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Cytochemical Analysis of Pollen Development in Wild-Type *Arabidopsis* and a Male-Sterile Mutant

Sharon M. Regan and Barbara A. Moffatt[1]

Department of Biology, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1

Microsporogenesis has been examined in wild-type *Arabidopsis thaliana* and the nuclear male-sterile mutant BM3 by cytochemical staining. The mutant lacks adenine phosphoribosyltransferase, an enzyme of the purine salvage pathway that converts adenine to AMP. Pollen development in the mutant began to diverge from wild type just after meiosis, as the tetrads of microspores were released from their callose walls. The first indication of abnormal pollen development in the mutant was a darker staining of the microspore wall due to an incomplete synthesis of the intine. Vacuole formation was delayed and irregular in the mutant, and the majority of the mutant microspores failed to undergo mitotic divisions. Enzyme activities of alcohol dehydrogenase and esterases decreased in the mutant soon after meiosis and were undetectable in mature pollen grains of the mutant. RNA accumulation was also diminished. These results are discussed in relation to the possible role(s) of adenine salvage in pollen development.

INTRODUCTION

Although the course of pollen development has been described for many plants, the process is poorly understood at a biochemical level (for recent review, see Mascarenhas, 1990). Molecular genetic analysis of male-sterile mutants may allow the identification of gene products involved in pollen development. Nuclear male-sterile mutants have arisen spontaneously in more than 175 plant species, and more have been mutagen induced (Kaul, 1988). In the majority of cases, male sterility is due to defects in the development of the microspore or the surrounding nutritive layer, the tapetum. During microsporogenesis, the tapetum supplies nutrients and growth substances to the developing sporogenous tissue and later degenerates to facilitate the release of the mature pollen. The defects in these male-sterile mutants vary widely, from premature degeneration of the tapetum to aberrant microspore mitosis. Often it has been difficult to pinpoint a specific time when pollen development aborts in these plants because the microspores do not die synchronously (Frankel and Galun, 1977). Furthermore, the molecular basis of the defects in almost all of these mutants remains unknown.

A mutant of *Arabidopsis thaliana* has been isolated recently that is male sterile because of a recessive, nuclear mutation (Moffatt and Somerville, 1988). The mutant, designated BM3, has less than 1% of the adenine phosphoribosyltransferase (APRT) activity found in wild-type plants. APRT is an enzyme of the purine salvage pathway that converts adenine to AMP. To our knowledge, this is one of the few nuclear male-sterile mutants for which the biochemical lesion causing its sterility has been identified. BM3 mutants grow somewhat more slowly than the wild type and are male sterile because of aborted pollen development sometime after meiosis (Moffatt and Somerville, 1988).

We are interested in understanding how APRT deficiency causes male sterility. As a first step, we have used cytochemical stains to investigate pollen development in wild-type *Arabidopsis* and the APRT-deficient mutant. The course of pollen development in *Arabidopsis* has been characterized and several phenotypic effects of APRT deficiency have been detected.

RESULTS

Pollen Development

The inflorescence of *Arabidopsis* has a helical arrangement of flowers that are initiated sequentially. When the oldest flower displays its petals and the anthers begin to dehisce, there are 10 to 15 separable buds in the inflorescence. These buds were embedded in epon, sectioned for light microscopy, and stained with toluidine blue O (TBO). TBO is a nonspecific, polychromatic stain that binds to most cellular components except starch and lipid (O'Brien et al., 1964). Ten stages of pollen development were arbitrarily defined, starting with the microsporogenous mass and ending with the mature pollen within the dehiscing anther. Figure 1 compares the gross anatomy of the mutant and wild-type anthers in buds of the same size, as detected by TBO. The two earliest stages, which occur in the smaller

[1] To whom correspondence should be addressed.

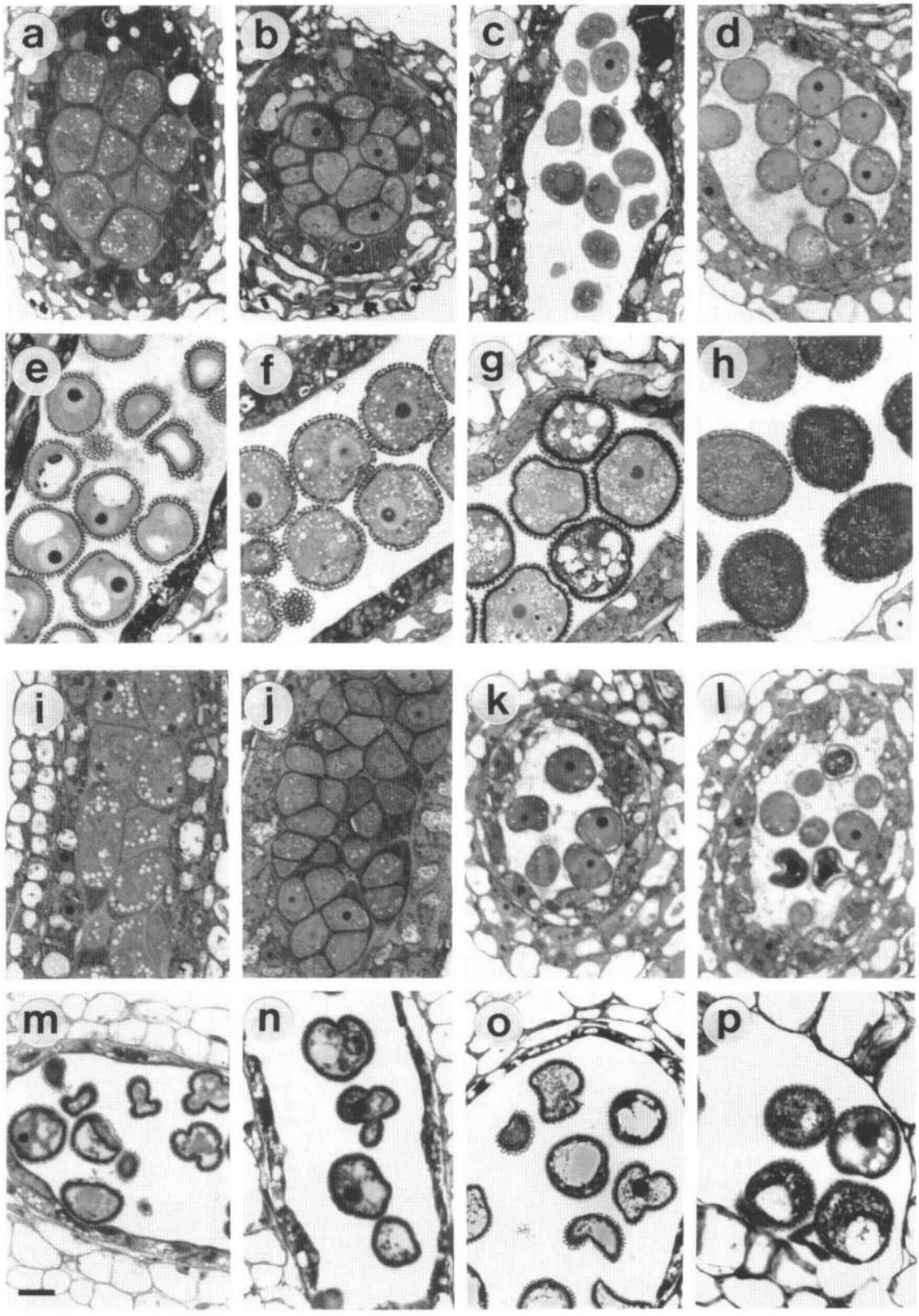

Figure 1. Pollen Development in Wild-Type and Mutant Flowers Stained with Toluidine Blue O.

buds, are not shown.

The pollen mother cells (PMCs), which developed from the microsporogenous mass, became surrounded by a callose wall that physically isolated them from the tapetum (Figures 1a and 1i). The callose has been identified by staining with aniline blue, which binds specifically to β-1,3-glucan (Eschrich, 1956; data not shown). Each PMC underwent meiosis to form a tetrad of microspores that were also encased in the callose wall (Figures 1b and 1j). After meiosis, the callose wall broke down in both mutant and wild-type anthers and the microspores were released. The just-released microspores in the mutant had a consistently darker stained wall compared with the wild type (Figures 1c and 1k). The wild-type microspores rounded up, small vacuoles appeared in their cytoplasm, and their walls thickened because of the formation of the exine (Figure 1d). Some of the mutant microspores also rounded up; others appeared to be somewhat collapsed and deeply stained. In addition, the walls of the mutant microspores did not develop the distinct bacula observed in the wall of the wild-type microspores (compare Figures 1d and 1l). In the next stage of development, a large vacuole formed in each wild-type microspore, which caused both a rapid increase in size and a displacement of the nucleus to one side (Figure 1e). Mutant buds of the corresponding stage contained irregularly shaped microspores with a thickened wall, which continued to lack distinct bacula. The cytoplasm within these microspores appeared diffuse (Figure 1m). Furthermore, vacuole formation was delayed in the majority of the mutant microspores and the tapetal layer appeared to deteriorate. Serial sections indicated that the first mitotic division occurred in the wild-type microspores, after the reabsorption of the vacuole (Figure 1f and data not shown). At the time of the first mitotic division in the mutant microspores, there was often a vacuole-like structure remaining in the cytoplasm and the microspores contained abnormal nuclei (Figure 1n). Concurrent with the second mitotic division, the cytoplasm in the wild-type microspores became more darkly stained and storage bodies were visible (Figure 1g), whereas in the mutant, the microspores and tapetum continued to degenerate (Figure 1o).

Mature, wild-type *Arabidposis* pollen is trinucleate and oval. TBO staining indicated that the cytoplasm of these pollen grains was very dense (Figure 1h). The tapetal layer, which had been thick up to this stage, rapidly disappeared just before dehiscence of the anther. The pollen in the mutant was either collapsed or intact with vacuole-like structures still present (Figure 1p). The fate of the tapetal layer in mutant anthers was variable; in some anthers, it disappeared completely, whereas in others, a remnant was still visible (Figure 1p and data not shown).

Based on these results, the course of microsporogenesis in the mutant appeared to be normal up to the time the microspores were released from the tetrads. After that point, there were several noticeable changes in the mutant relative to the wild type. These included aberrant development of the pollen wall, early degeneration of the tapetum, diffuse staining of the cytoplasm, the presence of collapsed microspores, and abnormal nuclei. Furthermore, the mutant microspores were smaller than the wild type in the later stages (compare Figures 1e with 1h and 1m with 1p). Stains specific for nucleic acids, vacuoles, and various cellular constituents were used to investigate these changes.

DNA and Vacuole

DAPI (4′,6-diamidino-2-phenylindole) specifically binds to double-stranded nucleic acids and fluoresces blue when excited with ultraviolet light (Coleman and Goff, 1985). In some cases, the vacuole can be distinguished as an unstained region. Staining with DAPI is an effective and fast method of staging pollen development according to DNA content. For all staining reactions of fresh material, duplicate sections were stained with DAPI so that the occurrence of cellular constituents, as monitored with cytochemical stains, could be correlated with stages of pollen development. Representative examples of DAPI-stained microspores at various stages of development are presented in Figure 2.

When wild-type microspores were released from the callose wall, a single nucleus was visible and the cytoplasm

Figure 1. (continued).

(a) to **(h)** Sections of wild-type flowers.
(i) to **(p)** Sections of mutant flowers.
Stages according to wild-type development. Bar = 10 μm.
(a) and **(i)** Pollen mother cells.
(b) and **(j)** Tetrads of microspores.
(c) and **(k)** Just-released microspores.
(d) and **(l)** Early-vacuolate microspores.
(e) and **(m)** Vacuolate microspores.
(f) and **(n)** First mitotic division.
(g) and **(o)** Second mitotic division.
(h) and **(p)** Mature pollen.

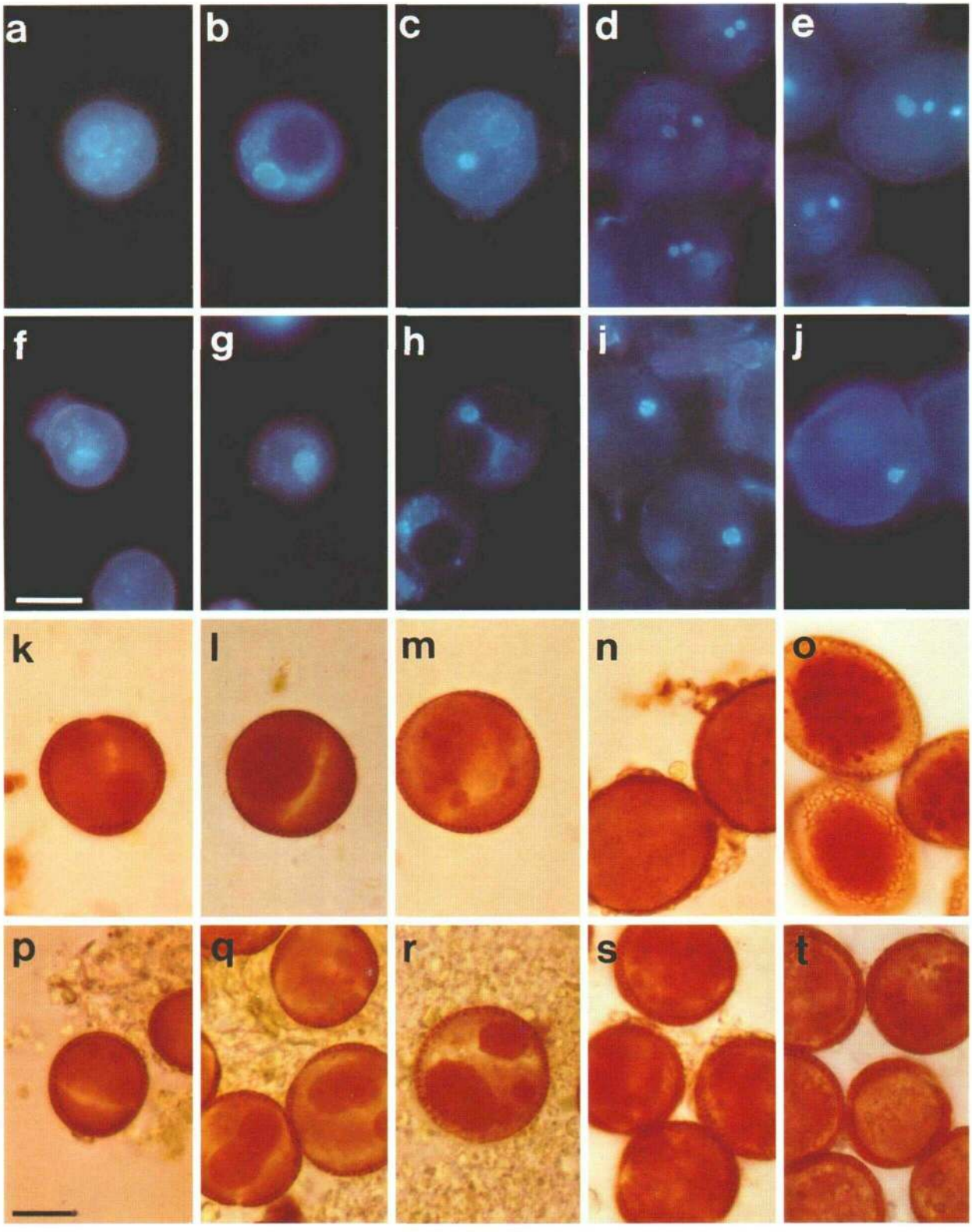

Figure 2. Staining of DNA and Vacuoles in Wild-Type and Mutant Pollen.

was speckled because of the staining of the mitochondrial and plastid DNA (Figure 2a). Displacement of the nucleus and cytoplasm by the developing vacuole produced a "signet ring" appearance (Figure 2b). The first mitotic division occurred after the vacuole disappeared and was easily distinguished by the presence of the generative and vegetative nuclei (Figure 2c). [The generative nucleus is smaller and more brightly stained than the vegetative nucleus (Stanley and Linskens, 1974)]. During the second mitotic division, there was no detectable DAPI-positive staining in the cytoplasm (Figure 2d). This apparent loss of plastid and mitochondrial staining during pollen development has been observed previously by several groups (Kirk and Tilney-Bassett, 1978; Miyamura et al., 1987; A. Coleman, personal communication) and may be associated with the mechanism of maternal inheritance of mitochondrial DNA.

The DAPI-stained mutant tetrads appeared indistinguishable from wild-type tetrads (data not shown). However, by the time of the first mitotic division, DAPI staining of mutant microspores differed. In contrast to wild-type microspores, when those in the mutant were stained with DAPI for 5 min, there was no visible fluorescence. As the staining time was increased, so did the appearance of DAPI-positive structures, up to a maximum at 12 hr. A similar effect was observed when the microspores were treated with the DNA-specific stain mithramycin (data not shown). Apparently, the DNA within the mutant microspores was less accessible to the stain by the time of the first mitotic division. The lack of staining may have been due to the changes in the microspore wall or may indicate that DNA in these cells was in a different condition or structure that did not stain as effectively as the wild-type DNA. For this reason, mutant microspores were stained with DAPI for 12 hr to monitor their DNA content. At the onset of the first mitotic division, there were still vacuoles present in some of the mutant pollen (Figure 2h), and the normally coordinated development of microspores within the anther was lost because some of the microspores failed to undergo mitosis. This was most pronounced after the second mitotic division when about one-half of the pollen was made up of equal amounts of trinucleate, dinucleate, or uninucleate grains and the remaining had no DAPI-positive staining (Figure 2i). The mature pollen grains increased in size slightly, but there was essentially no change in the frequency of dinucleate and trinucleate microspores (Figure 2j).

Neutral red is a dye that primarily accumulates in vacuoles and vesicles (Mahlberg, 1972; Stadelmann and Kinzel, 1972) but has also been shown to stain cell walls (Stadelmann and Kinzel, 1972). The color of neutral red staining can vary depending on the contents and pH of the vacuole or vesicle (Mahlberg, 1972; Stern et al., 1986). Dead or damaged cells will contain neutral red staining throughout their cytoplasm (Gahan, 1984). This stain was used to follow vacuole formation and dissolution during microsporogenesis in wild-type and mutant anthers. In the wild type, a single small vacuole formed within each microspore soon after its release from the tetrad (Figure 2k). The vacuole increased in size (Figure 2l) and finally dissipated into a number of small vacuoles that eventually became reabsorbed (Figure 2m). There were no vacuoles present during the first or second mitotic divisions (Figure 2n), but darkly stained bodies appeared in most of the mature pollen (Figure 2o). We are unsure of the identity of these bodies, but their darker staining (compare Figures 2m and 2o) suggested that they differ from the vacuoles present during early pollen development.

Somewhat later than in the wild type, the mutant developed a number of small vacuoles (Figure 2p) that increased in size (Figure 2q) but failed to reabsorb (compare Figures 2n and 2r). At the time of the mitotic divisions, diffuse neutral red staining was seen in the cytoplasm (Figure 2s). This disperse staining, which persisted in the mature pollen of the mutant, suggests that the vacuole is no longer intact or that the cells have died (Gahan, 1984). A few mature

Figure 2. (continued).

The presence of DNA was detected with DAPI, which specifically binds to DNA and fluoresces blue when exposed to ultraviolet light. Neutral red accumulates in vacuoles and appears red when viewed with white light. Bar = 10 μm.

(a) to **(e)** Wild-type pollen stained with DAPI.
(f) to **(j)** Mutant pollen stained with DAPI.
(k) to **(o)** Wild-type pollen stained with neutral red.
(p) to **(t)** Mutant pollen stained with neutral red.
(a) and **(f)** Microspores recently released from callose wall.
(b) and **(g)** Vacuolate stage.
(c) and **(h)** First mitotic division.
(d) and **(i)** Second mitotic division.
(e) and **(j)** Mature pollen.
(k) and **(p)** Early vacuolate stage.
(l) and **(q)** Mid-vacuolate stage.
(m) and **(r)** Late vacuolate stage.
(n) and **(s)** First or second mitotic division.
(o) and **(t)** Mature pollen.

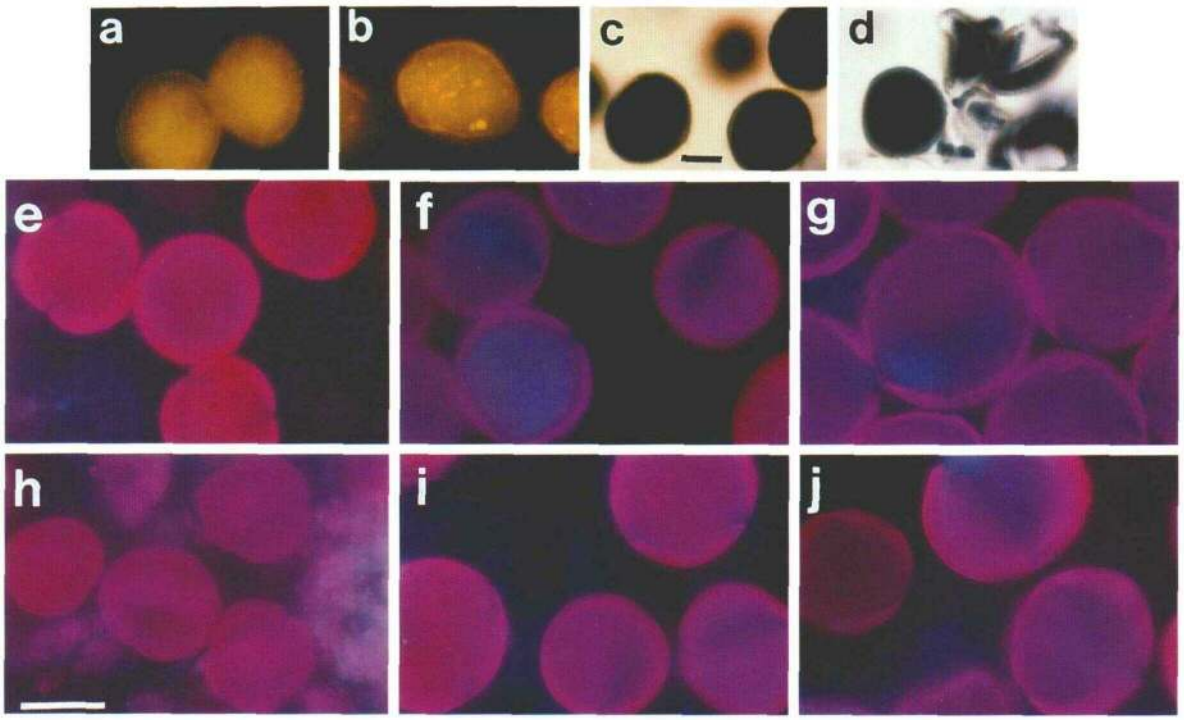

Figure 3. Cytochemical Staining for Lipid, Protein, Intine, and Exine.

Bar = 10 μm. Fluorol binds specifically to lipid and fluoresces yellow.
(a) Mature wild-type pollen stained with Fluorol.
(b) Mature mutant pollen stained with Fluorol.
Amido black stains protein black.
(c) Mature wild-type pollen stained with amido black.
(d) Mature mutant pollen stained with amido black.
Pollen was double stained with tinapol and $DiOC_2$, which stain intine blue and exine red, respectively.
(e) Vacuolate wild-type pollen stained with tinapol and $DiOC_2$.
(f) First mitotic division of wild-type pollen stained with tinapol and $DiOC_2$.
(g) Mature wild-type pollen stained with tinapol and $DiOC_2$.
(h) Vacuolate mutant pollen stained with tinapol and $DiOC_2$.
(i) First mitotic division of mutant pollen stained with tinapol and $DiOC_2$.
(j) Mature mutant pollen stained with tinapol and $DiOC_2$.

grains also contained the darkly stained bodies that were observed in the mature, wild-type pollen (Figure 2t).

Cellular Constituents

Pollen can be classified on the basis of its storage energy contents of lipids and starch. The dynamics of their synthesis and metabolism have been studied in only a few instances, the most extensive being in *Lilium* (Reznickova, 1978). In that case, starch is synthesized in anther tissue before meiosis and subsequently is hydrolyzed to provide energy for the synthesis of lipids in both the tapetum and the microspores. We examined the storage contents of wild-type and APRT-deficient *Arabidopsis* pollen to determine whether changes in the level of starch or lipid could account for the empty-looking microspores of the mutant. The results of staining for lipid are shown in Figure 3. Lipids, which were detected by Fluorol staining (Brundrett et al., 1990), existed as uniformly distributed bodies in wild-type pollen of all stages. Mutant microspores and pollen also contained lipid bodies throughout microsporogenesis. However, in later stages, larger aggregates of lipid were present in microspores of the mutant (Figure 3b). When wild-type and mutant pollen were stained for starch with periodic acid-Schiff reagent or with iodine potassium iodide, there was no evidence of starch in the mature grains, despite the success of these stains on corn pollen, which has a high starch content (data not shown; Stanley and Linskens, 1974). Thus, there was no obvious

difference in the lipid or starch contents of the maturing microspores of the mutant, relative to the wild type, that could account for their apparent emptiness.

Amido black, a protein stain, was used to detect overall levels of protein in the pollen; however, no significant difference was observed between the wild type and the mutant (Figures 3c and 3d). The wall of the mature pollen grains is known to be rich in protein and this likely blocked detection of the internal protein composition by staining. Thus, no conclusion could be made about the protein content of wild-type and mutant pollen based on this staining analysis.

Pollen Wall Formation

The darker staining of the microspore walls by TBO was one of the first differences observed in the course of microsporogenesis in the mutant. The timing of intine and exine deposition on the pollen wall was followed with the stains tinopal and diethyloxadicarbocyanine iodide ($DiOC_2$; Figures 3e to 3j). The former binds specifically to cellulose (Harrington and Raper, 1968), a constituent of intine, whereas the latter has been shown to bind to the exine and mitochondria (Swanson et al., 1990). In wild type, the exine began to develop soon after the microspores were released from the callose wall. By the vacuolate stage, there was intense staining of the exine (red), but the intine (blue) was barely visible (Figure 3e). As the pollen developed, more intine was deposited until the first mitotic division. At this time, intine development appeared complete (Figure 3f) and remained unchanged until maturity (Figure 3g). In the mutant, the exine became visible at the normal time (Figure 3h), and when intine synthesis began, the intensity of the exine staining was similar to the level seen in wild type (Figure 3i). However, intine deposition did not proceed normally in the mutant because mature pollen had either less intense staining of the intine or none at all (Figure 3j). This less intense staining of the intine with tinopal is consistent with the thinner walls of mutant microspores detected by TBO staining (Figures 1l to 1p).

Pollen Viability

Stains specific for RNA, mitochondrial activity, and enzymatic activity, as shown in Figure 4, were used to assess the metabolic potential of the mutant pollen versus the wild type. Azure B was used to localize RNA. This metachromatic dye binds to free anion groups of high-molecular-weight compounds and the resulting color is produced from the arrangement of the particular binding sites. In the case of nucleic acids, azure B binds to the phosphate groups and causes RNA to stain blue and DNA to stain blue-green (Flax and Himes, 1952). Lignin also reacts with azure B and stains green. Before wild-type microspores had undergone the first mitotic division, the majority of the RNA was found in the nucleolus (Figure 4a). (DNA in the nucleus is only visible as a lighter stained halo surrounding the nucleolus.) A similar pattern of staining was seen in early mutant microspores (Figure 4c). Sometime during or after the first mitotic division in the wild type, RNA accumulated in the cytoplasm to the level seen in the mature pollen (Figure 4b). There was very little azure B staining in the collapsed microspores of the mutant (data not shown), and at later times, the azure B staining appeared mottled, which suggested that RNA was less abundant in the mature pollen of the mutant (Figure 4d).

The presence of active mitochondria was assessed using rhodamine 123, which enters mitochondria in response to the membrane potential (Wu, 1987; Vannini et al., 1988). We have used the intensity of rhodamine 123 staining as an indicator of the metabolic activity of developing microspores. During wild-type pollen development, mitochondria could be found in all stages and appeared to be distributed evenly throughout the cytoplasm (Figures 4e and 4f). In the mutant, the distribution of mitochondria appeared normal until the first mitotic division (Figure 4g). After this time, mitochondria became increasingly limited to irregular-shaped areas and were absent in many of the mature pollen (Figure 4h). This result suggested that around the time of the first mitotic division the respiratory metabolism of the mutant microspores began to decrease.

Enzyme activities that could be detected by cytochemical stains were also used to monitor cellular viability. Alcohol dehydrogenase (ADH) activity can be monitored by its reduction of tetrazolium blue (Stinson and Mascarenhas, 1985). ADH activity was present in the tapetal cells of both wild-type and mutant anthers during meiosis. The level of ADH activity in these cells was undetectable by the time the microspores were released from the tetrads (data not shown). There was no apparent difference in the timing or extent of ADH activity in the mutant tapetal cells as compared with the wild type. This suggests that the tapetum in the mutant was responding normally in the early stages of development. ADH activity in wild-type microspores was initially detected during the first mitotic division (Figure 4i) and increased during development (Figure 4j) to a maximum level in mature pollen grains (Figure 4k). A different pattern of expression was observed during mutant pollen development. ADH activity was initiated at the first mitotic division to a level similar to that found in the wild type (Figure 4l), but decreased to an undetectable level in mature grains (Figure 4n). Occasionally, a single pollen grain with some ADH activity was observed in the mutant anthers (Figure 4n).

A second measure of cell viability is based on staining with the dye fluorescein diacetate (FDA), which is converted by nonspecific esterases to a fluorescent analog (Heslop-Harrison and Heslop-Harrison, 1970). Propidium iodide, which is excluded from live cells but stains the DNA and cytoplasm of dead cells, was used as a counter-stain (Huang et al., 1986). Whole anthers were cut across all

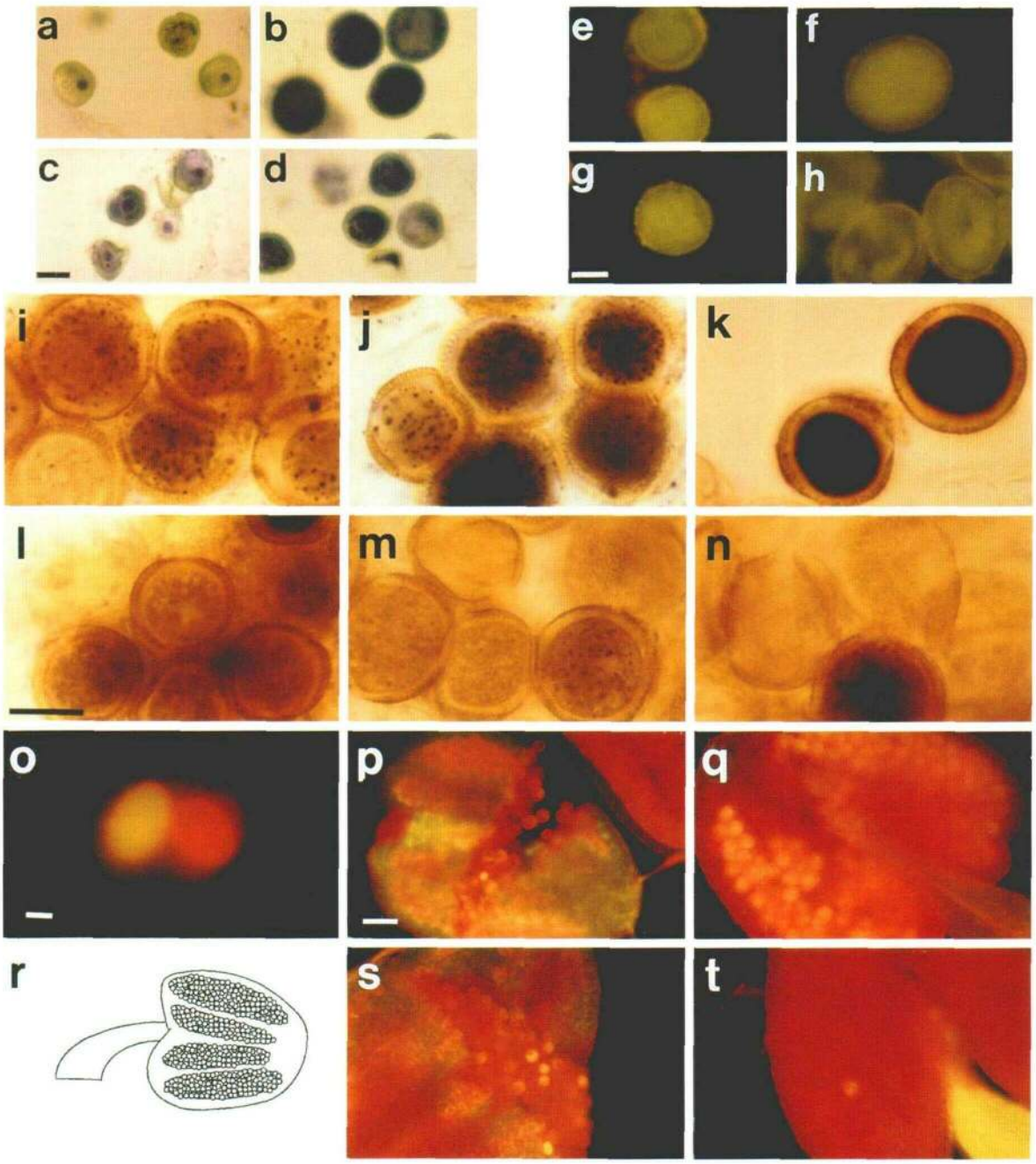

Figure 4. Pollen Viability as Determined by Histochemical Staining for RNA, Mitochondria, Alcohol Dehydrogenase, and Esterase Activity.

Azure B stains RNA blue and DNA blue-green. Lignified walls stain green. Bar = 10 μm.
(a) Just-released microspores of wild-type pollen stained with azure B.
(b) Mature wild-type pollen stained with azure B.
(c) Just-released microspores of mutant pollen stained with azure B.
(d) Mature mutant pollen stained with azure B.
Rhodamine 123 accumulates in viable mitochondria and fluoresces yellow. Bar = 10 μm.
(e) Just-released microspores of wild-type pollen stained with rhodamine 123.
(f) Mature wild-type pollen stained with rhodamine 123.
(g) Just-released microspores of mutant pollen stained with rhodamine 123.
(h) Mature mutant pollen stained with rhodamine 123.
ADH activity was detected with tetrazolium blue. The colorless dye becomes blue when reduced by ADH. Bar = 10 μm.

four locules to give the stains access to the pollen. In the case of anthers containing tetrads, the stain is excluded from the microspores by the presence of the callose (Knox and Heslop-Harrison, 1970), and, thus, FDA could not be used to monitor viability at this stage. The results of fluorescein diacetate and propidium iodide staining are shown in Figures 4p, 4q, 4s, and 4t. During the vacuolate stage, the wild-type (Figure 4p) and the mutant (Figure 4s) anthers exhibited similar staining of their microspores. (The microspores are sensitive to damage caused by cutting the anther; for this reason dead microspores appear at the cut site.) Numerous replications of this staining procedure indicated that the numbers of live pollen (as measured by FDA) in the wild type and the mutant were equal at this stage. The number of live pollen in the mutant dropped to almost zero in the mature anther (Figure 4t), whereas essentially all pollen in the wild type were alive. The most significant decrease in the number of live pollen occurred after the first mitotic division (data not shown). No obvious pattern was observed in the death of the microspores, relative to their position in the locule. The loss of esterase activity in the mutant pollen was similar in its timing to the reduction in ADH activity, suggesting an overall decrease in metabolic activity around the time of the first mitotic division.

DISCUSSION

We have studied microsporogenesis in wild-type *Arabidopsis* and a male-sterile mutant. The course of pollen development was divided into 10 stages, and each of these was examined using cytochemical stains. The developmental profiles of various pollen constituents have been described for both the wild type and the mutant.

Cytochemical Analysis of Pollen

Several stains are suitable for identifying cellular components in plant cells. When these staining reactions were used on pollen, several factors had to be considered. Stains had to be fluorescent or very intense to be clearly visible because of the color and thickness of the pollen wall. In addition, *Arabidopsis* pollen is relatively small, about one-fifth the size of *Zea mays* or *Tradescantia* pollen (Stanley and Linskens, 1974), which increased the importance of vivid staining reactions. However, using an intense stain sometimes had negative consequences. The pollen wall stained very heavily with many apparently specific dyes, thereby obscuring detection of the pollen contents. Staining of exine is likely due to its complex structure, which contains lipid, protein, lignin, polysaccharides, and other undefined substances (Stanley and Linskens, 1974). In some cases, the staining of the exine was minimized by decreasing either the concentration of the stain or the staining time. A final consideration for cytochemical analysis of pollen is the interpretation of a negative staining reaction. We found it necessary to test several different variations of staining conditions before concluding that the substrate for the stain was not present in the sample. For example, many stains were excluded from the developing microspores because of the presence of callose. Occasionally, increasing the staining time or the stain concentration would allow access into the microspore.

Pollen Development in BM3

Pollen development in the BM3 mutant paralleled that in the wild type until shortly after meiosis when the first difference observed was a darker staining of the microspore wall (Figure 1k). Because TBO binds very nonspecifically, this intense staining did not give any insight into what was different in the mutant wall. As development proceeded, the wall did not appear to increase in thickness in the same way as in the wild type (compare Figures 1e and 1m). Based on the results of intine-specific and exine-specific stains, it appeared that the thinness of the wall was caused by a reduced amount of intine (Figures 3e to 3j). The increased time required to stain the nucleic acid in the mutant also with DAPI suggested that the wall was abnormal by the time of the first mitotic division. Later, the mutant pollen grains either collapsed or sustained internal

Figure 4. (continued).

(i) First mitotic division of wild-type pollen stained with tetrazolium blue.
(j) Second mitotic division of wild-type pollen stained with tetrazolium blue.
(k) Mature wild-type pollen stained with tetrazolium blue.
(l) First mitotic division of mutant pollen stained with tetrazolium blue.
(m) Second mitotic division of mutant pollen stained with tetrazolium blue.
(n) Mature mutant pollen stained with tetrazolium blue.
When pollen is double stained with fluorescein diacetate and propidium iodide, live pollen appear yellow and dead pollen stain red.
(o) Live and dead pollen. Bar = 10 μm.
(p) Vacuolate microspores of pollen in wild-type anthers stained with fluorescein diacetate and propidium iodide. Bar = 50 μm.
(q) Mature pollen in wild-type anthers stained with fluorescein diacetate and propidium iodide. Bar = 50 μm.
(r) Interpretative diagram of anther showing location of the locules.
(s) Vacuolate microspores of pollen in mutant anthers stained with fluorescein diacetate and propidium iodide. Bar = 50 μm.
(t) Mature pollen in mutant anthers stained with fluorescein diacetate and propidium iodide. Bar = 50 μm.

morphological changes, such as deterioration of the nucleus, persistence of vacuole-like structures, and irregular staining of the cytoplasm. DAPI staining indicated that less than half the mutant microspores went through the mitotic divisions.

Several stains were used to follow pollen abortion in the BM3 mutant because each stain was informative only when its substrate was present within the tissue. A few of the substrates were present during a defined period of pollen development, whereas others were present continuously. In addition, the various stains ranged in sensitivity, allowing some to uncover more subtle changes than others. Stains specific for starch, lipid, and protein suggested that there was little or no change in the accumulation of these components in the mutant microspores at any time during microsporogenesis. Other stains, such as those monitoring intine, exine, and vacuole development, revealed changes at the vacuolate stage. Consistently, more than one vacuole was present in the mutant, and these vacuoles did not reabsorb normally. Later, however, there was diffuse neutral red staining of the cytoplasm in mutant microspores, which suggested that their vacuoles were no longer intact. Stains specific for esterase and ADH indicated a reduction in the activities of these enzymes, also around the vacuolate stage. Slightly later, the number of active mitochondria visibly declined in the mutant. The irregular pattern of stained mitochondria was reminiscent of the apparent emptiness of the microspores detected by TBO staining (compare Figures 1p and 4h). Lower RNA accumulation in the mature mutant pollen was also indicative of their reduced metabolic activity.

Our results indicated that the coordinated development of the mutant microspores was first lost after their release from tetrads. This loss of synchrony was most likely an indication that some of the microspores were dying. Asynchrony in microspore development is commonly observed in male-sterile mutants and may relate to the positions of the microspores relative to the tapetal layer (Frankel and Galun, 1977). Thus, the observation that the BM3 microspores did not die simultaneously does not necessarily reflect residual APRT activity in this mutant. Furthermore, although the first noticeable change in the mutant was seen soon after meiosis, this does not exclude the possibility that the mutation may have affected development at an earlier stage.

These results do not reveal the functional basis for the male-sterile phenotype of the APRT-deficient mutant. The primary role of APRT is to promote the reuse of adenine in the synthesis of adenine nucleotides. There are at least two stages of microsporogenesis that may be particularly dependent on purine salvage for nucleotides. One stage is during early pollen development, when the tapetal cells, which are providing nutrients to the developing PMCs, undergo endoreplication of their DNA (Chapman, 1987). The later period of meiosis, which involves rapid DNA replication, may also be sensitive to a lack of purine salvage activity. In the latter case, it is not known whether adenine salvage is required in the cells of the tapetal layer, the microspores, or both. The levels of adenine nucleotides have been examined in BM3 leaf tissue and found to be indistinguishable from those in wild-type leaves (B.A. Moffatt and M. Laloue, unpublished results). We are currently undertaking a similar analysis of anther tissue.

Abnormal pollen development in the APRT-deficient mutant may be due to a poisoning of the anther tissue, rather than a deficiency of nucleotide. In plant cells, free adenine produced from the breakdown of purine nucleotides and nucleotide cofactors is rapidly salvaged by APRT (Guranowski and Barankiewicz, 1979; Giovanelli et al., 1983), thereby maintaining low intracellular concentrations of adenine (Brown, 1975; Shimazaki and Ashihara, 1982). Adenine is toxic to animal cells (Hershfield et al., 1977) and presumably plant cells also because germinating seeds of *Arabidopsis* can be killed by exogenous adenine (B.A. Moffatt, unpublished data). The BM3 microspores are unable to salvage adenine, and because of the presence of the callose or exine, adenine or one of its degradation products may accumulate to toxic levels.

A third possible involvement of APRT in pollen development may relate to its role in the metabolism of cytokinin bases to their nucleotide forms (Chen et al., 1982; Burch and Stuchbury, 1986; B.A. Moffatt, C. Pethe, and M. Laloue, manuscript in preparation). Although the involvement of cytokinins in microsporogenesis is not understood (Rueda and Vasquez, 1985), the deficiency of APRT activity in this mutant may alter its metabolism of cytokinin bases and, thus, its profile of cytokinin metabolites.

The results presented here do not clearly favor any one of these explanations for the male-sterile phenotype of this mutant. However, they do define specific changes in pollen development of the BM3 mutant that will be critical in monitoring the effect of treatments designed to alter its sterility.

METHODS

Plant Material

Wild-type and mutant BM3 *Arabidopsis thaliana* Heynh. descended from Columbia wild type were maintained and grown as previously described (Moffatt and Somerville, 1988).

Reagents and Stains

All chemicals were of reagent grade. Epon-embedding reagents were purchased from Ernest F. Fullam, and Fluorol yellow 088 was a kind gift from Mark Brundrett. All other reagents were from Sigma, Fisher, and BDH (formerly British Drug House).

Fixing and Embedding Tissue in Epon Araldite

Arabidopsis buds from the primary inflorescence were teased apart and rapidly sorted according to size. Buds were fixed immediately in cold 2.5% glutaraldehyde (v/v) in 0.1 M phosphate buffer, pH 7.2, overnight, washed twice in cold phosphate buffer, and post-fixed in 1% osmium tetroxide (w/v) in 0.1 M phosphate buffer, pH 7.2, overnight. Buds were then washed in deionized water for 20 min and dehydrated with an increasing ethanol series for 20 min each and finally in 100% ethanol overnight. Ethanol was replaced with acetone by incubating buds in 4:1, 3:1, 2:1, and 1:1 ethanol:acetone mixtures for 1 hr each and finally in 100% acetone overnight. Acetone was gradually replaced with resin by immersing the buds in a 1:1 mixture of acetone and epon araldite (30.0 g dodecenylsuccinic anhydride, 11.1 g araldite epoxy resin 502, and 15.1 g epon poly/bed 812 embedding media) and rotating overnight. This infiltration was continued by transferring the buds to a fresh 1:1 mixture of acetone and epon araldite. These tubes were left open overnight to allow the acetone to evaporate. Buds were transferred to 100% epon araldite and placed on a rotating wheel for 10 hr. They were subsequently transferred to epon araldite with catalyst (1.4 g 2,3,6-tri-dimethylaminoethyl phenol) and again rotated overnight. Finally, buds were placed in fresh epon araldite with catalyst, rotated for 10 hr, and polymerized in Beem capsules by incubating at 37°C overnight and then at 60°C for 48 hr. Thin sections of 2 μm were made on a Reichert ultramicrotome using glass knives and were heat fixed to glass slides. Before staining with aniline blue, the epon resin was extracted from the sections by incubating in a saturated solution of sodium methoxide for 8 min (Sutherland and McCully, 1976).

Fixing and Embedding Tissue in Paraffin

Buds from the primary inflorescence were separated by size and then fixed in Carnoy's fixative (10:30:60, acetic acid: chloroform:ethanol, v/v) for 2 hr. The buds were dehydrated in an increasing ethanol series for 10 min each. The ethanol was replaced with xylene by immersing in a 1:1 mixture of ethanol and xylene for 10 min, then in 100% xylene for 10 min. Buds were gradually infiltrated with paraffin (Paraplast) by immersing in a solution of 1:1 xylene and molten paraffin for 10 min, then in 100% molten paraffin for 10 min. Buds were transferred to molds containing molten paraffin and left at room temperature until paraffin solidified. Sections (8 μm) were made on an American optical 820 microtome using a steel knife and then heat fixed to albumin-treated glass slides (Lillie, 1954). Sections were deparaffinized before staining by 5-min washes in 100% xylene, hydrated in a decreasing ethanol series, and finally washed in water.

Isolation of Anthers

Anthers were teased from buds of the primary inflorescence, cut with a razor blade across all four locules, and stained immediately.

Staining Procedures

A summary of most of the simpler staining procedures is given in Table 1. In addition, starch was detected with the periodic acid-Schiff procedure as described by Feder and O'Brien (1968) and the iodine potassium iodide procedure of Gahan (1984). Staining

Table 1. Summary of Staining Procedures

Stain	Specificity	Type of Section	Stain Conc.	Solvent	Staining Conditions	Optics	Reference
Toluidine blue O	None	A	0.05%	1% Boric acid	1 min, rt	Bright-field	Trump et al. (1961)
Aniline blue	Callose	A	0.05%	0.067 M PO_4, pH 8.5	5 min, rt	UV	Smith and McCully (1978)
DAPI	DNA	C	2 μg/mL	7% Sucrose	5 min, rt	UV	Coleman and Goff (1985)
Neutral red	Vacuole	C	0.1%	20 mM PO_4, pH 7.8	5 min, rt	Bright-field	Gahan (1984)
Fluorol yellow 088	Lipid	C	0.01%	50% PEG, 40% glycerol	1 hr, rt	Blue	Brundrett et al. (1990)
Rhodamine 123	Mitochondria	C	10 μg/mL	7% Sucrose	15 min, rt	Blue	Vannini et al. (1988)
Azure B	RNA	B	0.25 mg/mL	0.1 M Sodium citrate, pH 4.0	2 hr, 50°C	Bright-field	Flax and Himes (1952)
Amido black	Protein	B	0.2%	7% Acetic acid	30 sec, rt	Bright-field	Fisher (1968)
Tinopal	Intine	C	10 μg/mL	0.5 M NaCl, 7% sucrose	15 min, rt	UV	Swanson et al. (1990)
$DiOC_2$	Exine	C	5 μg/mL	0.5 M NaCl, 7% sucrose	30 min, rt	UV	Swanson et al. (1990)

Abbreviations and symbols are: A, epon-embedded; B, paraffin-embedded; C, fresh-cut anthers; rt, room temperature; PEG, polyethylene glycol. All percentages are w/v.

protocols too extensive for description in Table 1 are given below. For epon-embedded or paraffin-embedded sections, photographs presented here are representative of pollen development from at least five individual plants. All other staining procedures were performed on at least 10 plants, and photographs show typical results.

The presence of alcohol dehydrogenase (ADH) was detected by the method of Stinson and Mascarenhas (1985). Fresh-cut anthers were frozen at −20°C in 0.1 M phosphate buffer, pH 7.3, for 3 hr to 4 hr and thawed at room temperature while shaking. The anthers were stained while shaking in a solution containing 0.3 mM *p*-nitroblue tetrazolium chloride, 1.0 mM β-nicotinamide adenine dinucleotide, and 9.5% ethanol in 86 mM phosphate buffer, pH 7.6, in the dark for 3 hr to 4 hr. The staining reaction was stopped by replacing the stain with 100% methanol. Anthers were transferred to a drop of 80% glycerol on a glass slide and viewed using bright-field optics.

Double staining with propidium iodide and fluorescein diacetate was carried out by a modified procedure of Huang et al. (1986). A stock solution of 2 mg/mL fluorescein diacetate was made in acetone and diluted to 100 μL/mL with 7% sucrose (w/v). Propidium iodide was dissolved at 1 mg/mL in water and diluted to 100 μL/mL with 7% sucrose (w/v). Equal amounts of each diluted stain were mixed and used to stain anthers for 30 min. Anthers were rinsed in 7% sucrose and transferred to a fresh drop of 7% sucrose on a glass slide, and a coverslip was applied gently.

Photography

Epon-embedded sections were photographed with a Zeiss Photomicroscope III using bright-field optics and recorded on 32 ASA black-and-white print film. Propidium iodide and fluorescein diacetate double-stained anthers were photographed on the same microscope using epifluorescent illumination (excitation filter BP 546 peak emission, chromatic beam splitter FT 580, barrier filter LP420 and excitation filter BP 485, chromatic beam splitter FT 510, barrier filter 515 to 565) and recorded on 200 ASA Ektachrome color slide film. All other preparations were photographed on a Nikon Labophot microscope with a Microflex AFX-II photomicrographic attachment using bright-field or epifluorescent optics with a blue or ultraviolet filter and recorded on 200 ASA Ektachrome color film.

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