freon-TF, or through removal of perianth parts after CPD. Specimens then were mounted with silver paste on brass discs, coated with a uniform layer of gold/palladium in a Polaron E5100 sputter coater, and observed with a JEOL-JSM 35 scanning electron microscope at an accelerating voltage of 15kV.

Results

Anther development in male-fertile soybean has been described by Carlson (1973), Albertsen and Palmer (1979) and Buntman and Horner (1983). No deviations were detected from

the basic pattern they described. Using the terminology of Albertsen and Palmer (1979), though somewhat condensed, anther ontogeny may be divided into five stages: sporogenous, meiotic, tetrad of microspores, free microspore and pollen. Observations of male-fertile development are presented when direct comparisons with that of male-sterile anthers are warranted.

The pattern of anther development in male-sterile and male-fertile plants as seen with the light microscope is presented in Figures 1-8. MMCs were differentiated from sporogenous cells; those of male-sterile plants could not be distinguished from those of male-fertile plants (Figs. 5,6). The meiotic process was completed, resulting in the production of tetrads of microspores. The sheath of callose surrounding the tetrads failed to dissolve (Fig. 2) in male-sterile plants and microspores subsequently aborted (Fig. 3). In male-fertile plants, callose was degraded and individual microspores liberated (Fig. 7). At "maturity", only degenerate cells were present within the locules of male-sterile plants, while in male-fertile plants, engorged pollen filled the anther (Figs. 4,8).

Abnormalities of the tapetal and anther wall layers were observed. During the meiotic stage, tapetal cells of male-sterile plants often were characterized by the presence of large vacuoles (Fig. 1) not seen in the tapetal cells of

Fig. 1-8. Light microscopic observations of anther development in male-sterile (1-4) and male-fertile (5-8) anthers.

1. Male-sterile anther at meiotic stage. Meiocytes (M) are surrounded by tapetal layer (T) which demonstrates premature vacuolation. X 255.

2. Male-sterile anther at tetrad stage. Callose (C) surrounds tetrads; tapetal cells (T) contain little cytoplasm. X 260.

3. Male-sterile anther demonstrating degeneration of tetrads; callose remains evident. X 270.

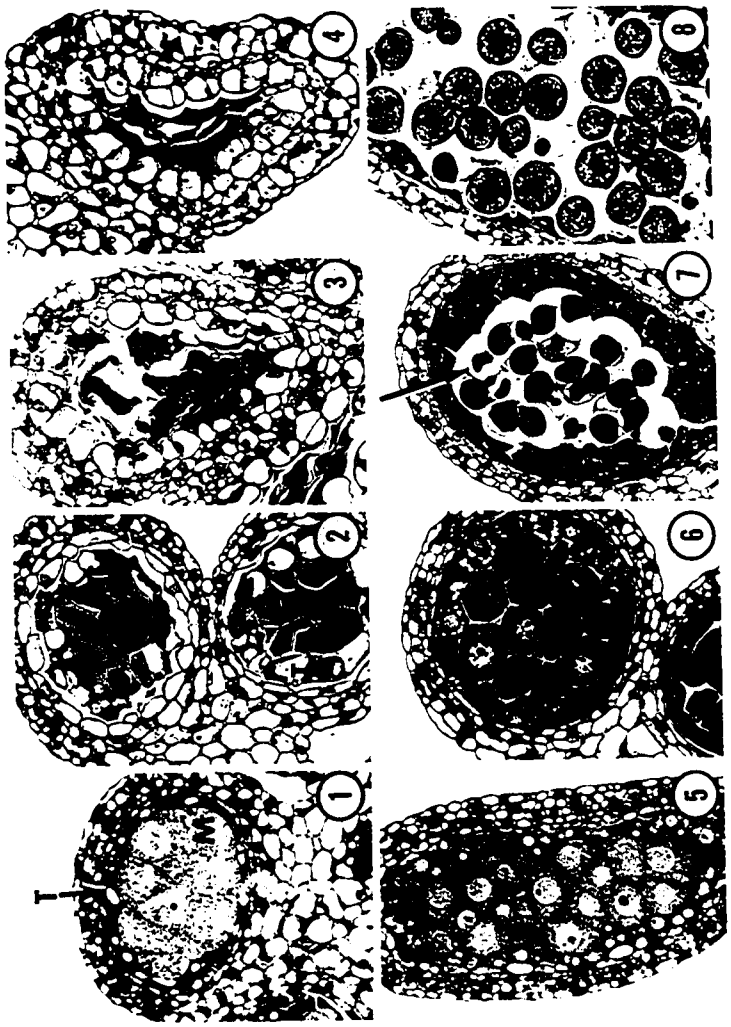
4. Male-sterile anther at maturity. Locule is empty except for the remains of degenerate cells. X 270.

5. Male-fertile anther at meiotic stage. X 280.

6. Male-fertile anther at late meiotic stage. X 280

7. Male-fertile anther at early microspore stage. Callose dissolves (arrow) liberating microspores. X 320.

8. Male-fertile anther at anthesis. Engorged pollen fills locule. X 240.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male-fertile plants (Figs. 5,6). These vacuoles increased in size as meiosis progressed. By the tetrad stage, little tapetal cytoplasm remained (Fig. 2). Tapetal cells of male-fertile plants remained cytoplasmically dense and contained only small vacuoles (Figs. 5-7). The tapetal cells of male-sterile plants degenerated along with the microspores (Figs. 3,4); tapetal cells of male-fertile plants did not collapse until the pollen stage (Fig. 8). The inner parietal layer of male-sterile plants expanded radially toward the locule (Fig. 4) while in male-fertile plants it was crushed (Fig. 7). The endothecial layer of male-sterile plants also expanded (Fig. 4), but secondary wall thickenings characteristic of male-fertile plants were lacking.

Ultrastructural observations did not demonstrate any differences between male-fertile and male-sterile anthers at the sporogenous stage (Figs. 9,10). Sporogenous and pretapetal cells appeared cytoplasmically dense with numerous organelles. Amoeboid plastids and dumbell-shaped mitochondria were common, and difficult to distinguish from each other. Sporogenous cells differentiated into MMCs that also were identical in male-fertile and -sterile plants. Early in development, channels were present between adjacent MMCs. These channels often were traversed by organelles (Fig. 11). Synaptonemal complexes formed, strands of endoplasmic reticulum (ER) became evident in the cytoplasm,

Fig. 9-13. Ultrastructural aspects of male-sterile anthers during sporogenous and meiotic stages.

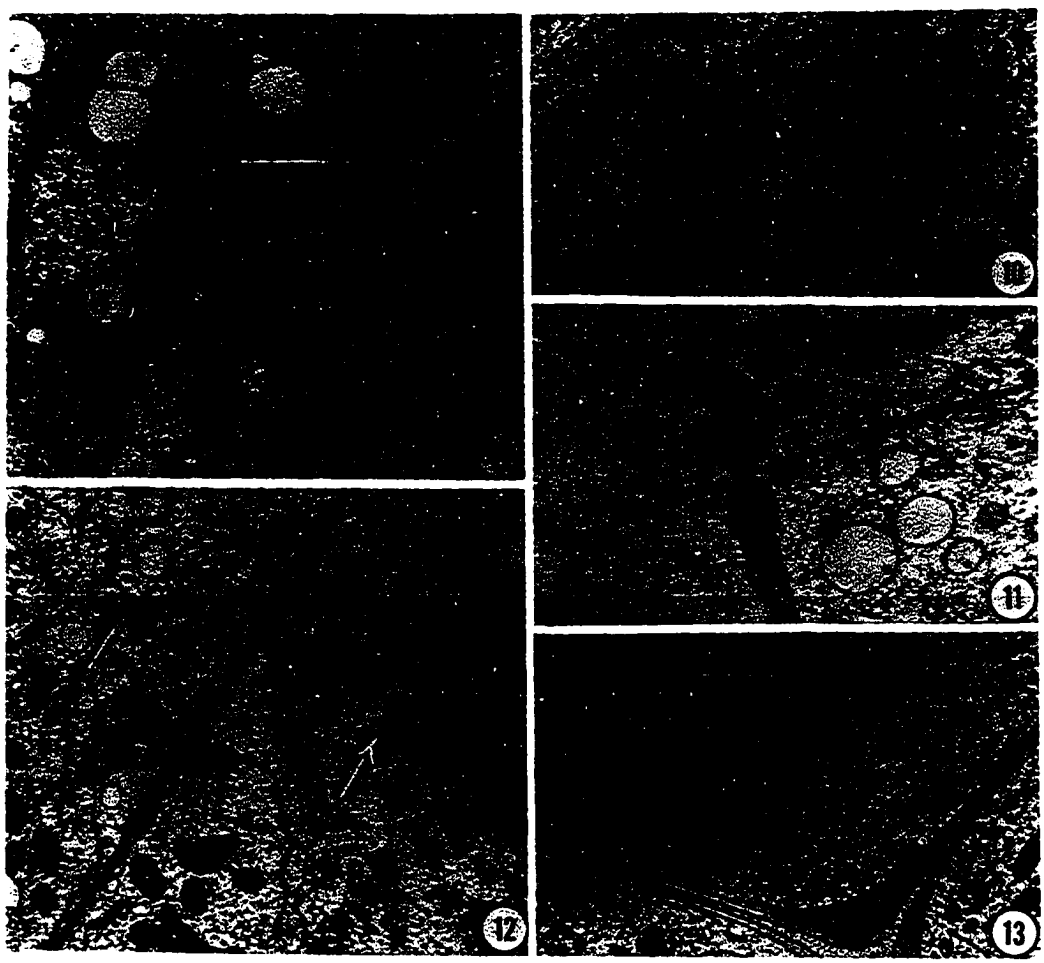
9. Sporogenous (S) and tapetal (T) cells appear meristematic in nature, with dense cytoplasms and numerous organelles. X 2,200.

10. Organelles of sporogenous cell. X 10,000.

11. Meiotic stage: channels occur between adjacent meiocytes and may be traversed by organelles. X 7,000.

12. Meiotic stage: strands of endoplasmic reticulum (ER) become common in cytoplasm; synaptonemal complexes (SC) are evident. X 4,420.

13. Meiotic cells secrete callose (C) within primary cell walls. X 2,610.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and mitochondria and plastids easily were identified (Fig. 12). Callose was secreted within the primary cell walls of the MMCs, effectively isolating them from each other and from the tapetal cells (Fig. 13).

Early in the meiotic stage, mitochondria of tapetal cells often were larger and contained less dense cristae than those of adjacent MMCs (Fig. 14). Tapetal mitochondria condensed as prophase I progressed; eventually they were identical to those of the MMCs (Fig. 15). The appearance of male-sterile tapetal cells at this time was variable. In most, numerous vacuoles, or a large central vacuole, were present (Figs. 14,16). During the meiotic stage, tapetal cells of male-fertile plants appeared isodiametric in cross-sectional view (Fig. 30). Tapetal cells of male-sterile plants were usually longer along radial than along tangential axes (Fig. 17).

Postmeiotic cytokinesis was identical in male-sterile and -fertile plants. Cleavage furrows originated in association with the plasmalemma (Fig. 18). Cells were severed through simultaneous cytokinesis that proceeded via the deposition of callose-laden vesicles. Multiple furrows met in the central portion of the cell (Fig. 19).

At the time of their initiation, microspores were bound only by their plasmalemmas, and retained as tetrads within a sheath of callose (Fig. 20). A fibrillar primexine was

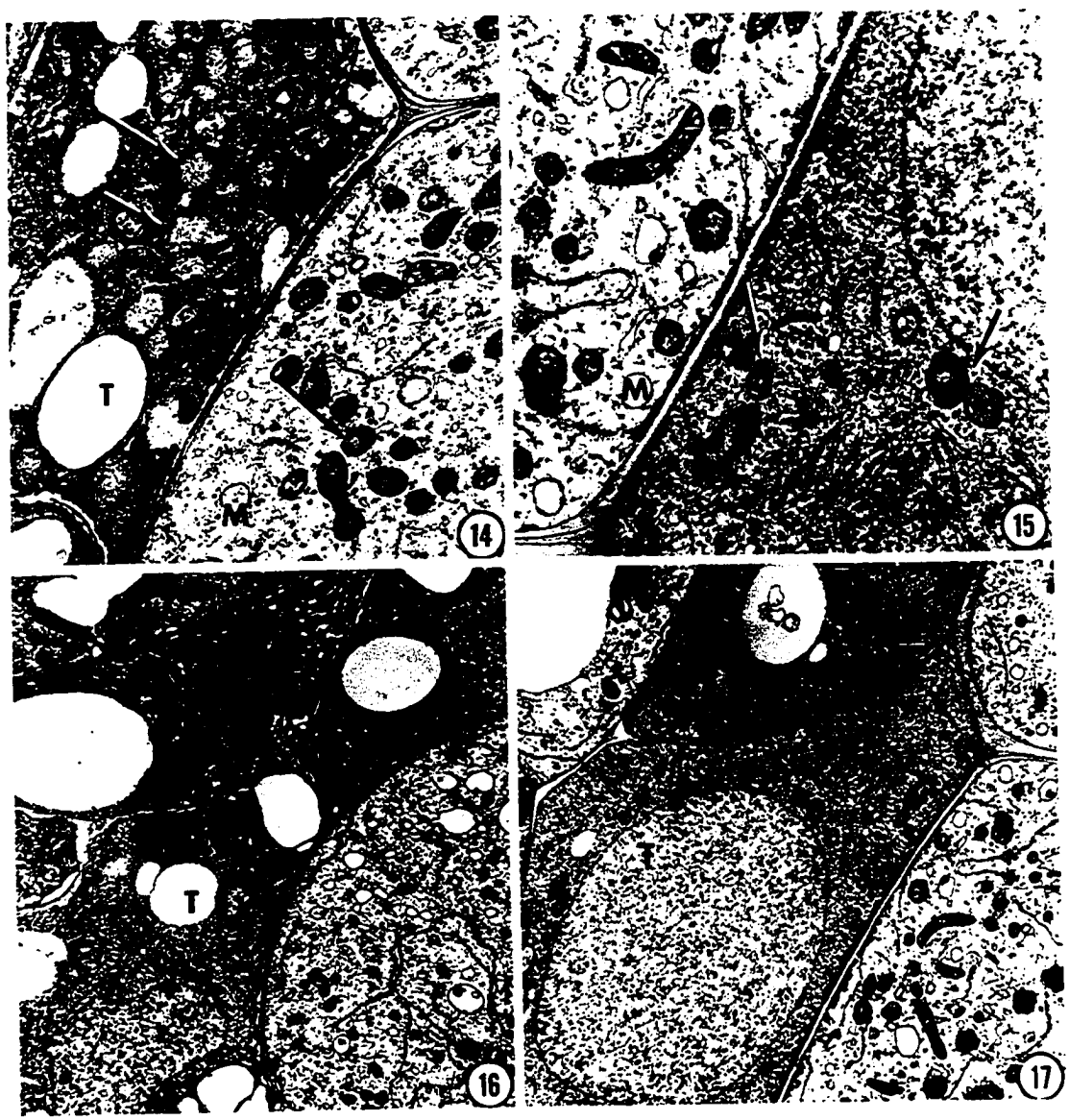
Fig. 14-17. Aspects of tapetal cells of male-sterile plants during meiosis.

14. Cytoplasm of tapetal cells (T) is more dense than that of adjacent meiocytes (M). Early in meiosis tapetal mitochondria (arrows) bear a less dense matrix than those of meiocytes. X 8,000.

15. Late meiotic stage: tapetal mitochondria (arrows) become as dense as those of meiocytes. X 7,500.

16. Tapetal cells (T) bear numerous large vacuoles not observed in male-fertile tapeta. X 2,000.

17. A minority of tapetal cells (T) lack large vacuoles during meiosis. X 2,400.


T
M
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M
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Fig. 18-23. Observations of postmeiotic reproductive cells of male-sterile plants.

18. Initiation of cell plate in association with the plasmalemma. X 4,750.

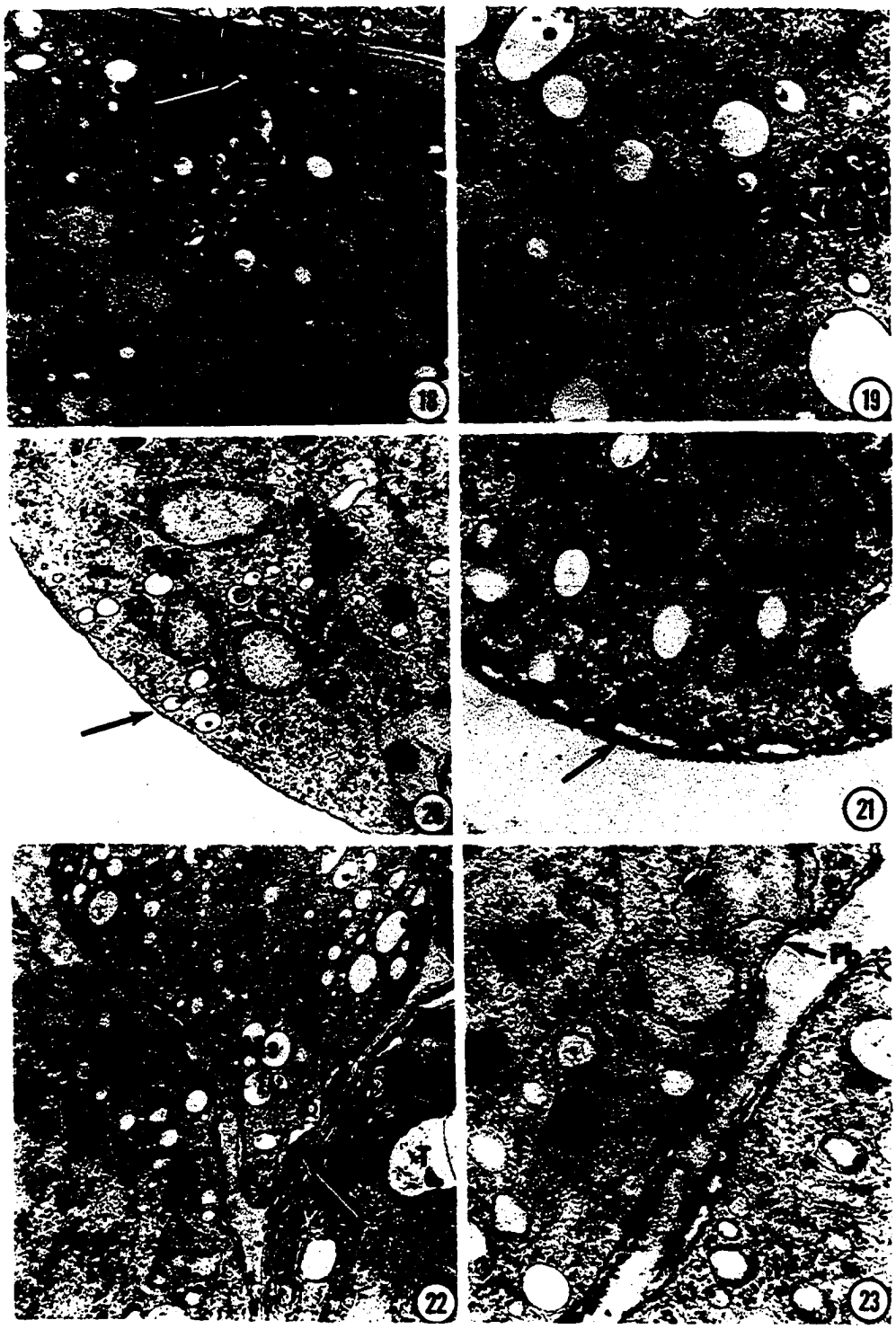
19. Cytokinesis involves union of cell plates in the central portion of the cell. X 10,080.

20. Microspores after cytokinesis are first bound only by the plasmalemma (arrow). X 10,340.

21. While still enclosed in callose, microspores secrete primexine (arrow) external to the plasmalemma. X 17,820.

22. Probacullae (arrow) are produced within the primexine. Microspores (Ms) collapse after this stage. X 5,300.

23. Cytoplasmic components of degenerating microspores: Mt = mitochondria, P = plastids, Pb = probacullae. X 11,950.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deposited external to the plasmalemma (Fig. 21). Probacullae then were formed within the primexine (Fig. 22). This was the last stage of microspore wall formation observed in male-sterile plants; subsequently, the microspores collapsed. At the onset of degeneration, microspore cytoplasmic components appeared similar to those of male-fertile plants. Mitochondria displayed one predominant crista; plastids contained one to three lamellae and one or more electron dense osmiophilic bodies (Fig. 23). The tetrads aborted while still enclosed in callose (Fig. 24). In male-fertile plants, uniform deposition of the tectum and columellae was attained before callose dissolution (Fig. 25). Completion of the ektexine by male-sterile plants was not achieved.

At the time of tetrad degeneration, tapetal cells of male-sterile plants were variable in nature. Most contained little cytoplasm, the bulk of the cellular volume being occupied by vacuoles (Fig. 26). The inner cell wall was persistent (Fig. 26,27). The tapetal cytoplasm was usually disorganized and appeared occluded when compared to that of adjacent tetrads and male-fertile tapetal cells (Figs. 27,32). Mitochondria, though infrequent, appeared normal, while plastids often were disrupted (Fig. 28). Infrequently, tapetal cells at this time appeared similar to those observed at the sporogenous stage (Fig. 29). The cytoplasm remained dense, with few vacuoles, numerous mitochondria and plastids.

Fig. 24-29. Aspects of tetrads and tapetal cells.

24. Tetrad of microspores of male-sterile plant collapsing while still enclosed in callose (C). X 1,900.

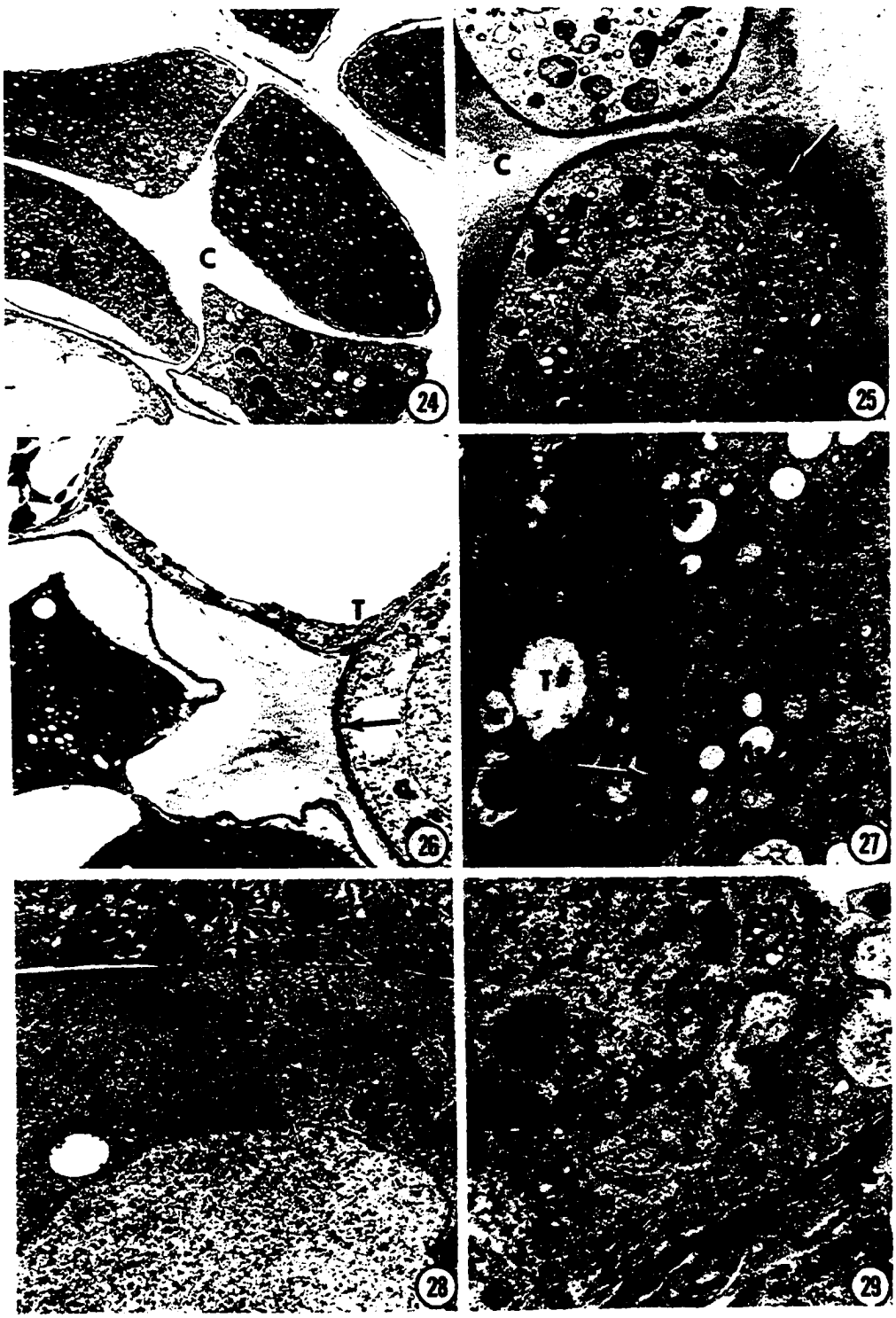
25. Microspores of male-fertile plants complete ektexine deposition (arrow) while still enclosed in callose (C). X 2,050.

26. Tapetal cells (T) and tetrad of male-sterile plant. Tapetal cells retain inner tangential cell walls (arrow) and become vacuolate. X 4,390.

27. Differential staining properties of male-sterile tapetum (T) and microspore (Ms). Inner tangential cell wall of tapetal cell is evident (arrow). X 12,200.

28. Infrequently, tapetal cells of male-sterile plants retain a dense cytoplasm at tetrad stage. X 3,000.

29. Tapetum of male-sterile plant at the tetrad stage: mitochondria (Mt) appear normal, but plastids (P) are disorganized. X 12,320.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Such cells did not mature in the manner of tapetal cells of male-fertile plants. Rather, they stagnated in development, remaining unchanged as anther maturation progressed.

The pattern of tapetal differentiation in male-fertile plants contrasted markedly with that of male-sterile plants (Figs. 30-35). During the meiotic stage, the inner tangential cell wall was degraded (Fig. 30), and dictyosomes and strands of ER accumulated (Fig. 31). At the tetrad stage the plasmalemma facing the locule was devoid of a cell wall and displayed an undulating contour (Fig. 32). Extensive profiles of ER occurred (Fig. 31,33). In the late microspore stage, tapetal cells became vacuolate (Fig. 34). By the pollen stage, the cytoplasm was disoraganized, but still bound by a plasmalemma (Fig. 35). Numerous cytoplasmic lipid bodies and plastid-bound osmiophilic bodies were present.

The ms2 mutant had no discernible effects on gross stamen morphology until advanced stages of development. Anthers were initiated as individual primordia (Fig. 36). Ten anthers were present at maturity; they developed in two whorls of five, with the outer whorl developing first (Fig. 37). Filaments did not elongate until one to three days pre-anthesis. The pistil developed by growing through the center of the staminal whorls. The first evidence of the pistil was the exertion of ovarian trichomes above the anthers (Fig. 37), and stigma elevation followed (Fig. 38). At this time,

Fig. 30-35. Development of tapetum of male-fertile plants.

30. During meiotic stage, inner tangential cell wall dissolves (arrow). X 6,320.

31. Tapetum during meiotic stage: dictyosomes (D) and endoplasmic reticulum (ER) become common. X 5,925.

32. Tapetum at tetrad stage: inner tangential wall is absent, plasmalemma bears an undulating profile (arrow). X 6,200.

33. Tapetum at tetrad stage: extensive profiles of endoplasmic reticulum (ER) are evident. X 10,400.

34. Tapetum at late microspore stage: cytoplasm becomes vacuolate, but organelles are still abundant. X 5,100.

35. Tapetum at engorged pollen stage: plastids accumulate electron dense inclusions; lipid bodies (L) become common. The cytoplasm appears disorganized but cells are still bound by a plasmalemma (arrow). X 5,925.

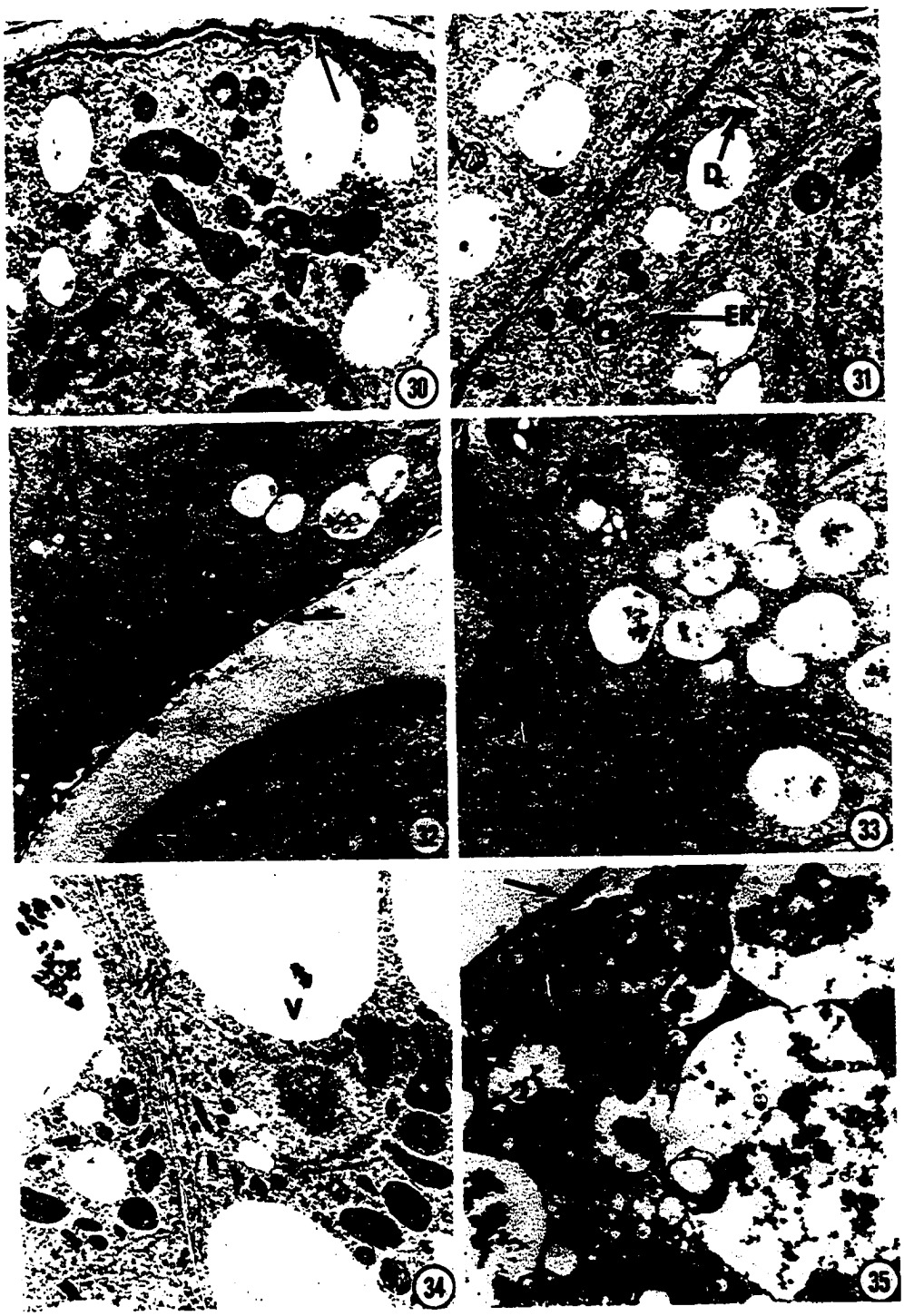
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Fig. 36-41. Morphologic features of anthers of male-sterile and male-fertile plants.

36. Initiation of anthers as individual primordia. X 270.

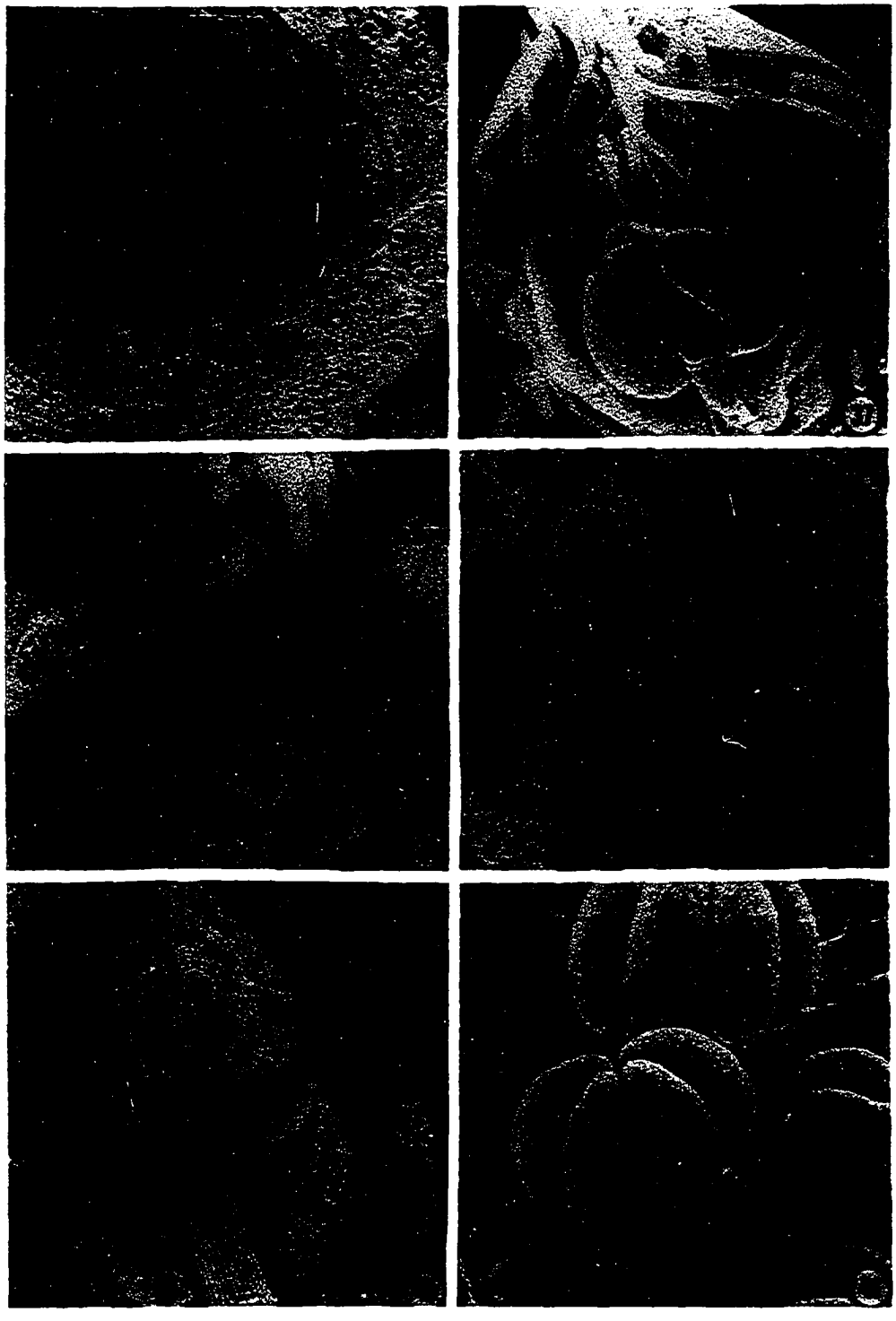
37. Exertion of ovarian trichomes through the center of anther whorls. X 94.

38. Exertion of stigma: male-sterile anthers begin to appear shriveled. X 132.

39. Continued degradation of male-sterile anther. X 225.

40. Male-sterile anthers at anthesis. X 120.

41. Male-fertile anthers at anthesis. X 85.



sterile anthers began to appear shriveled; internal events (i.e. tetrad abortion) had been completed. As floral development progressed, anthers became increasingly abnormal in form (Fig. 39). Approximately three days before flowering, their shrunken and degenerate appearance was obvious (Fig. 40). At anthesis, anthers of male-sterile plants appeared shrunken and collapsed, while those of male-fertile plants were full and plump (Fig. 41). Male-sterile anthers did not dehisce.

Discussion

Cytological effects of several male-sterile mutants of soybean have been reported (Albertsen and Palmer, 1979; Palmer et al., 1980; Delannay and Palmer, 1982; Stelly and Palmer, 1982; Buntman and Horner, 1983). Phenotypic expression of the ms2 locus showed similarities to that of the msp and ms3 loci. In msp, abortion of reproductive cells occurred at any stage of anther development, but was most common at prophase I or the tetrad stages (Stelly and Palmer, 1982). Tapetal abnormalities, in the form of excessive vacuolation or premature collapse of tapetal cells, were evident. Tapetal malfunction was usually obvious before germline cells aborted. In the ms3 mutant, premature tapetal degeneration occurred concurrently with the abortion of microspores (Palmer et al., 1980; Buntman and Horner, 1983). Microspores aborted while still enclosed in callose, but wall

formation progressed further than in the ms2 mutant (Buntman, 1983). The behavior of the endothecial and parietal layers was identical in ms2 and ms3. Gross anther morphology at anthesis also was similar.

The ms1 and ms4 mutants of soybean also have been analyzed cytologically. In the former, male-sterility was attributed to the failure of postmeiotic cytokinesis (Albertsen and Palmer, 1979). In ms4 cytokinesis either failed or was delayed (Delannay and Palmer, 1982). Orientation of divisions often was irregular so that cells possessing zero to four nuclei were present. Most subsequently aborted, though pollen-like cells were generated in some anthers.

The induction of male-sterility by the ms2 mutant may involve interactions between the tapetal cells and the MMCs and/or microspores. The differentiation of tapetal cells in male-sterile plants clearly deviated from the pattern in male-fertile plants. Microspores, however, were identical in male-sterile and male-fertile plants. No apparent structural abnormalities occurred until the onset of degeneration. Microspore abortion may have been the result of the absence of a functional tapetum. Aberrant tapetal development has been correlated with both NMS and CMS in many plant species (Colhoun and Steer, 1981; Delannay 1979; Dundas et al., 1981; Greyson et al., 1980; Horner, 1977; Horner and Rogers, 1974; Lee et al., 1979; Nakashima and Hosokawa, 1974; Overman and

Warmke, 1972; Scoles and Evans, 1979).

The concentration of organelles, especially ER and dictyosomes, and the frequent dissolution of inner tangential cell walls, suggest that the main function of the tapetum is the secretion of metabolites essential to pollen formation (Buchen and Sievers, 1981). Several precise functions have been attributed to the tapetum. Stieglitz (1977) identified a B-1,3-endoglucanase (callase), the maximum activity of which coincided with the dissolution of callose. The likely source of this enzyme was the tapetal layer. Tapetal cells may synthesize sporopollenin or its precursors for use in pollen wall deposition (Echlin, 1971; Horner and Pearson, 1978). Various substances that coat mature pollen grains (i.e. tryphine and "Pollenkitt") are of tapetal origin (Dickinson, 1973; Pacini and Casadoro, 1981; Stevens and Murray, 1981).

Most pollen grains, including those of soybean (Albertsen and Palmer, 1979) pass through a vacuolate stage as metabolites are expended in wall formation. By anthesis, however, pollen engorgement with food reserves has occurred. As the tapetum surrounds developing pollen grains it may synthesize or at least regulate the passage of substances necessary for pollen enrichment. The liberation of carbohydrates and lipids into anther locules by the tapetal cells has been documented (Gori, 1982; Reznickova and

Dickinson, 1982). Christensen and Horner (1974) observed that the single aperture of Sorghum pollen always was oriented toward the tapetum. Degeneration of tapetal cytoplasm coincided with pollen engorgement, and the accumulation of starch grains by pollen began in the cytoplasmic region adjacent to the pore.

The callosic sheath surrounding MMCs may present a barrier to the passage of material between MMCs and tapetal cells. However, small molecules such as acetate and glucose can traverse it (Albertini et al., 1981). Subsequent to meiosis, callose dissolves, liberating the microspores. In soybean, the timing of callose dissolution coincides with maximum tapetal development (Albertsen and Palmer, 1979). In ms2, callose was persistent, and microspores degenerated without being freed. The failure of callose to dissolve may be a consequence of the inability of the tapetal layer to produce and/or release enzymes necessary for its degradation. This may, in turn, prevent additional tapetal-derived compounds from reaching the microspores. However, sterility also may be a result of the cumulative effects of aberrant tapetal ontogeny.

Misbehavior of callose, perhaps indicative of tapetal malfunction, has been observed in several male-sterile systems. Premature breakdown of callose occurred in male-sterile Sorghum (Warmke and Overman, 1972) and the RM CMS

Petunia (Izhar and Frankel, 1971). Delayed or absent callose degradation was noted in ms3 NMS soybean (Buntman and Horner, 1983), Capsicum (Horner and Rogers, 1974), Helianthus (Horner, 1977), and the RM NMS Petunia (Izhar and Frankel, 1971). Izhar and Frankel found that the timing of callase activity was a function of locular pH. In male-fertile plants, callose dissolution coincided with a drop in pH from 7.0 to 6.0. Premature or delayed pH changes led to mistiming of callose degradation.

The ms2 mutant behaves in a similar manner to a NMS mutant of Cajanus cajan, another legume. In both, tetrads degenerate without being released from callose, tapetal cells vacuolate prematurely, and the inner parietal layer expands (Dundas et al., 1981). Thus, the function encoded by the ms2 locus may be essential for pollen maturation in legumes.

Ultrastructurally, the phenotypic behavior of the ms2 locus demonstrates similarities to CMS systems of Helianthus (Horner, 1977) and Capsicum (Horner and Rogers, 1974). In Helianthus, tetrads aborted while still enclosed by callose, and microspore wall formation progressed only as far as the deposition of primexine and a portion of the exine. The inner parietal layer enlarged. The tapetal layer was of the plasmodial type, so direct comparisons with the soybean parietal tapetum are not possible. However, tapetal dysfunction, evidenced by radial enlargement and premature

degeneration, occurred. In Capsicum, primexine was formed, but microspores degenerated without any further wall formation and while still enclosed in callose. Tapetal cells vacuolated prematurely, remained uninucleate (male-fertile tapetal cells became binucleate in Capsicum, but remained uninucleate in soybean), and retained their primary cell walls. Parietal layer expansion occurred. In ms2, and in the Helianthus and Capsicum CMS systems, microspores were normal in appearance until degeneration, while the tapetal layer developed improperly.

The similarities between ms2 and these other systems are of interest when one considers that the mode of inheritance differs. Though the molecular basis for sterility in Helianthus and Capsicum has yet to be elucidated, the steriltiy factor must reside in the cytoplasm in order to be uniparentally inherited. The ms2 mutation occurred in a nuclear gene. Thus, there may be some fundamental similarities in the biochemical causes of NMS and CMS, though the similarities observed in cellular phenotypes may be only indicative of the need for both nuclear and cytoplasmic functions in the genesis of pollen.

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SECTION II: ANEUPLOIDS FROM A MALE-STERILE MUTANT (ms2) OF SOYBEAN (GLYCINE MAX (L.) MERR.)

Abstract

The tendency of a male-sterile mutant (ms2) of soybean (Glycine max (L.) Merr., 2n=40) to produce aneuploids was investigated. The rate of spontaneous trisomy among hybrid progeny of male-sterile plants was 2.4%. Transmission of extra chromosomes to subsequent generations was studied. F1 trisomics produced F2 aneuploids at a rate of 36.0% 2n=41 plus 3.5% 2n=42 chromosome plants. F2 plants with 42 chromosomes produced 76.0% aneuploid progeny. Pollen analysis demonstrated that trisomy leads to no significant reduction in pollen fertility. The low frequency of F1 aneuploids may render the use of ms2 as a source of trisomics impractical. However, once trisomic lines are established, they are fairly stable.

Introduction

Primary trisomics may be used to establish linkage relationships between genetic loci and specific chromosomes (Khush, 1973). They may also provide information on the effects of individual chromosomes on plant phenotype, and may be employed in attempts to identify chromosomes involved in translocations. In soybean, only three of 20 possible

primary trisomics have been reported (Palmer, 1976; 1984). No linkage group has been assigned to a specific chromosome. To date, only one mutant, a chimeric chlorophyll-deficiency character, has been located on one of the three primary trisomics (Palmer, 1984). Thus, in order to more fully characterize linkage relationships in soybean, the discovery and isolation of additional primary trisomics are necessary.

In soybean, trisomics have been obtained from asynaptic and desynaptic mutants (Palmer, 1976), from irradiated material (Sadanaga and Grindeland, 1979), from seed produced by haploids obtained from the msl male-sterile mutant (Sorrells and Bingham, 1979), and from an F4 population derived from a cross between msl male-sterile plants and a translocation homozygote (Chen et al., 1984). Chen and Palmer (In Preparation) have obtained trisomics indirectly from triploids derived from msl male-sterile plants. Progeny of triploids ranged in chromosome number from 44 to 71. Upon selfing, some of these aneuploids produced trisomic progeny.

Triploids are one of the most common sources of trisomics in plants (Khush, 1973). Sadanaga and Grindeland (1981) unsuccessfully attempted to isolate triploids through natural cross-pollination between an induced tetraploid line and a diploid male-sterile (ms2) line. However, among 175 diploid hybrid progeny of the male-sterile plants, six trisomics, one tetraploid, and one mixoploid were isolated. Thus, the

occurrence of trisomics in progeny of ms2 male-sterile plants may circumvent the need to produce triploids.

This report summarizes observations on the tendency of the ms2 male sterile to produce aneuploids. Additional screening of the F1 population produced by Sadanaga and Grindeland (1981) was conducted in an attempt to isolate more trisomics. Trisomic lines were then evaluated in regards to transmission of extra chromosomes to subsequent generations. The effect of the additional chromosomes on pollen fertility also was studied.

Materials and Methods

Sadanaga and Grindeland (1981) selected hybrid progeny of green-seeded male-sterile plants (d1d1d2d2ms2ms2). Putative pollen parents were yellow-seeded and male-fertile (D1D1D2D2MS2MS2). Hybrid seed was recognized by virtue of its yellow cotyledons. Trisomics were isolated from four of six plots. In the present study, an additional 20 hybrid seed was selected at random from each of the same six plots. Chromosome counts were obtained on all seedlings by the method of Palmer and Heer (1973). To verify further the hybrid nature of the seed tested, F2 segregation ratios for cotyledon color were obtained. Each F1 plant would be expected to yield F2 seed segregating in a ratio of 15 yellow:1green. Chi-square analysis was used to test for

departures from the expected ratios. F2 plants were classified for male-sterility/fertility as well.

Trisomics isolated by Sadanaga and Grindeland (1981) and those found in the present study were analyzed further. Transmission of extra chromosomes via self-pollination was determined by obtaining chromosome counts on a minimum of 25 F2 plants per F1 trisomic. F1 plants produced seven F2 plants with 42 chromosomes. Chromosome counts were obtained on F3 and F4 progeny of these 42-chromosome plants to determine the stability of these lines.

The influence of extra chromosomes on pollen fertility was analyzed by squashing anthers in a dilute solution of I2KI. A minimum of 500 pollen grains per plant were counted. Grains appearing clear and shrivelled were considered abortive; dark staining grains were considered fertile. Pollen counts were obtained from both 40- and 41-chromosome F2 individuals. At least eight plants per family were examined. Differences in mean pollen fertility between 40- and 41-chromosome plants were tested by use of Student's t-test. The L.S.D. procedure (Steel and Torrie, 1980) was used to test for differences between families.

Results

One trisomic was isolated from the 120 F1 plants analyzed. Combined with the data of Sadanaga and Grindeland (1981), the

frequency of spontaneous trisomy was 7/295 or 2.4%. The trisomic arose in a plot that previously had not produced a trisomic. All 120 F1 plants produced both yellow and green seeds, and were male fertile. Thus, they were of hybrid origin. All but nine adhered to the expected segregation ratio of 15:1. Of the nine that deviated, one segregated at a ratio that fit a 3;1 ratio. This indicates that a pollen grain of genotype d1D2 or D1d2 must have participated in fertilization. The remaining eight deviations may have been due to random chance or errors in counting seed.

Transmission of extra chromosmomes by the seven trisomics following self-pollination is given in Table 1. The frequency of aneuploids among progeny of trisomics ranged from 16.0% to 57.5%, with the mean being 39.5%. Production of 42-chromosome plants was rare. However, seven plants were identified, indicating simultaneous transmission of extra chromosomes by both ovules and pollen.

Chromosome numbers of F3 and F4 progeny of six 42-chromosome plants are given in Table 2. Three of the seven F3 42-chromosome plants did not set seed. Two more produced two and three seeds, respectively, but seeds failed to germinate. The remaining two varied in both seed set and transmission of extra chromosomes (Table 2). F4 progeny also varied in the chromosome numbers of progeny produced. The frequency of aneuploidy in these plants was 76.0%. One 42-chromosome

Table 1. Chromosome numbers in progeny of F1 trisomic plants

Trisomic identity	Number of progeny with respective chromosome number					
	40	41	42	%41	%42	%aneuploid
KS106-22	16	13	0	44.8	0	44.8
KS106-25	16	9	4	31.0	13.8	44.8
KS408-27	23	7	0	23.3	0	23.3
KS408-38	21	4	0	16.0	0	16.0
KS504-24	16	15	0	48.5	0	48.4
KS606-25	18	9	3	30.0	10.0	40.0
BG-34	11	15	0	57.6	0	57.6
Total	121	72	7	36.0	3.5	39.5

Table 2. Chromosome numbers in progeny of 42-chromosome plants

Plant identity	Number seed set	Number of progeny with respective chromosome number						
		40	41	42	43	%41	%42	%aneuploid
KS606-25-4	51	7	9	6		40.9	27.3	68.2
BG-256*+	16	0	16	0		100.0	0	100.0
BG-274+	@200	4	3	8	1	18.8	50.0	75.0
BG-311+	@200	6	4	5		26.7	33.3	60.0
KS106-25-18	6	5	0	1		0	16.7	16.7
BG-291++	21	1	4	16		19.1	76.2	95.2
Total		23	36	36	1	37.5	37.5	76.0

* = male-sterile plant
+ = progeny of KS606-25-4
++ = progeny of KS106-25-18

plant was male-sterile. All of its progeny had 41 chromosomes.

Results of the analysis of pollen fertility are given in Table 3. When summed across families there was no significant difference (at the Ø.Ø5 level) in mean pollen fertility between 4Ø- and 41-chromosome plants. Within families, three showed no significant differences. In the remainder, three showed higher fertility in 4Ø-chromosome plants, while one had greater fertility in 41-chromosome plants.

In one family (KS1Ø6-22), pollen fertility in all plants was diminished when compared to that of all other families. As there was no difference in fertility within this family, mean fertility was compared to that of all other families by combining results from both 4Ø- and 41-chromosome plants. These results are given in Table 4. Mean pollen fertility differs between KS1Ø6-22 and all other families. The remaining other families did not differ significantly from each other. The reduction in fertility within this family was not due to the extra chromosome, and was an inherited trait. Progeny of one member of this family (KS1Ø6-22-4, 2n=41, pollen fertility = 69.7%) were analyzed for both chromosome number and pollen fertility. Ten plants with 4Ø chromosomes and six with 41 chromosomes were found. The respective pollen fertilities for these classes were 84.1%

Table 3. Pollen fertility of F2 40- and 41-chromosome plants

F1 trisomic parent	Mean pollen fertility (%)			
	40-chromosome plants	41-chromosome plants	t	p
KS106-22	76.90	80.27	0.19	>0.80
KS106-25	98.56	97.50	1.67	>0.10
KS408-27	99.27	94.67	6.64	<0.05
KS408-38	98.90	91.98	7.14	<0.05
KS504-24	99.43	96.30	2.51	<0.05
KS606-25	98.73	93.43	2.12	>0.05
BG-34	97.87	99.01	2.23	<0.05
Total	96.09	92.59	1.52	>0.05

Table 4. Pollen fertility by family; pooled results from 40- and 41-chromosome plants

Trisomic Parent	Mean pollen fertility (%)
KS106-22	79.06
KS106-25	98.17*
KS408-27	97.73*
KS408-38	95.44*
KS504-24	98.00*
KS606-25	95.20*
BG-34	98.55*

* Indicates means that do not differ at the 0.05 level.

and 85.4%.

Discussion

Khush (1973) does not list male-sterile mutants as a source of trisomics. In soybean, progeny of ms1 male-sterile plants often demonstrate increases or decreases in ploidy level (Kenworthy et al., 1973; Beversdorf and Bingham, 1977; Chen et al., 1984), but aneuploids have not arisen directly from diploid ms1 male-sterile plants. Buss and Autio (1980) found one twin seedling and a triploid among progeny of ms2 male-sterile plants. There is no significant reduction in female fertility associated with the ms2 locus (Graybosch et al., 1984). The tendency of the ms2 mutant to yield trisomic progeny may be unique. Graybosch et al. (1984) determined that male-sterility in the ms2 system was due to an abortion of microspores at the tetrad stage, but they did not note meiotic abnormalities. The origin of abnormalities associated with the female reproductive system of the ms1 mutant has been determined (Cutter and Bingham, 1977; Kennell, 1984). Observations on female reproduction in ms2 male-sterile plants are needed before the origin of trisomic progeny can be explained.

The frequency of trisomy (2.4%) associated with ms2 may not be great enough to allow ms2 to serve as a practical source of aneuploids. However, once aneuploids are isolated, trisomic lines are fairly stable. Plants with 41 chromosomes

yielded up to 57.6% aneuploid progeny, while 42-chromosome plants produced up to 100% aneuploid offspring. The transmission of extra chromosomes via self pollination by the trisomic lines was less than that reported by Palmer (1976) for Trisomics A, B, and C, and that reported by Sorrells and Bingham (1979) for trisomics isolated from haploids. In the present study, 36.0% of the F2 progeny had 41 chromosomes. Palmer (1976) found 49.3%, while Sorrells and Bingham (1979) isolated 41% trisomic progeny. Transmission rate may vary with the specific chromosome in question. The wide range in transmission rate in the present study (16.0% to 57.6%) may indicate that several different primary trisomics were involved.

Selfing by Trisomics A, B, and C yielded 3.0% 42-chromosome plants (Palmer, 1976). Sorrells and Bingham (1979) failed to obtain 42-chromosome plants in progeny of trisomics. In this study, 42-chromosome progeny occurred at a rate of 3.5%. Progeny of 42-chromosome individuals ranged in chromosome number from 40 to 43. True-breeding lines for 42 chromosomes were not located. Gwyn (1984) found that approximately 85% to 90% of the progeny of 42-chromosome plants also had 42 chromosomes. In this study, no more than 76.2% of the progeny of a 42-chromosome plant bore 42 chromosomes; the mean frequency was 37.5%. Again, the differences may be due to differential effects of various

chromosomes on plant development.

Selfing by trisomic plants did not give the theoretical ratio of 1 2n: 2 2n+1: 1 2n+2 progeny. This is a common phenomenom in plants. Khush (1973) attributed this to several causes, including elimination of chromosomes during meiosis, reduced viability of n+1 gametes or spores, abnormal development of aneuploid zygotes or endosperm, or poor or delayed germination of aneuploid seed. In this study, there was no significant reduction in pollen fertility associated with extra chromosomes when results from all families were summed. Even in those families in which pollen fertility was lower in 41-chromosome plants, at least 90% of the pollen from 41-chromosome plants appeared fertile. Thus, it does not seem likely that there is selection against aneuploid pollen before anther dehiscence. However, n pollen may participate in fertilization more often than n+1 if it exhibits more vigorous growth after germination.

The reduction in pollen fertility in family KS106-22 was not associated with the presence of the extra chromosome, as there was no difference in mean pollen fertility between 40- and 41-chromosome plants. It may be due to the presence of some other type of chromosomal abnormality, such as an inversion or translocation. Observations of meiotic divisions in this family were obtained but revealed no evidence that a translocation or paracentric inversion was

present. Further study is necessary to determine whether a pericentric inversion is present.

The need for a complete linkage map of soybean is obvious. The seven trisomic lines examined in this study may be useful in attaining that goal. Crossing experiments including the three known primary trisomics, and subsequent meiotic analysis of resultant 42-chromosome plants are necessary to first characterize these trisomic lines.

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SECTION III: PHENOTYPIC EXPRESSION OF A MALE-STERILE MUTANT (ms4) OF SOYBEAN (GLYCINE MAX (L.) MERR.)

Abstract

Observations on the reproductive biology of a male-sterile mutant (ms4) of soybean (Glycine max (L.) Merr.) demonstrated that male-sterility was the result of a syndrome of abnormalities that influenced the function of the postmeiotic microspore mother cells (MMCs). Cytokinesis following telophase II was absent, incomplete, or disoriented, resulting in cells with varying numbers of nuclei. Postmeiotic (PM) cells demonstrated various abnormalities in the formation of pollen walls. The nature of the exine ranged from absence through amorphous accumulations of sporopollenin to stratified layers. Infrequently, anther locules functioned properly and normal pollen was differentiated. The extent and frequency of cytokinesis varied both between and within locules of single anthers. Temperature influenced the frequency of cytokinesis. At temperatures of 35C/32C day/night, cytokinesis did not occur. Male-sterility was concluded to result from the cumulative effects of various abnormalities, all of which involve functions of the postmeiotic MMCs plasmalemma.

Introduction

Several nuclear male-sterile (NMS) mutants have been reported in the cultivated soybean (Glycine max (L.) Merr.) (Graybosch and Palmer, 1984a). All were inherited as monogenic recessive characters. Observations on the developmental reproductive biology of most of these mutants have been reported (Albertsen and Palmer, 1979; Palmer et al., 1980; Delannay and Palmer, 1982; Stelly and Palmer, 1982; Buntman and Horner, 1983; Graybosch et al., 1984). In two soybean NMS mutants, ms1 and ms4, male sterility was due, or related to, the failure of postmeiotic cytokinesis. The omission of cytokinesis, as a mechanism of male sterility in angiosperms, has been cited only infrequently (Laser and Lersten, 1972; Gottschalk and Kaul, 1974). Therefore, it is notable that two such mutants have been reported in the same species.

Light microscopic observations on the effects of the ms4 locus were reported by Delannay and Palmer (1982). They found that postmeiotic cytokinesis in microspore mother cells (MMCs) was either absent or delayed. Planes of division were often irregular, so that cells with variable numbers (0-4) of nuclei resulted. Subsequent development of reproductive cells was variable. Many degenerated, while some persisted until anthesis. Premature vacuolation of the tapetal cells often accompanied degeneration of germline

cells. At times, the orientation of cytokinesis was normal, and pollen-like cells were formed. These cells were suspected of being capable of effecting fertilization. Graybosch and Palmer (1984b) showed that ms4 male-sterile plants were capable of a low rate of seed production in the absence of pollinating vectors. The response was found to be temperature-dependent.

Albertsen and Palmer (1979) presented light- and electron-microscopic observations on anther development in ms1 male-sterile plants. Failure of cytokinesis was complete; cytokinesis was not observed in any cells. The resulting four-nucleate cells, termed coenocytic microspores (CMs), mimiced pollen grains through the formation of pollen-like walls and engorgement with starch and lipids. No tapetal abnormalities were detected.

The ms4 mutant was studied only through the use of FAA and CRAF III fixed material (Delannay and Palmer, 1982). Such fixatives often induce artifacts that make the interpretation of male-sterile characters difficult (Laser and Lersten, 1972). Also, no ultrastructural observations have been reported. Due to these reasons, and to the unique mechanism of male sterility, additional observations with the light and electron microscopes would be useful in understanding the mechanism of male-sterility in the ms4 system, and its relationship to the type of sterility induced by the ms1 mutant.

Materials and Methods

Plants studied were descendants of the original ms4 male-sterile plant isolated by Delannay and Palmer (1982). The strain is maintained as an isogenic line of cultivar Rampage. Reproductive buds from male-fertile (Ms4 ___) and male-sterile (ms4ms4) were harvested. Individual anthers were removed and processed for light-and electron-microscopic observations by previously described methods (Section I). Plants were either greenhouse-grown, or cultivated in a growth chamber under a 29C/23C day/night temperature regime. Photophase was initially 16 hours, reduced to 14 hours after three weeks to induce flowering. Lighting was provided by fluorescent and incandescent lights.

Fluorescence microscopy was used to analyze the nature of cytokinesis in male-sterile plants. Buds were fixed in a 3:1 mixture of absolute ethanol:acetic acid. Anthers were removed and squashed in a Ø.ØØ5% solution of aniline blue in Ø.15 M phosphate buffer, pH 8.2 (Jensen, 1962) to detect the presence/absence of callose. Observations were obtained through use of a Zeiss Standard WL microscope equipped with a high pressure mercury lamp, using heat filter BG38, exciter filter UG1 and a 41Ø nm barrier filter.

To investigate the effects of temperature on the frequency of cytokinesis, flower buds were harvested one day pre-

anthesis from male-sterile plants grown under three temperature regimes for the duration of their life cycle. Day/night temperatures used were: 24C/21C, 29C/23C and 35C/32C. Lighting conditions were cited above. Buds were fixed in 3:1, and anthers squashed in a dilute solution of I_2KI.

Results

Light microscopy

Anther and pollen development in male-fertile soybean have been described by Carlson (1973), Albertsen and Palmer (1979) and Buntman and Horner (1983). Observations of male-fertile development are herein presented only when pertinent to the present discussion. Results presented will demonstrate the production of normal pollen by ms4ms4 individuals. To simplify terminology, all homozygous recessive individuals will be refered to as "male-sterile" regardless of whether pollen was formed.

Anther development was normal during early stages of microsporogensis. During the sporogenous stage (Fig. 1), cytoplasmically dense sporogenous cells were surrounded by the tapetal, parietal, endothecial and epidermal layers. MMCs were differentiated from sporogenous cells (Fig. 2). As meiosis progressed, MMCs became isolated by callose. The tapetal layer enlarged, and cells of the anther wall became

vacuolate (Fig. 3). Chromosome movement during meiosis was normal, and four daughter nuclei were formed. Subsequent to telophase II, development of postmeiotic (PM) cells was highly variable both between and within locules of a given anther.

In many locules, cytokinesis failed, and daughter nuclei migrated to the center of the cell (Fig. 4). Behavior of the resulting quadrinucleate cells (coenocytic microspores or CMs) also was highly variable. Many (Fig. 5-7) were incapable of organizing any sort of pollen-like walls, and soon degenerated (Fig. 7). Material with staining properties similar to those of sporopollenin was observed surrounding CMs (Fig. 5,6), accumulating on the outer tangential surface of the tapetal cells (Fig. 6), or filling the locules after CMs had degenerated. In other cases, CMs formed a thick pollen-like wall (Fig. 8-12). Such cells typically became vacuolate and degenerated (Fig. 8-10). The behavior of the tapetum in such cases was variable. In some locules, CMs degenerated while the tapetum retained its cytoplasm and appeared functional, while in others, the tapetal cells became highly vacuolate and devoid of cytoplasm. Tapetal vacuolation only occurred concurrent with PM cell degeneration. Tapetal malfunction prior to PM cell abortion was not detected.

CMs often retained a dense cytoplasm and persisted to

Fig. 1-12. Anther development in male-sterile plants.

1. Sporogenous stage: sporogenous cells (S) are cytoplasmically dense. X 350.

2. Meiotic stage: meiocytes (M) are distinguished by a less dense cytoplasm than adjacent tapetal cells. X 300.

3. Meiotic stage: callose (arrow) is secreted by meiocytes. X 340.

4. Postmeiotic anther: failure of cytokinesis leads to the production of four-nucleate coenocytic microspores (arrow). X 330.

5. Coenocytic microspores (CM) may become disorganized without organizing pollen-like walls. Sporopollenin-like deposits are evident (arrow). X 360.

6. Sporopollenin-like deposits occur along outer tangential surface of tapetal cells (arrow) and surrounding degenerating CMs. X 390.

7. Sporopollenin-like deposits filling locule (arrow). X 415.

8. CMs forming pollen-like walls, but becoming vacuolate. X 365.

9. Aborted CMs, surrounded by intact tapetum (T). X 330.

10. Simultaneous vacuolation of tapetum (T) and CMs. X 370.

11. CMs retaining dense cytoplasm. Fusion of nuclei is evident (arrow). X 360.

12. Mitotic divisions in CMs (arrows). X 330.

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anthesis. In such cells, fusion of nuclei (Fig. 11) and mitotic divisions (Fig. 12) were observed. It was not determined whether these divisions occurred after nuclear fusion or represented division by one of the CMs nuclei.

The nature and extent of cytokinesis was inconsistent, with variation occurring among locules of the same anther, and within locules. Cytokinesis was often incomplete or irregular (Fig. 13) resulting in the production of cells bearing Ø-4 nuclei. However, divisions of normal extent and orientation did occur. Typical and atypical divisions were observed within the same locule (Fig. 15,16). Cells resulting from such divisions degenerated (Fig. 16) or persisted to maturity (Fig. 17). Incomplete divisions resulted in the occurrence of fused cells (Fig. 17).

Infrequently, pollen grains were formed, though some still degenerated (Fig. 18). Within an anther, the nature of the locular contents at maturity varied. In one case, three adjacent locules were characterized by, respectively: pollen, cells with incomplete cytokinesis, and cells with complete but irregular cytokinesis (Fig. 19). In another case, pollen was differentiated in one locule, while the adjacent locule bore degenerate CMs (Fig. 2Ø).

Aniline blue staining demonstrated that cytokinesis occurred via the deposition of crosswalls containing callose (Fig. 21). Pollen produced was capable of <u>in vitro</u>

Fig. 13-22. Postmeiotic development in male-sterile plants.

13. Irregularly oriented and partial cytokinesis. X 330.

14. Properly oriented cytokinesis. X 370.

15. Occurrence of normal, abnormal and partial cytokinesis within a single locule. X 430.

16. Degeneration of partitioned cells. X 350.

17. Partial cytokinesis resulting in the production of fused cells. X 330.

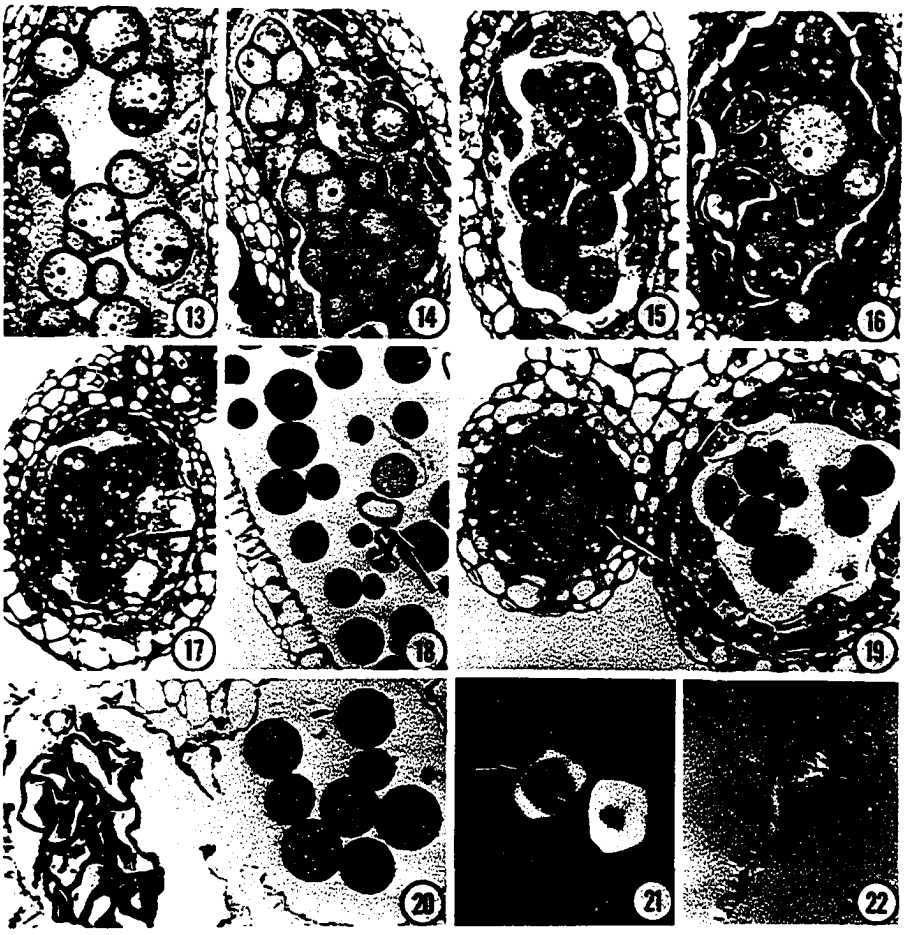
18. Pollen produced by male-sterile plants. Pollen may degenerate before anthesis (arrow). X 335.

19. Occurrence of normal cytokinesis in one locule, with partial cytokinesis in the adjacent locule (arrow). X 415.

20. Occurrence of pollen in one locule with degenerate CMs in adjacent locule. X 330.

21. Septa fluoresce with aniline blue staining, indicative of the presence of callose. X 330.

22. *In vitro* germination of pollen from male-sterile plants. X 280.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germination (Fig. 22). Cytokinesis occurred in all buds sampled from temperature regimes of 24C/21C (9 buds) and 29C/23C (18 buds). In 27 flower buds sampled from the hottest environment (35C/32C), cytokinesis did not occur.

Electron Microscopy

No ultrastructural differences were detected between anther development in male-sterile and male-fertile plants until meiosis had been completed. Behavior of reproductive cells subsequent to telophase II was again variable. Hence, it was not possible to predict the behavior of a given cell had it developed to maturity. Thus, it was not possible to present a complete developmental sequence of events in male-sterile anthers.

Organelles within PM cells were consistent in appearance, and resembled those of male-fertile plants. Mitochondria usually bore one enlarged predominant crista each. Plastids contained one to three lamellae, and included one or more electron dense osmiophilic bodies. The most obvious difference between PM cells was in the nature of the pollen-like wall. At least four degrees of wall formation were attained by PM cells. These were: 1) randomly scattered tufts of sporopollenin; 2) stratified walls, but layers amorphous; 3) stratified walls, with at least one layer, usually the endexine, organized; 4) normal, with an organization consisting of ektexine (tectum, columellae and

pedium), endexine, intine and evident colpi, identical to the pollen walls of normal microgametophytes. This last type occurred only in uninucleate cells; the remainder were found in multi-, uni- and a-nucleate cells.

While still enclosed in callose, PM cells were capable of producing a fibrillar primexine identical to that of male-fertile plants (Fig. 23). Callose dissolution occurred in all anthers examined. Infrequently, PM cells were able to organize only localized deposits of sporopollenin (Fig. 24). In such cells, the primexine was not present between areas of sporopollenin; however, it could not be determined whether primexine deposition had been omitted or primexine had been degraded. These cells were destined to degenerate. Numerous autophagic vacuoles enclosed portions of the cytoplasm (Fig. 24). These coalesced to form large vacuoles, and abortion followed (Fig. 25).

Stratified wall layers were formed, but often were devoid of any type of organization (Fig. 26). Such cells either retained a dense organelle-rich cytoplasm similar to that of male-fertile microspores and persisted to anthesis (Fig. 27) or became vacuolate and degenerated (Fig. 28).

In other cells, stratification of wall layers occurred, and at least one layer was organized (Fig. 29). This layer usually resembled the endexine of normal microspores (Fig. 39). The ektexine was absent or represented by scattered

Fig. 23-27. Variable aspects of postmeiotic cells.

23. Deposition of primexine (arrow). X 20,790.

24. Random deposition of sporopollenin-like particles (arrow) along plasmalemma: autophagic vesicles (A) are common. X 2,660.

25. Cells characterized by localized sporopollenin deposits accumulate vacuoles (V) and degenerate. X 2,000.

26. Postmeiotic cell with stratified but disorganized walls. X 12,000.

27. Postmeiotic cells with poorly organized walls may retain a dense cytoplasm and persist to maturity. X 2,700.

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particles of sporopollenin. The outer surface of such endexine-like walls bore lamellations similar to those observed at the interface of the pedium and endexine of fertile microspores (Albertsen and Palmer, 1979) (Fig. 30).

Pollen wall material often was observed to extend across cleavage furrows when cytokinesis had occurred (Fig. 31), indicating that cytokinesis had been delayed. It does not seem possible that sporopollenin could align itself in this manner unles a membrane was present to assimilate it. Thus, the deposition of sporopollenin occurred along the membrane face, followed by cytokinesis, leaving sporopollenin spanning empty space. In male-fertile plants, pollen wall deposition was not initiated until the completion of cytokinesis; layers of sporopollenin were not observed to traverse cleavage furrows (Fig. 32). In male-sterile plants, sporopollenin deposition occurred within the cleavage furrows (Fig. 33). Daughter cells were joined by common walls across the cell plates (Fig. 33). When cytokinesis was partial, cleavage furrows merely were brought to a halt at some point within the cytoplasm (Fig. 34). No cytoplasmic abnormalities were detected within cells characterized by this type of division.

Pollen identical to that of male-fertile plants was generated in 3.3% (8/220) of all PM locules examined. Pollen contained generative and vegetative cells (Fig. 35), and engorged with lipids (Fig. 36) and starch. The pollen wall

Fig. 28-33. Additional properties of postmeiotic cells.

28. Postmeiotic cells with poorly organized walls often accumulate vacuoles (V) and eventually degenerate. X 5,650.

29. Cell producing endexine (arrow) and pedium but lacking ektexine. X 9,390.

30. Cell producing pedium with lamellations (arrow) typical of pedium. X 17,800.

31. Mitochondria (Mt) and plastids (P) of postmeiotic cells resemble those of male-fertile plants. Pollen wall material (arrow) extends across cleavage furrow. X 8,140.

32. Tetrad of male-fertile plant: Cleavage furrows (arrow) are not traversed by sporopollenin. X 1,700.

33. Postmeiotic cell of male-sterile plant. Pollen wall material (arrow) traverses and fills cleavage furrow. X 2,000.

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was normal, with stratification and colpi distribution identical to that of male-fertile pollen (Figs. 37-39). Colpi were produced only by normal pollen; PM cells with abnormal walls did not form apertures. Only uninucleate cells produced by properly timed and oriented cytokinesis possessed pollen characteristics, but proper cytokinesis did not ensure normal development. PM cells with abnormal walls often persisted to anthesis with dense cytoplasms, but engorgement was not detected.

The only abnormality detected in tapetal cells was excessive and premature vacuolation, and this only occurred when PM cells degenerated early, without undergoing cytokinesis and wall formation. The correlation between early PM degeneration and tapetal malfunction was not absolute; approximately 50% of the locules with this pattern of development displayed tapetal abberrancies.

In most anthers, the postmeiotic tapetum was indistinguishable from that of male-fertile plants. The cytoplasm contained numerous mitochondria, dictyosomes and extensive complexes of endoplasmic reticulum (ER) (Fig. 40, 41). The inner tangential and radial cell walls dissolved. Locules usually were filled with electron dense globules that seemed to erupt from the intercellular spaces between tapetal cells, growing larger as they extended away from tapetal surface (Fig. 40). These globules were absent when normal

pollen was formed. Tapetal cells of completely normal appearance were frequently adjacent to degenerate or disorganized PM cells (Fig. 42,43).

At the time of endothecial differentiation, tapetal cytoplasm became less dense and more vacuolate, a pattern also displayed by male-fertile plants (Fig. 44). One unique feature of male-sterile tapeta was the accumulation of electron-dense sporopollenin-like deposits along the outer tangential cell walls, and within the cytoplasm (Fig. 45).

The ms4 mutant had little influence on gross anther morphology. At two days pre-anthesis, anthers were identical to those of male-fertile plants (Fig. 46), though at anthesis anthers were more shrivelled than normal (Fig. 47). Anther dehiscence occurred, even when the locular contents were degenerate (Fig. 48). Anthers did not open as completely as those of male-fertile plants (Fig. 49). Pollen-like cells were released, but they showed a tendency to adhere to each other in groups of two to four (Fig. 50). Colpi were not present on the surface as in male-fertile pollen (Fig. 50,51). Because of the low frequency of their occurrence, observations of external features of normal pollen generated by male-sterile plants were not obtained.

Fig. 34-39. Additional observations of postmeiotic cells.

34. Cell demonstrating partial cytokinesis. X 4,500.

35. Pollen produced by male-sterile plant. Generative (GC) and vegetative (VC) cells are evident. X 5,400.

36. Lipid bodies (L) are common in male-sterile pollen. X 6,600.

37. Pollen wall of male-sterile plants with intine (I), endexine (En) and ektexine (Ek). X 9,630.

38. Colpus of male-sterile pollen grain. X 10,870.

39. Colpus of male-fertile pollen grain. X 6,170.

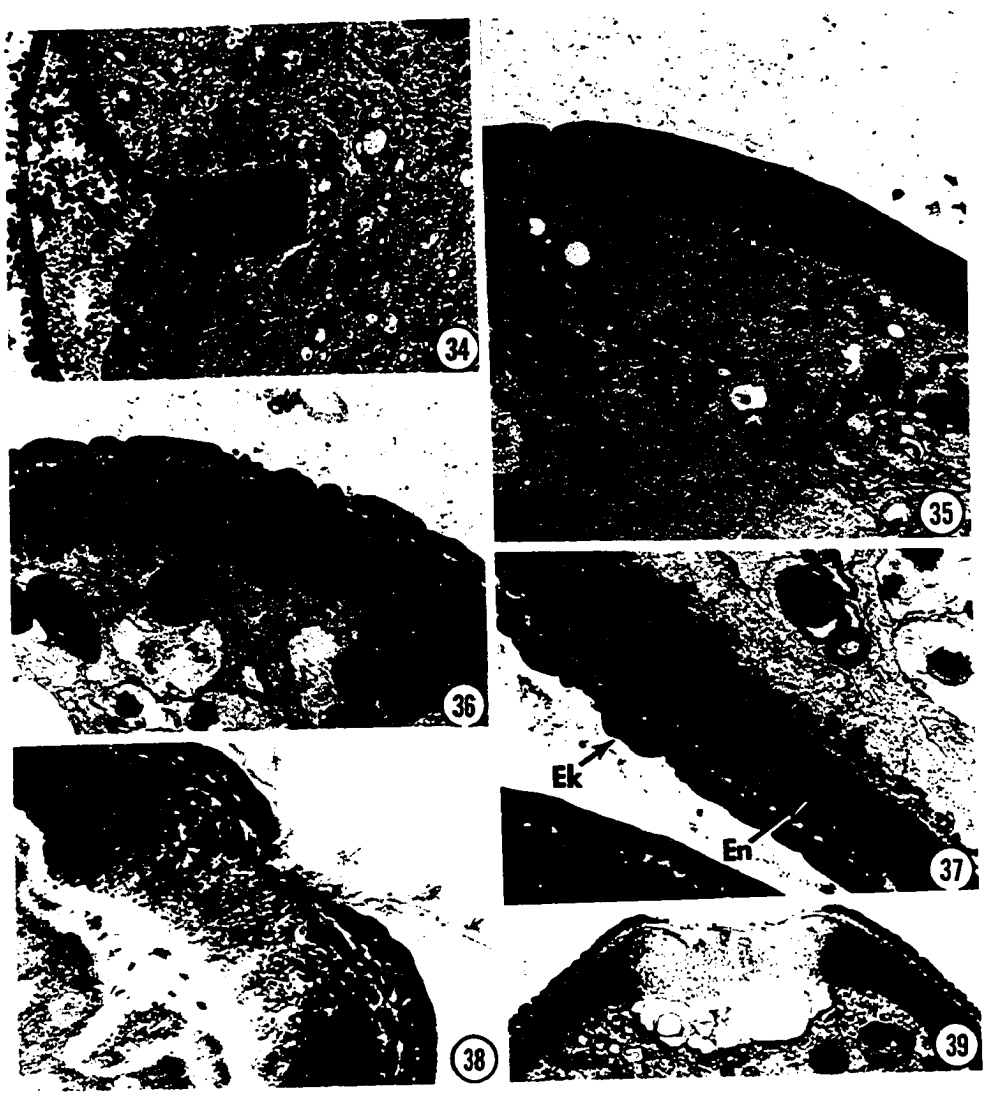
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Fig. 40-45. Aspects of the postmeiotic tapetum of male-sterile plants.

40. Tapetal cells bear numerous mitochondria (Mt) and dictyosomes (D). Sporopollenin-like particles (arrow) accumulate in locule and appear to originate from the tapetum. X 3,000.

41. Endoplasmic reticulum (ER) and dictyosomes (D) are common cytoplasmic components. X 11,200.

42. Tapetal cells (T) of normal appearance adjacent to degenerating postmeiotic (PM) cell. X 4,600.

43. Tapetal cell (T) adjacent to aborted PM cell. X 4,550.

44. Vacuolation (V) of tapetal cell. X 2,500.

45. Accumulation of sporopollenin-like particles (arrows) within and adjacent to tapetum. X 2375.

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Fig. 46-51. Morphological features of anthers and pollen.

46. Male-sterile anthers, two days pre-anthesis. X 60.

47. Male-sterile anther, one day pre-anthesis. X 220.

48. Male-sterile anther at anthesis. X 260.

49. Male-fertile anther at anthesis. X 225.

50. Pollen-like cells released by male-sterile anthers. X 550.

51. Male-fertile pollen. X 1,125.

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Discussion

The results of this study essentially confirm those of Delannay and Palmer (1982) on the nature of male sterility in the ms4 system. In the present report, degeneration of PM cells without the organization of any sort of pollen-like wall, and the occurrence of partial cytokinesis, was first demonstrated. Delannay and Palmer (1982) observed the engorgement of CMs with starch, and their persistence to maturity as giant pollen grains with vegetative and generative nuclei. Both studies noted the fusion of CM nuclei, and in the present one it was demonstrated that mitotic divisions occurred within CMs. It is likely, then, that the bicellular condition of CMs noted by Delannay and Palmer (1982) was the result of such divisions. It is still not known whether these giant pollen grains can effect fertilization. Ultrastructural observations failed to detect any CMs with normal pollen walls.

The results of this study indicate that male sterility induced by the ms4 mutant is not due to failure of cytokinesis per se . Cytokinesis did occur, but its presence and nature varied, both between and within locules of single anthers. Cytokinesis was either present or absent, partial or incomplete, or regular or irregular in orientation. Postmeiotic cell division occurred through the formation of cell plates containing callose, as in most flowering plants

(Buchen and Sievers, 1981). However, the initiation of cytokinesis often was delayed. In soybean microsporogenesis, cytokinesis is initiated in association with the plasmalemma (Section I). In ms4 PM cells, pollen wall material spanned the cleavage furrows, indicating that cytokinesis occurred after its deposition.

Proper orientation of division plates was not sufficient stimulation for the production of viable pollen. Normal pollen was generated only when both timing and orientation were proper. Daughter cells of degenerate PM cells always were joined by a common wall across cleavage furrows. In normal pollen such walls were lacking, but the possible breakdown of such walls in male-sterile anthers producing normal pollen can not be disregarded. Properly oriented cytokinesis did not ensure the genesis of normal pollen walls; many examples of abnormal PM cells generated by properly oriented cell plates were observed.

The correlation of abnormal cytokinesis and abnormal pollen wall formation may indicate that male sterility is related to the function of the postmeiotic MMCs plasmalemma. Plant cytokinesis has been described as a "topographically organized secretion process" (Lopez-Saez et al., 1982) that involves the deposition of cell plate and nascent membrane components through the fusion of vesicles derived from dictyosomes. Nakamura and Miki-Hirosige (1982) demonstrated

that cytokinesis in MMCs of Lilium proceeded in the following manner. Coated vesicles produced by dictyosomes were gathered together through the activity of microtubles. Vesicles coalesced to form cell plate subunits, similar to those observed in soybean (Section I). Subunits then fused, releasing their contents (pectin and callose) into the cell plate while contributing membrane components for the expansion of the plasmalemma. Postmeiotic cytokinesis thereby involves the synthesis of new membrane and cell plate material through the activity of the endomembrane system. The process is similar to somatic cytokinesis, except that cell plates often develop in a centripetal manner, rather than the centrifugal pattern of somatic divisions (Esau, 1965). In soybean, PM cytokinesis is initiated in association with the plasmalemma and proceeds in a centripetal manner (Albertsen and Palmer, 1979; Section I).

Aspects of pollen wall formation, including the stratification of wall layers, and the distribution of apertures, involves activity of the plasmalemma (Heslop-Harrison, 1971; Horner and Pearson, 1978; Buchen and Sievers, 1981). Cytoplasmic components, especially ER, dictyosomes and microtubules have been observed to interact with the plasmalemma during pollen wall deposition. Pollen wall material is initially secreted across the microspore membrane, but later arises in the tapetum (Buchen and

Sievers, 1981). The pollen wall layers develop in a centripetal fashion (Horner and Pearson, 1978; Buchen and Sievers, 1981) with each successive layer being formed adjacent to the plasmalemma. Vegetative cell wall synthesis also may involve activity of the plasmalemma. Cellulose synthesis and microfibril orientation may be due to the activity of membrane-bound enzyme complexes, and the plasmalemma interacts with dictyosome-derived particles in the deposition of noncellulosic components (Leonard and Hodges, 1980).

Thus, the MMCs plasmalemma is important to pollen ontogeny in several respects. The sites for the initiation of cytokinesis reside on the membrane, and the plasmalemma exerts some level of control over pollen wall formation. In ms4, both cytokinesis and pollen wall deposition may be inhibited or rendered abnormal. The ms4 mutant may have an affect on the structure and/or function of the PM plasmalemma. The precise abnormality may be in an intrinsic protein(s) involved in pollen wall synthesis and cytokinesis, or in a diffusible metabolite that typically acts on the plasmalemma. As both pollen wall production and cytokinesis involve secretory functions, the ability of the PM cells to assimilate and export materials also may be impaired. Compounds such as caffeine and deoxyguanosine inhibit somatic cytokinesis, perhaps through interference with ATPase

activity required for the membrane fusion of dictyosome-derived vesicles (Lopez-Saez et al., 1982; Becerra and Carmona, 1983). Whatever the lesion induced by ms4, its effects are conditional and localized. Normal pollen was generated in 3.3% of the PM locules examined. This correlates well with the observations of Graybosch and Palmer (1984b) that under optimal growth conditions, seed set occurred at a rate of 3.9% that of male-fertile plants grown in the same environment.

Malfunction of the tapetal layer often has been cited as a cause of male sterility (Laser and Lersten, 1972; Gottschalk and Kaul, 1974). In ms4, premature vacuolation of tapetal cells may occur, but only when PM cells aborted before any sort of pollen wall was organized. Such cells may have degenerated early because of tapetal malfunction; however, tapetal vacuolation was not observed to precede PM cell abnormalities. The behavior of tapetal and PM cells indicates a gradient in response of anthers to the ms4 mutant. In the most severely affected, both tapetal cells and PM cells develop abnormally. In most, only PM cells malfunction, and in the minority, all cells function properly. Tapetal cells also may suffer from a deficient or defective gene product, but may have greater tolerance. The consistent association of normal tapetal cells with degenerate PM cells does not argue well for a role of tapetal

abnormalities in the induction of male sterility in this system.

Sporopollenin-like deposits frequently accumulated in locules, adjacent to tapetal plasmalemmas, and within tapetal cells. The tapetum is the likely site of sporopollenin synthesis (Echlin, 1971; Horner and Pearson, 1978). In ms4, the tapetal cells seem capable of synthesizing sporopollenin precursors, but the inability of the PM cells to properly organize sporopollenin into pollen walls causes sporopollenin accumulation. In the ms3 male-sterile mutant of soybean, pollen wall formation also was abnormal (Buntman, 1983; Section V). Material exhibiting fluorescence properties of sporopollenin (Nakashima et al., 1984) accumulated in tapetal cells (Buntman and Horner, 1983). In this case, the transport of sporopollenin precursors may have been inhibited, while in ms4 transport was successful, but assimilation by the PM cells was not.

Colhoun and Steer (1983) found that postmeiotic application of the gametocide RH-531 induced abnormal sporopollenin processing within anthers of Hordeum. Tapetal cells were not affected, but orbicules were either absent or disorganized, and sporopollenin-like particles accumulated within cytoplasmic vesicles. Microspores either degenerated without exine formation, or only an amorphous wall was generated, similar to some observed in ms4. RH-531 acts as a

growth retardant, and Colhoun and Steer (1983) postulated that its effect on pollen maturation was due to interference with growth regulators. Additional studies have found correlations between growth regulators and male sterility. Saini and Aspinall (1982) were able to induce male sterility in Triticum through the application of absciscic acid to flowering spikes. Ahokas (1982) found that cytokinin translocation from root to shoot was depressed in male sterile Hordeum. Excessive deposition of sporopollenin in anthers was a common feature of male-sterile plants in the line analyzed (Ahokas, 1978). Thus, it is possible that the abnormalities associated with the ms4 mutant may be due to disruption of the hormonal status of the plant or anther.

Failure of cytokinesis has been cited as a cause of male sterility in Solanum (Abdalla and Hermsen, 1972), Medicago (McCoy and Smith, 1983), and the ms1 mutant of soybean (Albertsen and Palmer, 1979). Partial cytokinesis occurred in a radiation-induced mutant of Pisum (Klein, 1969). In Zea mays, the va mutant led to variable cytokinesis and partial male sterility (Beadle, 1932). Variable cytokinesis also occurred naturally in Magnolia soulangeana, with some microspores arising via the typical dicotyledonous mode of simultaneous division, but the majority being produced by successive divisions usually characterisitc of monocotyledons (Gabara, 1971). Both absent and incomplete cytokinesis were

observed in Kniphofia (Moffett, 1932). Castetter (1925) described a condition in Melilotus that resembled ms4. Cytokinesis was absent, partial or irregular in orientation. Pores were not formed in the exine. Many male-sterile systems either prevent pollen wall formation (Horner, 1977; Horner and Rogers, 1974) or lead to abnormal wall formation (Audran and Bouillot, 1981). With the possible exception of the situation in Melilotus, the ms4 mutant may be unique in that it induces abnormalities in the processes of cytokinesis and pollen wall formation.

The only similarity detected between the ms1 and ms4 mutants of soybean is the failure of PM cytokinesis. In ms1, CMs were formed consistently; cytokinesis was not observed (Albertsen and Palmer, 1979). CMs produced walls identical to those of normal pollen. All CMs engorged with starch and lipids. Internal partitioning occurred at the time of intine deposition, and the septa were ultrastructurally similar to the intine. Colpi were formed, but their distribution was irregular. The generation of bicellular pollen grains did not occur, though nuclear fusions, mitotic divisions, and the production of pollen-like tubes by CMs was noted. This contrasts markedly with the pattern observed in ms4 male-sterile plants.

The ms1 mutant has a pleiotropic effect in that polyembryonic seedlings and polyploids occur among progeny of

male-sterile plants (Kenworthy et al., 1973; Beversdorf and Bingham, 1977; Chen et al., 1984). It has not been firmly established whether polyploidy is the result of abnormalities in the megagametophyte, or is due to fertilization by CMs acting as pollen grains. However, studies by Cutter and Bingham (1977) and Kennell (1984) demonstrated the presence of supernumerary nuclei, and fusion of such nuclei, in embryo sacs of male-sterile plants. Polyembryony is the result of multiple eggs (Kennell, 1984). Similar abnormalities of female reproduction have not been detected in ms4 male-sterile plants. Chromosome counts on 108 progeny resulting from putative self-pollination by male-sterile plants failed to isolate plants with chromosome complements other than the normal diploid state of 2n=40 (Graybosch, unpublished results).

The ms4 mutant either has no effect on female reproduction, or the frequency of abnormalities is less than 1/108 (<0.1%). The frequencies of polyembryonic seedlings and polyploids among progeny of ms1 male-sterile plants ranged from 0.1% to 3.6%, and 0.12% to 7.76%, respectively, depending on the genetic background in which the ms1 mutant resided (Chen et al., 1984). The lack of female abnormalities associated with ms4 is further indication of a fundamental difference between it and ms1.

In ms4, nuclear fusion and mitotic divisions within PM

cells can lead to the formation of giant bicellular pollen. If these functioned in fertilization, polyploidy would result. The failure to isolate polyploid progeny may indicate that these cells are unable to germinate, or can not effect fertilization. However, fertilization may occur, but embryos subsequently abort due to endosperm failure. Successful generation of triploid progeny as a result of pollination between tetraploid and diploid lines of soybean has not been achieved (Sadanaga and Grindeland, 1981). Endosperm formation in inter-ploidy crosses may depend on the presence of the proper maternal:paternal ratio of either genomes (Lin, 1984) or Mendelian factor(s) (the EBN hypothesis of Johnston et al., 1980). In ms4, polyploid pollen may function, but the resulting endosperm may abort due to abnormal maternal:paternal contributions. Recovery of polyploids from the ms1 mutant may be due to the ability of the embryo sac to support egg cells with elevated ploidy level in association with normal endosperm, or to the presence of polyploid fusion nuclei that allow the generation of functional endosperm after fertilization by polyploid pollen (Kennell, 1984).

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SECTION IV: PARTIAL FERTILITY IN A "MALE-STERILE" MUTANT (*ms4*) OF SOYBEAN (*GLYCINE MAX* (L.) MERR.)

Abstract

Investigations on the reproductive biology of a male-sterile mutant (*ms4*) of soybean (*Glycine max*) demonstrated that male-sterility is not complete. Field-grown male-sterile plants gave rise to sterile progeny at a frequency of up to 96%. Seed set occurred in the absence of pollen vectors. The effect of temperature on this response was studied. Warm temperatures lead to complete male-sterility. Seed set did occur at medium and cool temperatures. The potential use of the *ms4* system in hybrid seed production was discussed.

Introduction

Male-sterile mutants are of interest to plant breeders and geneticists as a means by which hybrid seed, be it for experimental or commercial use, may be obtained. Cytoplasmically inherited male sterility has been utilized to produce commercial hybrid seed in many crop species (Pearson, 1981). However, in several species (i.e. soybean) cytoplasmic male-sterility is not available. Several nuclear (genetic) male-sterile mutants have been reported in soybean (Graybosch and Palmer, 1984). These mutants have been used

to obtain limited quantities of hybrid seed for the evaluation of heterosis (Nelson and Bernard, 1984) and have served as source populations for recurrent selection programs (Brim and Stuber, 1973). To date, no attempts have been made to obtain commercial quantities of hybrid seed via male sterility in soybean.

Before male-sterile plants could be used in hybrid production, three conditions must be satisfied: 1) heterosis must be of sufficient magnitude to warrant the use of F1 hybrids over parental lines, 2) outcrossing must occur at a rate that ensures maximum seed set on male-sterile plants, and 3) commercial sized plantings of male-sterile plants must be obtained.

The presence of significant F1 heterosis in soybean has yet to be firmly established. Weber et al. (1970) compared F1 hybrids to parental lines during four growing seasons. The midparent heterosis for seed yield, averaged across all years, was 25.1%. Nelson and Bernard (1984) tested 27 combinations over one to three growing seasons. Statistically significant high-parent heterosis was observed for five F1 hybrids. The % heterosis in these lines ranged from 13% to 19%. However, in no case was heterosis demonstrated over two consecutive growing seasons. Heterotic lines possibly may be produced if enough parental lines are evaluated for combining ability.

Though most seed results from autogamy, soybean flowers do possess features that render them attractive to insects (Erickson and Garment, 1979). If adequate populations of pollen vectors are maintained in production fields, male-sterile plants may yield seed at a rate as high as 90% that of male-fertile plants (Carter et al., 1983).

If consistently high yielding hybrids can be produced, a problem that might prevent their exploitation is the difficulty involved in obtaining homogeneous populations of male-sterile plants. With nuclear male-sterility conditioning obligate allogamy, the maximum frequency of male-sterile plants in any given population is 50%. This maximum is obtained only if pollen parents are always heterozygous for the male-sterile allele, and if there is no selection against pollen carrying the fertility restoring allele. In other words, crosses between individuals of genotype msms and Msms will produce an F1 population segregating in a ratio of 1Msms:1msms. To isolate hybrid seed from such a population, male-fertile plants would have to be eliminated before flowering. Male-fertile plants of a line segregating for male sterility would contaminate hybrid seed through self-pollination and outcrossing with desired female parents. To obtain maximum yields from seed production fields, double-density plantings of male-sterile lines would be necessary to ensure a desirable population after roguing. Presently,

roguing is far too labor intensive for practical application. There are no seed or seedling characters linked to male-sterile loci that would facilitate the identification of male-fertile plants before flowering.

The maintenance of populations of male-sterile plants might be possible through the use of partial male-sterile mutants. In such systems, pollen may be produced under certain environmental conditions. The optimal case would be a line that is able to consistently set enough self-pollinated seed to maintain a male-sterile line, but still produce enough male-sterile flowers to allow for outcrossing. Such a system may exist in the ms4 mutant of soybean.

In the ms4 mutant, male-sterility has been shown to result from numerous abnormalities of reproductive cells that appear subsequent to telophase II (Delannay and Palmer, 1982; see also Section III). Two observations indicated that male-sterility was not complete. Infrequently, anthers produced pollen of normal appearance. Also, Delannay and and Palmer noted that up to 75% of the progeny of field-grown male-sterile plants could be male sterile. The maximum expected frequency of male-sterile progeny from open-pollinated male-sterile plants is 50%.

In this report the ability of ms4ms4 individuals to self-perpetuate their genotype is examined. The effect of temperature on this response also is presented. Finally, the

potential use of the ms4 system in hybrid seed production is discussed.

Materials and Methods

In the fall of 1982, seed was randomly harvested from five populations of male-sterile plants grown at Ames, Iowa. Populations were segregating at one of the following loci: ms1, ms2, ms3, or ms4. Seed was also obtained from a new male-sterile mutant from cultivar Beeson. The frequency of male-sterile individuals in progeny of each male-sterile line was determined in the summer of 1983. To assess the consistency of the phenotypic expression of the ms4 locus, seed was harvested from 35 male-sterile plants in 1983 and grown at the ISU-UPR Soybean Breeding Nursery, at the Isabela Substation, Isabela, Puerto Rico.

In order to determine whether seed set was possible by ms4 male-sterile plants in the absence of pollen vectors, 65 progeny of field-grown male-sterile individuals were greenhouse-grown in the summer of 1983. Six individuals homozygous for ms2 were grown in the same house as controls. The number of seeds produced per plant and per pod was determined. Progeny were greenhouse-grown in the winter of 1983-84 and classified as male fertile or male sterile at maturity.

Growth chamber experiments were conducted to investigate

the possibility that temperature affects the expression of the ms4 mutant. Three day/night temperature regimes were used: 35C/32C, 29C/23C and 24C/21C. Photophase was 16 hours, reduced to 14 hours after three weeks in order to induce flowering. Illumination was provided by fluorescent and incandescent lights. Male-fertile plants of cultivar Rampage were included as controls.

Results

The frequency of male-sterile individuals in progenies of each of the male-sterile lines is given in Table 1. A frequency in excess of 50% would indicate either self-pollination by "male-sterile" plants or apomictic seed production. The frequency of male-sterile plants in progeny of the 1982 ms4 male-sterile plants was 96%. For all other male-sterile lines, the frequency was less than 50%.

Seed produced by the ms4 male-sterile plants in 1983 did not yield as high a frequency of male-sterile progeny. A total of 416 individuals grew to maturity. Of these, 206 (49.5 %) were male sterile.

Of 65 greenhouse-grown progeny of ms4 male-sterile plants, 62 were male-sterile. Twenty-three male-sterile plants set seed in the greenhouse (Table 2). Male-sterile plants averaged 0.97 seed per plant, or 0.6 % that of fertile plants. Thirty-two percent of the pods produced by male-

Table 1. Frequency of male-sterile individuals in progeny of male-sterile plants

Male-sterile line	No. plants	Ratio of fertile to sterile plants	Frequency of sterile progeny
ms1	87	69:18	Ø.21
ms2	79	47:32	Ø.41
ms3	69	51:18	Ø.26
ms4	174	7:167	Ø.96
Beeson	65	33:32	Ø.49

Table 2. Seed production by greenhouse-grown male-fertile and male-sterile plants

Genotype	No. plants	No. producing seed	Seed/plant (mean)	Seed/pod (mean)
ms2ms2	6	2	Ø.33	1.ØØ
ms4ms4	62	23	0.97	1.67
Ms4ms4	3	3	163.ØØ	2.27

sterile plants were one-seeded; male-fertile plants produced only 15% one-seeded pods. Sixty seed were recovered from ms4ms4 individuals. Forty grew to maturity, 38 were male sterile plants, and two were male-fertile plants. Of the six individuals homozygous for ms2, two produced a single seed each, which gave rise to male-fertile plants. Male-fertile progeny could arise from male-sterile plants only if insects had gained access to the greenhouse. Some of the seed produced by the ms4 plants may have arisen in the same manner.

Results of growth chamber experiments are given in Table 3. Only the warm temperature regime (35C/32C) completely inhibited seed set. The medium temperature was optimal for seed production, although restoration of fertility was incomplete. Seed set by male-sterile plants in this environment was only 3.9% that of male-fertile plants.

Discussion

From the results presented, it is evident that the ms4 mutant is capable of some form of self-perpetuation. Two observations support this statement. The frequency of male-sterile progeny arising from seed harvested from male-sterile plants can exceed the maximum expected frequency of 50%. Secondly, individuals homozygous for ms4 can set seed in the absence of insect pollinators. Even though pollen vectors contaminated the greenhouse experiment, the frequency of

Table 3. Seed set on male-sterile (ms4ms4) plants in three temperature regimes

Temperatures (day/night)	No. plants	No. producing seed	Seed/plant (mean)	Seed/pod (mean)	%+
35C/32C	16	Ø	Ø	Ø	Ø
29C/23C	7	7	6.57	1.44	3.9
24C/21C	14	5	Ø.5Ø	1.2Ø	2.4

+ = Seed set by male-sterile plants expressed as a percentage of seed set by male-fertile plants grown in the same environment.

male-sterile progeny from seed obtained was 38/40 or 95%. Also, seed was produced in the growth chamber experiments which were conducted during winter months, when pollen vectors were not available.

At this time, it is not possible to discern whether seed production by male-sterile plants is due to self-pollination or apomixis. A genetic test using the chlorophyll-deficient mutant yl1 is currently being conducted. This will discriminate between these two alternative hypotheses. It is known, however, that approximately 3.0% of the anthers produced by ms4 male-sterile plants contain normal-appearing pollen (Section III). Thus, self-pollination seems likely.

The ms4 mutant is, therefore, a temperature-sensitive partial male-sterile mutant. Male-sterile mutants exhibiting temperature-dependent phenotypic effects have been reported in maize (Beadle, 1932), tomato (Rick, 1948; Rick and Boynton, 1967), broccoli (Dickson, 1970) and sorghum (Brooking, 1979). Two other partial male-sterile mutants have been reported in soybean, the "Arkansas" (Caviness et al., 1973) and msp (Stelly and Palmer, 1980). In its response to temperature, ms4 is more similar to the "Arkansas" mutant. Caviness et al.(1973) determined that daytime temperature of 35C lead to complete male-sterility. Medium and low temperatures allowed seed set to occur. The msp mutant was characterized by a different response. Stelly

and Palmer (1980) found that in each of two experiments, seed set was lowest at cool temperatures, and greatest at high temperatures. The "Arkansas" mutant was able to produce seed at a rate of 60% that of male-fertile, while msp could attain yields as high as 80%. Based on field, greenhouse and growth chamber experiments, it is doubtful that seed set in excess of 10% is possible in the ms4 system.

In order for a partial male-sterile mutant to be useful in breeding programs, its response should be consistent and predictable. One would need to obtain enough selfed seed to maintain the male-sterile line, but selfing must be low enough to allow outcrossing for the production of hybrid seed. Although observations on the consistency of self-pollination under field conditions are not extensive, the results presented above are encouraging. Seed harvested from male-sterile plants in 1982 produced 96% male-sterile progeny. The 1983 planting yielded only 50% male-sterile plants. The growth chamber experiment demonstrated that high temperatures lead to complete male sterility. It should be noted that plants were exposed to high temperatures for the duration of their life cycle, a condition not likely to occur under field conditions. The summer of 1983 was one of the hottest and driest on record in Iowa. The decrease in male-sterile progeny may have been due to unfavorable conditions for self-perpetuation.

Another factor that may have influenced the frequency of male-sterile progeny was the availability of heterozygous pollen parents. In this study, no attempt was made to discriminate in the field between progeny resulting from selfing by ms4ms4 individuals and plants resulting from outcrossing between male-sterile plants and fertile heterozygotes. In 1982, seed was harvested from a large segregating population. In 1983, less than 200 individuals were grown, of which only four were heterozygous male-fertile plants. Thus, in 1982, a higher frequency of heterozygous pollen parents may have contributed to the higher frequency of male-sterile progeny.

The ms4 mutant may be useful if it were coupled with a character that allows one to separate selfed seed from hybrid seed. Sadanaga and Grindeland (1981) used the green cotyledon mutant d1d1d2d2 to recognize hybrid seed. Pollen parents were D1 D2 , female parents were d1d1d2d2. Thus, any hybrid seed bore yellow cotyledons while selfed seed had green cotyledons. With male-sterile plants of genotype ms4ms4d1d1d2d2, seeds with green cotyledon would indicate selfing and could be separated from the yellow-cotyledon hybrid seed. Green seeds could then be used to perpetuate the male-sterile line. The only potential problem preventing employment of such a scheme is that selfing by the male-sterile plants may be too infrequent to perpetuate large

populations of male-sterile plants. Additional studies on ms4 in field conditions over several growing seasons are warranted.

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SECTION V: ANALYSIS OF A MALE-STERILE MUTANT IN 'WABASH' SOYBEAN

Abstract

A male-sterile mutant that arose spontaneously in soybean (Glycine max (L.) Merr.) cv. Wabash was shown to be an independent mutation at the ms3 locus. The Wabash male-sterile mutant (Wms3) was found to be nonallelic to five additional male-sterile mutants. Cytological comparisons between Wms3 and the original ms3 line (Cms3) demonstrated that the two mutants displayed similar phenotypic effects, even though they were present in different genetic backgrounds. Mature pollen grains were not differentiated. The sheath of callose that surrounds MMCs (microspore mother cells) and microspores failed to dissolve properly, and microspores subsequently aborted. Abnormalities of the tapetal layer were found to associate with microspore degeneration.

Introduction

In recent years, several male-sterile mutants of soybean (Glycine max (L.) Merr.) have been reported (Graybosch and Palmer, 1984). To date, six male-sterile mutants have been assigned gene symbols. All are nuclear mutations and are inherited as monogenic recessive traits. Observations on the

cytological effects of most of these mutants have been presented (Albertsen and Palmer, 1979; Palmer et al., 1980; Delannay and Palmer, 1982; Stelly and Palmer, 1982; Graybosch et al., 1984). In the majority, male sterility was due to an abortion of reproductive cells at a mutant-specific stage. However, in the msp mutant, the time at which degeneration occurred was variable (Stelly and Palmer, 1982). In all mutants except msl, tapetal dysfunction associated with male sterility.

Several additional male-sterile mutants have been reported but not yet assigned gene symbols (Caviness et al., 1970; Patil and Singh, 1976; Chaudhari and Davis, 1977). One of these mutants arose spontaneously in cv. Wabash (Chaudhari and Davis, 1977). The "Wabash male-sterile" was inherited as a monogenic recessive character, and anthers were devoid of pollen at maturity. This report summarizes additional observations on the Wabash male-sterile mutant. Allelism tests were conducted to determine whether this mutant defines a new locus, or represents an independent mutation at a previously described locus. Cytological observations were obtained to assess the effects of this mutant on the developmental reproductive biology of male-sterile plants.

Materials and Methods

Seeds of the Wabash male-sterile line were obtained from Dr. W.H. Davis, Ring Around Products Co., Plainview, Texas. Allelism tests were conducted with the male-sterile mutants ms1, ms2, ms3, ms4, and ms5 (Soybean Genetic Type Collection numbers T266H, T259H, T273H, T274H and T277H, respectively). Crosses were obtained using homozygous recessive individuals as female parents, and known heterozygotes as pollen parents. Reciprocal combinations were obtained when possible. If the mutations arose at different loci, the F1 plants from each cross would all be male fertile. Independent mutations at the same locus would be indicated by an F1 population segregating in a ratio of 1 male-fertile plant:1 male-sterile plant. Parental lines were grown at Ames, Iowa. F1 populations were grown at the ISU-UPR Soybean Breeding Nursery, at the Isabela substation, Isabela, Puerto Rico. All F1 plants were classified for male sterility/fertility at maturity.

Light and transmission electron microscopy were used to obtain cytological observations. Individual anthers were removed from buds of both male-fertile and male-sterile plants. Techniques for the fixation and preparation of material for microscopic examination have been described previously (Section I).

Results

Classification of the F1 plants derived from crosses between the Wabash male sterile and five additional male-sterile lines is given in Table 1. In the F1 population obtained through crosses with the ms3 mutant, 12 of 27 F1 plants were male sterile (X^2 1:1 = 0.333, $p > 0.90$). Thus, the Wabash male-sterile represents an independent mutation at the ms3 locus, and is hereafter designated as Wms3. The original mutation at the ms3 locus arose in an F3-derived line from the cross 'Calland' X 'Cutler' (Palmer et al., 1980). This strain will be referred to as Cms3. All F1 hybrid seed from the remaining combinations gave rise to male-fertile plants.

Even though cytological observations have been presented for Cms3 (Palmer et al., 1980; Buntman, 1983; Buntman and Horner, 1983), it was deemed desirable to conduct similar studies with Wms3, in order to assess any possible effects of a different genetic background on the phenotypic expression of the ms3 locus. Observations are presented in Figures 1-16. Anther development in male-fertile plants has been characterized previously (Albertsen and Palmer, 1979; Buntman and Horner, 1983). Observations of male-fertile plants are given only when pertinent to the present discussion.

Table 1. Results of allelism tests between the Wabash male-sterile mutant and five additional male-sterile mutants. Classification of F1 populations.

Male-sterile parent	Number of F1 plants	Number male sterile	Number male fertile	X^2	p
ms1	24	Ø	24	Ø	Ø
ms2	22	Ø	22	Ø	Ø
ms3	27	12	15	Ø.333	>Ø.9Ø
ms4	21	Ø	21	Ø	Ø
ms5	25	Ø	25	Ø	Ø

Microsporogenesis was normal through the tetrad stage (Fig. 1); no meiotic disorders were detected. As the microspores matured, various abnormalities were noted. The callose sheath, which dissolves at the end of the tetrad stage in male-fertile plants, was persistent (Figs. 2 and 3). Microspore cytoplasm then either disorganized (Fig. 4) or became vacuolate. Vacuoles gradually increased in size, so that at maturity, no cytoplasm remained (Figs. 4-8). The microspores were represented only by empty cell walls (Fig. 8). Even at advanced stages of degeneration, microspores were still encased with callose (Fig. 7). Walls of adjacent microspores showed a tendency to fuse (Fig.5). Microspore mitosis did occur, as binucleate cells were evident (Fig. 6).

Premature degeneration of the tapetal cells also occurred, although the time (in relation to microspore development) at which it was evident varied. In some anthers, the tapetum aborted before the microspores (Fig. 3). In others, microspores degenerated while the tapetum remained intact (Fig. 4). In later stages, the tapetum usually collapsed into a mass of darkly stained material (Figs. 5-8). However, many tapetal cells remained intact and accumulated a densely stained refractive material of unknown origin (Fig. 7).

Aberrant development of anther wall layers also was noted Fig.8). The endothecial layer expanded, but did not develop the secondary wall thickenings characteristic of male-fertile

Fig. 1-8. Postmeiotic development in anthers of Wms3 plants.

1. Early tetrad stage. X 460.

2. Late tetrad stage: arrow indicates persistent callose. X 370.

3. Early microspore stage: arrow indicates degenerating tapetal cells. Callose remains evident between microspores. X 332.

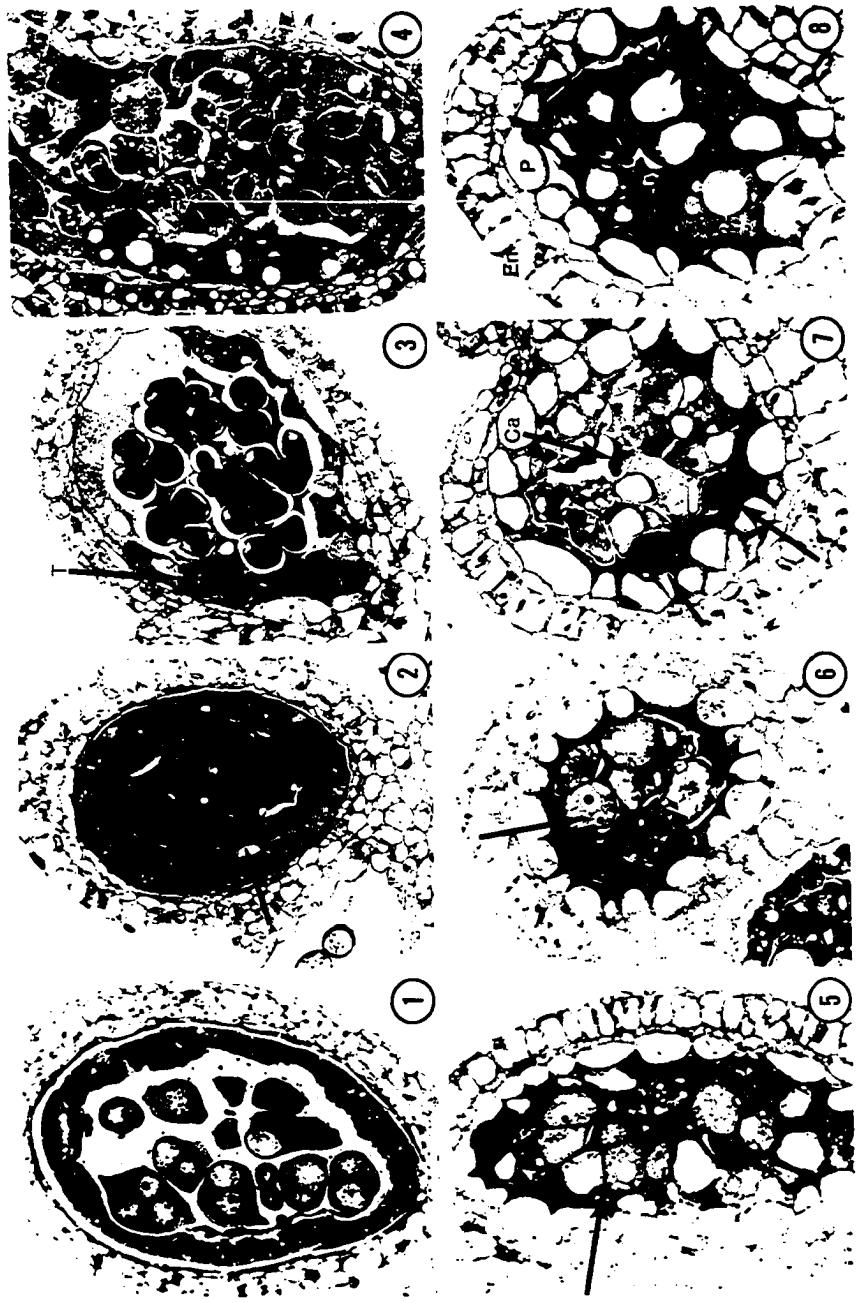
4. Microspore stage: degenerating microspores are evident whereas tapetal remain normal. X 310.

5. Microspore stage: arrow indicates fusion of adjacent microspore walls; tapetum has degenerated. X 370.

6. Microspore stage: binucleate condition (arrow) indicates that microspore mitosis has occurred. X 390.

7. Anther at maturity: persistent callose (Ca) remains about degenerate cells; dense refractive material (arrows) accumulates in tapetal cells. X 400.

8. Mature anthers: arrow indicates empty microspore. Inner parietal layer (P) expands in radial planes; endothecial layer (En) expands but does not bear secondary wall thickenings. X 330.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plants (Albertsen and Palmer, 1979). The inner parietal layer expanded toward the locule. In male-fertile plants, this layer was crushed.

Ultrastructurally, the only detected difference between young microspores of male-fertile and male-sterile plants was in the nature of the microspore wall (Fig. 9-12). By the late tetrad stage, microspores of male-fertile plants have initiated the tectum and columellae of the ektexine, and the endexine (Fig. 11). In male-sterile plants, the columellae were poorly defined (Fig. 9). After the release of microspores from callose, the columellae of fertile microspores were distinct (Fig. 12). At a comparable stage in male-sterile plants, columellae were absent (Fig. 10).

In general, subcellular tapetal development was similar to that of male-fertile plants until the tetrad stage. However, the extensive profiles of endoplasmic reticulum (ER) seen in tapetal cells of male-fertile plants were absent (Figs. 13-15). Features common to tapetal cells of male-sterile and male-fertile plants include: the dissolution of the inner tangential cell wall; the presence of an undulating plasmalemma, and the accumulation of a fibrillar material within invaginations of the plasmalemma; and the presence of numerous mitochondria with distinct cristae (Figs. 13-16). The persistent callose wall may be seen appressed to the tapetal layer of male-sterile plants (Fig. 13).

Fig. 9-12. Ultrastuctural aspects of microspore walls in male-sterile (9,10) and male-fertile (11-12) anthers.

9. Microspore at late tetrad stage: arrow indicates absence of columellar layer. X 17,820.

10. Late microspore stage: a thick exine has been deposited but columellae are absent. X 8,400.

11. Microspore of male-fertile plant at late tetrad stage, before callose dissolution. Columellar deposition (arrow) is evident. X 11,880.

12. Microspore of male-fertile plant after callose dissolution: columellae (arrow) are distinct. X 10,750.

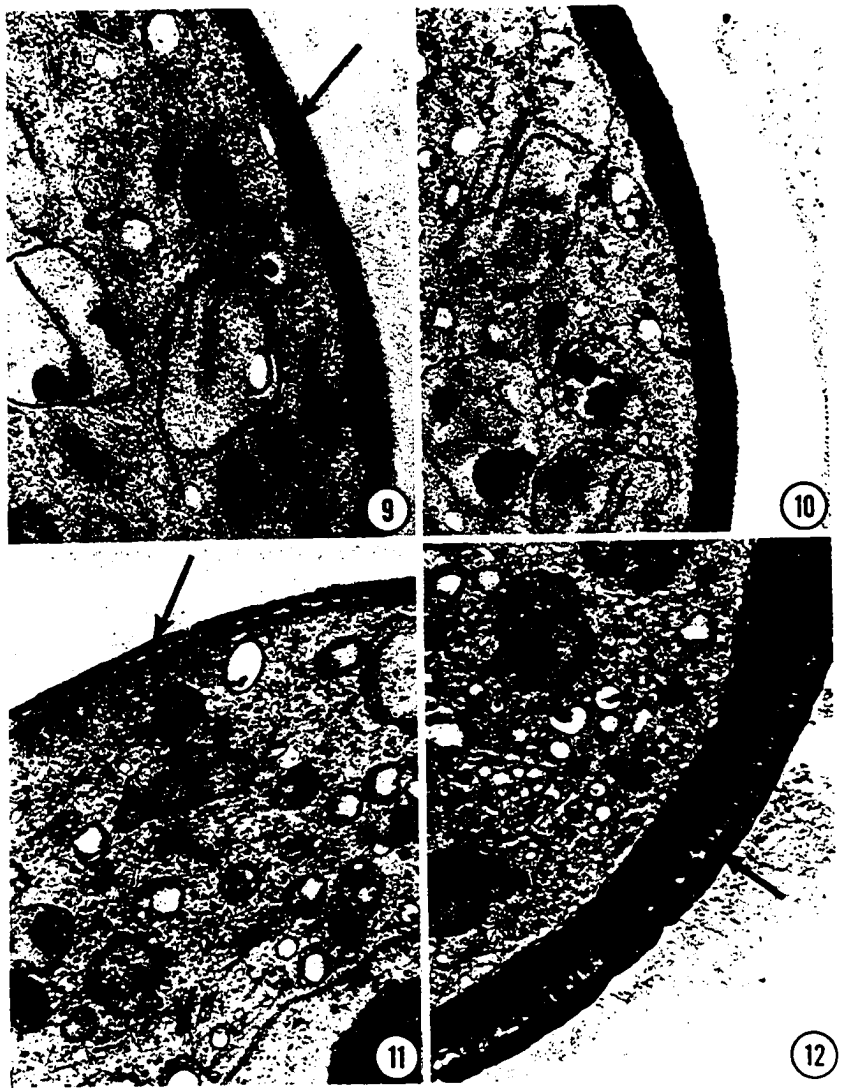
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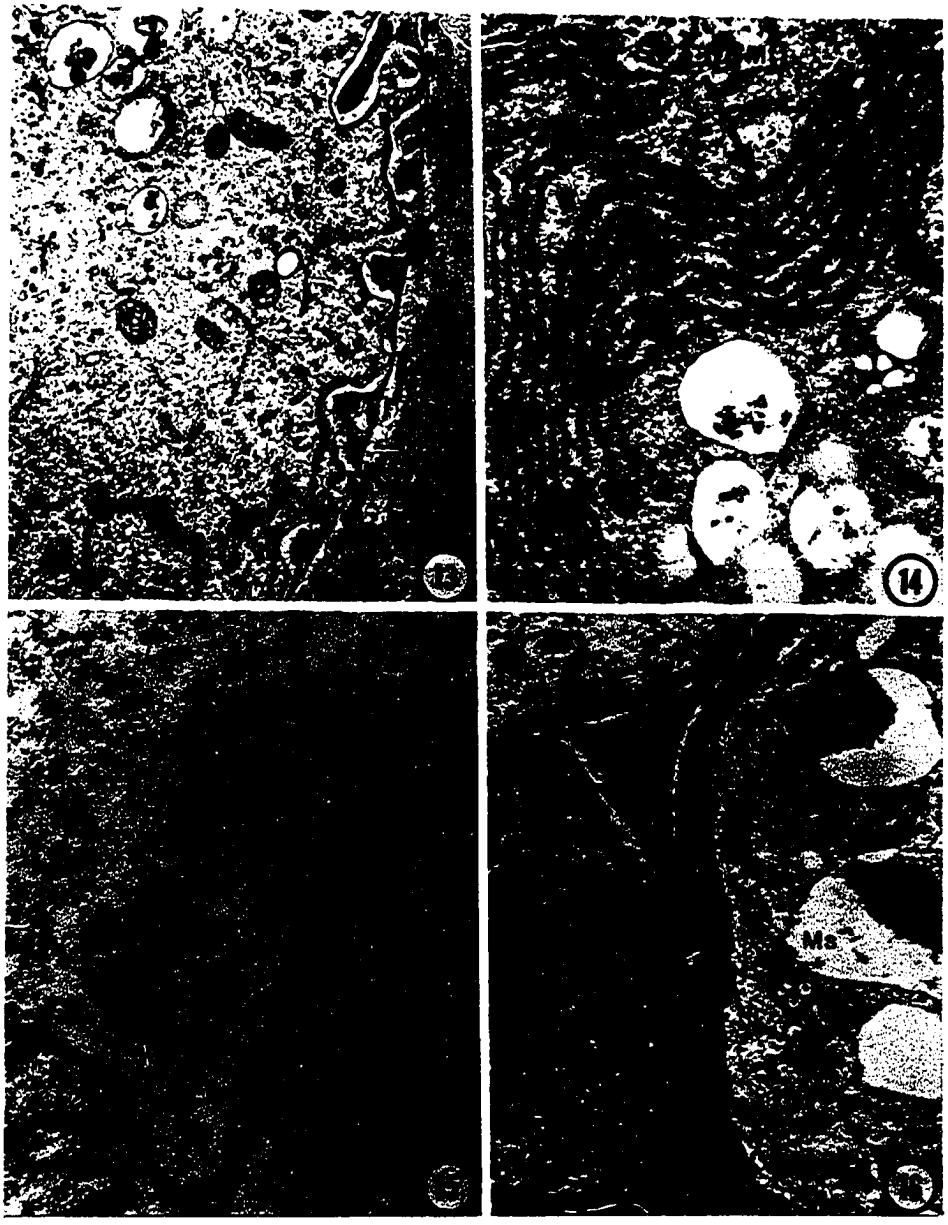
Fig. 13-16. Aspects of the tapetum of male-sterile and male-fertile plants.

13. Tapetal cell at late tetrad stage: inner tangential cell wall has degraded and plasmalemma develops an undulating profile (arrow). X 8,750.

14. Tapetal cell of male-fertile plant at microspore stage: numerous mitochondria (Mt) and extensive profiles of endoplasmic reticulum (arrow) occur. X 11,000.

15. Tapetal cell of male-sterile plant at late tetrad stage: mitochondria (Mt) identical to those of male-fertile plants are abundant. X 15,300.

16. Tapetal cell (T) and microspore (Ms) of male-sterile plant at maturity: tapetal cytoplasm is disorganized, whereas microspore cytoplasm becomes vacuolate. X 5,270.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In most anthers, the tapetal layer degenerated in a manner that differed from that of the microspores (Fig. 16). The tapetal cytoplasm became disoraganized, and organelles were barely distinguishable. In the microspores, organelles remained intact; however, vacuoles accumulated and expanded, gradually replacing and eliminating the cytoplasm.

Discussion

The genetic data presented led to the conclusion that the Wabash male-sterile mutant represents an independent mutation at the ms3 locus. Although many nuclear male-sterile mutants are known in flowering plants, few cases of independent mutations at one locus have been reported (Gottschalk and Kaul, 1974). In soybean, four independent mutations have occurred at the ms1 locus (Palmer et al., 1978b). These have been designated NCms1 (T260H), Tms1 (T267H), Ams1 (T268H), and Ums1 (T266H). The synaptic mutant st2 (conditioning both male and female sterility) has arisen in two different populations (Winger et al., 1977).

The nuclear backgrounds in which these two mutations at the ms3 locus arose were genetically distinct. The Coefficient of Parentage between Wabash and progeny of the cross Calland X Cutler is 0.11 (Delannay et al., 1983; X. Delannay, Monsanto, St. Louis, Mo., personal communication), or, in other words, only 11% of the genetic constitutions of

these two lines were theoretically derived from a single common ancestor. The cytoplasms of these two sources may not be as diverse. Based on the pedigrees given by Hymowitz et al. (1977), Wabash bears 'Dunfield' cytoplasm, which was derived from Plant Introduction (PI) 36846, from NE China. Calland cytoplasm traces to 'Mukden', also descended from a PI (5Ø523Q) from NE China. Fragment patterns generated by the restriction endonucleases BamI and SalI for Dunfield and Mukden mitochondrial DNA's were identical (Sisson et al., 1978). Shoemaker (1984), using five restriction enzymes, found no differences in chloroplast DNA's of these two cultivars. Therefore, the two ms3 mutations arose in different nuclear, but possibly identical cytoplasmic backgrounds.

Despite the different nuclear backgrounds, the Cms3 and Wms3 mutants exert similar effects on anther development. The Cms3 mutant was studied by Palmer et al. (198Ø), Buntman (1983) and Buntman and Horner (1983). In both Cms3 and Wms3 male-sterile plants, two outstanding abnormalities were noted. Tapetal cells often accumulated a dense refractive material, and callose surrounding the microspores did not dissolve. The appearance of tapetal cells and microspores at the time of degeneration was similar. In both lines, the inner parietal and endothecial layers expanded, and secondary wall thickenings of the endothecial layer were absent.

Buntman (1983) described microspore walls as being aberrant, but did not elaborate on the nature of the abnormality. In Wms3, microspores lacked the columellar layer.

Buntman and Horner (1983) noted differences in the mitochondria of tapetal cells of male-sterile and male-fertile plants. At the late sporogenous - early meiotic stages, mitochondria of male-sterile plants were described as being larger and with less dense cristae than those of male-fertile plants. H.T. Horner Department of Botany, Iowa State University (personal communication) has found that these differences do not persist. Mitochondria of tapetal cells condense dramatically, and develop more extensive cristae, as sporogenous cells move toward meiosis. In male-sterile plants, this response was delayed. In Wms3, tapetal mitochondria at the tetrad stage appeared normal. A more extensive analysis would be required in order to determine whether mitochondria of Wms3 behave in a manner similar to those of Cms3.

The nature of male sterility induced by mutants at the ms3 locus does not appear to be influenced by the genetic background. As in most male-sterile mutants of angiosperms, be they cytoplasmic (Laser and Lersten, 1972) or nuclear (Gottschalk and Kaul, 1974), tapetal abnormalities associated with abortion of reproductive cells. The most obvious defects of the tapetal layer in both Wms3 and Cms3 were

premature degeneration and the accumulation of an unknown refractive material. This refractive material, based on auto- and primuline-induced fluorescent properties, was suspected to be either sporopollenin, or its immediate precursors (Nakashima et al., 1984). The tapetum is thought to be the site of sporopollenin precursor synthesis (Echlin, 1971). The failure of callose to dissolve may also be indicative of tapetal malfunction, as the tapetum is regarded as the source of a callose-degrading enzyme (callase) (Buchen and Sievers, 1981). These observations may indicate an inability of the tapetal cells to transport materials (i.e. sporopollenin precursors and enzymes) to the locule. The inner tangential cell wall did dissolve in both Wms3 and Cms3 male-sterile plants, but the mutations may have altered the structure and integrity of the tapetal membrane, leading to blockage of transport mechanisms.

The persistence of the callose wall also may be involved in the abortion of microspores by acting as a barrier to tapetal-derived products bound for the microspores. Izhar and Frankel (1971) observed similar behavour of callose in a male-sterile mutant of Petunia. Callose dissolution was retarded due to a delay in the timing of callase activity.

In Wms3, microspores also demonstrated abnormalities, concurrent with those of the tapetal layer. The most obvious was in the formation of microspore walls. This may have been

due to the absence of a critical tapetal-derived product, or, perhaps, the microspores and tapetal cells were exhibiting independent reactions to the deficiency of the same gene product.

Little information has been published on the influence of genetic background on the expression of male-sterile mutants. As mentioned previously, four independent mutations have been reported at the msl locus. Palmer et al. (1978a) noted slight differences in the number of coenocytic microspores (four-nucleate cells produced by the failure of cytokinesis during microsporogenesis) formed by male-sterile anthers of the four msl lines. Albertsen and Palmer (1979) did not note any differential effects of these mutants on microsporogenesis, although specific comparisons were not made. The msl locus also has a pleiotropic effect on female reproduction. Progeny of male-sterile plants were frequently polyembryonic or polyploid (Kenworthy et al., 1973; Beversdorf and Bingham, 1977; Chen et al., 1984). The magnitude of the effect on female reproduction did vary when these source populations were compared. Boerma and Cooper (1978) found that Umsl male-sterile plants displayed greater female fertility than NCmsl plants. Umsl plants produced a greater number of three-seeded pods when the lines were grown in the same environment. This phenomenon occurred even when the Umsl and NCmsl mutants were placed in new genetic

backgrounds. Boerma and Cooper (1978) postulated that this effect was due either to the influence of closely linked modifier genes, or to Ums1 and NCms1 being different alleles.

Chen et al. (1984) found differences in the frequency of polyembryony and polyploidy among the four ms1 source populations. They also studied the effects of the Ams1 and Ums1 mutants in F4 populations derived by crossing male-sterile plants with two different translocation homozygotes. The Ums1-derived F4 population differed from Ums1 in frequencies of both polyembryonic seeds and polyploid progeny. The Ums1-derived F4 population differed from its male-sterile parental line only in the frequency of polyploid progeny produced. The differential effect of the various ms1 mutants on female reproduction may be a consequence of their being different alleles. However, the differences displayed between Ums1, Ams1 and their derived populations indicates that the genotypic background may exert an effect on gene expression.

The Cms3 and Wms3 mutants behaved in a similar manner, even though they arose in different nuclear backgrounds. The cytoplasmic backgrounds may have been similar. No nuclear-cytoplasmic interactions have been reported for male-sterile mutants of soybean, so it is not likely that a common cytoplasm diluted any potential differences in expression of these two mutants. The nature of the ms3 gene product must

be such that it is essential to the differentiation of male gametophytes.

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SUMMARY AND DISCUSSION

Microsporogenesis and microgametogenesis are complex processes in which both nuclear and cytoplasmic functions must occur and interact at precise times for pollen differentiation to be successful (Albertsen and Palmer, 1979). Pollen formation also depends on the maturation and function of two distinct tissue layers, the tapetum and the sporogenous cells. Coordinated interaction between these two cell types (or their descendants) is required for the generation of functional male gametophytes.

Male sterility can result when one, or both, of these tissues fails to fulfill a requisite function. The analyses of male-sterile mutants reported in preceding chapters illustrate this point. In both ms2 and Wms3 (Sections I and IV), abnormalities of the tapetal layer were instrumental in the induction of male sterility. In the ms2 mutant (Section I), germline cells displayed no aberrancies until after the completion of meiosis and cytokinesis; the tetrads of microspores then rather suddenly aborted. Tapetal cells, however, were completely atypical in development. After the sporogenous stage, the tapetum made no attempt to develop normally (excuse the teleological digression). The function of the angiosperm tapetum is largely secretory in nature (Buchen and Sievers, 1981). The absence of a functional tapetum, then, deprives the microspores of metabolites

necessary for successful pollen maturation.

In the ms4 mutant (Section III), the nature of the abnormality conditioned by the mutant was of a different sort. In the majority of anthers, tapetal cells functioned properly. Reproductive cells, however, displayed aberrancies related to the functions of the plasmalemma. In a minority of anthers, premature and excessive vacuolation of tapetal cells was noted. This may indicate that both tapetal and germline cells were influenced by the ms4 mutant, but the tapetal cells are more tolerant. Whatever the biochemical nature of the lesion in the ms4 system, it is only conditional. Anthers generated normal pollen grains, although at a low frequency. Seed was set in the absence of insect pollinators, and the ability to produce seed was shown to be influenced by temperature (Section IV). Likewise, the behavior of anthers of male-sterile plants may be influenced by microenvironmental features.

In Wms3 (Section V), abnormalities of both germline and tapetal cells occurred. Microspores were not released from callose, and were incapable of generating normal walls. Callose dissolution may be due to the activity of tapetal cells (Stieglitz, 1977). Tapetal cells also accumulated sporopollenin-like deposits. Thus, male-sterility in the ms3 system may be related to an interruption of normal transport phenomenon between tapetal cells and microspores. The

barrier to translocation may be an abnormal tapetal membrane, or the persistent callose sheath. The Wms3 mutant is of interest because it represents an independent mutation at a previously defined locus. Independent mutations have occurred at the ms1 locus in soybean (Palmer et al., 1978). Nuclear male-sterile mutants have been reported in many plant species, but the occurrence of independent mutations at single loci is rare (Gottschalk and Kaul, 1974). Despite rather different genetic backgrounds, the two mutations at the ms3 locus exerted similar phenotypic effects. Thus, the function of the ms3 locus is vital to pollen differentiation, regardless of the genotype of the plant.

By definition, male-sterile mutants do not impair female reproduction. However, the ms1 mutant of soybean induces polyploidy and polyembryony (Kenworthy et al., 1973; Beversdorf and Bingham, 1977; Chen et al., 1984). Such influences on the female reproductive system may prevent the utilization of ms1 in crop improvement programs. The ms2 mutant induced spontaneous trisomy (Section III) but the frequency (2.4%) was low. Trisomy does not significantly reduce seed set in soybeans (Palmer and Heer, 1976). No female abnormalities have been reported to associate with the ms3 and ms4 mutants. Thus, there are no barriers to the use of ms2, ms3 and ms4 in breeding programs.

The ms2 mutant displayed phenotypic similarities to

cytoplasmically inherited male-sterile systems. This may indicate a common biochemical origin of nuclear male sterility (NMS) and cytoplasmic male sterility (CMS). However, there is no reason to suppose that identical cellular phenotypes could not be due to fundamentally different molecular processes. Birds and butterflies have wings, but obviously the means by which wings arose and develop in such diverse organisms must be very different. CMS itself may have many different molecular origins, depending on the species in question. The behavior of viruses (Grill and Garger, 1981), mitochondrial DNAs (Leaver and Gray, 1982) and nucleolar-cytoplasmic interactions (Burns and Gerstel, 1981) have been correlated with CMS.

A common attribute of both CMS and NMS may be the involvement of plant growth regulators in the induction of sterility. Ahokas (1982) linked CMS in Hordeum to cytokinin levels, while Saini and Aspinall (1982) found a possible role for absciscic acid in male sterility. Colhoun and Steer (1983) observed that gametocidic compounds mimic both plant growth regulators and male-sterile mutants (both nuclear and cytoplasmic). CMS most often arises via interspecific and interracial hybridizations, and presumably is due to nuclear-cytoplasmic incompatibilities. Such incompatibilities may be due to the inability of a cytoplasmic component to respond to a growth regulator produced by a foreign nucleus. Likewise,

nuclear mutations in loci involved in the production and/or regulation of growth regulators might induce sterility.

Male sterility provides a means by which the rate of outcrossing in plants that lack self-incompatibility systems is increased. This function has been employed through the use of male-sterility systems (usually cytoplasmic) in the production of commercial F1 hybrid cultivars of several crop species (Pearson, 1981). The occurrence of male sterility also has evolutionary significance. Male sterile mutants are not restricted to cultivated plants (Ross, 1969; Kheyr-Pour, 1980). Male-sterile mutants have been linked to the origin of dioecy within the genus Cirsium (Delannay, 1979). However, dioecy is rare in flowering plants; approximately 4% of angiosperms are completely dioecious, with an additional 10% of all species containing both unisexual and bisexual individuals (Frankel and Galun, 1977). Male sterility, with the concurrent potential for restoration of fertility, may be more selectively advantageous, especially in self-pollinated species. With obligate dioecy, heterozygosity is high, but propagation of the species depends on the continual availability of pollen and ovulate parents. NMS would allow individuals to increase outcrossing for one or two generations, but fertility can be restored if pollen parents carry fertility-restoring alleles. Though self-pollinated plants do not suffer from inbreeding depression, the

potential for occasional genetic recombination via male sterility would not be detrimental.

CMS, being associated with interspecific and/or interracial hybridization, may have evolutionary implications from another standpoint. Unless fertility restoring genes are present in at least one of the hybridizing populations, male-sterile individuals would be incapable of producing progeny with restored fertility. Thus, the male-sterile genotypes would be eliminated from the populations. This would limit the rate of gene flow between adjacent populations or species. NMS usually arises through spontaneous recessive mutants (Gottschalk and Kaul, 1974). Thus, dominant restorer genes should be available, unless there was selection against such genotypes.

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APPENDIX

Procedures for preparation of material for microscopy

I. Resin embedded material for light and transmission electron microscopic observations.

A. Fixation: Place buds in fixative, dissect and remove individual anthers. Fixative consists of 2% paraformaldehyde plus 2.5% glutaraldehyde in Ø.1 M phosphate buffer, pH 7.25. Refrigerate material in fixative for 2-4 hrs, then remove anthers and place in a mixture of 2.5% glutaraldehyde in Ø.1 M phosphate buffer, pH 7.25, for an additional 16-2Ø hrs at 4C.

B. Postfixation: Wash anthers for 45-6Ø minutes in three changes of Ø.1 M phosphate buffer, pH 7.25. Place in 1.Ø% OsO_4 in Ø.1 M phosphate buffer, pH 7.25, for 1-2 hrs. Repeat washings in phosphate buffer.

C. Dehydration: Dehydrate in a graded acetone series. Begin with 1Ø% acetone in water; change solutions at 1Ø-15 minute intervals, increasing the concentration by 1Ø% acetone with each change. When specimens are in 1ØØ% acetone, three or four changes are required to remove any residual water.

D. Infiltration: Infiltrate material with Spurr's resin, using the following schedule:

Ratio acetone: resin	Time in solution
7:1	Ø.25 hr
5:1	Ø.5Ø hr
4:1	Ø.75 hr
3:1	2.ØØ hr
2:1	3.ØØ hr
1:1	8.ØØ hr
1:2	8.ØØ hr
1:3	8.ØØ hr
pure resin (I)	8.ØØ hr
pure resin (II)	8.ØØ hr

Cast and polymerize at 7ØC overnight.

II. Material for scanning electron microscopic observations of anthers and floral parts.

A. Fixation: Use the procedure cited above. Whole buds or buds less perianth parts can be used. When placed in the first fixative, specimens must be kept under vacuum for 3Ø minutes to ensure penetration of fixative.

B. Dehydration: Dehydrate in a graded ethanol series. Begin with 2Ø% ethanol in water, increase concentration of ethanol in 2Ø% increments, changing solutions every 2Ø minutes. When 1ØØ% ethanol is reached, change solution three times, and place specimens in 5Ø% ethanol: 5Ø% Freon TF for 3Ø minutes (2 changes, 15 minute intervals). Place specimens in 1ØØ% Freon TF, change solution three

times, and dry specimens with carbon dioxide in a critical point apparatus.

C. Exposure of anthers: Anthers may be exposed by placing buds in liquid nitrogen before the introduction of Freon TF, and freeze-fracturing with a razor blade at the plane of the anthers. Alternatively, anthers may be observed by mounting buds on brass discs with silver paste, and subsequent removal of perianth parts from the dried buds. The latter method gives better results but is also a royal pain in the dupa.