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TETRASPORE is required for male meiotic cytokinesis in *Arabidopsis thaliana*

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SUMMARY

In flowering plants, male meiosis occurs in the microsporocyte to produce four microspores, each of which develops into a pollen grain. Here we describe four mutant alleles of *TETRASPORE* (*TES*), a gene essential for microsporocyte cytokinesis in *Arabidopsis thaliana*. Following failure of male meiotic cytokinesis in *tes* mutants, all four microspore nuclei remain within the same cytoplasm, with some completing their developmental programmes to form functional pollen nuclei. Both of the mitotic divisions seen in normal pollen development take place in *tes* mutants, including the asymmetric division required for the differentiation of gametes; some *tes* grains perform multiple asymmetric divisions in the same cytoplasm. *tes* pollen shows a variety of abnormalities subsequent to the cytokinetic defect, including fusion of nuclei, formation of ectopic internal walls, and disruptions to external wall patterning. In addition, ovules fertilized by *tes* pollen often abort, possibly because of excess paternal genomes in the endosperm. Thus *tes* mutants not only reveal a gene specific to male meiosis, but aid investigation of a wide range of processes in pollen development and function.

Key words: *TETRASPORE*, *Arabidopsis*, meiosis, cytokinesis, tetrad, callose, microspore, pollen development, pollen mitosis, fertilization, endosperm

INTRODUCTION

The male gametophyte of flowering plants, the pollen grain, contains only three cells. Despite this apparent simplicity, pollen differentiation encompasses many of the major developmental events in the life of a plant. These include meiosis, the switch from the sporophyte to the gametophyte generation, and gamete formation.

In all plants, meiosis initiates the transition from sporophyte to gametophyte. In angiosperms (flowering plants), the sporophyte includes stems, leaves, roots and most tissues of the floral organs, while the gametophyte generation comprises the embryo sac, which produces the egg, and pollen, which generates sperm (Dickinson, 1987, 1994). Pollen development in angiosperms, including the model species *Arabidopsis thaliana* (Cruciferae), has been described previously (see e.g. Maheshwari, 1950; Regan and Moffatt, 1990; Scott et al., 1991; McCormick, 1993; Owen and Makaroff, 1995). The sporophytic microsporocytes (also termed pollen mother cells or meiocytes) that give rise to pollen differentiate within each anther locule. These cells secrete a wall of the β-1,3 glucan callose and then undergo meiosis, producing tetrads of four haploid microspores which become separated by callose walls. When meiosis is complete, the callose microsporocyte and intersporal walls are degraded to release the microspores, each of which develops into a pollen grain. Early microspores contain a single, central nucleus. This is subsequently displaced to the periphery of the cell as the microspore becomes vacuolate and undergoes an asymmetric division, pollen mitosis I (PMI), to produce a small generative cell, with condensed chromatin and few organelles, and a larger vegetative cell. The generative cell moves away from the inner wall of the microspore, becoming completely enclosed in the vegetative cytoplasm, and at pollen mitosis II (PMII) divides to form two sperm. In some species, including *Arabidopsis*, PMII occurs before pollen is shed; in others (the majority) the generative cell divides on pollen germination. After release from the anther, pollen that lands on a receptive stigma extends a pollen tube, formed from the vegetative cell, which grows through the style and into the ovary. The two sperm are transported along the pollen tube into the embryo sac, where one fertilizes the haploid egg to produce the embryo and the other fuses with the diploid central cell to form the nutritive endosperm (Maheshwari, 1950; Knox, 1984).

Thus, from the time of microsporocyte formation, every cell division during angiosperm pollen development involves a change in cell identity. Despite decades of research on pollen development, little is yet known about the mechanisms by which these changes occur: for example it is poorly understood how microsporocytes differentiate, how meiosis is initiated and

regulated in plants, what controls the switch from sporophytic to gametophytic development, or how microspore polarity is established before the asymmetric division required for generative cell formation. Progress is now being made through the analysis of mutants defective in some of these processes (Kaul, 1988; Regan and Moffatt, 1990; Aarts et al., 1993; Dawson et al., 1993; Chaudhury et al., 1994; Preuss et al., 1994; Chen and McCormick, 1996; He et al., 1996; Peirson et al., 1996).

In this paper we report the isolation of four mutant alleles of the *Arabidopsis* gene *TETRASPORE* (*TES*), and show that this locus plays an essential role in pollen development. We present evidence that the primary defect in *tes* mutants is failure of cytokinesis at the end of male meiosis, and describe other abnormalities in *tes* pollen, including fusion of nuclei, ectopic internal walls, disruptions to aperture formation in the external wall, and aberrant fertilizations resulting in seed abortion. The primary defect causes all four microspore nuclei to begin development in a common cytoplasm; nevertheless, we find that some of these nuclei complete their developmental programmes to form functional vegetative nuclei and sperm. Finally, we discuss how *tes* mutants contribute to our understanding of various fundamental aspects of wild-type pollen development and function.

MATERIALS AND METHODS

Genetics

The origins of the four *tetraspore* alleles described in this paper are set out in Table 1.

Wild-type pollen used for crosses and developmental comparisons was taken from the following ecotypes: Ws2 (NASC), Col-0 (NASC and Lehle Seeds), or L*er* (NASC and Lehle Seeds).

Due to the low fertility of pollen produced by homozygous *tes* plants, allelism tests were performed by testing for a 1:1 segregation of wild-type:mutant plants in the F_1 of crosses between heterozygous *tes* pollen parents and homozygous *tes* seed parents (data not shown). χ^2 tests on all crosses were consistent with 1:1 segregation ($0.9<P<0.5$).

The following cross was used for mapping the *TES* locus:

$$\frac{\textit{tes-1 gl1}}{\textit{tes-1 gl1}}\ \text{(Col)} \times \frac{\textit{TES-1 GL1}}{\textit{TES-1 GL1}}\ \text{(L}\textit{er}\text{)}$$

To map *TES* relative to *GL1*, plants from the F_2 generation of the mapping cross were scored for both single and double mutant phenotypes. This cross was also used to map *TES* relative to the molecular marker m249. DNA was extracted from *tes* mutants and amplified by PCR using m249 primers (kindly supplied by Jim Beynon and Peter Bittner-Eddy), as described by Bell and Ecker (1994).

Chromosome counts

The somatic chromosome number of *tes* plants was determined as described by Bailey and Stace (1992). Mitotic cells were obtained from root tips, shoot apices, or premeiotic inflorescences.

Transmission electron microscopy

Buds and whole inflorescences were dissected on ice and vacuum infiltrated with Karnovsky's fixative (4% paraformaldehyde, 3% gluteraldehyde, buffered in 0.05 M potassium phosphate pH 7.0; some fixations also included 0.01% Tween 20). Specimens were fixed for 4 hours at 4°C, washed in phosphate buffer as above, postfixed in 2% osmium tetroxide, dehydrated through an acetone series, and embedded in TAAB Embedding Resin (Hard). Ulrathin sections were stained with uranyl acetate and lead citrate, and viewed in a JEOL 2000EX microscope operating at 80 kV.

Scanning electron microscopy

Anthers were dissected into 30% ethanol, dehydrated through an ethanol series, dried in hexamethyldisilazene (Polysciences) for 2×1 hour, air-dried, mounted on stubs and sputter-coated with gold to a depth of 20 nm. Specimens were viewed with a Cambridge Stereoscan S150 microscope.

Fluorescence microscopy

Inflorescences were fixed in ethanol:acetic acid (3:1) and stored in 70% ethanol, or in ethanol:acetic acid (1:3) and dehydrated through an ethanol series to 70% ethanol. The latter, unconventional fixation resulted in the strongest fluorescence when grains were stained with DAPI (see below). Anthers were crushed on a coverslip to release their contents, and inverted on to a drop of mountant mixed with fluorochrome on a microscope slide. For staining nuclei, DAPI was used at a final concentration of 1-2 mg/ml in Vectashield (Vector Laboratories, Peterborough, UK); for staining callose, a mixture of one part decolorized 0.1% aniline blue in 0.1 M $K_3PO_4H_2O$ to one part glycerol was used. Specimens were viewed with a Zeiss Axiophot microscope using a 50 W mercury lamp and the following filter set: 365 nm excitation, 395 nm dichroic, 420 nm long-pass emission.

Confocal laser scanning microscopy

Pollen

Specimens were prepared as for epifluorescence microscopy as described above, and imaged at the John Innes Centre (Norwich, UK) using an MRC 1000 confocal laser scanning microscope controlled by COMOS software (Bio-Rad Microscience Ltd). DAPI- or aniline blue-stained specimens were excited using an argon ion laser at 363 nm (UV), and emissions detected at either 455±30 nm, or ≥460 nm. Serial optical sections were collected at 1 μm intervals through each specimen, averaging over 5 scans per image.

Seeds

Specimens were prepared according to the method of Braselton et al. (1996) and imaged at the University of Leicester (UK) using an MRC 600 confocal laser scanning microscope controlled by COMOS

Table 1. The origins of the four *tetraspore* alleles

Allele	Ecotype	Mutagenesis	Source
tes-1	Col-0 with *gl1* phenotypic marker	Fast neutron	Lehle Seeds (M2F-1A-1, batch no. 92D)
*tes-2**	Col-0 with *gl1* phenotypic marker	EMS	Lehle Seeds (M2E-1A-2, batch no. 92G)
tes-3	L*er*	EMS	R. J. Scott
tes-4	Ws2	T-DNA (Feldmann, 1991); Feldmann no. 4401-4420†	Nottingham Arabidopsis Stock Centre (NASC)

*Some data for this allele was from mutants recovered from the F_2 of a cross into L*er*. These plants are designated *tes-2* (Col/L*er*).
†Assays of *tes-4* progeny for kanamycin resistance indicated this mutation is not T-DNA tagged (data not shown).

software (Bio-Rad Microscience Ltd). Feulgen-stained specimens were excited using an argon ion laser at 488 nm, and emissions detected at ≥515 nm. Images were collected using a Kalman filter.

In vitro pollen germination

In vitro pollen germination was carried out in covered Petri dishes on a medium containing 15% sucrose, 0.36 mg/ml $CaCl_2$, 0.08 mg/ml H_3BO_3, 0.01 mg/ml myo-inositol, 1% agar, pH 5.8-6.0 (modified from Pickert, 1988 and Chen and McCormick, 1996). The dishes were inverted and placed in a growth chamber at 20°C for at least 4 hours. For microscopy, squares of medium were mounted on slides, stained with DAPI in Vectashield (see above), and viewed with a Zeiss Axiophot microscope as above.

Photometry

Pollen was germinated and stained with DAPI as above, and DNA content of sperm nuclei measured using a Newcastle Photometric Systems photon-counting photometer attached to the side port of a Nikon Diaphot TMD inverted microscope, equipped with a 100W mercury lamp attenuated to 0.4% with neutral density filters. Specimens were viewed with a Zeiss 100× oil immersion objective using the following filters: 360±10 nm excitation, 400 nm dichroic, 420 nm long-pass emission. For each measurement, the aperture was adjusted to the size of the nucleus, and a corresponding background reading was taken of a segment of adjacent pollen tube. Each slide included pollen from a *tes* plant and, as controls, pollen from 2x and 4x wild-type plants, which had been germinated at the same time as the mutant pollen. Individual calibration curves were prepared for each slide based on linear regression through the means of the two control groups, as DAPI-stained pollen nuclei fluoresce in proportion to DNA content (Coleman and Goff, 1985).

Seed clearing

To clear seeds for light microscopy, siliques were fixed overnight in ethanol:acetic acid (3:1), followed by two washes in 90% and 70% ethanol, respectively; and then cleared with chlorohydrate: glycerol:water (8:1:2 w/v). Whole-mount preparations were examined with a Zeiss Large Universal microscope using dark-field illumination.

RESULTS

Identification and initial characterization of *tes* mutants

In order to identify genes involved in pollen development, mutagenized populations of *Arabidopsis* were screened for plants with pollen defects. We identified four mutants with similar phenotypes; the alleles responsible were all subsequently found to reside at the same locus, which we named *TETRASPORE* (*TES*). The most immediately noticeable features of the mutants were (1) reduced fertility following self-pollination, resulting in abnormally short siliques, and (2) production of relatively small numbers of pollen grains approximately twice the diameter of wild type. DAPI staining combined with epifluorescence microscopy showed that these enlarged pollen grains did not contain the three nuclei typical of wild type, but instead had variable numbers of nuclei (see below). Aside from slight abnormalities in the size and shape of anthers, the floral organs of *tes* plants appear normal. Vegetative growth is indistinguishable from that of wild type.

Self-pollinated *tes* plants produce a small amount of viable seed, which give rise to 100% mutant progeny. Wild-type pollen on *tes* stigmas produces normal seed set, while the reverse cross yields relatively little viable seed. Therefore *tes* mutants have reduced male fertility but female fertility is not impaired. All pollen grains produced by *tes* homozygotes are abnormal in size and shape, while all grains (n=200) from *tes* heterozygotes have a wild-type appearance, and contain three nuclei. One-quarter of the progeny of self-pollinated *tes* heterozygotes have the mutant phenotype (χ^2, $0.9<P<0.5$), indicating that *tes* alleles are recessive mutations at a single nuclear locus. These segregation ratios, and the production of phenotypically wild-type pollen by heterozygotes, shows that the *tes* mutations act sporophytically.

The F_2 of the cross *tes-1 gl1* (Col-0) × L*er* was used to assay *TES* for linkage to several markers. We have located *TES* on chromosome 3, between *GL1* and m249. Both mapping and complementation tests indicate that *tes* is not allelic to the *quartet* mutations described by Preuss et al. (1994), which affect tetrad separation.

TES is required for male meiotic cytokinesis

To determine when pollen development is perturbed in *tes* mutants, we examined wild-type and mutant pollen at various stages. The earliest detectable defect in *tes* mutants is the failure of cytokinesis at the end of male meiosis. Microsporocytes appear to proceed normally through the nuclear divisions of meiosis (data not shown), but intersporal walls are absent in tetrads of *tes-1*, *-3* and *-4*, and form only partially in *tes-2* tetrads (Figs 1 and 2). The incomplete walls in *tes-2* consist of ingrowths of callose from the parent microsporocyte wall. Ultrathin sections show apparently isolated fragments of callose between microspore nuclei (Fig. 2D); however, serial sectioning (not shown) reveals that these are ultimately connected to the parent wall. The partial cross-wall formation in *tes-2* homozygotes, combined with the observation that microsporocytes with the *tes-2*/*tes-4* genotype also form partial walls (data not shown), suggests that *tes-2* mutants retain some TES function.

We propose that two possible causes of the cytokinetic defect can be ruled out. First, it is unlikely that *TES* is involved in synthesis or deposition per se of an anther- or pollen-specific callose, since *tes* mutants can produce callose in both the sporophyte generation (i.e. the callose wall surrounding the microsporocyte; Figs 1 and 2) and in the gametophyte (callose plugs are formed in pollen tubes, as in wild type; data not shown). In addition, microsporocyte cytokinesis without callose has been observed in mutants or genetically altered plants in species that would normally form callose intersporal

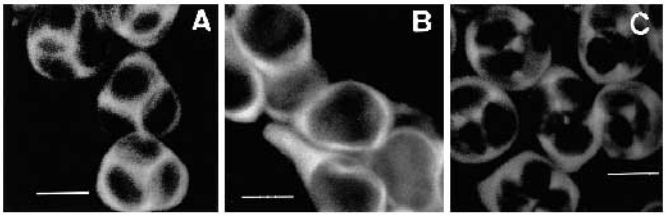

Fig. 1. Callose walls in wild-type and *tes* tetrads, stained with aniline blue and observed using confocal microscopy. (A) Wild-type (Ws2) tetrads; the outer, microsporocyte wall and intersporal walls are present in each. (B) *tes-4* tetrads; the microsporocyte wall but no intersporal walls are present. (C) *tes-2* (Col/L*er*) tetrads, showing partial formation of intersporal walls. Scale bars, 10 μm.

walls (Worrall et al., 1992; Peirson et al., 1996). In these, membranes or non-callosic walls separate the microspores, while in *tes* no intersporal membranes or walls are observed in the absence of callose. Secondly, *TES* is also unlikely to encode a product required for cell-cycle progression, since the ability of at least some microspore nuclei in the mutant to produce vegetative nuclei and sperm (see below) shows that they have exited meiosis and re-entered the mitotic cycle.

Other mutants which fail to partition the microsporocyte after meiosis have previously been reported. Both the ms_1 mutant of soybean and *jp* mutant of alfalfa lack male meiotic cytokinesis, resulting in production of coenocytic microspores with four nuclei (Albertsen and Palmer, 1979; McCoy and Smith, 1983). However, both of these mutants also show disruptions to female gametogenesis, and some *jp* clones produce eggs with ploidies of 2n or higher due to defects in female meiotic cytokinesis (Mariani et al., 1993). To test *tes* plants for such defects, we identified diploid *tes-4* individuals by counting chromosomes in sporophytic cells, crossed these as seed parents with diploid (2x) or tetraploid (4x) wild-type plants, and subsequently determined the ploidy of the progeny. All scored progeny (n=18) of *tes-4* × 2x crosses were diploid and all scored offspring (n=6) of *tes-4* × 4x were triploid, as would be expected if *tes* plants produce only haploid (1n) eggs. In addition, aniline blue staining of carpels from stage 11 buds (Bowman, 1994) reveals multiple callose cross-walls within each ovule (data not shown), characteristic of those that separate megaspores, the products of female meiosis (Kapil and Tiwari, 1978): this is in contrast to the partial or complete failure of megasporocyte cytokinesis in some alfalfa *jp* mutants. Taken together, the evidence so far suggests that female meiosis is unaffected in *tes* plants. Mitotic cytokinesis also appears normal in these mutants. Therefore we conclude that *TES* encodes a product required for cytokinesis following the nuclear divisions of male meiosis, but not for cytokinesis following either mitosis or female meiosis.

Post-meiotic development of nuclei in *tes* mutants

We used DAPI staining combined with epifluorescence or confocal microscopy to investigate the number of male meiotic products in *tes* mutants. All *tes* tetrads examined (n=60) contained four nuclei, as in the wild type (not shown). In wild-type tetrads, the primexine layer of the pollen wall forms around each microspore while it is still encased in callose (Heslop-Harrison, 1968); in *tes*, the primexine instead encloses all four meiotic products in a single cell (coenocytic microspore) (Fig. 2B). At the end of the tetrad stage in wild-type plants, dissolution of the outer and intersporal callose walls releases uninucleate microspores into the anther locule. Callose walls disappear at the same stage from *tes* mutants, releasing coenocytic microspores (not shown). The partial walls in *tes-2* tetrads are apparently not sufficiently extensive to allow complete separation of the meiotic products, though the formation of exine projections within the cytoplasm (see 'Aberrant wall phenotypes', below) results in some partitioning. We therefore conclude that all *tes* microspores begin with four nuclei in a common cytoplasm.

To determine how multiple microspore nuclei behave when they remain within the same cytoplasm, we followed the stages of pollen development in *tes* mutants. As in the wild type, *tes* microspores became vacuolate and nuclei moved to the periphery of the grain in preparation for PMI; in some sections it appeared that *tes* microspores had formed multiple vacuoles (Fig. 3). Asymmetric divisions are observed in *tes* microspores, producing walled-off cells, which appear identical to wild-type generative cells, and nuclei in a larger cytoplasm, which resemble vegetative nuclei (Fig. 4). Confocal microscopy shows nuclei with two distinct staining patterns: dim and diffuse, or compact and bright, which in wild type are diag-

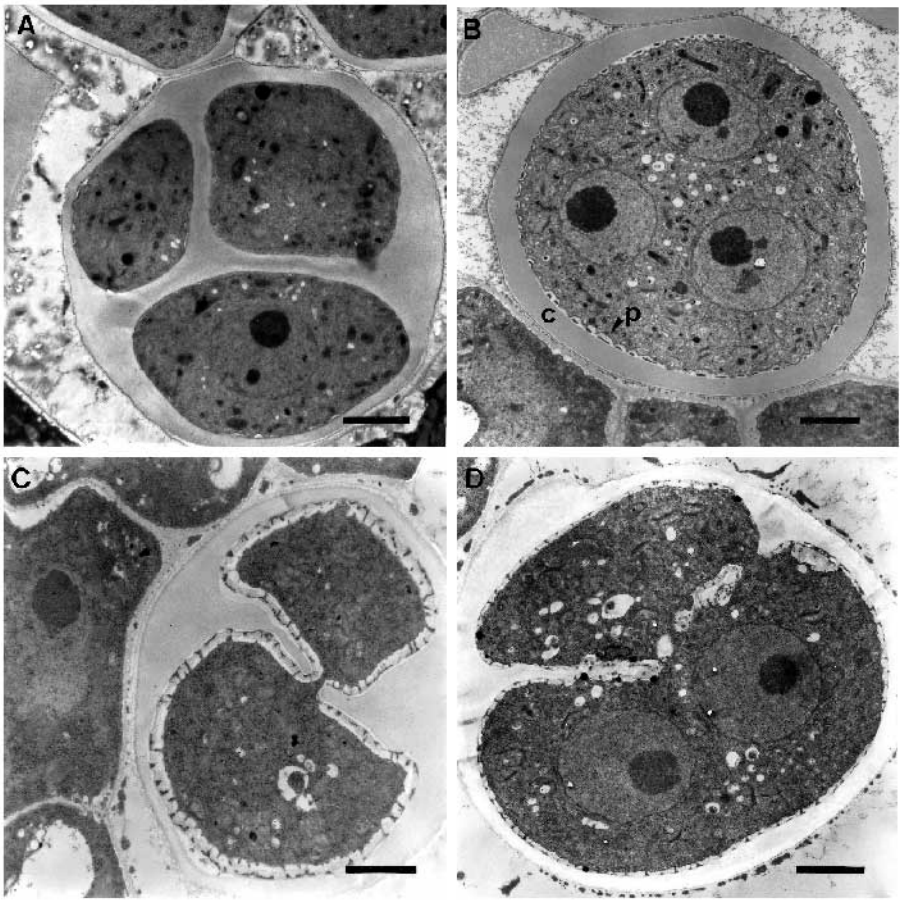

Fig. 2. Transmission electron micrographs of wild-type and *tes* tetrads. (A) Wild-type (Col-0) tetrad; three microspores are visible in this section. (B) *tes-4* tetrad; three microspore nuclei are visible in this section. The primexine layer (p) of the pollen wall has begun to form under the microsporocyte callose wall (c). (C,D) *tes-2* (Col/L*er*) tetrads showing infurrowing of the microsporocyte wall. What appear to be isolated callose fragments in (D) are most likely projections from the parent wall. Scale bars, 2 μm.

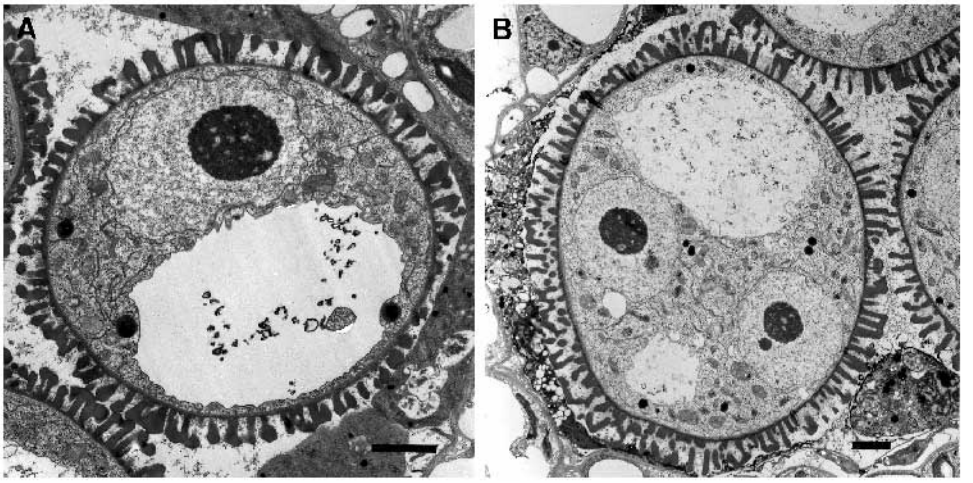

Fig. 3. Transmission electron micrographs of (A) wild-type (Ws2) and (B) *tes-4* vacuolate microspores. Scale bars, 2 μm.

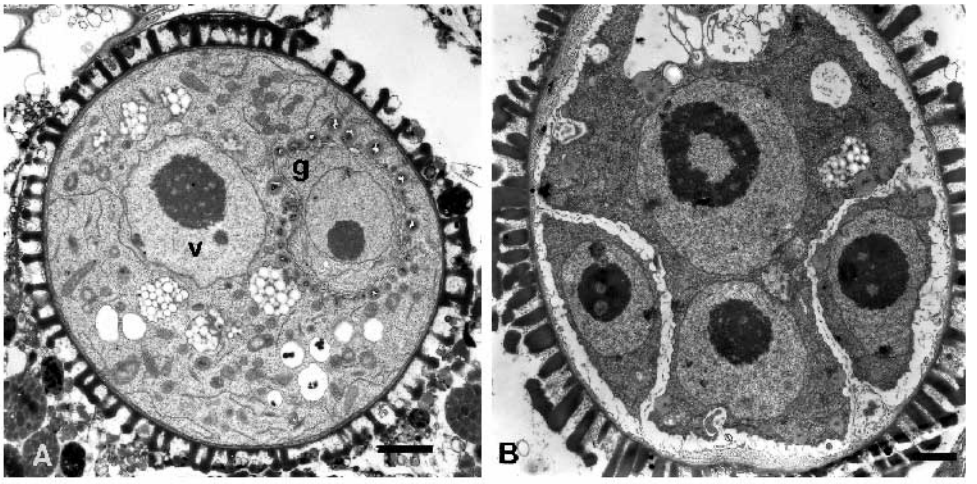

Fig. 4. Transmission electron micrographs of wild-type and *tes-4* pollen after PMI. (A) Wild-type (Ws2) binucleate pollen. The generative cell (g) has already moved away from the microspore wall and is enclosed in the vegetative cytoplasm. (v), vegetative nucleus. (B) *tes-4* pollen at a slightly earlier stage; the two generative cells visible in this section are still walled off. The cytoplasm has shrunk slightly in this preparation. Scale bars, 2 μm.

nostic of vegetative and generative nuclei respectively (Fig. 5). Therefore, in *tes* mutants multiple microspore nuclei can undergo PMI in the same cytoplasm.

If all microspore nuclei in *tes* tetrads developed normally, *tes* pollen grains would contain eight nuclei after PMI. However, by this stage the number of nuclei in each *tes* grain is variable, indicating that some nuclei have degraded, fused, or ceased to develop. In microspores before PMI, nuclei are occasionally seen very close together, with membranes interacting, perhaps as a prelude to fusion (Fig. 6). The possibility that nuclei fuse in *tes* microspores is supported by evidence

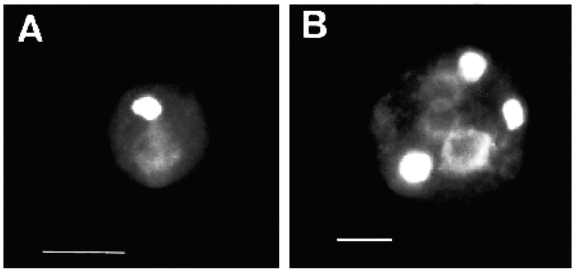

Fig. 5. Nuclei in wild-type and *tes-4* pollen after PMI, stained with DAPI and observed using confocal microscopy. (A) Wild-type (Ws2) pollen. Dim and bright staining are characteristic of vegetative and generative nuclei, respectively. (B) *tes-4* pollen, with three vegetative and three generative nuclei visible. Scale bars, 10 μm.

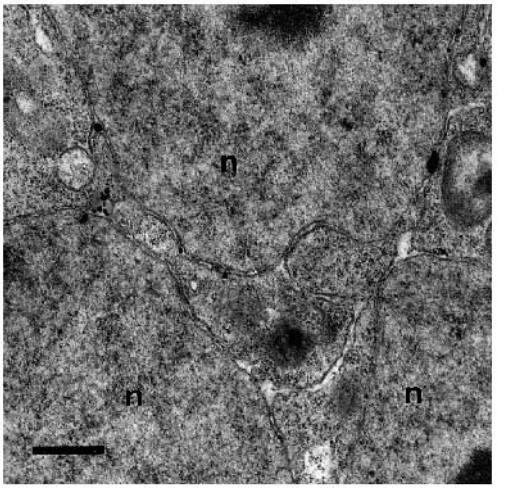

Fig. 6. Interaction of *tes-4* nuclei. Transmission electron micrograph of *tes-4* microspore, showing three nuclei (n) in close association. Some regions of the nuclear envelopes appear to be interacting, possibly prior to fusing. Scale bar, 500 nm.

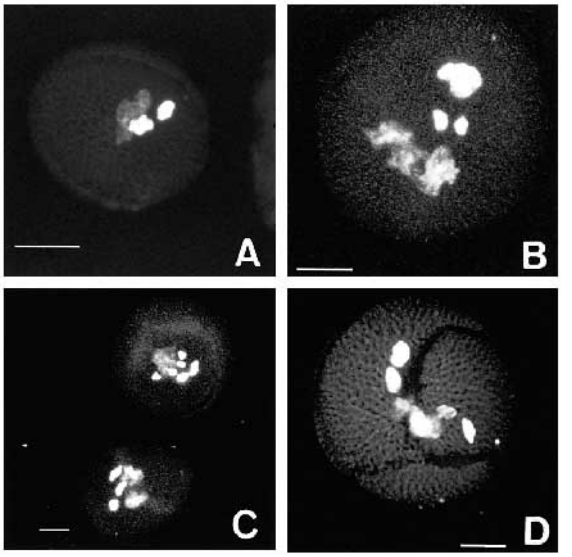

Fig. 7. Nuclei in wild-type and *tes-4* mature pollen, stained with DAPI and observed using confocal microscopy. (A) Wild-type (Ws2) grain containing a lobed vegetative nucleus and two bright, compact sperm. (B-D) *tes-4* grains containing variable numbers of nuclei. A and D also show autofluorescence from the pollen wall. Scale bars, 10 μm.

that *tes* sperm often contain more DNA than wild-type haploid sperm, and can give rise to progeny with extra chromosome sets (see below).

Mature *tes* pollen contains variable numbers of nuclei. Some resemble vegetative nuclei, generative nuclei, or sperm in size, shape and staining pattern, while others are not so readily classified (Fig. 7). Confocal microscopy of DAPI-stained *tes-4* pollen showed that the mean number of nuclei per grain was 5.6±2.4 (*n*=26, range 2–12).

During our investigations of pollen development it became apparent that some *tes* lines were much more likely than others to produce coenocytic pollen grains containing only three nuclei at maturity. To explore this further we used epifluorescence microscopy to determine the number of sperm in DAPI-stained pollen grains. Unlike confocal microscopy, epifluorescence microscopy does not necessarily allow vegetative nuclei to be seen clearly in the coenocytic pollen grains; however, this method is ideal for examining large numbers of grains. We found that the numbers of sperm per grain varied not only between the weak allele *tes-2* and the three strong alleles, but among the strong alleles themselves in their respective original genetic backgrounds (Table 2).

To investigate the process by which the number of nuclei becomes variable during *tes* pollen development, we measured the DNA content of sperm in *tes* pollen tubes. Some pairs of sperm in *tes* pollen tubes are larger than those produced by wild-type plants; in addition, some *tes* pollen tubes contain multiple sperm pairs, which can vary in size within a single tube (Fig. 8A-C). To quantify the DNA content of *tes* sperm, we used photometry to measure fluorescence from sperm pairs stained with DAPI.

For two allele/background combinations, *tes-1*/Col and *tes-4*/Ws2, we compared DNA content of sperm within pairs, and between pairs. Pollen for photometry was taken from

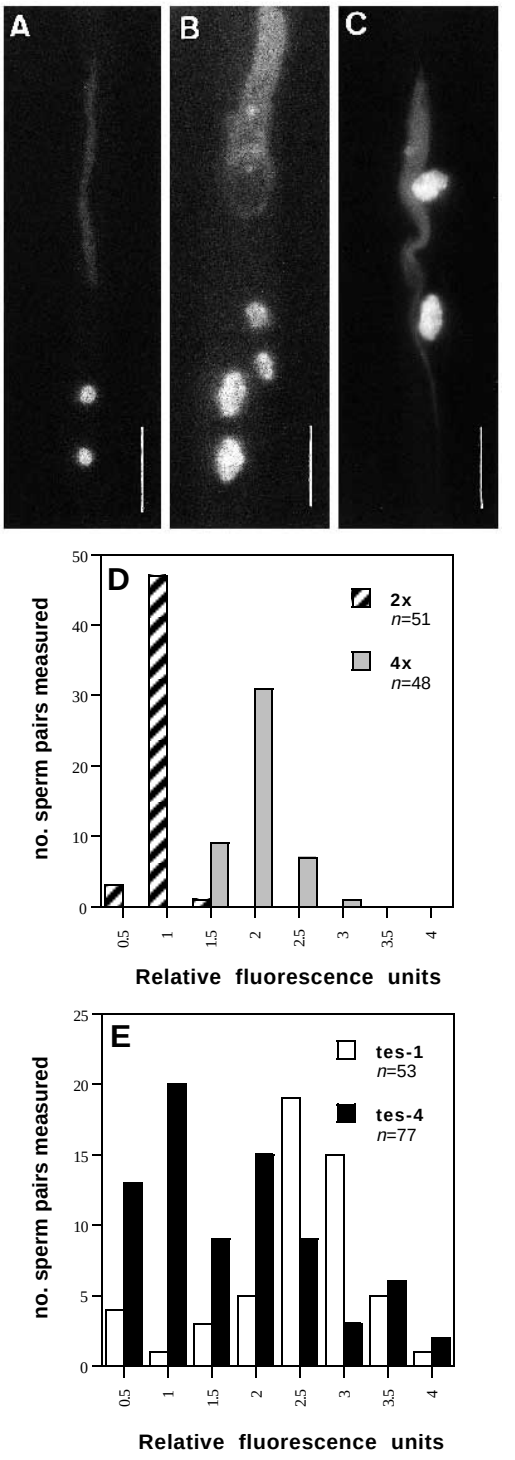

Fig. 8. Sperm nuclei in wild-type and *tes* pollen tubes. (A-C) Pollen tubes germinated in vitro, stained with DAPI, and viewed with epifluorescence microscopy, all at the same magnification. (A) Wild-type (Col-0) pollen tube. The vegetative nucleus appears as a streak; the two bright dots are sperm. (B) *tes-4* pollen tube with two pairs of sperm. Photometry of similar pollen tubes shows that different sizes of sperm reflect different DNA contents. (C) *tes-1* pollen tube. Scale bars, 10 μm. (D,E) Relative DNA content of (D) control (2x and 4x wild type) and (E) *tes-1* and *tes-4* sperm pairs, as measured by photometry. Values along the x-axis represent relative DNA content of sperm pairs, compared to values measured for 2x and 4x sperm pairs, which were standardized to 1 and 2 fluorescence units, respectively.

Table 2. Numbers of sperm in *tes* pollen grains

Genotype	*n*	no. sperm* (mean±s.d.)	% with other than 2 sperm
tes-1 (Col)	179	2.35 (0.98)	21
tes-2 (Col)	235	2.05 (0.48)	6
tes-3 (L*er*)	66	4.27 (1.20)	91
tes-4 (Ws2)	119	3.58 (1.71)	65

*Compact nuclei staining brightly with DAPI were counted as sperm. The great majority were arranged in pairs, typical of sperm; however, some single bright nuclei, which may represent undivided generative cells, were also counted.

plants which were confirmed to be diploid by chromosome counts. For both *tes-1*/Col and *tes-4*/Ws2, sperm within each pair showed about the same variation in DNA content as for wild-type controls (Table 3). Therefore, it seems likely that PMII is normal in *tes* pollen, with generative cells dividing to form two sperm containing equal numbers of chromosomes. For comparisons between pairs, pollen from control plants (2x and 4x wild type) was used to standardize measurements of DNA content of mutant nuclei (absolute measurements of fluorescence from sperm pairs from 2x and 4x plants were converted to 1 and 2 relative fluorescence units, respectively). Fig. 8D,E shows the relative DNA content of sperm pairs from control and *tes* pollen tubes. Most values for *tes* sperm pairs fell in the range of 1 to 4 relative fluorescence units. This is consistent with a model in which some sperm arise from haploid generative nuclei which in turn are derived from haploid microspore nuclei, while other sperm arise from polyploid nuclei resulting from fusion of two to four haploid microspore nuclei (i.e. before PMI). However, *tes* sperm pairs show fractional values as well as entire multiples of 1 fluorescence unit, including some below 1 unit, indicating that the variation in DNA content cannot be fully explained by fusion of nuclei. Possibilities include incorporation of incomplete chromosome sets into dividing nuclei at PMI, or abnormal DNA replication during pollen development or in the pollen tube.

tes-1/Col plants produce fewer sperm per pollen grain (mean 2.35) than *tes-4*/Ws2 plants (mean 3.58). If fusion of microspore nuclei is one mechanism by which the total number of nuclei is reduced during *tes* pollen development, then *tes-1*/Col sperm would be expected to contain on average more DNA than *tes-4*/Ws2 sperm. This is borne out by the photometry data, which show that the mean relative DNA content of *tes-1*/Col germ units is 2.47±0.77, compared with 1.73±0.99 for *tes-4*/Ws2 germ units.

Photometry shows DNA content of nuclei, but not ploidy. Therefore, to investigate further whether fusion of nuclei could be responsible for DNA content greater than 1 fluorescence unit in *tes* sperm, we counted chromosomes in the progeny of self-pollinated *tes-4* plants. If some sperm have more than 5 chromosomes (the 1x complement), as would result from fusion of nuclei during pollen development, we would expect to see some progeny with more than 10 chromosomes in sporophytic cells, since *Arabidopsis* can produce viable seeds when 2n sperm fertilize embryo sacs derived from megaspores (see Discussion). A diploid *tes-4* plant produced offspring with the following ploidies: 9 diploid, 2 triploid, 1 tetraploid. Since all progeny of *tes-4* × wild-type crosses showed ploidies consistent with 1n egg production in *tes* (see 'TES is required for male meiotic cytokinesis', above), it is likely that higher ploidies in *tes* self progeny are due to higher ploidies in the sperm. It is improbable that polyploid *tes* embryos result from fusion of the egg with more than one sperm, since in vitro fertilization experiments suggest that there is a block to polyspermy in eggs of higher plants, as there is in mammals (Faure et al., 1994).

Although the photometry data indicated that some progeny might have partial chromosome sets, no such plants have yet been found. Failure to recover progeny with partial chromosome sets could be due to impaired transmission of aneuploid karyotypes through pollen. It has previously been reported that the transmission of male gametes with an extra chromosome is usually low (Koornneef, 1994).

Table 3. Relative DNA content of sperm within pairs

Genotype	*n*	% within-pair variation (mean±s.d.)
2x	17	6.30 (4.52)
4x	9	6.28 (3.09)
tes-1/Col	22	5.62 (4.43)
tes-4/Ws2	28	6.60 (4.91)

Aberrant wall phenotypes in *tes* mutants: clues to aperture positioning and cytokinesis

Wall apertures

Pollen grains have species-specific patterns of wall apertures, which provide outlets for the pollen tube on germination. The positions of these apertures are established at the tetrad stage, when forming apertures can be seen as regions where the plasma membrane is appressed to the callose wall, resulting in gaps in the primexine (Heslop-Harrison, 1968). The mechanism of aperture placement is not understood but it has

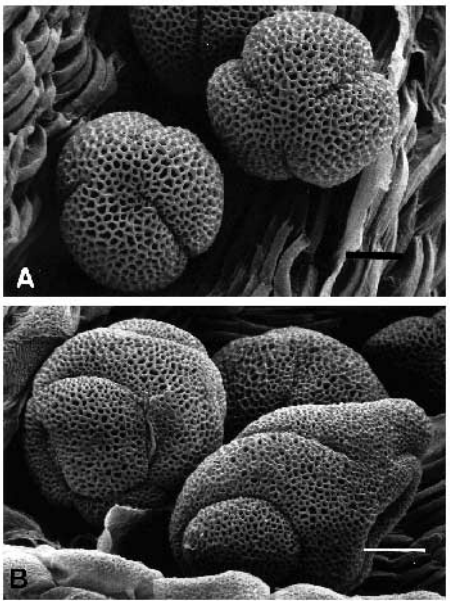

Fig. 9. Scanning electron micrographs of wild-type and *tes-4* mature pollen. (A) Polar view of wild-type (Ws2) grains, showing the three equidistant apertures in the pollen wall. (B) *tes-4* grains showing irregular number, placement, and shape of apertures. Scale bars, (A) 5 μm, (B) 10 μm.

been linked to the process of microsporocyte cytokinesis (Blackmore and Barnes, 1990). Since abnormalities in the tetrad would therefore be expected to disrupt aperture patterning, we examined the pollen wall in *tes* mutants. Wild-type *Arabidopsis* pollen has three longitudinal apertures (Fig. 9A). In *tes*, apertures become visible at the normal time (not shown), but in the coenocytic pollen grains the number, orientation and shape of apertures are aberrant (Fig. 9B). To some extent the extra apertures can be explained by the observation that each *tes* coenocytic pollen grain represents an aggregate of four normal grains. In addition, it is possible that the mechanism responsible for aperture placement in each microspore is able to spread throughout the entire *tes* coenocytic microspore, due to absence of intersporal walls. Interaction between these potential patterning elements could result in misshapen or irregular apertures.

Internal walls

In order to investigate the possibility that meiotic cytokinesis is delayed until after the tetrad stage in *tes* mutants, we looked for the formation of internal walls during pollen development. In wild-type pollen a transient wall separates the generative and vegetative cells at PMI, but mature angiosperm pollen lacks internal walls. In contrast, many *tes* microspores and pollen grains contain ectopic walls. Some are fibrillar, resembling cellulosic cell walls such as the pollen intine (Knox, 1984), and appear continuous with the intine (Fig. 10A). Walls resembling intine have also been reported within the coenocytic microspores of *ms1* mutants in soybean by Albertsen and Palmer (1979). These researchers suggested internal walls could represent either persistent generative cell walls, or a late attempt at meiotic cytokinesis. If cytokinesis were sufficiently delayed, it is conceivable that the cell would no longer be competent to make callose walls and instead would separate nuclei with the wall material being produced at that developmental stage, i.e. intine in the microspore.

In the weak *tes-2* allele, nearly all grains observed contain an additional type of wall within the cytoplasm that is not strictly ectopic; rather, it is exine that has formed on what would have been the exterior of the microspores had they separated completely (Fig. 10B). Serial sectioning indicates that these, like callose fragments at the tetrad stage, are projections from the outer exine (not shown). *tes-2* tetrads with partial callose walls show primexine surrounding callose projections, as well as beneath the parent callose wall where it would be expected. This suggests that exine formation begins wherever there is callose at the tetrad stage, reinforcing previous evidence that callose is required for normal exine deposition (Worrall et al., 1992).

In some *tes-2* microspores, exine fragments are joined by intine-like walls to the layer of intine surrounding the entire coenocytic microspore (Fig. 10C,D). Such a structure could represent a continuous wall that was formed in two stages, with the exine projections deriving from sites of partial callose wall deposition in the microsporocyte, and the intine-like regions being formed later. This would support the hypothesis that *tes* causes a delay in cytokinesis.

tes pollen can fertilize ovules but most seeds abort

Fertilization by *tes* pollen is sometimes successful, but more often seed set fails at one of two stages. Siliques of self-pollinated *tes* plants contain many ovules which appear to be unfertilized; in addition, a variable proportion of fertilized ovules abort (mean 94% [*n*=40 siliques] in *tes-1*/Col and 52% [*n*=29 siliques] in *tes-4*/Ws2), resulting in a mixture of healthy and collapsed seeds in

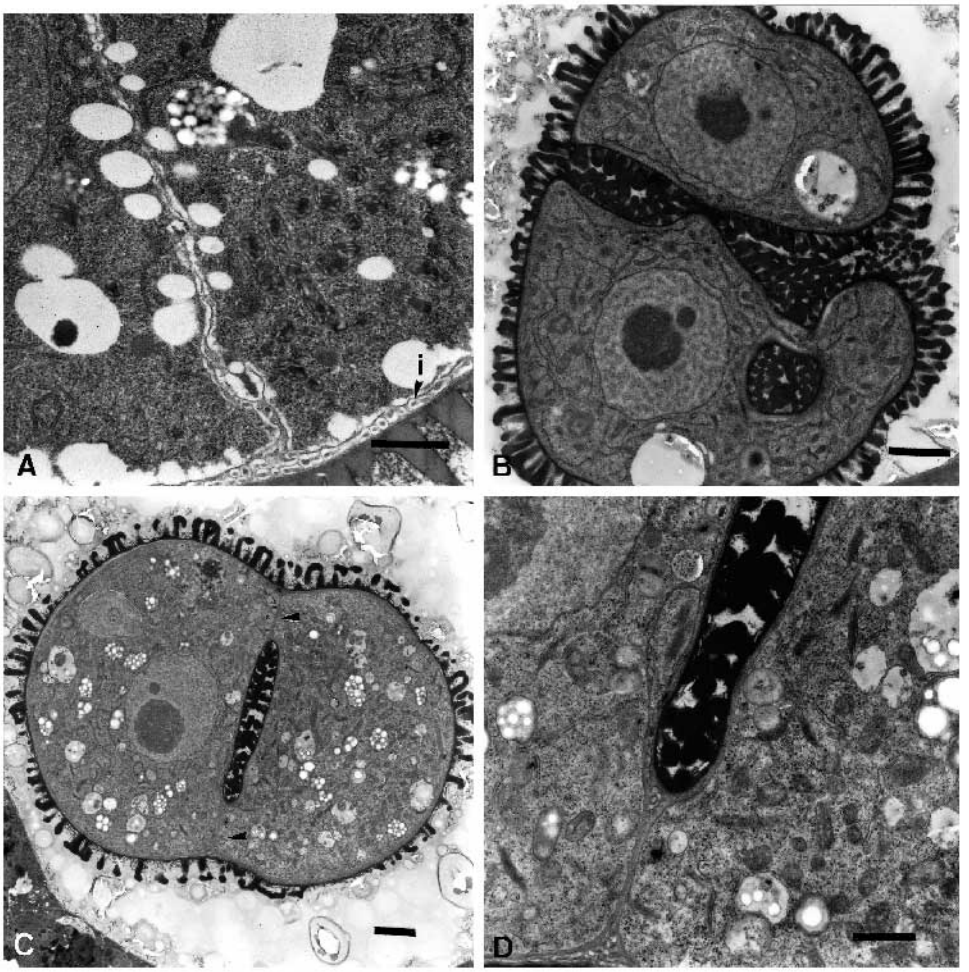

Fig. 10. Transmission electron micrographs showing internal walls in *tes* microspores and pollen. (A) *tes-4* pollen after PMI; the ectopic wall appears continuous with the intine (i). (B) 'Internalized' exine in a *tes-2* (Col/L*er*) microspore. (C) Dual exine/intine wall in a *tes-2* (Col/L*er*) microspore. Regions of intine-like wall (arrowheads) connect an exine projection with the parent wall. (D) Portion of C at higher magnification. Scale bars, (A, D) 1 μm, (B, C) 2 μm.

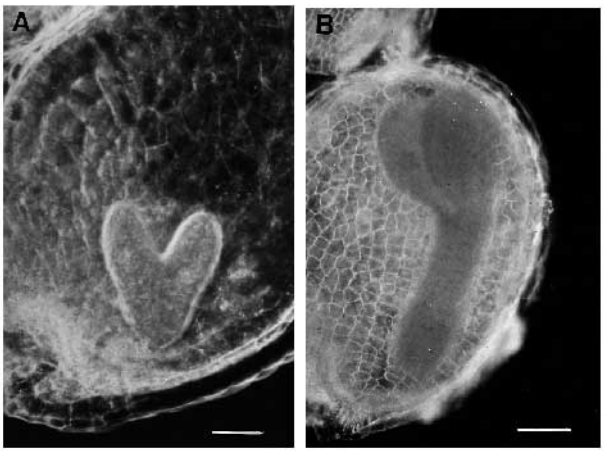

Fig. 11. Embryos taken from (A) aborted and (B) apparently healthy immature seeds in the same silique from a self-pollinated *tes-3* plant. A has arrested at heart stage. Scale bars, (A) 40 μm, (B) 70 μm.

each pod. One cause of seed abortion that has been documented for several angiosperm species is excess of paternal genomes in the endosperm, which normally requires a ratio of 2 maternal (m):1 paternal (p) genomes for successful development (reviewed by Haig and Westoby, 1989, 1991). Since some *tes* pollen tubes transport extra sperm, and/or contain sperm with extra DNA, we investigated the seed abortion phenotype in more detail.

Examination of cleared immature seeds (n=171) from self-pollinated *tes* mutants revealed that in all *tes* alleles, aborted seeds contained embryos that had arrested at heart stage (Fig. 11; see Bowman, 1994 for descriptions of embryo and endosperm development in *Arabidopsis*). Confocal microscopy of developing *tes* seeds shows that some appear normal (Fig. 12A,B). In these apparently healthy *tes* seeds, as in wild type, the nuclei of the micropylar endosperm first proliferate around the periphery of the embryo sac, then begin to cellularize, and fill the interior of the embryo sac, when the embryo is at the late globular/early heart stage. In other *tes* seeds, the micropylar endosperm seems to arrest before moving into the embryo sac and becoming cellular (Fig. 12C). In many preparations of *tes* seeds the endosperm peels away from the inner integument; this is never observed in wild-type seeds prepared for microscopy. Therefore preliminary evidence suggests that the micropylar endosperm is defective in some *tes* seeds, possibly because of paternal excess resulting from fusion of polyploid or multiple sperm with the central cell.

DISCUSSION

The role of *TES* in pollen development

We have identified a gene, *TETRASPORE*, that acts early in pollen development, and we describe four mutant alleles. The phenotypes of *tes* homozygotes indicate that *TES* encodes a product necessary for cytokinesis following the nuclear divisions of male meiosis, but not for cytokinesis following either mitosis or female meiosis. Although *Arabidopsis* mutations affecting mitotic cytokinesis have been reported (Assaad et al., 1996; Lukowitz et al., 1996; Liu et al., 1997), *TES* is the first gene directly involved in meiotic cytokinesis to be reported in this species.

The earliest defect we can detect in *tes* is failure of intersporal wall formation at the end of male meiosis. In *Arabidopsis*, as in other dicots, microsporocytes divide by simultaneous cytokinesis, with intersporal walls forming only after meiosis II (Owen and Makaroff, 1995). In simultaneous cytokinesis, intersporal wall formation generally begins as ingrowths from the callose wall surrounding the microsporocyte (Brown and Lemmon, 1988, 1991). Unlike the case in mitotically dividing cells of the sporophyte, division sites are not marked by a preprophase band of microtubules. Instead, post-meiotic cleavage planes are thought to be determined by 'spore domains' defined by arrays of microtubules radiating from each microspore nucleus; vesicles fusing at the interfaces of these arrays supply membranes and wall components.

Evidence presented above rules out a defect in callose production per se in *tes* mutants, as well as a failure to exit meiosis. However, the *tes-2* phenotype suggests two other possibilities. Tetrads in this allele form ingrowths of the microsporocyte callose wall to varying extents, suggesting that microsporocytes with partial *TES* function are to varying degrees able to deliver and assemble components necessary for constructing intersporal membranes and walls, but are unable to continue these processes until complete walls are formed. This is reminiscent of the seedling-lethal *knolle* mutation of *Arabidopsis*, which results in a (mitotic) cytokinetic defect characterized by

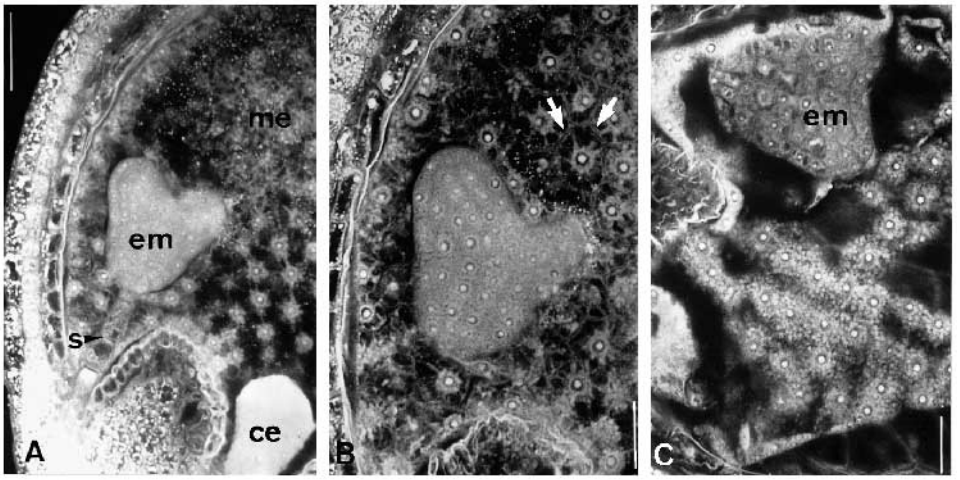

Fig. 12. Immature seeds from self-pollinated *tes* siliques, stained with Feulgen and observed using confocal microscopy. (A) *tes-4* seed showing heart stage embryo (em) and suspensor (s), surrounded by nuclei of the micropylar endosperm (me). The brightly staining structure is chalazal endosperm (ce). (B) As A, in a different optical section and at higher magnification. Walls separate some of the micropylar endosperm nuclei (arrows). (C) *tes-4* seed showing heart stage embryo and collapsing micropylar endosperm which has not cellularized. Scale bars, (A, C) 50 μm, (B) 25 μm.

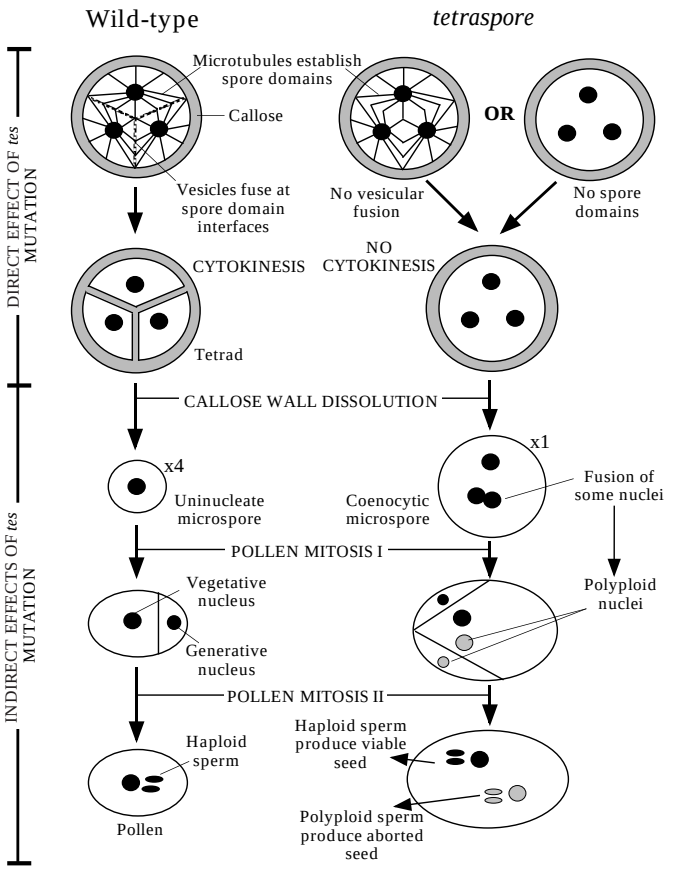

Fig. 13. Model of possible explanations for some aspects of the *tes* phenotype. (1) Direct effect of *tes* mutation (top). Failure of cytokinesis is the only direct effect of *tes* mutations. In wild-type tetrads (left), cytokinesis requires establishment of microtubule-based spore domains, and fusion of vesicles at the domain interfaces. In *tes* (right), failure of cytokinesis could be caused by defects in the spore domains or in vesicular function. In both wild-type and mutant tetrads, only three of the four microspore nuclei are shown. (2) Indirect effects (bottom). The other differences observed between developing pollen grains in wild-type and *tes* plants are indirect effects of the *tes* mutation, resulting from all microspore nuclei beginning development in the same cytoplasm. These include fusion of nuclei resulting in polyploid sperm (which in turn might cause seed abortion in self-pollinated *tes* siliques when sperm ploidy is sufficiently high), and multiple asymmetric divisions in the same cytoplasm. Abnormal internal and external walls observed in *tes* pollen are not shown.

fragmentary cross-walls. The wild-type *KNOLLE* product has homology to T-SNARES, which are involved in fusion of vesicles (Lukowitz et al., 1996; DuPree, 1996). Therefore, one possibility is that *TES* could be involved in a step of vesicular trafficking or fusion specific to microsporocyte cytokinesis (represented in Fig. 13, top).

Another possibility is that the *TES* product is required for or interacts with the specialized cytoskeletal apparatus involved in simultaneous microsporocyte cytokinesis (Fig. 13, top). Although plants and animals use different mechanisms for cytokinesis, it is noteable that a *Drosophila* mutation resulting in failure of meiotic cytokinesis during spermatogenesis is due to a defect in a kinesin-like protein that normally localizes to the meiotic spindle (Williams et al., 1995).

Some *tes* microspores form internal walls after the tetrad stage. In the strong *tes-4* allele, the ectopic walls resemble intine, and partly or completely divide the cytoplasm; these may represent a delayed attempt at cytokinesis. In the weak *tes-2* allele, intine-like walls can be seen joining regions of internalized exine to the parent wall. These structures suggest that cytokinesis has occurred in two stages, with part of the intersporal wall formed of callose in the tetrad (this portion leaves behind an area of exine after microspore release), and part formed later, using intine. The possibility that some *tes* microspores perform cytokinesis after the tetrad stage is consistent with both of the models for *TES* function presented above. If *TES* is involved in vesicle trafficking, then a *tes* mutation could impair vesicle function so that wall development is delayed. Alternatively, if *TES* is involved in establishment of the microtubule-based spore domains, a *tes* mutation could delay their formation.

The *tes* alleles described in this paper behave sporophytically: pollen grains produced by *tes* heterozygotes all appear wild type, indicating that expression of the mutant phenotype depends on the genotype of the diploid microsporocyte rather than that of the haploid microspore. However, it remains possible that the *TES* gene is expressed in the very early gametophyte, as mutations in a gene expressed before intersporal wall formation could be masked in the heterozygote. Conclusive evidence for whether *TES* is transcribed from the sporophytic or gametophytic genome must await cloning of the gene and examination of its timing and pattern of expression.

tes mutants show several other abnormalities in addition to the defect in microsporocyte cytokinesis, including variable numbers of pollen nuclei, which are caused at least in part by nuclear fusion leading to polyploid nuclei, and extra apertures in abnormal orientations in the pollen wall. We propose that these are indirect effects of the mutation, resulting from multiple meiotic products attempting to develop in the same cytoplasm (represented in Fig. 13, bottom). Even if *TES* is gametophytically expressed, *tes* mutations could no longer be masked in the gametophyte after separation of the meiotic products; yet all pollen grains in *tes* heterozygotes appear normal. Furthermore, the 3:1 segregation of wild type:*tes* phenotypes in the F_2 of outcrosses to wild type shows that pollen grains carrying the *tes* mutation are not at a disadvantage. Therefore it seems most likely that of all the defects seen in *tes* pollen, only failure of cytokinesis is directly attributable to lesions in the *tes* gene.

Nuclei in *tes* microspores can follow a normal developmental programme, including pollen mitosis I

The ability of *tes* pollen to germinate and fertilize ovules shows that some microspore nuclei complete development, differentiating functional vegetative nuclei and sperm. Particularly striking is the ability of *tes* microspores to perform multiple asymmetric divisions within the same cytoplasm.

Asymmetric divisions result in daughter cells with different fates. Studies of asymmetric divisions in a variety of model organisms show they are controlled in several steps, which include establishment of an asymmetric cue in the mother cell, distribution of factors to specific locations in this cell, and positioning of the plane of division so these factors are segregated differentially to daughter cells (Gönczy and Hyman, 1996). In

developing pollen, differentiation of the generative cell depends on division asymmetry at PMI: if the division is made symmetric or abolished altogether, the resulting cells either differentiate as vegetative cells (Eadey et al., 1995) or can even be induced to develop directly into haploid embryos without fertilization (Zaki and Dickinson, 1991). Although there have been some investigations of the mechanism by which the plane of division is controlled at PMI (e.g. Terasaka and Niitsu, 1990; Hause et al., 1991), there is currently no molecular information on the initial asymmetric cue, or on localized factors involved in generative cell differentiation. However, a clue to the time at which asymmetry is established in the microspore could come from species in which developing pollen grains remain attached in tetrad formation. Studies of pollen development in such plants (Geitler, 1935; Maheshwari, 1950; and references therein) show that generative cells always form at sites characteristic for each species — e.g. the proximal (inner) or distal (outer) pole in relation to the microspore's position in the tetrad — which suggests that nuclear migration responds to a polarizing signal established by the tetrad stage. We are currently using the *qrt1* mutant, in which microspores remain in tetrads (Preuss et al., 1994), to investigate whether the site of generative cell formation in *Arabidopsis* is likewise correlated with the position of the microspore in the tetrad. Preliminary evidence, from reconstruction of serial sections through *qrt1* microspores at PMI (not shown), indicates that generative cells may form distally or radially, but are never found at the proximal pole. Thus in *Arabidopsis*, it seems that the microspore is also polarized with respect to the tetrad, and it is therefore likely that the initial asymmetry required for nuclear migration and generative cell differentiation is established by the tetrad stage, by a mechanism as yet unknown.

The *tes* phenotype is consistent with such an early establishment of microspore polarity. The ability of *tes* microspores to orchestrate migration, division, and differentiation of multiple nuclei in a shared cytoplasm suggests that multiple polarizing signals are present in the grain. If polarization is established by the tetrad stage in *Arabidopsis*, then *tes* tetrads could retain four axes of polarity, allowing four asymmetric divisions (if all the microspore nuclei survive; see below). Existence of multiple polarities in *tes* coenocytic microspores resulting from their failure to partition is further supported by the observation that *tes* pollen grains have extra sets of wall apertures, which are known to be established at the tetrad stage (Heslop-Harrison, 1968). As the molecules involved in microspore polarity are identified, it will become possible to test directly the hypothesis that *tes* microspores have multiple polarities; likewise, *tes* should provide a useful system for exploring the mechanism of the asymmetric pollen division.

Behaviour of microspore nuclei in *tes*

Tetrasporic development is found in female gametogenesis in some angiosperm species (Maheshwari, 1950), with all four megaspore nuclei giving rise to various nuclei of the embryo sac; in these cases there is no meiotic cytokinesis in female development. However, *tes*, and other mutants lacking microsporocyte cytokinesis (Albertsen and Palmer, 1979; McCoy and Smith, 1983), are the only plants reported in which more than one meiotic product contributes to a single pollen grain. In most coenocytic microspores produced by alfalfa *jp* mutants, all four microspore nuclei fuse following failure of microsporocyte cytokinesis and before PMI, resulting in 4n pollen grains with one vegetative and one generative nucleus (McCoy and Smith, 1983). In contrast, several lines of evidence (variability in numbers and DNA content of sperm in *tes* pollen grains, as well as in ploidies of progeny of selfed *tes* plants) suggest that variable numbers of microspore nuclei fuse in each *tes* coenocytic microspore.

tes sperm do not show the high DNA content found in sporophytic nuclei of *Arabidopsis* mutants with defects in mitotic cytokinesis (nuclei in *knolle* embryos contain up to eight times the diploid chromosome complement, and *tso1* cells have C-values of up to 63 C, compared with 2 or 4 C for normal cells: Lukowitz et al., 1996; Liu et al., 1997). This suggests that endoreduplication can be ruled out as a major cause of elevated DNA content of *tes* sperm.

Pollen grains from different *tes* alleles in their respective genetic backgrounds ultimately contain different numbers of sperm (Table 2). While the relatively low number of sperm in the weak allele *tes-2* could be due to indirect effects of the primary phenotype, this cannot explain the differences among the three strong alleles. As these alleles are all in different ecotypes, it is possible that genes other than *tes* affect the behaviour of nuclei (e.g. fusion) in coenocytic microspores.

Seed abortion in *tes* mutants is linked to the abnormal pollen phenotype

Of the ovules that are fertilized in self-pollinated *tes* siliques, only about 5-50% produce mature seeds while the rest abort by heart stage. In some developing *tes* seeds, the micropylar endosperm begins to cellularize when the embryo is at globular to heart stage, as in wild type (Bowman, 1994). However, in others the endosperm fails to cellularize. We presume that the former type would develop into viable seeds and the latter would abort.

In other species in the Cruciferae, an excess of paternal genomes in the developing seed has been observed to result in abortion of the embryo at globular or heart stage (Håkansson, 1956; Nishiyama and Inomata, 1966). Similarly, the failure of cellularization seen in some *tes* endosperms is a typical feature of endosperm with extra paternal genomes (reviewed by Haig and Westoby, 1991). The photometry data suggest that many endosperms in self-pollinated *tes* plants do contain a paternal excess, since most *tes* sperm contain more DNA than wild-type haploid sperm. Unlike some other species, *Arabidopsis* endosperms can tolerate a degree of paternal excess, as diploid plants pollinated by tetraploids (resulting in 2m:2p endosperm) produce viable seeds (Koornneef, 1994; and our unpublished observations). However, crosses between hexaploids and diploids are reported to fail (Koornneef, 1994), so it is likely that paternal excess in the endosperm greater than 2m:2p is not tolerated. The high frequency of seed abortion in *tes-1*/Col plants compared to *tes-4*/Ws2 may thus be due in part to the high mean DNA content (i.e. almost always more than twice the 1n value) of *tes-1*/Col sperm.

Endosperm formation has been studied in detail in barley (Olsen et al., 1995), where, as in *Arabidopsis*, endosperm begins as a syncytium and later cellularizes. This study reports that in the late syncytium each endosperm nucleus is surrounded by a radial array of microtubules, which maintain the spacing of nuclei and later establish the pattern of cellularization. Since similar microtubule arrays are involved in inter-

sporal wall positioning during male meiotic cytokinesis, and since some endosperms produced by self-pollinated *tes* plants fail to cellularize, it is tempting to speculate that *tes* mutations have a direct effect on endosperm development as well as male meiosis. However, this is unlikely to be the case as self-pollinated *tes* heterozygotes produce homozygous *tes* progeny in the expected ratio, despite the fact that all of these would have developed in seeds containing endosperm with no wild-type copies of *TES*. Instead, the reduced fertility phenotype of *tes* mutants always coincides with use of a pollen donor with the *tes* phenotype, rather than with the presence of *tes* alleles per se in the sperm, egg, or central cell. Therefore it seems more likely that seed abortion is caused by fertilization by multiple, polyploid, aneuploid, or otherwise abnormal sperm indirectly resulting from the *tes* meiotic defect.

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