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Bcp1, a gene required for male fertility in *Arabidopsis*

(pollen development/antisense gene/male sterility/*Brassica*)

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ABSTRACT **Male fertility in flowering plants is dependent on production of viable pollen grains within the anther. Genes expressed exclusively in the anther are likely to include those that control male fertility. On the basis of their tissue specificity, such genes have been isolated, yet in none of them has this function been demonstrated. Here we report that one such gene, *Bcp1*, is active in both diploid tapetum and haploid microspores and is required for pollen fertility. Perturbation of this gene in either tapetum or microspores prevents production of fertile pollen in transgenic *Arabidopsis* plants. When tapetum expression of this gene is perturbed, mature anthers contain dead shriveled pollen. On the other hand, when microspore expression is perturbed, anthers show 1:1 segregation of viable/aborted pollen. These findings identify a class of sporophytic/gametophytic genes controlling male fertility and, hence, reproduction in flowering plants.**

Male fertility in flowering plants is dependent on the production of viable pollen grains, which produce and deliver the male gametes, the sperm cells, to the embryo sac (1). The result is successful fertilization and, ultimately, seed set. Pollen development occurs in the anther and depends on interactions between two different cell types. First, diploid microsporocytes undergo meiotic cell divisions to produce haploid microspores, which mature into the male gametophytes, pollen grains (2). Second, tapetal cells, the innermost layer of the anther wall, surround the microspores during their development and produce proteins and other molecules necessary for pollen development (3, 4). Selective ablation of tapetal cells by cell-specific expression of cytotoxin molecules blocks pollen development (5). Mutations have been identified that prevent normal pollen development and result in male sterility (6). Developmental analysis of such *ms* mutants shows that pollen ontogeny is blocked at different end points during microsporogenesis (6–10). Genetic complementation studies have shown that each *ms* mutant defines a single recessive gene (8).

We isolated an anther-specific gene, *Bcp1*, from *Brassica campestris* that showed a unique pattern of expression in the diploid tapetum and haploid microspores (11). Here we report that coordinated expression of this gene in both cell types is essential for production of functional pollen. This has been demonstrated by specifically downregulating its activity by the antisense RNA approach (12). Transgenic *Arabidopsis* plants in which the *Bcp1* gene is perturbed either in tapetum or in developing microspores show arrest in pollen development leading to pollen abortion. Such aborted pollen grains lack cytoplasmic contents at maturity and appear as empty, flattened exine shells. Thus, *Bcp1* is a diploid/haploid male fertility gene which provides a tool for gaining an understanding of the molecular control of pollen development.†

MATERIALS AND METHODS

Isolation and Nucleotide Sequence Analysis of Genomic Clone. The *Arabidopsis Bcp1* gene was isolated by screening an *Arabidopsis thaliana* ecotype Landsberg *erecta* genomic library with a probe derived from a *Brassica Bcp1* cDNA clone (13). The 4.2-kb *Bam*HI fragment containing *Bcp1* was subcloned into pBluescript KS(+) (Stratagene). DNA sequencing was performed by the dideoxy method using a T7 DNA sequencing kit (Pharmacia LKB). Specific oligonucleotide primers were used to obtain the complete sequence along both strands. Sequence analysis was performed with the Melbot/Angis database, which incorporates the GenBank, EMBL, and NBRF nucleic acid libraries and NBRF PIR and Swiss-Prot protein libraries.

RNA Gel Blot Analysis. Total RNA was extracted (14) from various tissues. Because it is extremely difficult to obtain a large amount of anthers from the tiny flowers of *Arabidopsis*, flowers rather than anthers were used. mRNA purification was carried out with Dynal Beads (Dynal, Great Neck, NY). Total RNA (20 μg) or mRNA (0.2 μg) was electrophoresed and transferred onto Hybond N^+ nylon membrane (Amersham) under vacuum with a Vacugene blotting system (Pharmacia). *Bcp1* sense and antisense RNA was detected with [α-^{32}P]UTP-labeled single-stranded RNA probes generated by *in vitro* transcription from the *Brassica Bcp1* cDNA clone. Hybridizations and washing were conducted as described (15).

DNA Gel Blot Analysis. Genomic DNA was extracted from leaf tissues of appropriate plants (16) and digested with *Bam*HI. DNA fragments (5 μg) were separated in a 0.7% agarose gel and transferred onto a Hybond N^+ nylon membrane under alkaline conditions. Blots were hybridized with a labeled probe derived from the *Bcp1* cDNA clone in 2× SSPE (1× is 0.18 M NaCl/10 mM sodium phosphate, pH 7/1 mM EDTA)/0.5% (wt/vol) nonfat milk/1% (wt/vol) PEG 20,000/7% (wt/vol) SDS with denatured salmon sperm DNA at 50 μg/ml at 65°C for 12–14 hr. Blots were washed in 2× SSC (1× is 0.15 M NaCl/15 mM sodium citrate, pH 7)/0.1% SDS at room temperature for 30 min, 0.1× SSC/0.1% SDS at 37°C for 30–60 min, and 0.1× SSC/0.1% SDS at 65°C for 30–60 min. Parallel blots were hybridized with an *NPTII* gene-specific probe.

***In Situ* Hybridization.** Flowers at two different developmental stages were fixed and embedded in LR gold resin (17). Biotin-labeled sense and antisense RNA probes were generated by *in vitro* transcription from the *Brassica Bcp1* cDNA clone and hybridization was performed (18). Hybridization signal was detected on sections by colloidal gold (15 nm)-conjugated goat anti-biotin antibody (1:15 dilution), followed by silver enhancement (17). Sections were viewed by dark-field microscopy.

Construction of Antisense Gene and Plant Transformation. Two antisense constructs were prepared. (*i*) *Bcp1* promoter-antisense construct. The 0.5-kb (nt 1–500, ref. 11) cDNA was excised from the *Brassica Bcp1* cDNA clone as a *Sma* I–*Sau*3A1 fragment and fused with 0.77 kb of *Bcp1* promoter (19) in the reversed orientation. This fusion fragment was ligated into the *Bam*HI and *Sma* I sites of the pBluescript KS(+). DNA sequenc-

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†The *Brassica* and *Arapidopsis Bcp1* sequences have been deposited in the GenBank data base (accession nos. X68209 and X68211).

ing confirmed the antisense orientation of *Bcp1*. The chimeric *Bcp1* antisense gene was excised as a *Hin*dIII–*Sac* I fragment and cloned into a binary vector, pBI101 (20). The *GUS* gene present in the vector was removed during this process. (*ii*) *LAT52* promoter-antisense construct. *LAT52* promoter (kindly provided by Sheila McCormick, U.S. Department of Agriculture Plant Gene Expression Centre, Albany, CA) was fused with 0.5 kb of *Bcp1* cDNA insert in the reversed orientation and cloned into pBI101. The resulting antisense constructs were then introduced into *Agrobacterium tumefaciens* LBA4404 by triparental mating (21). These constructs were introduced into *A. thaliana* (ecotype Landsberg *erecta*) by *A. tumefaciens*-mediated transformation (22). The transformants were selected on medium containing kanamycin (50 μg/ml).

Microscopic Analyses. Fluorochromatic reaction was used as a test of plasma membrane integrity. Fresh pollen grains from wild-type and transformed plants were incubated with fluorescein diacetate (in 15% sucrose) and viewed with fluorescence microscopy under UV excitation (23). Alexander's stain, which differentially stains pollen wall (green) and cytoplasm (purple), was also used to indicate the presence or absence of cytoplasm in pollen grains as a measure of sterility. Mature anthers were mounted directly in Alexander's stain and examined by bright-field microscopy (24). To prepare anther sections, flowers at various developmental stages were fixed, embedded in Spurr's resin, and sectioned (8). For fluorescence microscopy, semithin (2-μm) sections were stained with periodic acid/Schiff reagent and viewed with blue excitation filters (25). Ultrathin sections were stained and viewed with a JEOL transmission electron microscope (8). Dehiscing anthers were collected and observed with a JEOL JSM 840 scanning electron microscopy.

RESULTS

Homologue of the *Brassica Bcp1* Gene Is Expressed in *Arabidopsis*. RNA gel blot studies indicated that transcripts homologous to *Bcp1* of *Brassica* were present in the closely related model organism *A. thaliana* (11). The specificity and pattern of expression in anthers of *Arabidopsis* mirror those of oilseed rape (Fig. 1*a*). *In situ* hybridization studies with anther sections showed *Bcp1* mRNA in tapetal cells and developing microspores and pollen grains in *Arabidopsis* (Fig. 1 *b–e*). Expression in tapetal cells was evident shortly after microspore formation and continued until tapetum degeneration. Expression in unicellular microspores was detected from late vacuolate stage and high levels of transcripts were present in mature and germinating pollen. Southern blot analysis indicated a single gene copy in the *Arabidopsis* genome (13).

The *Arabidopsis Bcp1* gene was obtained by screening a genomic library with the *Brassica Bcp1* cDNA clone and is highly homologous to the *Brassica* gene, showing 73% sequence identity at the amino acid level (Fig. 2). The coding region contains an open reading frame of 137 aa (compared with 119 in Brassica) and encodes an alanine-rich (16%) protein of 14 kDa (compared with 12 kDa in *Brassica*). The nucleotide and deduced amino acid sequences of the *Bcp1* genes show no homology with other known genes or proteins in the databases.

Inhibition of *Bcp1* Gene Expression Leads to Arrest in Pollen Development. We used the antisense RNA approach to ascertain the role of *Bcp1* in pollen development. To achieve this we fused 0.77 kb of the *Bcp1* gene regulatory region with 0.5 kb of the cDNA insert (nt 1 to 500) in reverse orientation to create an antisense *Bcp1* construct. Using a *GUS* reporter gene, we have demonstrated that the same promoter region directs high-level gene expression in both tapetum and developing pollen (19). This spatial and temporal pattern of *GUS* expression in transgenic *Arabidopsis* plants was consistent with endogenous *Bcp1* expression in both tapetum and pollen.

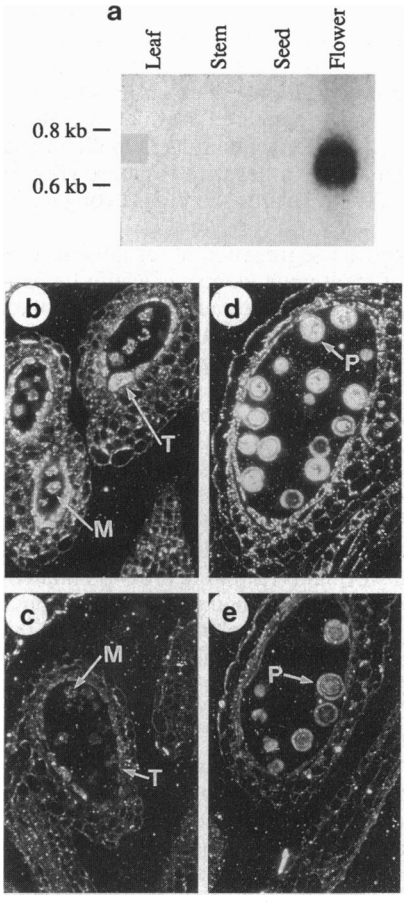

FIG. 1. Organ-specific expression patterns of *Arabidopsis Bcp1* gene. (*a*) RNA gel blot showing differential expression in vegetative and reproductive tissues. RNA from four different organs was hybridized with a labeled probe derived from *Brassica Bcp1* cDNA. Transcripts of ≈0.7 kb were detected in flowers, but not in the vegetative tissues tested. (*b–e*) *In situ* hybridization with biotin-labeled *Bcp1*-specific antisense RNA probe, with sense RNA probe as a control. RNA·RNA hybridization signal appears as light-scattering particles when viewed by dark-field microscopy. M, microspore; T, tapetum; P, pollen. (*b*) Longitudinal section of flower buds containing immature anthers (early vacuolate microspore stage), showing strong hybridization signal in tapetal cells and weaker signal in microspores. (*c*) Sense control for *b*. (*d*) Longitudinal section of flowers with maturing anthers (early tricellular pollen stage), showing strong signal in pollen whereas tapetal cells have now degenerated (low level of signal at site of tapetal remnants). (*e*) Sense control for *d*.

Thus, we expected antisense RNA to be expressed in both tapetum and pollen. The chimeric antisense construct was introduced into *Arabidopsis* plants by *Agrobacterium*-mediated transformation.

Of 42 primary transgenic plants (T_0) obtained from two independent transformation events, 16 failed to produce the

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Brassica     MGRQNVVVVFGLVFLAVLGLAAAAASSPSPSASPSKAPSTS 40
             ||||| ||| ||| ||||||||||||||||||| |
Arabidopsis  MGRQNIVVVVALVFIRIIGLAAAAASSPSPSASPSKAPAAS

Brassica     TPE*VEAPVSEDTIGTTDDDAAASPGDDDVAVAGPLGSDS 79
             ||||| | |||||||||| ||| ||||||||||||
Arabidopsis  KTDHVEAPVT*DQIGTTDDDAAPTPGDGDVAVAGPLGSDS

Brassica     SYGSNGPSGSADSADSGAAALGVSAVVVGVTSIVGSFLFF 119
             || |  |||||| ||||||||||||||||||
Arabidopsis  SYD*NAATGSADSAKSGAAALGVSAVVVGVTSLLVLSCYS

Brassica     ****************Z                        137
Arabidopsis  SAFYDKKVILNEDYYMZ
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FIG. 2. Alignment of the deduced amino acid sequences of *Brassica* and *Arabidopsis Bcp1* genes. The *Arabidopsis Bcp1* gene sequence was determined from a genomic clone. Asterisks indicate gaps inserted to obtain maximum homology.

elongated siliques and seeds characteristic of wild-type plants (Fig. 3*a*), indicating complete loss of fertility (Fig. 3*b*). Microscopic examination of flowers of the sterile transgenic plants (T_0) showed that the pollen grains were shriveled while other floral tissues appeared normal. In fluorochromatic reaction tests for pollen viability (23), pollen from the sterile transgenic plants gave a negative response (Fig. 3*d*), whereas pollen from wild-type plants showed a strong positive reaction (Fig. 3*c*).

There was no 1:1 segregation of fertile and sterile pollen in the anthers of transgenic plants, as would be expected if the male sterile phenotype were caused by inhibition of *Bcp1* gene expression in haploid pollen grains. This indicates that sterility is the result of downregulating *Bcp1* gene expression in the diploid tapetum.

Genomic analysis of four transgenic plants with severe male sterile phenotype indicated that, in addition to the 6.3-kb DNA fragment corresponding to the endogenous *Bcp1* gene, they contained two or more insertions of the transgene (Fig. 4*a*).

Male Sterile Phenotype Shows Mendelian Inheritance. To determine whether male sterility is a heritable trait, primary antisense transformants (T_0) were cross-pollinated with pollen from wild-type plants. Normal silique formation and seed set occurred in all transgenic plants, confirming that antisense transformants are male sterile since their pistils are capable of normal pollen recognition and male gamete transmission, their female fertility being unaffected. We conducted detailed analyses on the progeny (T_1) of a single T_0 plant. This T_0 plant carried two insertions of the antisense gene. Eight T_1 plants examined showed significant reduction in the seed set, producing ≈5 seeds per silique as compared with 50–70 seeds in wild-type plants. Microscopic analysis showed that anthers of these plants contained shriveled dead pollen similar in appearance to that in the primary transformants. However, a very small percentage of the pollen had normal fertile appearance. DNA gel blot analysis showed that all eight plants had inherited a single copy of the antisense transgene (data not shown).

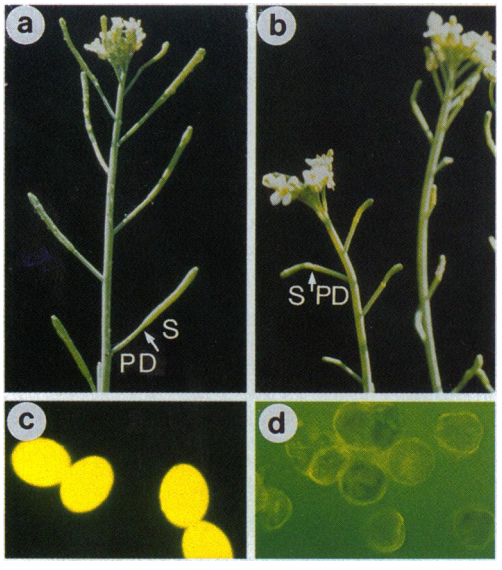

FIG. 3. Male sterile phenotype of *A. thaliana* plants carrying *Bcp1* promoter-antisense transgene. *A. thaliana* is self-fertilizing and the male sterile phenotype is characterized by short empty siliques. (*a*) Wild-type plants showing elongated siliques produced following self-pollination. (*b*) Male sterile transgenic plants carrying short empty siliques. Arrow indicates the junction between the silique (S) and pedicel (PD). (*c* and *d*) Fluorochromatic reaction for determination of pollen viability. This is a test of plasma membrane integrity in which only viable grains accumulate fluorescein (fluoresce yellow). Pollen from wild-type plants shows a positive reaction (*c*), whereas pollen from transgenic plants does not (*d*).

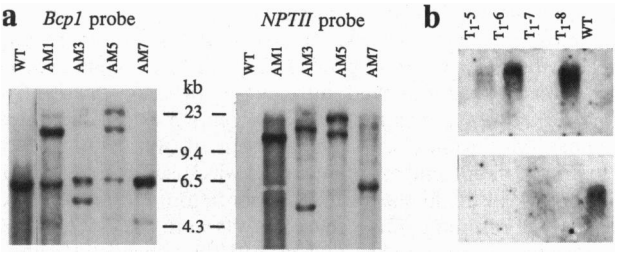

FIG. 4. DNA and RNA gel blots of the *Bcp1* promoter-antisense transgenic plants of *Arabidopsis* showing integration and expression of the *Bcp1* antisense gene. (*a*) *Bam*HI-digested genomic DNA from wild-type (WT) and four individual primary transformants was used for parallel hybridizations with a *Bcp1* gene-specific probe (*Left*) and a kanamycin-resistance gene (*NPTII*)-specific probe (*Right*). A 6.3-kb fragment corresponds to the endogenous *Bcp1* gene. (*b*) RNA gel blot analysis of four individual T_1 plants obtained by cross-pollinating male sterile transformants with pollen from a wild-type plant. Expression of endogenous (*Lower*) and antisense (*Upper*) *Bcp1* gene in T_1 plants was determined with specific probes.

The production of a few seeds by T_1 plants with this "leaky" phenotype allowed the transmission of the antisense transgene to the T_2 generation. A leaky phenotype has been reported for the T-DNA-tagged and other mutants of *Arabidopsis* (26, 27).

We further examined 10 T_2 progeny generated from a single T_1 plant after selfing. These T_2 plants showed simple Mendelian segregation of the antisense transgene. Three were completely fertile, 5 showed the leaky phenotype similar to T_1 plants, and 2 were completely sterile. DNA gel blot analysis revealed that two copies of the antisense transgene were present in plants with the complete male sterile phenotype, whereas a single copy was present in plants with the leaky male sterile phenotype (data not shown). Three fully fertile T_2 plants did not inherit the antisense transgene. Thus, the male sterile phenotype and presence of antisense insert cosegregated completely.

Antisense RNA Is Expressed in Anthers of Male Sterile Plants. We then confirmed whether the pollen abortion in these plants was due to inhibition of *Bcp1* expression. mRNAs from plants showing the male sterile phenotype were tested with probes specific for both endogenous and antisense *Bcp1* genes. A mRNA of ≈0.7 kb was detected in flowers of wild-type plants by using the probe specific for the endogenous *Bcp1* (Fig. 4*b*). The endogenous *Bcp1* mRNA was not detected in either primary transformants or T_1 plants with male sterile phenotype, indicating a dramatic reduction of the endogenous *Bcp1* gene transcripts by the antisense gene in these plants. However, with an antisense-specific probe, a strongly hybridizing transcript of ≈0.75 kb was detected in flowers of three male sterile transformants tested (T_1-5, T_1-6, T_1-8). This indicates that high levels of steady-state antisense transcripts were present in these plants. Thus, the male sterile phenotype is linked with specific reduction of endogenous *Bcp1* mRNA by antisense RNA.

Expression of *Bcp1* in Microspores Is Essential for Pollen Development. Since 100% pollen abortion obtained in the *Bcp1* promoter antisense transformants is due to perturbation of this gene in diploid tapetal cells, the question remains as to the role of *Bcp1* expression in haploid microspores. To address this question, we used a pollen-specific promoter, *LAT52*, to direct antisense expression in developing pollen, leaving *Bcp1* expression in the tapetum unperturbed. *LAT52* promoter is active postmeiotically in haploid pollen as shown by *GUS* fusion analysis (28), and its temporal expression is identical to the pattern of *Bcp1* expression in developing microspores (29). Transgenic *Arabidopsis* plants carrying the *LAT52* promoter-antisense gene construct set seeds with normal silique elongation, comparable to wild type, but microscopic analysis showed that 50% of their pollen was aborted (Fig. 5*b*). Among

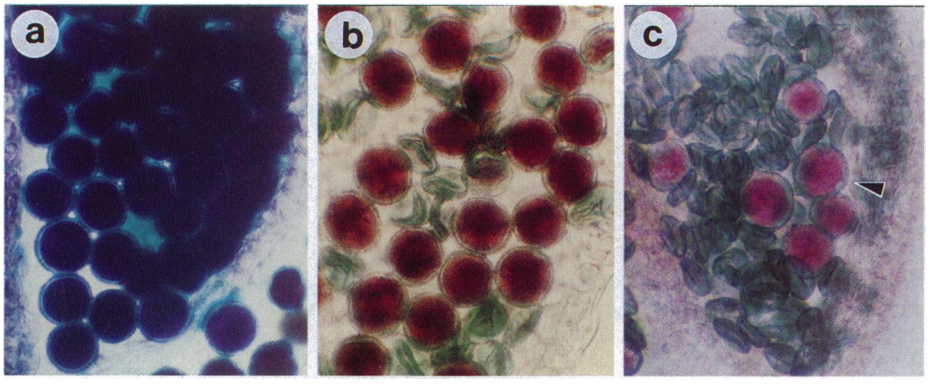

FIG. 5. Differential staining with Alexander's stain of pollen in anthers of wild-type (*a*), *LAT52* promoter-antisense (*b*), and *Bcp1* promoter-antisense (*c*) plants. Aborted pollen appears empty (green walls only), degenerating pollen appears pink (vacuolated cytoplasm, arrowheads), and normal pollen appears dark purple (green walls and densely staining cytoplasm). In *LAT52* promoter-antisense plants (*b*), 50% of pollen grains are aborted, whereas most pollen grains are aborted in *Bcp1* promoter-antisense plants (*c*).

the T_1 sibling progeny of these plants, 50% of the plants showed 1:1 segregation of fertile vs sterile pollen. These inheritance data show that the *LAT52* promoter-antisense gene is transmitted only via female gametes, confirming that the pollen carrying this construct is nonfunctional.

Timing of Pollen Arrest Differs in Transgenic Plants with Two Different Promoter-Antisense Constructs. Wild-type and aborted pollen showed normal structure and patterning of the outer exine wall, and the collapsed phenotype of aborted pollen resulted from complete loss of cellular contents (Fig. 6 *a–d*). The first signs of cytoplasmic degeneration appeared at different stages of anther development in transgenic plants carrying the two different antisense constructs. Microspore development proceeded normally until the uninucleate microspore stage, characterized by the presence of a large vacuole (Fig. 6 *e, i,* and *m*). In the *LAT52* promoter-antisense plants, the first signs of cytoplasmic degeneration appeared at the late bicellular pollen stage after formation of the generative cell (Fig. 6*k*), and in mature anthers, 50% of pollen grains had no cytoplasmic contents (Fig. 6*l*). In contrast, in the *Bcp1* promoter-antisense plants, degeneration

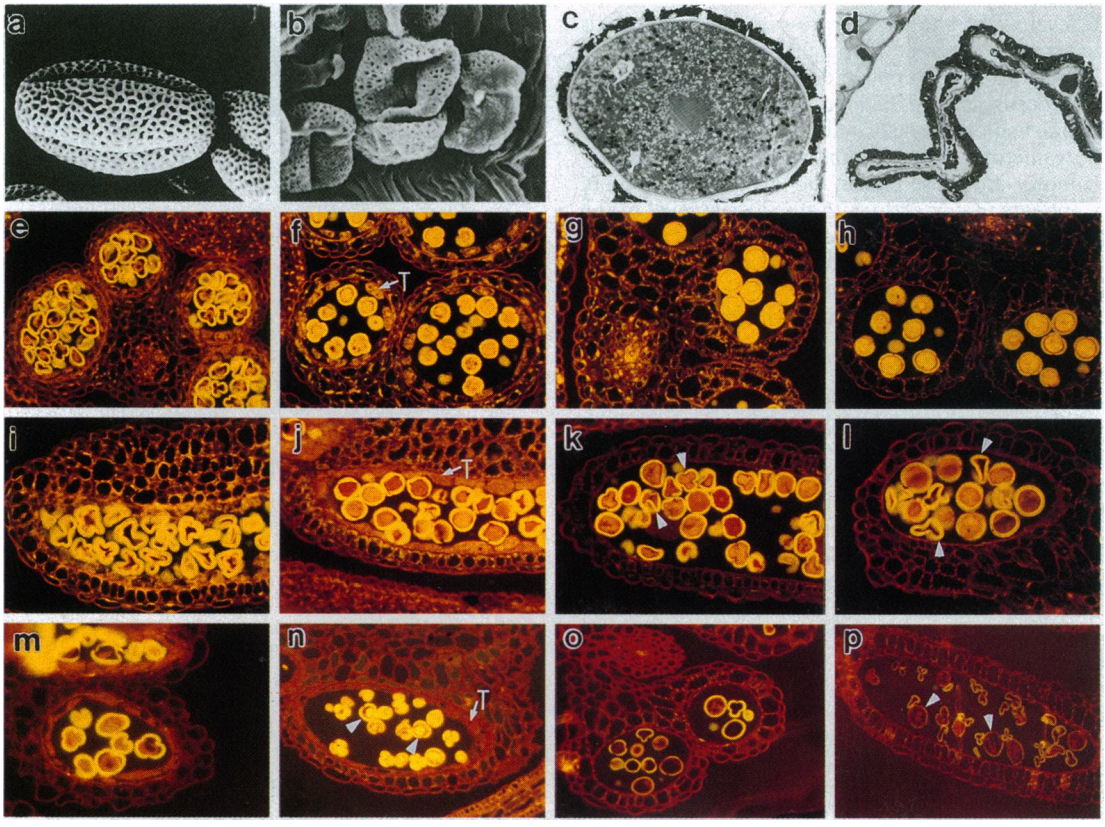

FIG. 6. Microscopic comparison of pollen development in wild-type, *LAT52* promoter-antisense, and *Bcp1* promoter-antisense plants of *Arabidopsis*. (*a* and *b*) Scanning electron micrographs of wild-type pollen (*a*) and shriveled sterile grains from transgenic *Bcp1* promoter-antisense plants (*b*). (×1350.) (*c* and *d*) Transmission electron micrographs of mature pollen of wild-type plants (*c*) and shriveled sterile grains from transgenic *Bcp1* promoter-antisense plants (*d*). In contrast to the spherical, electron-opaque grains of wild type (*c*), the sterile grains are generally completely empty, their collapsed walls forming a characteristic dumbbell shape (*d*). (×3600.) (*e–p*) Comparison of four developmental stages (determined by DNA fluorochrome stain, 4′,6-diamidino-2-phenylindole; ref. 30) in wild-type plants (*e–h*), *LAT52* promoter-antisense plants (*i–l*) and *Bcp1* promoter-antisense sequence (*m–p*). Stages of development shown: sections of uninucleate microspores with large vacuoles (*e, i,* and *m*), transition between uninucleate microspores and bicellular pollen (*f, j,* and *n*), late bicellular pollen (*g, k,* and *o*), pollen in mature anthers (*h, l,* and *p*). Periodic acid/Schiff fluorescent staining has been used to indicate the presence of cytoplasmic contents of microspores and pollen (yellow to red fluorescence). Cytoplasmic degeneration is evident at the late bicellular stage in *LAT52* promoter-antisense plants (arrowheads, *k*) and at the equivalent of uninucleate microspore stage in *Bcp1* promoter-antisense plants (arrowheads, *n*). In mature anthers, 50% of pollen has no cytoplasmic contents in *LAT52* promoter-antisense plants (arrowheads, *l*), while most grains lack contents and are collapsed with only a few retaining traces of contents (arrowheads, *p*) in *Bcp1* promoter-antisense plants. T, tapetum.

commenced earlier, at late uninucleate microspore stage (Fig. 6*n*). At the bicellular pollen stage, most grains were empty and only a few had residual highly vacuolated cytoplasm (Fig. 6*o*). In mature anthers, most grains had shriveled, but some retained a spherical shape with traces of residual cytoplasm (Fig. 6*p*).

DISCUSSION

Our results show that *Bcp1*, an anther-specific gene expressed in both tapetum and microspores, is essential for pollen development. Inhibition of its expression in either diploid tapetum or haploid microspores leads to arrest of pollen development.

Inheritance studies on male sterile mutants in various plant species, including *Arabidopsis*, have shown the existence of sporophytic genes controlling male fertility (6–10). In such cases, the genes exert their control on pollen development by functioning in parental sporophytic tissues. The most likely sporophytic tissues showing transcription of these genes are diploid microsporocytes or tapetal cells. Evidence has also been obtained for the existence of gametophytic genes controlling male fertility (31). The expected site of transcription of such genes is in haploid microspores following meiotic division. Antisense inhibition analysis has shown that *LAT52*, a pollen-specific gene from tomato, is one of such genes which control fertility at the gametophytic level (32). Unlike these cases, the *Bcp1* gene controls male fertility at both sporophytic and gametophytic levels. The timing of action of sporophytic control by *Bcp1* occurs earlier than that at the gametophytic level. When tapetal expression of this gene is perturbed, the arrest in pollen development occurs at the uninucleate microspore stage. When pollen expression of this gene is perturbed, arrest occurs later at the bicellular pollen stage. Thus, *Bcp1* has a diploid/haploid mode of action and represents a class of sporophytic/gametophytic genes controlling male fertility.

In *Bcp1* promoter-antisense plants, the level of reduction in male fertility appears to correlate with the dosage level of the antisense transgene. This was observed in the T_2 generation, where plants homozygous for the antisense gene were completely male sterile, while hemizygous plants which carried a single copy of antisense gene showed a leaky male sterile phenotype. Plants that did not inherit the transgene were fully male fertile. A similar gene dosage effect has been reported during antisense inhibition of the pTOM13 gene, which is involved in ethylene biosynthesis in ripening tomato fruits (33, 34). The tomato plants carrying two copies of the antisense gene showed increased inhibition of ethylene production. In the case of *Bcp1*, at least two copies of the antisense genes are required to induce complete male sterility. It would be of great interest to define threshold levels of *Bcp1* protein required for maintaining full male fertility. Such experiments will be more feasible in *Brassica*, where sufficient anther material can be obtained for such quantitative analyses.

In conclusion, our results show that the *Bcp1* gene is essential for male fertility in *Arabidopsis*. A challenge for the future will be to unravel the physiological basis of action of the protein product encoded by this sporophytic/gametophytic male fertility gene. A potential benefit of our work may be the development of a male sterile system for the production of hybrid seed in related crop plants.

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