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sidecar pollen, an *Arabidopsis thaliana* male gametophytic mutant with aberrant cell divisions during pollen development

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SUMMARY

During pollen development each product of meiosis undergoes a stereotypical pattern of cell divisions to give rise to a three-celled gametophyte, the pollen grain. First an asymmetric mitosis generates a larger vegetative cell and a smaller generative cell, then the generative cell undergoes a second mitosis to give rise to two sperm cells. It is unknown how this pattern of cell divisions is controlled. We have identified an *Arabidopsis* gene, *SIDECAR POLLEN*, which is required for the normal cell division pattern during pollen development. In the genetic background of the NoO ecotype, *sidecar pollen* heterozygotes have about 45% wild-type pollen, 48% aborted pollen and 7% pollen with an extra cell. Homozygous *sidecar pollen* plants have about 20% wild-type pollen, 53% aborted pollen and 27% extra-celled pollen. Similar ratios of *sidecar pollen* phenotypes are seen in the Columbia ecotype but *sidecar pollen* is a gametophytic lethal in the Landsberg *erecta* ecotype. Thus this allele of *sidecar pollen* shows differential gametophytic penetrance and variable expressivity in different genetic backgrounds. The extra cell has the cell identity of a vegetative cell and is produced prior to any asymmetric microspore mitosis. Pollen tetrad analysis directly demonstrates that *SIDECAR POLLEN* is indeed expressed in male gametophytes. To our knowledge, *scp* is the first male gametophytic mutation to be described in *Arabidopsis*.

Key words: *sidecar pollen*, *Arabidopsis thaliana*, male gametophyte, pollen development, vegetative cell, generative cell, asymmetric cell division

INTRODUCTION

The life cycle of flowering plants consists of two phases: the haploid gametophytic phase and the diploid sporophytic phase. The gametophytic phase begins at the completion of meiosis. Unlike the case in animals, the meiotic products in plants undergo mitotic divisions and develop into multicellular structures, the gametophytes, which bear the gametes. The male gametophytes (or microgametophytes) are pollen grains and the female gametophytes (or megagametophytes) are embryo sacs. Upon the fusion of sperm and egg to form a zygote, the gametophytic phase ends and the sporophytic phase begins, thus completing the alteration of generations that is characteristic of the sexual life cycle of plants.

Pollen development consists of several distinct stages (for reviews see Mascarenhas, 1975, 1989; Bedinger, 1992; McCormick, 1993). A microspore mother cell undergoes meiosis to give rise to a tetrad of four microspores which are encased in a callose (β-1,3-glucan) wall. These uninucleate microspores are released upon the dissolution of the callose wall. After quickly increasing in size, each uninucleate microspore undergoes an asymmetric mitotic division (the microspore mitosis) to give rise to two cells with distinct fates – the vegetative cell and the generative cell. The larger vegetative cell is transcriptionally active (Mascarenhas, 1990) and is thought to provide most of the proteins of the pollen grain. The vegetative cell forms the pollen tube during pollen germination but does not undergo any more cell divisions and therefore adopts a terminal cell fate. The generative cell is completely enclosed within the cytoplasm of the vegetative cell and is relatively transcriptionally quiescent (Mascarenhas, 1990), but will divide one more time to produce two sperm cells (the pollen mitosis). In most species (such as tomato and lily), this second mitosis takes place in the pollen tube. In other species (such as *Arabidopsis* and maize) this mitosis takes place before pollen is released from anthers. Mature *Arabidopsis* pollen therefore contains three cells – one vegetative cell and two sperm cells enclosed within the cytoplasm of the vegetative cell.

Pollen offers an excellent system with which to study developmental processes such as cell fate determination and cellular differentiation. Pollen has a consistent and simple cell lineage which is relatively easy to follow when compared with the lineages that derive most other plant structures. Many cDNA clones of genes that are gametophytically expressed during pollen development have been isolated (reviewed by McCormick, 1991, 1993) and can be used as cell-specific markers. Pollen development requires a coordination of gene expression in the sporophytic cells of the anther and in the developing pollen grains. For example, the callase that releases the uninucleate microspores from the callose wall is sporophytically expressed in the tapetum cells of the anther (Chasan, 1992 and references therein). Furthermore, a cytogenetic study of maize pollen development in aneuploid stocks that lacked

particular chromosome arms showed that 50% of the meiotic products arrested at specific developmental stages, depending on which chromosome arm was missing (Kindiger et al., 1991). This work implies that there are gametophytically-acting genes required for the development of male gametophytes on almost every chromosome.

Although pollen-specific genes have been studied extensively (reviewed by McCormick, 1991, 1993) in most cases it remains unclear what roles these genes play. We have used a mutational approach to identify genes involved in the control of pollen development. Here we describe a male gametophytic mutation in which some of the pollen produces an extra cell in the pollen grain, and hence we have named this mutation *sidecar pollen.* Using a cell-specific marker and in vitro pollen tube germination we show that the extra cell possesses a vegetative cell identity. *sidecar pollen* (*scp*) affects both the number of cell divisions in pollen and pollen viability. We show that the expressivity and gametophytic penetrance of *scp* varies in different genetic backgrounds. Finally, we show directly that *scp* is indeed a gametophytic mutation by crossing with the mutant *quartet1* (Preuss et al., 1994), in which the four microspores from one meiosis stay attached throughout pollen development.

MATERIALS AND METHODS

Plant materials and growth conditions

Homozygous seeds of LAT59-GUS-transformed *Arabidopsis*, NoO ecotype (Twell et al., 1990), were bombarded with fast neutron at the International Atomic Energy Agency, Vienna, Austria. Mutagenized seeds show reduced germination, therefore mutagenized seeds were planted on seed germination medium containing 1× Murashige and Skoog salt mixture (GIBCO BRL), 1× *Arabidopsis* vitamins (Feldmann, 1991), 1% sucrose and 2.6 mM MES buffer (pH 5.6) in 0.8% agar. Two weeks after germination, the seedlings were transplanted to soil, and grown as described below. Plants grown from the mutagenized seeds were termed the M_1 population. Wild-type *Arabidopsis* lines of Landsberg *erecta* (Ler), Columbia-O (Col) and NoO ecotypes were used to outcross putative mutations. Except for the M_1 population of mutagenized lines, all the *Arabidopsis* plants in this study were sown in soil and grown in the greenhouse under continuous fluorescent light at 22°C. All plants in soil were fertilized weekly with nutrient solution which contained 5 ml 1 M KNO_3, 2.5 ml 1 M KH_2PO_4 pH 5.6, 2 ml 1 M $MgSO_4$, 2 ml 1 M $Ca(NO_3)_2$, 2.5 ml 4.5 mM Fe-EDTA stock solution and 1 ml micronutrient mix per liter of nutrient solution. The micronutrient mix is 70 mM H_3BO_3, 14 mM $MnCl_2$, 0.5 mM $CuSO_4$, 1 mM $ZnSO_4$, 0.2 mM $NaMoO_4$, 10 mM NaCl and 0.01 mM $CoCl_2$.

Identification of male gametophytic mutants

A PAP PEN (Research Products International Corp.) was used to draw 5 mm diameter circles on glass microscope slides to retain the staining solution. Single flowers from three branches of each M_1 plant were placed into circles on the slide and dissected under a Zeiss Stemi SV6 dissecting microscope. Anthers were pulled out and the rest of the flower was discarded. 1 μl of DAPI (4′, 6-diamidino-2-phenylindole) solution (1 μg/ml; Regan and Moffatt, 1990) was added and the anthers were dissected with a needle to further release pollen. An additional 10 μl of DAPI was added to each sample to stain for 30 minutes to 2 hours. The pollen was examined (see below) to identify samples with half normal pollen and half abnormal pollen. Additional flowers from such plants were examined, and if the phenotype was consistent, the plant was crossed to wild-type to test for transmission through the female and/or male. The *scp* mutant was found in the first 2,000 M_1 plants screened.

Confirmation of male gametophytic mutation

Heterozygous *scp* plants were crossed to wild-type plants as males and as females. The F_1 progeny were scored as described above. F_2 self progeny of those F_1 plants showing the pollen abnormality were then planted to score the segregation of the mutant phenotype. For pollen tetrad analysis, heterozygous *scp* plants were crossed as females by homozygous *quartet1* (Preuss et al., 1994) plants in the Ler ecotype background. F_1 progeny were scored and those F_1 plants showing the *scp* pollen phenotype were then selfed and the F_2 progeny were planted to score for the *qrt1*; *scp* double mutant.

Histology

Microscopy

Scanning electron microscopy was performed as described by Preuss et al. (1993). Light and fluorescent microscopy (for DAPI staining) was performed using a Zeiss Axiophot compound microscope with 40× and 20× objectives. Nomarski (differential interference contrast) optics were used for images of pollen tubes and sections. For plastic sections, whole inflorescences were fixed (4% formamide, 1% glutaraldehyde) then dissected and sorted according to the sizes of the buds. Fixed buds were dehydrated in an ethanol series of 35%, 50%, 75%, 80%, 95% and 100% for 1 hour each. Buds in 100% ethanol were then infiltrated with resin (JB-4 embedding kit, Polysciences, Inc.) by immersing in a 2:1 mixture and 1:2 mixture of ethanol and solution AC (0.9 gm catalyst C in 100 ml solution A) for 2 hours each, then in AC (3×, 2 hours). Buds were finally transferred to fresh AC with solution B (25:1) for embedding and polymerized overnight. Sections (2 μm) were cut on a microtome (Microm, Heidelberg) using a tungsten knife and were heat fixed to poly-L-lysine-coated glass slides. Sections were stained with Toluidine Blue O and were photographed with Kodak TMX 100 black-and-white film. All the color images were taken with Kodak Ektachrome 160 color slide film.

GUS assay

Flowers were dissected as described above and pollen was stained with 1 mM X-Gluc as described previously (Twell, 1992) for 45 minutes. DAPI solution was then added to the pollen to stain it for 1 hour.

Pollen germination

Pollen was germinated in vitro on medium containing 0.36 mg/ml $CaCl_2$, 0.08 mg/ml H_3BO_3, 20% sucrose, 0.01 mg/ml myo-inositol, 1% gelatin and 1% noble agar (Pickert, 1988). Glass slides were dipped into warm unsolidified medium, then cooled to form a thin layer on the slide. Pollen was tapped onto these slides and germinated in a humid chamber for 8-16 hours. DAPI solution was then added for 30 minutes to stain the pollen.

Genetic mapping

We crossed homozygous *scp* in NoO background as female by wild-type plants of Col or Ler ecotype. We generated self progeny of the F_1 and backcrossed the F_1 plants as females by wild-type plants of Col or Ler ecotype to obtain backcross progeny. These populations were used for mapping. DNA was prepared and analyzed with simple sequence length polymorphisms (SSLP), as described by Bell and Ecker (1994); co-dominant cleaved amplified polymorphic sequences (CAPS), as described by Konieczny and Ausubel (1993); and the *Arabidopsis* RFLP mapping set (ARMS), as described by Fabri and Schäffner (1994).

RESULTS

Identification of *scp* and *scp* mutant phenotype

Mutant screens in diploid organisms are usually carried out on M_2 or subsequent generations, because M_1 plants are usually

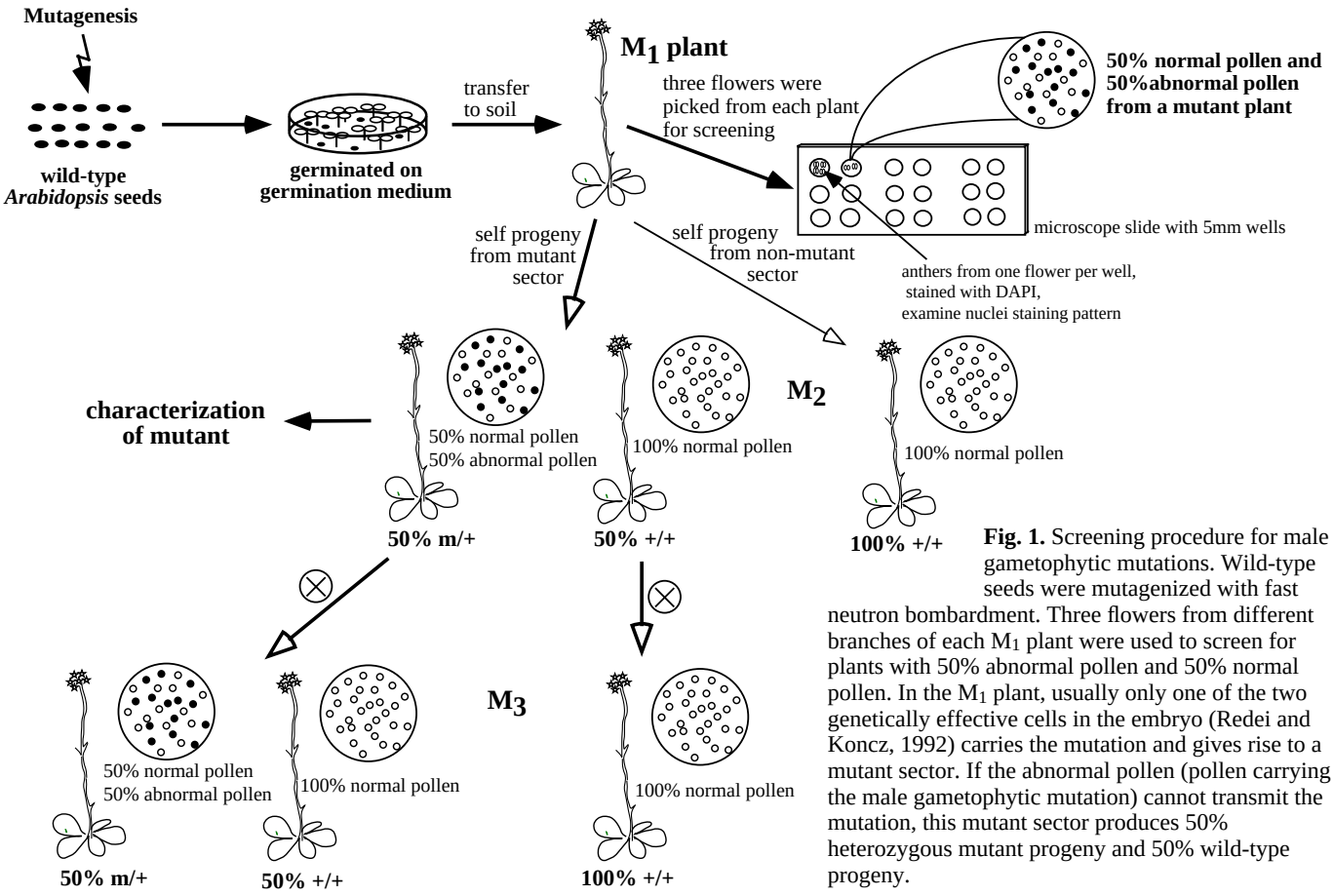

Fig. 1. Screening procedure for male gametophytic mutations. Wild-type seeds were mutagenized with fast neutron bombardment. Three flowers from different branches of each M_1 plant were used to screen for plants with 50% abnormal pollen and 50% normal pollen. In the M_1 plant, usually only one of the two genetically effective cells in the embryo (Redei and Koncz, 1992) carries the mutation and gives rise to a mutant sector. If the abnormal pollen (pollen carrying the male gametophytic mutation) cannot transmit the mutation, this mutant sector produces 50% heterozygous mutant progeny and 50% wild-type progeny.

heterozygous and mosaic for the mutation. However, since gametophytic genes are expressed postmeiotically, mutant phenotypes of gametophytes can be screened for and detected in either M_1 mutant sectors or in M_2 plants. For a male gametophytic mutation (which may not have a sporophytic phenotype) the screening usually requires microscopic examination. We therefore screened flowers from different branches of individual M_1 plants (see Fig. 1), rather than an equivalent screening of an M_2 family of 24 plants (Redei and Koncz, 1992). We have identified several mutations with approximately 50% aborted pollen (unpublished), demonstrating the feasibility of an M_1 screen for gametophytic mutations.

The M_1 plant carrying the *scp* mutation was initially identified as a plant with a branch that had approximately 50% aborted pollen in each flower. Upon closer examination (Figs 2, 3), we noticed that plants carrying the *scp* mutation in the NoO genetic background actually had 3 types of pollen grains: wild type (Figs 2A, 3A), aborted (Fig. 2B) and extra-celled (Figs 2B, 3B). The affected flowers of this M_1 plant had about 45% wild-type pollen, 48% aborted pollen and 7% extra-celled pollen (Fig. 2C). In the self progeny of this M_1 plant, we then identified occasional (as infrequent as 5%) M_2 plants that showed a higher incidence of pollen with an extra cell. These M_2 plants had about 20% normal pollen, 53% aborted pollen and 27% extra-celled pollen (Fig. 2C). To test whether these M_2 plants were homozygous for the *scp* mutation, we crossed them to wild-type and obtained 35 F_1 plants. All 35 F_1 plants showed a phenotype similar to that of the affected flowers of the M_1 plant. Moreover, all the self progeny (M_3) of these M_2 plants showed the same phenotype as the M_2 parent. These results indicate that the M_2 plants with a higher incidence of pollen with an extra cell were homozygous for *scp*.

The aborted pollen in *scp* plants is shrunken and shows no staining of cytoplasm, the inner pollen wall and nucleus (data not shown). DAPI staining of the nucleus of the extra cell is similar to the diffuse staining typical of a vegetative cell nucleus (Fig. 3B). Other pollen phenotypes are occasionally (<1%) observed in *scp* plants (Fig. 3C-F), including mature pollen with only one vegetative-like nucleus (Fig. 3C), pollen with only two vegetative-like nuclei (Fig. 3D), and pollen with an extra vegetative nucleus but no cell wall between the two vegetative nuclei (Fig. 3E,F). Such pollen grains (Fig. 3E,F) indicate that nuclear divisions and cytokinesis can sometimes be uncoupled in *scp*.

The additional cell in the mutant pollen of *scp* has a vegetative cell identity

Because the extra cell of *scp* shows DAPI staining typical of a vegetative cell (Fig. 3A,B), we tested whether the extra cell showed other characteristics of vegetative cells. The LAT52 and LAT59 promoters have been shown to direct pollen-specific reporter gene expression in *Arabidopsis* (Twell et al., 1990). LAT52 is transcribed specifically in the vegetative cell but not the generative cell (Twell, 1992) and LAT59 is transcribed in the vegetative cell (Ursin et al., 1989). We were able to directly test gene expression of the extra cell with the LAT59

promoter-directed GUS reporter gene (LAT59-GUS; Twell et al., 1990) since *scp* was isolated from a LAT59-GUS transformed population. In order to test expression of the LAT52 reporter gene construct, we first crossed LAT59-GUS; *scp* plants to wild-type plants and identified F_2 progeny with the *scp* phenotype but without blue-staining pollen. We then crossed these to plants carrying a LAT52 promoter-directed GUS reporter gene with a nuclear targeting signal sequence (NIa) (LAT52-GUS-NIa; Twell, 1992). GUS staining indicates that the extra cell and the normal vegetative cell in a *scp* mutant pollen grain both express these two LAT gene constructs (Fig. 4B and data not shown). This result suggests that the extra cell of *scp* has a vegetative cell identity.

The vegetative cell of a wild-type pollen grain germinates a pollen tube in order to deliver the two sperm cells to the embryo sac. The cytoplasm of the vegetative cell extends into the newly synthesized tube and the vegetative nucleus and the two sperm cells move into the pollen tube (Fig. 4C). The extra cell of the *scp* mutant pollen can germinate and grow a pollen tube and the nucleus of the extra cell also moves into the pollen tube (Fig. 4D). In *scp* pollen with an extra cell, the vegetative cell of the normal three-celled part can also grow a pollen tube (data not shown), but an *scp* pollen grain with two pollen tubes was never seen.

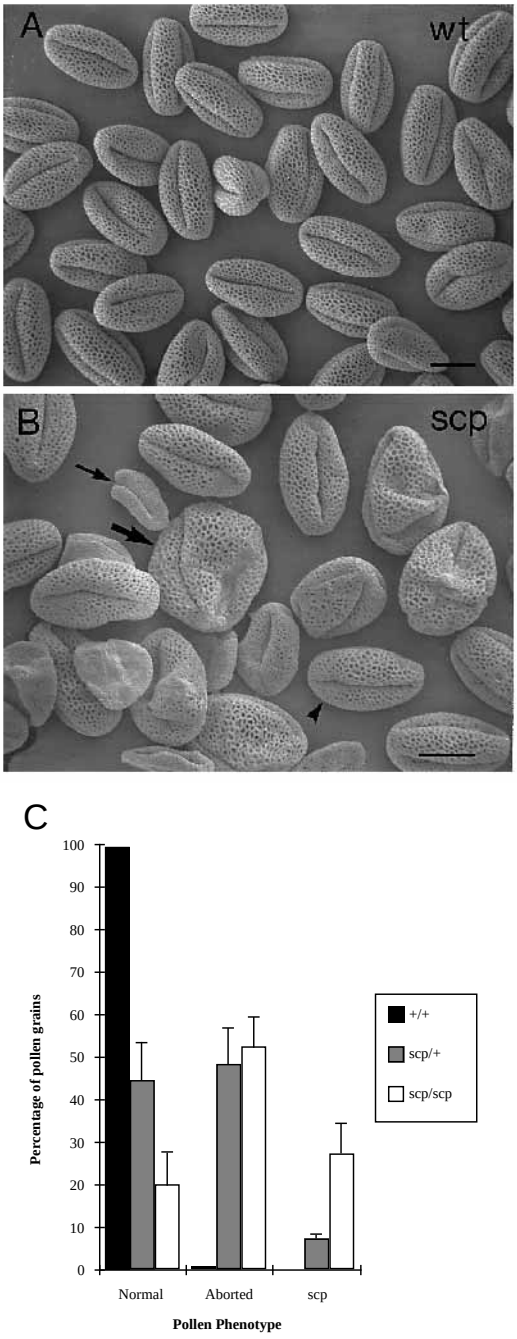

Fig. 2. Pollen phenotypes. Scanning electron micrographs of pollen grains from wild-type plants (A) and from homozygous *scp* plants (B). Notice that there are three types of pollen grains in *scp*: aborted (shrunken pollen grains, thin arrow), extra-celled (swollen pollen grains, thick arrow) and normal (arrowhead). (C) Percentage of different types of pollen grains in heterozygous *scp* and homozygous *scp* plants. Scale bar 10 μm.

scp is male-specific and shows differential penetrance among ecotypes

Since wild-type pollen can still be observed in homozygous *scp* plants (Fig. 2), the *scp* mutation in the NoO genetic background is incompletely penetrant at the gametophytic level. The phenotype of aborted pollen cannot be segregated from the extra-celled pollen phenotype after several rounds of selfing and outcrossing to wild-type plants (data not shown), indicating that the pollen abortion phenotype is also tightly linked to the *scp* locus.

Self progeny of heterozygous *scp* plants give a higher than expected percentage of wild-type progeny (Table 1A). To test the origin of this transmission bias, heterozygous *scp* plants were crossed as male or as female to wild-type and scored for the segregation of *scp*. Table 2 shows that when a heterozygous *scp* plant is crossed as a female, the transmission of *scp* is 100% relative to the wild-type allele. However, when a heterozygous *scp* plant is crossed as a male, the transmission of *scp* relative to the wild-type allele is only 37%, indicating that

Table 1. Self progeny segregation of *scp*/+ in NoO and in Ler/NoO background

Parental cross	Number of WT (+/+) progeny	Number of *scp*/– progeny	Total	χ^2 value‡ for *scp*/– : +/+ = 3 : 1	χ^2 value for *scp*/– : +/+ = 1 : 1
A. *scp*/+ (NoO) × *scp*/+ (NoO)	110* (33.1)†	222 (66.9)	332 (100)	11.711	37.783
B. *scp*/+ (NoO/Ler) × *scp*/+ (NoO/Ler)	52 (52.0)	48 (48.0)	100 (100)	38.88	0.16

*number of progeny scored.
†percentage of progeny with a given phenotype.
‡P=0.005 when χ^2=7.879.

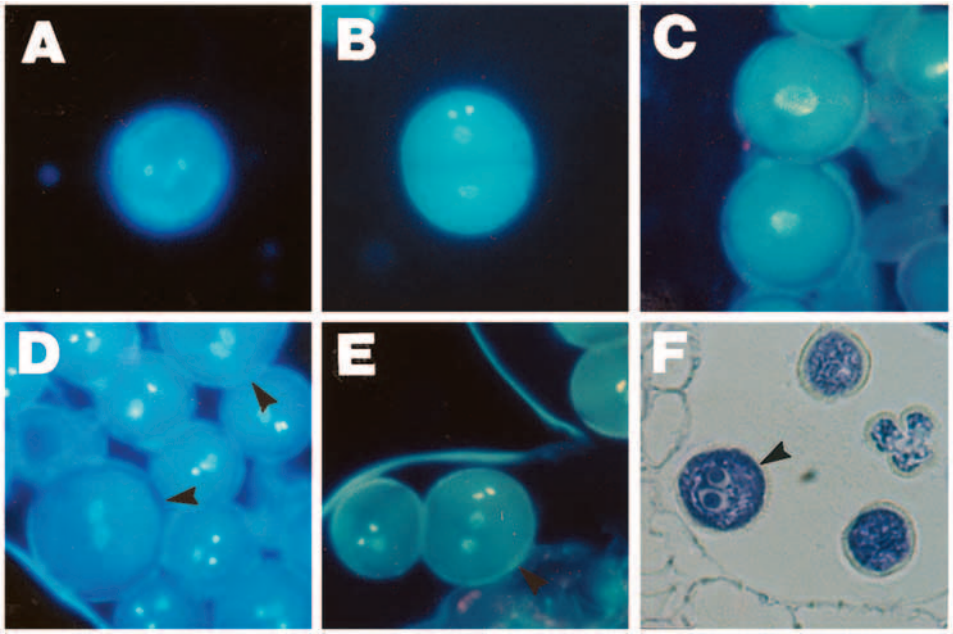

Fig. 3. The nuclear constitution of mature pollen from wild-type *Arabidopsis* plants (A) and *scp* plants (B-F). (A) Mature wild-type pollen. The two more brightly stained small nuclei are sperm nuclei and the larger and more diffusely stained nucleus is the vegetative cell nucleus. (B) Four-celled *scp* pollen. The nucleus of the extra cell stains diffusely, like a vegetative nucleus. There appears to be a cell wall between the 'normal' content of the pollen (two sperms and one vegetative cell) and the extra cell. (C-F) Mutant pollen phenotypes occasionally seen in *scp* plants. (C) Pollen with only one diffusely stained nucleus. (D) Pollen with two diffusely stained nuclei (arrowhead). (E) Pollen with four nuclei but no evident cell wall between the two diffusely stained nuclei (arrowhead), compare with B. (F) 2 μm section through a mature *scp* anther. Arrowhead indicates a pollen grain with no cell wall separating the two vegetative-like nuclei. (A-E) Pollen stained with DAPI; (F) Toluidine blue O staining.

Table 2. Male gametophyte-specific effect of *scp* mutation

Parental cross	Number of WT (+/+) progeny	Number of *scp*/+ progeny	Total	χ^2 value‡ for *scp*/+ : +/+ = 1 : 1
A. +/+ female × *scp*/+ male	158* (72.8)†	59 (27.2)	217 (100)	45.166
B. *scp*/+ female × +/+ male	144 (48.5)	153 (51.5)	297 (100)	0.273

*number of progeny scored.
†percentage of progeny with a given phenotype.
‡P=0.005 when χ^2=7.879.

this mutation does not transmit as well as a wild-type copy of the gene. These results demonstrate that *scp* affects only the male but does not compromise the function of the female reproductive structure.

When homozygous *scp* plants are outcrossed as females by wild-type plants of the Col ecotype, no significant deviation from the phenotype and transmission frequency observed in the NoO background is found. In contrast, when they are outcrossed as females by wild-type plants of the Ler ecotype, the F_1 progeny, which are all *scp* heterozygotes in the genetic background of NoO/Ler, show a 50% pollen abortion phenotype (Fig. 5 and Table 4B). In rare cases, a pollen grain with the infrequent *scp* phenotype of two vegetative nuclei is found in these NoO/Ler F_1 plants (Fig. 8C). We are not able to obtain *scp* homozygous plants in the self F_2 progeny of a F_1 *scp*/+ plant in this NoO/Ler background because almost all the pollen carrying the *scp* mutation is aborted in this background. Therefore, only wild-type and heterozygous *scp* progeny are produced from the selfing of heterozygous *scp* plants in the genetic background of NoO/Ler and the segregation of these two types of F_2 progeny is about 1:1 (Table 1B). We backcrossed the F_1 heterozygous *scp* plants in the NoO/Ler background to homozygous *scp* plants in the NoO background in order to obtain homozygous *scp* plants in partial Ler background (a further reduction in the contribution from the Ler genome) (see Fig. 5). For convenience, we will use the term 'NoO/Ler' to indicate the genetic background of half NoO and half Ler and the term 'partial Ler' to indicate the genetic back-

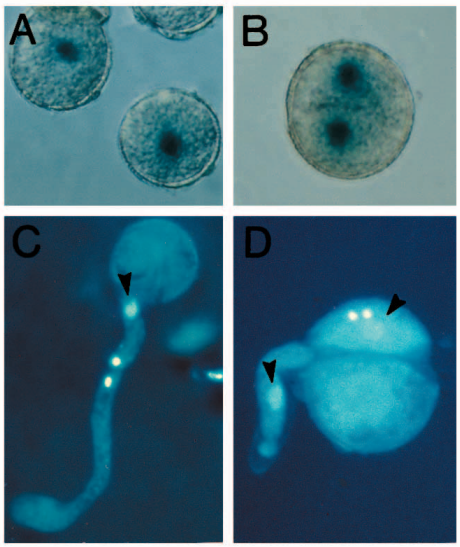

Fig. 4. LAT52-GUS-NIa expression and pollen tube growth. (A,B) Pollen incubated with X-Gluc. (C,D) Pollen stained with DAPI. (A) Wild-type pollen expressing the LAT52-GUS-NIa reporter gene. (B) *scp* pollen expressing the LAT52-GUS-NIa reporter gene. Both the vegetative cell and the extra cell express the LAT52-GUS-NIa reporter gene. (C) Germinating wild-type pollen. The vegetative nucleus and sperm all move into the growing pollen tube. (D) The germinating extra cell of *scp* pollen. Arrowheads: vegetative nuclei.

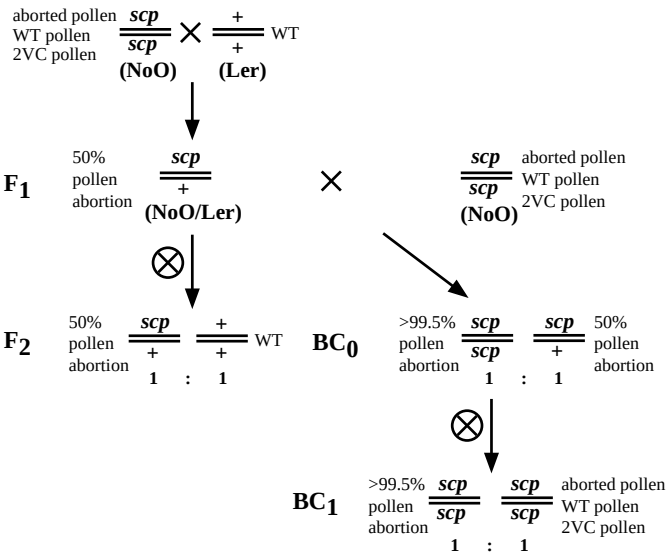

Fig. 5. Scheme for outcrossing *scp* in NoO genetic background to Ler genetic background. (For details see text.)

ground with a further reduction of Ler genome. The backcross used the F_1 as the female parents and the homozygous *scp* NoO plants as the male parents, because homozygous *scp* in NoO background still produces some viable and functional pollen but the *scp* mutation in the F_1 (NoO/Ler) can only be transmitted through females. Half of the backcross progeny (BC_0) produced about 50% aborted pollen and 50% wild-type pollen and were similar to their female parent (heterozygous *scp* in NoO/Ler background) (Fig. 5 and Table 3). The other half of the backcross progeny were homozygous for *scp* and had strongly reduced fertility (much less than one seed produced per silique), presumably due to the fact that more than 99.5% of the pollen is aborted (Fig. 5, Fig. 7C and Table 3). Nonetheless, a few self seeds were obtained from the homozygous *scp* plants in partial Ler background and were planted. This small population (BC_1, Fig. 5) segregated out two gametophytic phenotypes: plants with >99.5% pollen abortion (10 plants) and plants which restored the variable pollen phenotype (wild-type pollen, aborted pollen and pollen with an extra cell) seen in the NoO background (10 plants). These results all indicate that in the genetic background of Ler, *scp* shows uniform expressivity (pollen abortion phenotype) and is almost completely penetrant at the gametophytic level. Except for the pollen abortion and the subsequent low-fertility phenotype, there is no sporophytic phenotype observed in the homozygous *scp* plants in partial Ler background.

Pollen development in *scp* mutants

A vegetative cell is one of the two products of the microspore mitosis, the first postmeiotic mitosis. Since the extra cell produced in the *scp* mutant pollen possesses a vegetative cell identity, we sought to determine when this extra vegetative cell is produced. Both wild-type and *scp* buds at all developmental stages were sectioned and the salient results are shown in Fig. 6.

At the mature stage, wild-type pollen contains three cells, one vegetative cell and two sperm cells enclosed within the vegetative cell cytoplasm (Fig. 6A). The microgametophyte is surrounded by two layers of pollen wall: intine and exine. The

Table 3. Progeny phenotypes of *scp/+* (Ler/NoO) crossed by *scp/scp* (NoO)

	Plant fertility and pollen viability		
	Male fertile (100% normal pollen)	Semi-male sterile (50% normal pollen and 50% aborted pollen)	Male sterile (> 99.5% aborted pollen)
scp/+ progeny	0	15	0
scp/scp progeny	0	0	16

intine is the inner pollen wall, whose components are mainly synthesized by the vegetative cell. The exine is the outer pollen wall, whose components are mainly secreted by the sporophytic tapetum cells of the anther (Mascarenhas, 1975). In *scp*, the extra vegetative cell and the 'normal' microgametophyte are separated by intine but are enclosed in the same exine (Fig. 6B); this finding was confirmed by histochemical staining (Regan and Moffatt, 1990) for exine and intine (data not shown). Many aborted pollen grains with very little or no cytoplasm can be seen at this stage (Fig. 6B). At the binucleate pollen stage, wild-type pollen contains one vegetative cell and one generative cell enclosed in the vegetative cell (Fig. 6C). In *scp*, the extra vegetative cell was present in the pollen before the pollen mitosis of the generative cell (Fig. 6D). Notice that in *scp*, fewer aborted pollen grains are found at the binucleate stage than at the mature stage. The wild-type uninucleate microspores are shown in Figure 6E, while an extra cell appeared in some microspores of *scp* at this stage (Fig. 6F). Almost no aborted microspores are found at this stage in *scp* (Fig. 6F and see Fig. 7). Tetrads of *scp* do not differ significantly from those of wild-type plants (Fig. 6G,H). Thus the extra cell of *scp* pollen appears to be produced after tetrad stage and prior to any asymmetric microspore mitosis. We noticed that the plane of cell division of this mitosis is perpendicular to the division plane in wild-type microspores. To illustrate, consider that the pollen grain is equivalent to the globe, and the nucleus is appressed towards Antarctica. In wild-type the plane of division of the microspore mitosis occurs along a southern latitude, resulting in a larger and a smaller cell (Fig. 6C). However, in *scp* microspores the plane of division of the first mitosis occurs along a longitude and results in two approximately equal-sized cells (Fig. 6F).

To investigate when and possibly how the aborted pollen arrests during development, we compared the nuclear staining (DAPI) patterns in wild-type plants, homozygous *scp* plants (in NoO background) and homozygous *scp* plants (in partial Ler background). The results are shown in Fig. 7. At mature stage, wild-type has almost no pollen abortion (Figs 7A, 2A,C). There are aborted pollen grains, pollen with an extra vegetative cell and normal pollen in *scp* of NoO background (Figs 7B, 2B,C). In *scp* in the partial Ler background, almost all the pollen grains are aborted, resulting in very little DAPI staining (Fig. 7C). At the binucleate pollen stage, there is some aborted pollen in both *scp* backgrounds (Fig. 7E,F) when compared with wild-type (Fig. 7D), but the pollen abortion is not as much as at the mature stage. Some pollen with an extra nucleus can be seen at this stage in *scp* of both backgrounds (Fig. 7E,F). At the microspore stage, no significant abortion of microspores is seen in wild-type (Fig. 7G) or in either *scp* background (Fig. 7H,I). At tetrad stage, *scp* in either genetic background (Fig.

7K,L) is identical to wild-type (Fig. 7J). These results indicate that pollen abortion caused by the *scp* mutation does not take place at early pollen developmental stages but occurs in some pollen grains between the binucleate and mature stages, after producing an extra cell.

scp is a male gametophytic mutation

quartet1 (*qrt1*) is a sporophytic recessive mutation which causes the four microspores produced from one meiosis to stay attached for the remainder of pollen development (Preuss et al., 1994; Fig. 8A and Table 4A). The *qrt1* mutation can be used to perform tetrad analysis without interfering with the normal function and development of pollen grains (Preuss et al., 1994). We crossed *scp* to *qrt1* in a Ler background in order to perform tetrad analysis on the *scp* mutation. In *qrt1/qrt1*; *scp/+* plants, each 'quartet' of mature pollen grains contained two normal pollen grains and two aborted pollen grains (Fig. 8B; Table 4B). This clearly demonstrates that the *scp* mutation is gametophytically expressed in the pollen since the lethal phenotype, typical of *scp* in the NoO/Ler background, segregates 2:2 in the tetrads.

Mapping of the *SCP* locus

scp (in NoO background) was outcrossed as female by wild-type Col or Ler plants and the F_1 progeny were selfed to obtain F_2 progeny and backcrossed by the wild-type parents to obtain backcross progeny. The F_2 and backcross progeny were tested for linkage of *scp* to SSLP (Bell and Ecker, 1994), CAPS (Konieczny and Ausubel, 1993) and ARMS (Fabri and Schäffner, 1994) markers. We were able to localize the *SCP* gene on chromosome 3 near markers nga6 and BGL1.

DISCUSSION

scp causes the production of an additional vegetative cell in pollen

To examine the cell identity of the extra cell produced in *scp*, we tested the ability of the extra cell to grow a pollen tube and to express two vegetative cell-specific reporter genes: LAT52-GUS-NIa and LAT59-GUS. It is reasonable to assume that the growth of a pollen tube is supported by the transcriptionally active vegetative cell rather than by the generative cell or sperms, since the rapid growth of a pollen tube requires a significant amount of biosynthetic activity. Although there is no direct evidence showing that pollen tube growth is directed by

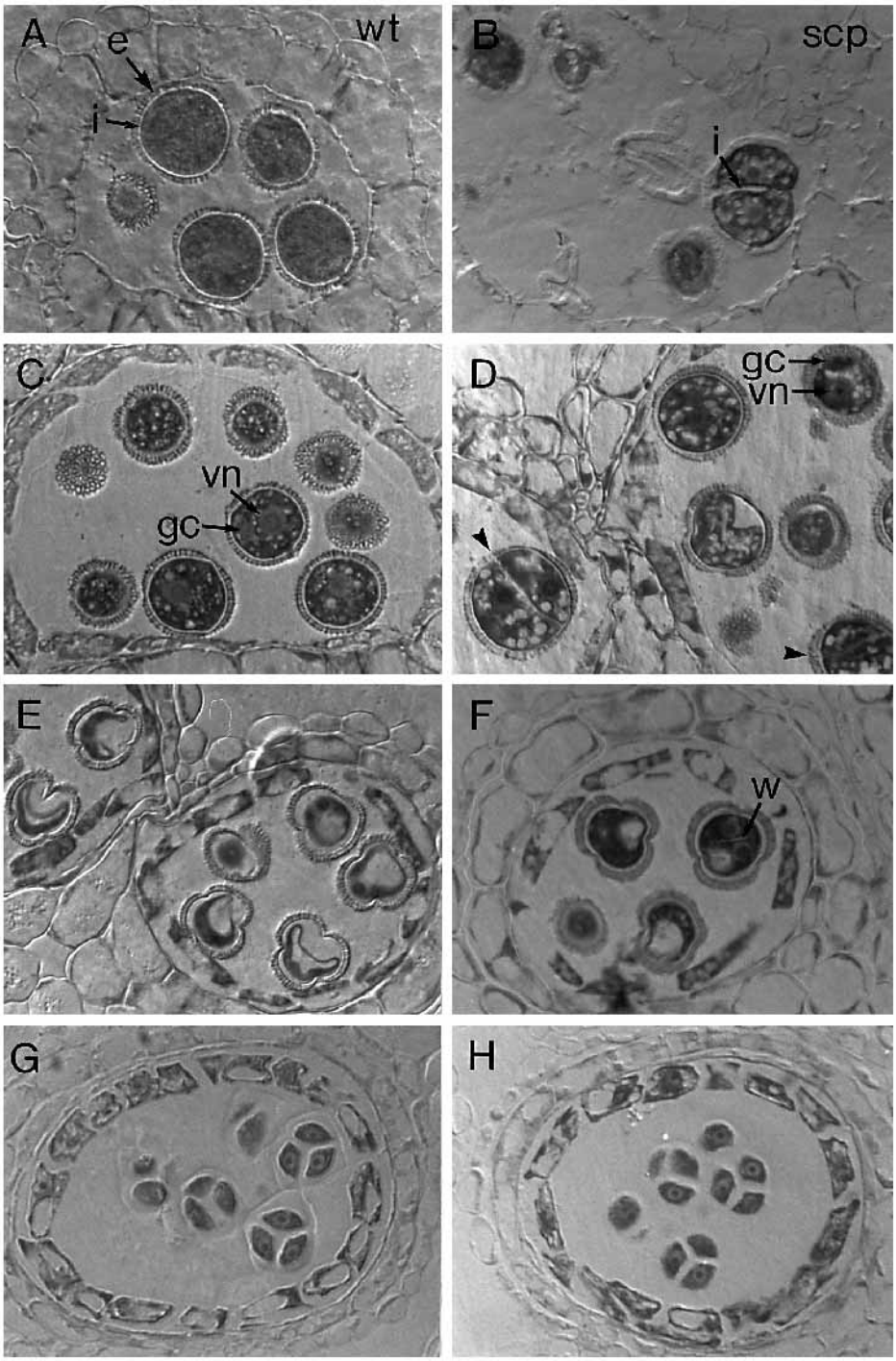

Fig. 6. Pollen development in wild-type and *scp* plants. (A) Mature wild-type pollen. (B) Mature mutant *scp* pollen with two vegetative nuclei and cell wall (intine) separating the two vegetative cells. (C) Wild-type binucleate pollen. (D) *scp* pollen at the same stage as C. (E) Wild-type uninucleate microspores. (F) *scp* microspores at the same stage as E. Before the asymmetric microspore mitosis, an extra cell can already be seen in some microspores of *scp*. w, cell wall between the two cells in the microspore. (G) Wild-type tetrads. (H) *scp* tetrads. i, intine; e, exine; gc, generative cell; vn, vegetative nucleus.

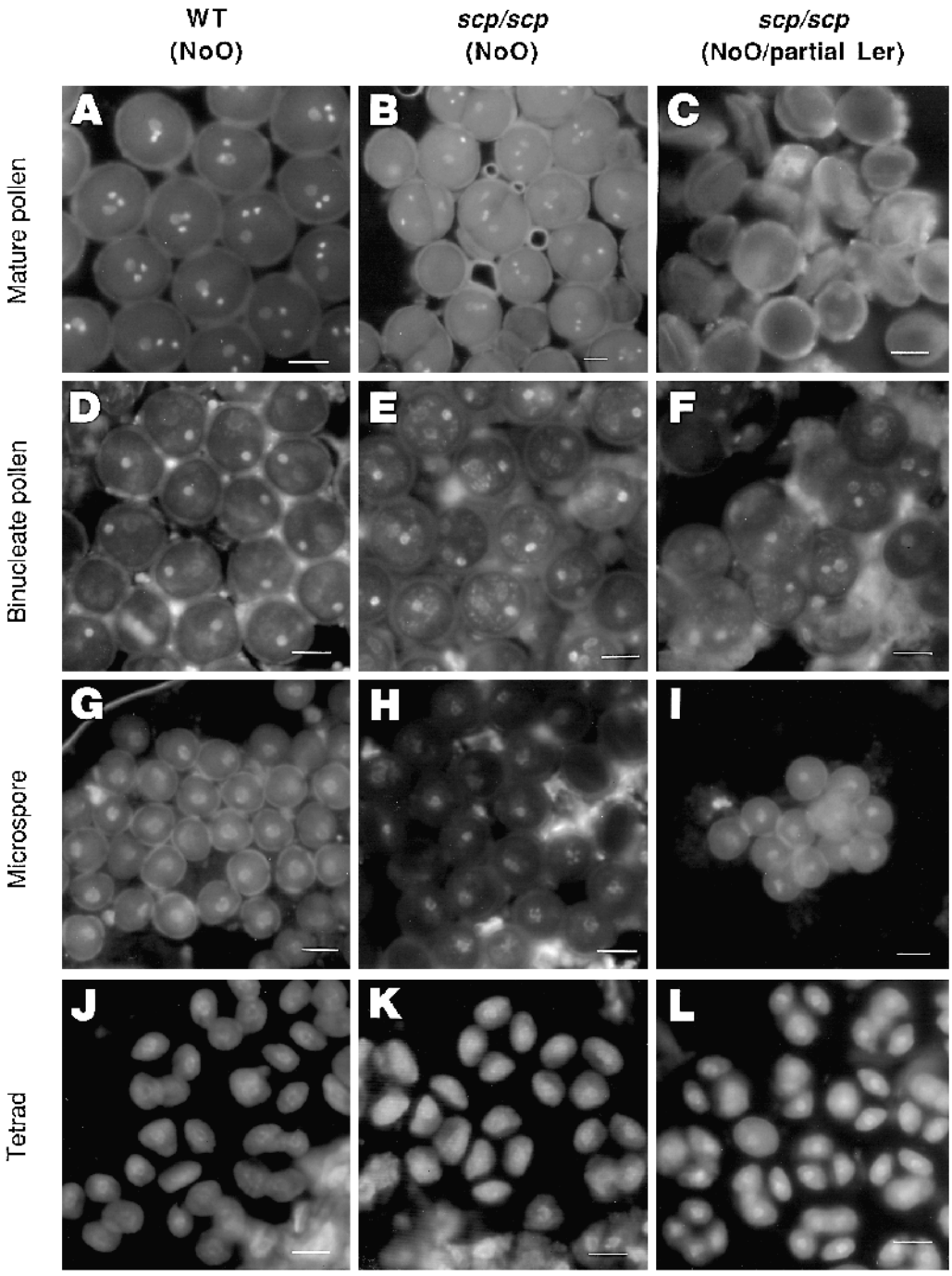

Fig. 7. Nuclear DAPI staining of tetrads, microspores and pollen in wild-type (WT (NoO)), homozygous *scp* in NoO background (*scp/scp* (NoO)) and homozygous *scp* in partial Ler background (*scp/scp* (NoO/partial Ler)). (A) Wild-type mature trinucleate pollen. (B) NoO *scp* mature pollen. Notice that there are three types of pollen: aborted, extra-celled and normal. (C) Partial Ler *scp* pollen at mature stage. No viable pollen is seen. (D) Wild-type binucleate pollen. The generative nucleus is stained intensely and the vegetative nucleus is stained more diffusely. (E) NoO *scp* pollen at the same stage as D. Many pollen grains show an extra vegetative-like nucleus. (F) Partial Ler *scp* pollen at the binucleate stage. Notice some pollen also shows an extra vegetative-like nucleus. There is some pollen abortion but not as much as at the mature stage. (G) Wild-type microspores. (H) NoO *scp* microspores, at a slightly later stage than the wild-type shown in G. An extra nucleus can be seen in some of the microspores. (I) Partial Ler *scp* microspores. Almost no abortion is seen at this stage. (J) Wild-type tetrads. (K) NoO *scp* tetrads. (L) Partial Ler *scp* tetrads. Scale bar, 10 μm.

the vegetative nucleus, there are several reports that support this hypothesis. In trying to determine the role of the vegetative cell and the generative cell in pollen tube growth, Bishop and McGowan (1953) used colchicine to block the microspore mitosis of *Tradescantia paludosa* pollen in culture and obtained uninucleate pollen which were still capable of germinating and growing pollen tubes. Eady et al. (1995) used a similar approach to show that colchicine-induced uninucleate pollen of tobacco is capable of germinating. They further demonstrated that the nucleus of the uninucleate pollen stained diffusely with DAPI, similar to that of a vegetative cell, and that this uninucleate pollen expressed the vegetative cell-specific LAT52-GUS-NIa reporter gene (Twell, 1992). Lastly, laser ablation of the generative nucleus of *Amaryllis* pollen did not affect subsequent pollen tube growth (cited by Mascaren-

Table 4. Tetrad analysis of *scp*

	Tetrad				
	4+(oooo)	3+(ooox)	2+(ooxx)	1+(oxxx)	0+(xxxx)
A. +/+; *qrt1/qrt1*	425* (98.0)†	8 (1.8)	0	1 (0.2)	0
B. *scp*/+; *qrt1/qrt1*	0	0	639 (96.5)	23 (3.5)	0

o, wild-type pollen grain; x, mutant pollen grain.
*number of tetrads scored.
†percentage of tetrads with a given phenotype.

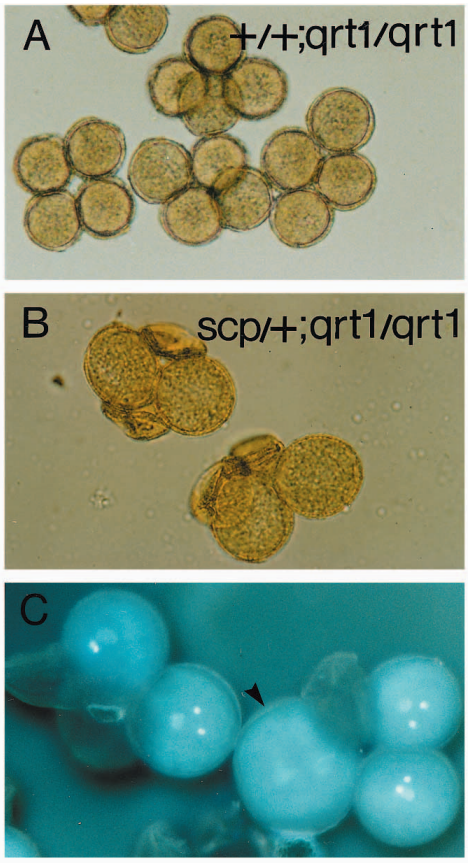

Fig. 8. Pollen phenotypes in +/+; *qrt1/qrt1* and *scp*/+; *qrt1/qrt1* plants. (A) +/+; *qrt1*/*qrt1* pollen, showing that the four products of one meiosis stay attached even at the mature pollen stage. (B) *scp*/+; *qrt1*/*qrt1* pollen in Ler/NoO genetic background, showing a 2 : 2 segregation of normal pollen : mutant (aborted) pollen. (C) DAPI staining of two pollen tetrads from *scp*/+; *qrt1*/*qrt1* pollen in Ler/NoO genetic background. Arrowhead: pollen with two diffusely stained nuclei.

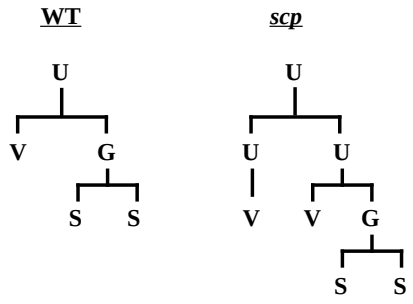

Fig. 9. Cell lineages during pollen development of wild-type and *scp*. U, (undetermined) uninucleate microspore. V, vegetative cell; G, generative cell; S, sperm cell.

has, 1978). These results indicate that a vegetative cell is sufficient to grow a pollen tube and that the generative cell (or sperm) is dispensable for the growth of the pollen tube. Since the extra cell produced in *scp* pollen behaves like a vegetative cell morphologically (DAPI staining), physiologically (pollen tube growth) and molecularly (LAT59-GUS and LAT52-GUS-NIa expression), we conclude that in *scp*, the male gametophyte produces an extra vegetative cell.

Do the pore positions of *scp* pollen define differences between the two vegetative cells? An *Arabidopsis* pollen grain has three symmetric pores and therefore *scp* pollen with two vegetative cells is likely to be polar relative to the position of the pores. However, the sections and whole mount analyses of *scp* show that the cleavage orientation is not always consistent relative to the position of the pores. Thus determining whether the number of pores associated with a vegetative cell affects the ability to undergo the subsequent asymmetric division and/or tube germination may require serial sections and three-dimensional reconstructions.

scp displays variable expressivity and differential penetrance at the gametophytic level in different genetic backgrounds

scp exhibits variable phenotypes in the genetic background of NoO and Col ecotypes. In addition to pollen with an extra vegetative cell and aborted pollen, *scp* produces wild-type pollen. Perhaps the array of mutant phenotypes of *scp* in both backgrounds is due to a variable expressivity of this mutation so that it is expressed weakly in some pollen grains but strongly in others. However, even though wild-type pollen grains are produced in the homozygous *scp* mutant in the NoO background (Fig. 2C), all of the self progeny of a homozygous plant are phenotypically homozygous (i.e. about 25% pollen with an extra cell). Furthermore, when a homozygous *scp* plant is outcrossed as either a male or a female to a wild-type plant, all of the progeny are phenotypically heterozygous (i.e. about 7% pollen with an extra cell). In our terms, *scp* shows an incomplete penetrance at the gametophytic level, but each sporophyte (plant) carrying the *scp* mutation will predictably (according to the genotype) produce a certain percentage of microspores showing mutant phenotypes. The *Arabidopsis* female gametophytic mutation *prolifera* is also fully penetrant at the sporophytic level but not at the gametophytic level (Springer et al., 1995). In their study, the mutation *prolifera* was termed a ‘leaky’ female gametophytic mutation.

In contrast to the phenotype in a pure NoO (or in a NoO/Col) genetic background, *scp* exhibits a pollen abortion phenotype in a NoO/Ler genetic background. Since the same allele of *scp* (in the NoO background) results in a wide range of phenotypes, including pollen abortion, but results in nearly complete pollen abortion in the Ler background, the pollen lethal phenotype is probably a severe phenotype of *scp*. This modification of mutant phenotype in the NoO/Ler background is observed in the F_1 plants, suggesting that at least a dominant sporophytic gene is involved in the modification of the phenotype of *scp*. However, no BC_0 plants give rise to the variable pollen phenotype seen in the NoO background (Fig. 5 and Table 3), indicating that there are likely multiple genetic factors involved as well. The genetic interactions resulting in variable expressivity of a mutation in different backgrounds can lead to the identification of other factors that function in the same developmental processes (Becraft and Freeling, 1994). Two other *Arabidopsis* mutations, *sin1* (Lang et al., 1994) and *FRI* (Lee et al., 1994) were also found to have modified phenotypes in the Ler genetic backgrounds and two modifying loci, *mod1* and *FLC*, respectively, were identified. The variable pollen phenotype of *scp* is restored at the BC_1 generation (Fig. 5),

indicating that the number of modifying factors in Ler genetic background is limited. We also found that none of these factors are linked to the *erecta* locus (data not shown), although the modifier, *mod1*, of the mutation *sin1* was inseparably linked to the *erecta* locus (Lang et al., 1994). Despite the restoration of the variable pollen phenotype in the BC_1 generation, we cannot yet determine how many genetic factors are involved in the penetrance and expressivity of *scp*, since only one line was analyzed.

We have seen no evidence of a visible mutant phenotype in the sporophyte or in the female gametophyte in homozygous or heterozygous *scp* plants in any of the genetic backgrounds we have tested. The transmission of *scp* through female gametophytes is 100% (Table 2), indicating that female gametophytes of *scp* function normally. These results suggest that *SCP* may function specifically in male gametophytes. However, since we do not know whether this allele of *scp* is a null allele we cannot exclude the possibility that the *SCP* gene has a sporophytic or megagametophytic function that is not disrupted by this particular allele.

SCP may play an important role in the asymmetric cell division of pollen development

There are generally two types of asymmetric cell division: division that is intrinsically asymmetric and asymmetry that is determined by cell signaling (Horvitz and Herskowitz, 1992). The normal microspore mitosis is likely to be an intrinsically asymmetric cell division, with unequal distribution of cellular components leading to different cell fates in the two cells. Fig. 9 illustrates the different cell lineage patterns of pollen development in wild-type and *scp*. In the development of *scp* pollen, an extra cell is seen at the uninucleate microspore stage (Fig. 6F). This phenotype is seen even in the homozygous *scp* plants of partial Ler background, where almost all the pollen is aborted at the mature stage (Fig. 7). The two cells in these *scp* microspores cannot be distinguished by the staining of nuclei or the size of the cells. Since an extra cell is seen before a generative cell can be seen in the microspore, this abnormal cell division giving rise to the extra cell certainly takes place first instead of the typical asymmetric microspore mitosis. Nonetheless, in the NoO genetic background, one of the two cells produced from this abnormal division can recapitulate the fate of a uninucleate microspore and undergo an asymmetric division to give rise to the normal complement of a three-celled gametophyte. This indicates that the *scp* mutation does not simply prevent asymmetric division per se.

Several models could potentially account for the formation of the extra vegetative cell. First, *SCP* could be involved in the partitioning and/or localization of a determinant for the asymmetric microspore mitosis. For instance, *SCP* could function by determining the orientation of the division plane of the microspore mitosis so that in *scp* mutant plants, an incorrectly oriented division plane gives rise to two equal-sized, undetermined cells. It has been shown in mammalian neurogenesis that the orientation of the cleavage plane (or the orientation of spindle) during mitosis may be exploited to control the asymmetry (or symmetry) of the cell division by differentially partitioning an asymmetrically localized gene product (Chenn and McConnell, 1995, for review see Rhyu and Knoblich, 1995). If there is a cytoplasmic determinant which confers the ability to undergo the asymmetric cell division in microspores, it might be distributed randomly in *scp* pollen after the abnormal division. The daughter cell that acquires sufficient determinant could subsequently divide asymmetrically, while the other one becomes a vegetative cell by default. Another model is that *SCP* could be a developmental checkpoint for the asymmetric microspore mitosis; for example, it could be a cell division inhibitor which prevents the microspore from dividing until the cell is ready to undergo an asymmetric division. In *scp*, the microspore divides prematurely before the cell is competent for an asymmetric division, resulting in an equal division. However in later developmental stages when microspores are normally ready for the asymmetric division, one of the two daughter cells acquires the ability to undergo an asymmetric division while the other daughter cell becomes a vegetative cell by default. In a third scenario, *SCP* could be a gene whose product is sufficient for the cell to divide but that normally would not be expressed until the microspores are ready for the asymmetric mitosis. In this scenario SCP is ectopically expressed at an earlier stage and induces microspores to divide early but symmetrically. All the models propose that the vegetative cell identity could be the default state. One line of evidence that supports this idea is that occasional pollen grains with only two vegetative-cell-like nuclei but no sperm cells are always found in *scp* plants (Fig. 3D). The two vegetative-cell-like nuclei in these pollen grains both express the LAT52-GUS-NIa reporter gene (data not shown) so they probably both have vegetative nuclei identities. The formation of such pollen grains might occur if after cell division neither of the daughter nuclei acquired the ability to divide again and therefore both became vegetative nuclei by default. The models do not directly account for the significant pollen abortion in *scp*. Perhaps the *SCP* gene product is also required for later development, because pollen abortion only occurs at later stages of development (Figs 6, 7), or perhaps it is just physiologically difficult for all the pollen with an extra cell to survive to the mature stage, since pollen grains are known to compete for nutrients in the locule (Gambier and Mulcahy, 1996; Ottaviano and Mulcahy, 1989).

One way to discriminate between these models will be to precisely time the production of the extra cell in *scp* microspores. Since there is no change of division timing involved in the first model (in which the *scp* mutation only changes the partitioning of a determinant) but the other two models predict that the cell division producing the extra cell is premature, a division shown to occur earlier than a normal asymmetric mitosis in wild-type microspores would support the latter two models. One way to test the second and third models would be to apply cell division inhibitors to dividing *scp* microspores in culture. If a pulse of cell division inhibition at early stages restores normal development, it may indicate that a delay of cell division is necessary for the asymmetric mitosis. However, since *scp* is a gametophytic mutation acting in the haploid genome, further tests to discriminate between a gain-of-function or a loss-of-function mutation would be required.

In many different plant species the symmetry of the microspore mitosis can be manipulated in culture. For example, in the related cruciferous species *Brassica napus*, cell divisions in pollen development can be manipulated with temperature (Custers et al., 1994) or colchicine (Zaki and Dickinson, 1991). These studies showed that disruption of the

asymmetric microspore mitosis can switch the gametophytic developmental pathway to a sporophytic developmental pathway that eventually gives rise to haploid plants. The mutant phenotype of *scp* microspores somewhat mimics the first step of the sporophytic switching and thus *scp* may provide an alternative with which to analyze this switch.

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