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TECHNIQUES FOR MOLECULAR ANALYSIS

A compendium of methods useful for characterizing *Arabidopsis* pollen mutants and gametophytically-expressed genes

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Summary

This article provides detailed protocols for collecting pollen and outlines genetic crosses and phenotypic assays that are useful for characterizing mutants that affect pollen development.

Keywords: gametophyte, mutant, *quartet*, *tetraspore*, segregation distortion, pollen germination.

Introduction

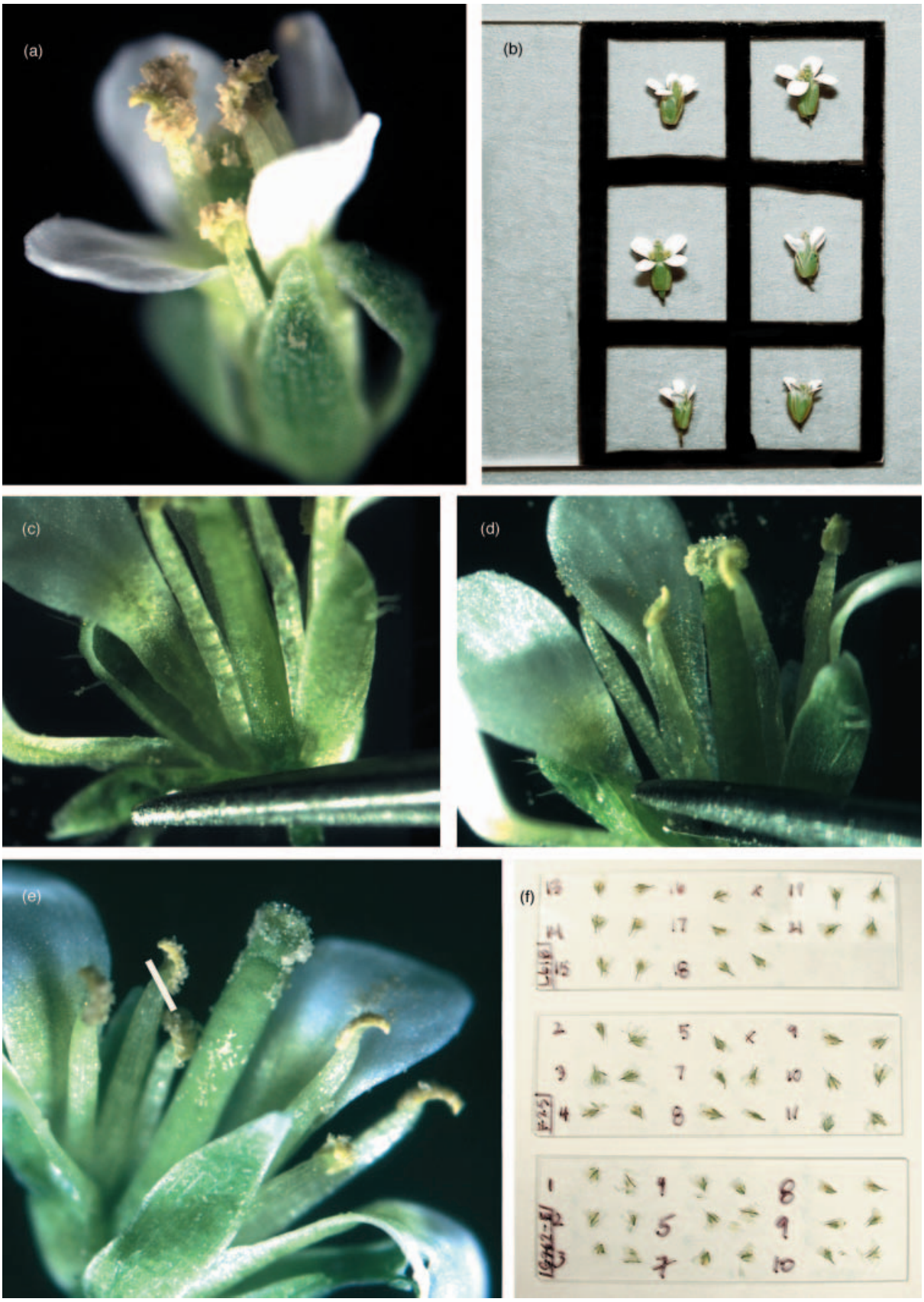
The male gametophyte is an ideal system for studying a number of processes, including tip growth and cell–cell interactions. Mature pollen of *Arabidopsis* has three cells: a larger vegetative cell that will extend to grow the pollen tube, and two sperm cells that are enclosed within the cytoplasm of the vegetative cell. The pollen vegetative cell has two cell walls: an intine or inner wall and an exine or outer wall; the sperm cells lack cell walls. In *Arabidopsis*, microarray hybridizations suggest that about 10% of the genes are selectively pollen-expressed (Becker *et al.*, 2003; Honys and Twell, 2003), a percentage similar to earlier estimates for pollen in other species (reviewed in Mascarenhas, 1990). Whether genes are expressed both in pollen and in the diploid sporophyte, or are pollen-specific, mutational analysis is frequently the first step in functional characterization. To facilitate such approaches, there are now over 900 000 T-DNA and transposon insertion lines (Alonso *et al.*, 2003; Kuromori *et al.*, 2004; Pan *et al.*, 2003; Parinov and Sundaresan, 2000) and ethyl methanesulfonate (EMS) mutagenized populations (McCallum *et al.*, 2000) available.

This article is aimed at several different groups of researchers. Some researchers might be characterizing T-DNA insertion lines for genes expressed in the sporophyte, but find to their dismay that they cannot obtain a homozygous line with the T-DNA insertion. Others might have identified a phenotype that affects pollen development and might be trying to determine the molecular basis for this phenotype. Still others might know only that the gene they are studying is expressed in pollen and not whether its gene product plays an important role during pollen development. Here, we provide detailed protocols for preparing flowers for microscopic observation of pollen and for collecting pollen in bulk for germination or for RNA or protein isolation. We outline crossing schemes that will help confirm or refute hypothesized roles for the gene of interest, and we outline phenotypic characterizations that are useful in determining whether a gene product is important during one or more phases of pollen development or germination.

Harvesting pollen

Preparing flowers for observations of pollen

Arabidopsis flowers have four upper stamens and two lower stamens (Figure 1a) that differ slightly in maturation; occasionally flowers will have only four or five stamens. To obtain dehiscent anthers containing mature pollen or younger anthers that contain immature pollen grains or microspores, it is convenient to remove anthers from flowers by dissection, using forceps with narrow tips (Fine Science Tools, Foster City, CA, USA; see Table S1 for detailed supplier information). Dissecting tools that are suitable for


(a)
(b)
(c)
(d)
(e)
(f)

dissecting young anthers can also be fashioned from disposable hypodermic needles (#23 gauge; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) attached to 1-ml disposable syringes.

In general, mature pollen is present in the flower once white petals are visible, coinciding with stage 13 of flower development (Smyth *et al.*, 1990). For mature but non-dehiscent anthers, flowers can be collected and left to dry slightly on a glass slide to promote anther dehiscence (Figure 1b). If pollen will be hydrated in water or a histochemical stain, a Super HT PAP pen (Research Products International Corp., Mt Prospect, IL, USA) can be used to draw ~5-mm diameter wells on glass microscope slides (Figure 1b,f) to retain the solution. To prepare a slide, single flowers are placed in each well, anthers are dissected and the remaining flower parts are removed. For photography, dissect anthers from a flower, on a standard glass slide, and then transfer, onto a new glass slide, only the anthers to a PAP pen well, to reduce the presence of cellular debris or stray flower parts (debris can obscure images; some flower parts have intense autofluorescence).

To dissect anthers from a flower, first press, gently wiggle and then hold a forceps or needle along the calyx, just above the pedicle margin, so that the forceps is perpendicular to the pistil (Figure 1c,d). The forceps will stabilize and splay the flower, making removal of anthers easier (Figure 1e). Before splaying a young bud, lift the calyx free by sliding a forceps along the calyx margin and lifting upward. In either case, pollen can be released into solution by gently squashing open the anthers using a probe (Fine Science Tools) or a similar microprobe (Fisher Scientific, Pittsburgh, PA, USA). To facilitate squashing, apply a small amount of liquid (up to 5 μl), squash and then add solution to fill the well. The dispersion of pollen within a PAP pen well can easily be controlled by changing the size of the well or the volume of liquid added to the well.

Whole flower preparations are useful for rapidly scoring mature pollen phenotypes from many individual plants (Figure 1f). For example, in a mapping population it might be necessary to score 1000 plants to determine whether each has mutant or normal pollen, and it is advisable to score at least two flowers from each plant to confirm that the pollen phenotype is consistently observed. To screen pollen, flowers with dehiscent anthers are placed on slides with PAP pen wells (Figure 1b,f). Square ($100 \times 100 \times 15$ mm) Petri plates (Fisher Scientific) are useful for holding up to three standard slides (Figure 1f) of harvested flowers when in transit from the greenhouse or growth chamber. If using square plates, apply a drop of water to the inside surface of the lid and wipe across the surface until dry to eliminate static electricity; otherwise, when the lid is put back on after flower collection, the flowers might fly off and stick to the lid. In preparation for flower squashes, staining solution or water (if staining is not desired) is added to each well. Pollen is released from the flower by splaying it open and then gently rolling over the flower and dabbing the flower and anthers using either a pipette tip, reusable plastic toothpick or stirring rod (BioSpec Products, Inc., Bartlesville, OK, USA). Whole flower preps interfere with level placement of a cover glass onto the slide, but for low-magnification observations, a cover slip is not required. To add a cover glass, first gently draw up and eject the solution from a well several times, in order to agitate the pollen into the solution and away from the flower parts. Then, draw up the solution, remove the flower parts from the well and return the solution to the well. Alternatively, transfer the staining solution that contains the pollen into a new PAP pen well on a clean slide; this is best for closer inspection of one or a few plants.

In some cases, slides can be stored for later re-examination, depending on the type of stain used and the cellular component being stained. For example, air-dried slides can be re–scored by re–hydrating the pollen to confirm the phenotype of putative recombinants in a mapping population. An easy way to determine if slides stained with a particular stain will be suitable for storing is to test whether the staining pattern can be reproduced when the stain (or water, in the presence of previously applied stain) is applied to the sample. We have had success storing and rescoring slides with flowers stained with decolorized aniline blue to detect callose (see histology section for further discussion on decolorized aniline blue). However, viability stains, such as Alexander's stain (see histology section for further discussion) or rhodamine 123, which stains active mitochondria, are only useful on fresh tissue. Nonetheless, some preparations can be retained for several days if coverslips are applied and sealed with fingernail polish. When this procedure is followed, the slides are stored in the dark at 4°C to prevent the sample from drying out. Mature dehiscent pollen can be easily released from dehiscent anthers into mineral oil or glycerol by dabbing or squashing. Pollen dispersed in oil is

Figure 1. Preparation of mature flowers for screening pollen on slides or for dissection of anthers.
(a) Close-up of flower showing dehiscent stamens.
(b) Freshly picked flowers to be used for screening; wells drawn on slide correspond to score sheet with identifying plant numbers.
(c, d) Procedure to open a flower for access to anthers and pollen. (c) A forceps is placed at the calyx–pedicel junction and gently wiggled left and right, (d) in order to splay open the flower.
(e) A splayed-open flower. White line indicates optimum point of dissection, to minimize transfer of filament cells when anther is squashed to release pollen.
(f) Examples of slides used for screening pollen. Each slide contains 18 PAP pen-outlined wells.
Care should be taken not to disrupt the continuity of the PAP pen edge, so that the solution remains inside. The plant line is recorded on the lower left corner of each slide. Two flowers from each plant (e.g. –13, –14) are placed in adjacent wells. Such slides can be stored and rehydrated for examination later.

ideal for analysis of surface features of pollen but interferes with pollen germination assays. Alternatively, thin strips of double-sided tape can be placed onto a glass slide and dehiscent anthers touched to the sticky surface to release and adhere the pollen to the tape. An advantage of the double-sided tape is that the pollen grains are fixed in place and can be monitored over time. Pollen adhered to the tape will hydrate when staining solution or germination medium is added; a disadvantage is that it is impossible to remove a cover slip from such microscope slides, as they stick too firmly to the tape. This method is suitable for fluorescence microscopy as long as the filters used will block autofluorescence from the tape; different brands of tape should be tested.

Large-scale pollen isolation

Maize pollen is easily collected in bulk by shaking tassels into paper bags, and pollen from Solanaceous plants is fairly easy to collect by agitating flowers or flower pedicels with a mechanical buzzer. Because *Arabidopsis* flowers are so small, bulk harvesting of mature pollen might seem impossible but is not. One way to isolate *Arabidopsis* pollen in bulk is to cut off the primary inflorescences from several flats of plants. It is not necessary to collect each plant carefully, a gardening shears cutting across the canopy works well. The collected tissue is agitated in liquid (e.g. TE buffer) to release the pollen into solution, for example, by stirring in a large flask. The solution is then filtered through Miracloth (Calbiochem, La Jolla, CA, USA), transferred into 50 ml disposable centrifuge tubes, pelleted by low-speed centrifugation, then the supernatant is decanted. To conserve centrifuge tubes, additional solution can be added to tubes with already pelleted pollen and re-centrifuged, so that the pellets accumulate. The pelleted pollen can be resuspended in a small amount of TE, and examined with a microscope for potential contaminants. Depending on the application, additional filtration can eliminate contaminants, or fluorescence-activated cell sorting (Becker *et al.*, 2003) can be used to separate fully hydrated pollen from partially hydrated and non-hydrated pollen. This liquid agitation method was used for bulk isolation of pollen for RNA isolation (Becker *et al.*, 2003; Honys and Twell, 2003; Kulikauskas and McCormick, 1997), and for a second round screen for *raring-to-go*-like mutants (Johnson and McCormick, 2001). The disadvantages of this method are the time required for repeated centrifugation and that the main inflorescence stalks of the plants are removed, although eventually the side branches grow out and flower again.

It is much easier and significantly less time-consuming to use a vacuum cleaner to collect pollen from flowers of intact plants. Figure 2(a) shows a vacuum cleaner that has been modified for large scale pollen isolation; the protocol described here is based on the one developed in Jose Feijo's laboratory (Oeiras, Portugal). The pollen vacuum consists of three different-sized Nitex® meshes (Sefar America, Inc., Depew, NY, USA) held together in sequence, using plumbing fittings that are readily available at a hardware store; see Figure S1 for assembly instructions. This 'wand' is then attached to a handheld vacuum cleaner with duct tape. The 80 micron mesh traps the flower parts and the 35-micron mesh traps debris, such as soil and vermiculite particles. The pollen is trapped on the 6-micron mesh. For vacuuming pollen, each flat is planted with ~250 seeds. The seeds are prepared by mixing 10 mg seeds with 50 ml 0.1% agar, shaking to eliminate seed clumps, and then dribbling 25 ml of the agar/seed mixture uniformly over the soil surface. Figure 2(b) shows pollen collected on the 6-μm mesh after vacuuming 10 flats. In the summer, the yield from 10 flats planted at this density is ~10 mg pollen day^{-1}, in the winter the yield is ~7 mg pollen day^{-1}. Pollen can be harvested from the same plants repeatedly (four to five times) during their flowering cycle. Damage to the plants can be avoided by controlling the suction force of the vacuum (note: the vacuum cleaner used in the Feijo laboratory has a rheostat that can control the suction force; the suction force of inexpensive hand-held vacuums can be modified by adding extra plumbing fittings to extend the length of the wand). It is important that the plants be well-watered before harvest, to avoid detaching the plants from the soil. It is also important to hold the vacuum near the top of the canopy (Figure 2c) and stay away from the soil level, in order to minimize/prevent collection of fungal spores that might be present in the flat (fungal spores are about the same size as pollen grains). Maximum yield is obtained when pollen is vacuumed from open flowers. The best time of day should be determined empirically, given the growing conditions in the greenhouse or growth chamber.

We found that substantial amounts of pollen get trapped on the 35-micron mesh and that some adheres to the inside of the plumbing fittings. We tried using anti-static cloth or sprays inside the plumbing parts, but they did not help. However, a small paintbrush can be used to release residual pollen from the mesh and plumbing parts. For example, while the vacuum is applied, the 35-micron mesh is dabbed with the paintbrush to push residual pollen through onto the 6-micron mesh. Disassemble the wand in reverse order and examine the 6-micron mesh filter under a dissecting microscope to estimate yield and confirm purity. To remove collected pollen from the 6-micron mesh, cover the wide opening of a 200 μl pipette tip (where the micropipettor would seat) with a small section of 6-micron mesh. Insert the mesh-facing side of the pipette tip into the rubber tubing attached to the suction, such as that provided by a NALGENE® vacuum pump aspirator (Nalge Nunc International, Rochester, NY, USA) attached to a water faucet. To avoid loss of the mesh filter by suction into the vacuum, the filter can be secured to the pipette tip with a rubber band.

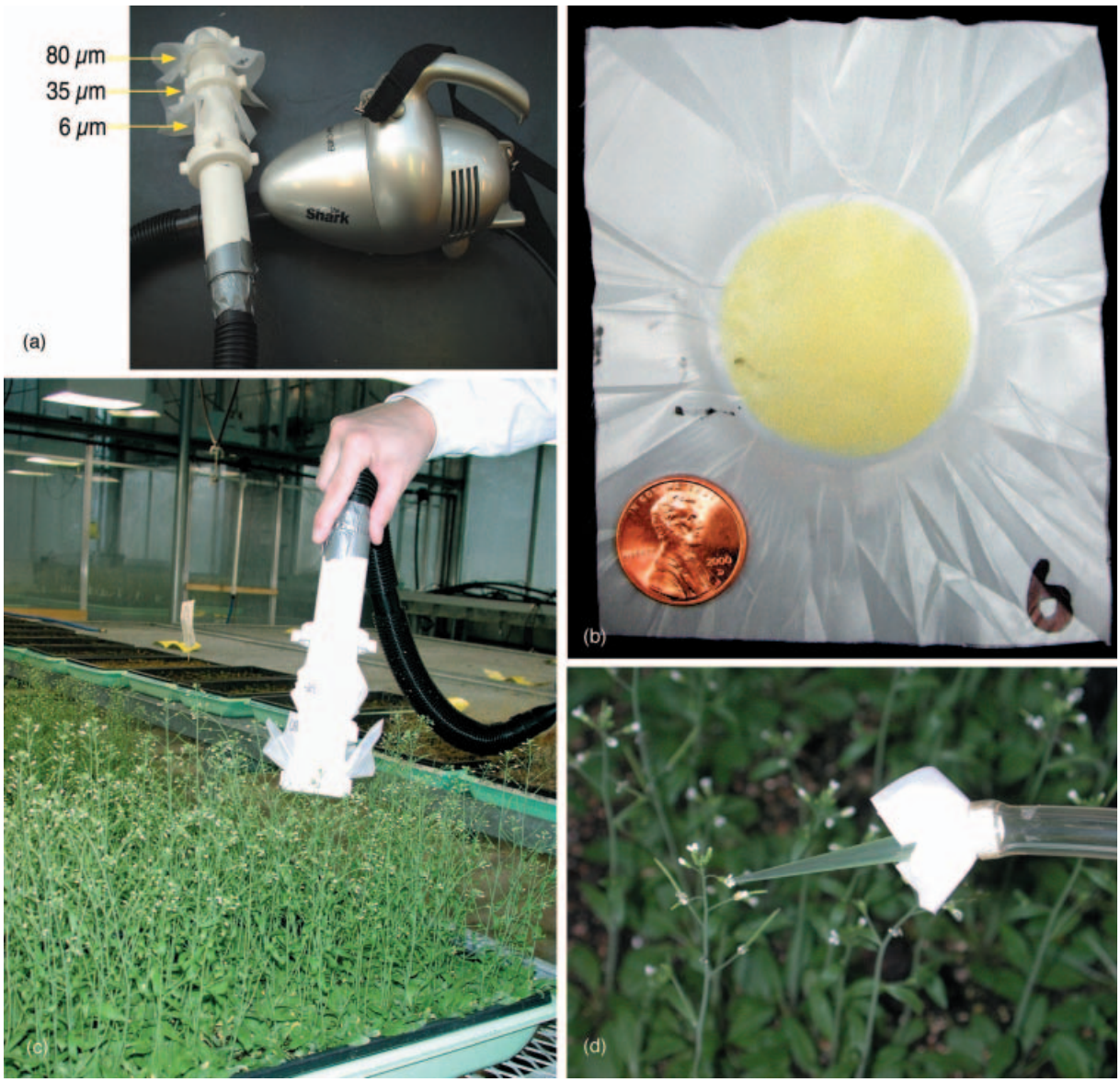


Figure 2. Harvesting *Arabidopsis* pollen with a vacuum cleaner.
(a) Vacuum with the filter attachment used to isolate pollen in bulk. The collection tube is constructed from plumbing parts that screw together, with interspersed mesh filters. The size of each mesh filter is indicated to the left. Unwanted plant tissue and debris collects on the 80- and 35-micron mesh filters. Pollen passes through these mesh filters and collects on the 6-micron mesh.
(b) Pollen obtained on the 6-micron mesh after vacuuming 10 flats. Penny placed in lower left for scale.
(c) Pollen is vacuumed from plants by passing the filter 'wand' over flowers.
(d) Isolating pollen from individual plants. A 6-micron mesh filter is fitted over the wide opening of a pipette tip (200 μl) and inserted into tubing connected to a water aspirator. A gentle vacuum is created by turning on the faucet, before placing the open end of the pipette tip near a flower.

Apply the vacuum and then vacuum the pollen from the larger 6-micron mesh. In order to transfer the pollen adhering to the inside of the pipette tip, successively clip the end of the tip and use the paintbrush to push pollen onto the small section of the 6-micron mesh.

To harvest pollen from individual flowers, use a pipette tip version of the vacuum as shown in Figure 2(d). After vacuuming, carefully disassemble the setup. To remove pollen from the filter used with the pipette tip setup, turn the pollen-containing side of the filter into a 1.5-ml microfuge tube and secure the mesh to the tube with a rubber band. Attach a pipette tip to the rubber tubing connected to a laboratory air supply. Turn on the air supply and adjust to a gentle air current, holding the pipette tip away from the filter until air is adjusted appropriately. To avoid having too strong of an air current and potentially blowing off the filter with the pollen on it, hold the pipette tip above the filter and then slowly bring it toward the filter. Apply the end of the

pipette tip to or near the filter and make several passes over the filter to ensure that all the pollen is transferred into the tube, lowering the air flow if necessary. Once the filter mesh is removed, the pollen can be pelleted by brief, low-speed centrifugation. The pelleted pollen can be used for immediate RNA or protein extraction, germination or microscopic examination. For RNA or protein isolation, store pelleted pollen at −80°C until needed. To store pollen for later *in vivo* or *in vitro* germination, Pickert (1988) added a drying agent to pollen and allowed the pollen to dry at room temperature before storing it at −20°C. When pollen was stored in this way for 10 months and then rehydrated (100% humidity at 24°C for 30 min) before use, it was reported that ~80% of the pollen germinated *in vitro* and was comparable with wild-type pollen when used for crosses (Pickert, 1988). If imaging and quantifying of pollen tube lengths is planned, a convenient feature of collection onto mesh is that the pollen can be transferred directly from the mesh onto solid germination medium. From a sparse monolayer (from vacuuming a few flowers; Figure 2d) pollen grains will form tubes that will be easily separated, instead of forming an overlapping, intertwined mass of tubes.

Genetic analyses

Determining transmission ratios

When the ratio of the expected progeny from a selfed pollination deviates significantly from the expected 1:2:1 Mendelian segregation ratio, it can be inferred that a mutant has impaired transmission (Howden *et al.*, 1998). Gametophytic mutations affect the haploid life cycle and thus it is typical for gametophytic mutations to show segregation distortion, that is, altered transmission ratios through male, female, or both. Accordingly, it can be difficult to obtain a homozygote, but gametophytic mutations can be maintained as heterozygotes (see Figure S2 for potential outcomes of crosses).

Many gametophytic mutants have been identified from segregation distortion screens, whereby altered transmission of a T-DNA-linked antibiotic resistance gene was noted first, and then the individual lines were examined for potential phenotypes (Bonhomme *et al.*, 1998; Howden *et al.*, 1998; Page and Grossniklaus, 2002). One such example is the *kinky pollen* mutant (Procissi *et al.*, 2003); the mutant pollen grains exhibit aberrant pollen tube growth, accounting for the altered transmission through the male parent. Another segregation distortion screen looked for EMS mutants with altered transmission of visible markers using a multiply-marked chromosome 1 (Grini *et al.*, 1999); mutants affecting the female, the male or both gametophytes were identified. Of the four male-specific mutants identified, three were arrested at different stages of development and one was defective in pollen tube growth. Still other gametophytic mutants exhibiting altered transmission have been identified by brute-force screens. For example, microscopic inspection of pollen from individual M_1 or M_2 plants yielded the gametophytic mutants *sidecar pollen* (Chen and McCormick, 1996), *raring-to-go* (Johnson and McCormick, 2001), *gemini pollen* (Park *et al.*, 1998; Twell *et al.*, 2002), *germ unit malformed* (*gum*) and *male unit displaced* (*mud*) (Lalanne and Twell, 2002). Analysis of T-DNA insertions in genes implicated in pollen development have also shown altered transmission through the male parent. For example, the *no pollen germination1* mutant, a T-DNA insertion in a gene that encodes a calmodulin-binding protein, appears normal at pollen maturity but the mutant pollen fails to germinate (Golovkin and Reddy, 2003). Occasionally mutants might exhibit perturbed transmission through the male, although they show no apparent defect in pollen tube growth, as was observed with a Rop GTPase mutant in maize (Arthur *et al.*, 2003). Some mutants with striking pollen phenotypes (*gum* and *mud*) exhibit little or no effect on transmission through the male (Lalanne and Twell, 2002).

It is important to test explicitly for gametophytic expression/function; it should not be inferred solely by observations of percentage affected pollen. For example, the *tardy asynchronous meiosis* mutant (Magnard *et al.*, 2001) was first identified in a screen to identify lines in which about 50% of the pollen appeared abnormal. However, further characterization of *tam* revealed that the primary defect occurred during meiosis and that *tam* is sporophytically-expressed. Mutants obtained from screens conducted to search for specific sporophytic phenotypes have also been shown to have gametophytic phenotypes. For example, *tip1* is a mutant that affects root hair morphology but also has defects in pollen tube growth (Schiefelbein *et al.*, 1993). Furthermore, from a screen to identify suppressors of the trichome branching mutant *zwi-3*, the *suz1 zwi-3* double mutant showed suppression of the trichome branching defect and showed very poor seed set (Krishnakumar and Oppenheimer, 1999), although the single mutants were completely fertile. Further analysis and reciprocal outcrosses of the *suz1 zwi-3* double mutants demonstrated that the defect was caused by aberrant pollen germination and tube growth.

Reciprocal crosses should be carried out when segregation distortion is observed in the selfed F_1 progeny, in order to determine if one or both parents are affected. The absence of a homozygous mutant cannot, in and of itself, be used to infer that the mutation is homozygous lethal. This should be concluded only if transmission through both the male and female is normal, yet the progeny of a selfed heterozygote yields no homozygotes (Figure S2c). To determine transmission of the mutation through the female, cross mutant plants as females to wild-type plants (Figure S2d). Transmission of the mutation through the female is unaffected if the segregation ratio for the F_1 progeny is 1:1. To test transmission of the mutation through the male, conduct the

reciprocal cross by pollinating wild-type females with pollen from mutant plants (Figure S2d). Transmission of the mutation through the male is affected if the segregation ratio is less than 1:1 (Figure 2Se,g). For example, when F_1 plants with the *rtg* phenotype (*rtg*/+) were allowed to self-pollinate, the F_2 plants segregated 1:1:0 (wild-type:*rtg* heterozygote:*rtg* homozygote), a significant deviation from the expected 1:2:1 ratio. Reciprocal crosses showed that female transmission of the mutant allele was normal, while crosses using *rtg*/+ as the male donor yielded only wild-type progeny (Johnson and McCormick, 2001). Transmission efficiency, or the percentage of gametes that can successfully transmit the mutant allele, can be calculated by determining the ratio of mutant to wild-type in the F_1 progeny and multiplying by 100. For example, the *gem1* mutant had 8.6% transmission efficiency through the male but 29.5% through the female (Park *et al.*, 1998).

It is advisable to perform more than one cross in each direction, and it may prove useful to keep track of the position of the seeds within the silique. For example, seeds in a particular region of the silique might be the only ones to have obtained the mutant allele from the male parent. The quickest growing pollen tubes generally fertilize the ovules closest to the stigma (Hülskamp *et al.*, 1995b), and pollen tubes arriving later have to grow further down the transmitting tract to other ovules. It is easy to plant seeds in order, by placing the silique (nearly mature but not yet shattered) on a piece of double-sided tape and removing the seeds from top to bottom, one by one.

Crosses with quartet *and* tetraspore

If a pollen mutant is outcrossed to wild-type and some of the F_1 progeny show the mutant pollen phenotype, this suggests that the mutation is either a gametophytic mutation or, less likely, a dominant sporophytic mutation. Because pollen is haploid, it is not easy to determine if a gametophytic mutation is dominant or recessive. One way to test whether a mutation is dominant or recessive is to cross the mutant female with diploid pollen from a tetraploid plant. However, the resulting F_1 progeny are triploid. Pollen from triploid plants typically exhibits pollen abortion that might confound reliable scoring of the pollen phenotype being tested. Luckily, in *Arabidopsis*, two mutants, *quartet* (Copenhaver

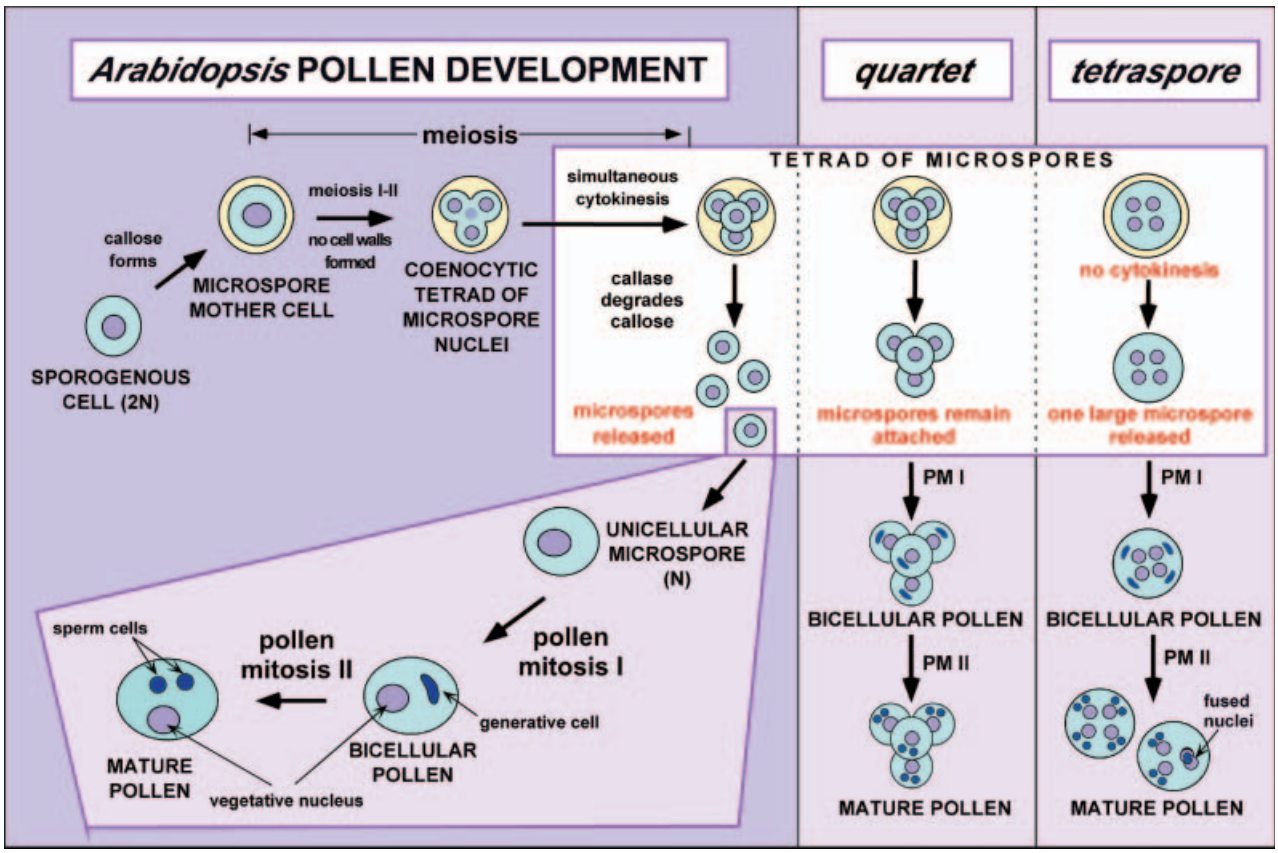


Figure 3. *Arabidopsis* pollen development in wild-type, *quartet,* and *tetraspore* mutants.
The white box highlights the developmental differences between wild-type and the *quartet* and *tetraspore* mutants. In *quartet,* microspores remain attached after the tetrad stage but otherwise complete development normally. In *tetraspore,* simultaneous cytokinesis is inhibited at meiosis II so that a large, multinucleate microspore is formed; subsequent nuclear divisions occur but in some cases polyploid sperm form by nuclear fusion. Such sperm give rise to polyploid progeny or fail to fertilize, partially accounting for the poor seed set of *tetraspore* plants.

et al., 2000; Preuss *et al.*, 1994) and *tetraspore* (Spielman *et al.*, 1997; Yang *et al.*, 2003), make it possible to answer these questions. The stages of pollen development in wild-type are shown in Figure 3, left panel. Differences from the normal development pathway occur in the mutants.

To test whether observed pollen phenotypes result from a gametophytic mutation or dominant sporophytic mutation, tetrad analysis is performed using *quartet1 (qrt1)*, a sporophytic recessive mutation that keeps all the products of a single meiosis together throughout pollen development (Preuss *et al.*, 1994). If a mutation is sporophytically expressed but has low expressivity, the numbers of normal and affected pollen might vary in a given quartet. However, in a plant that is heterozygous for the pollen mutant being tested, the ratio of normal to affected pollen in each quartet should be 2:2 if the mutation is gametophytic. To perform pollen tetrad analysis, *mutant*/+ plants are crossed as females to homozygous *quartet* plants. Those F_1 progeny with the mutant phenotype are selfed, and the F_2 progeny is scored to identify the *m*/+; *qrt1/qrt1* double mutants. Figure 4 illustrates the crossing scheme, and illustrates the phenotypes of three mutants that were confirmed to be gametophytic by such crosses. There can be differences in the expressivity of pollen mutants in different genetic backgrounds. For example, the *sidecar pollen* mutant (in No-0) exhibited a more severe phenotype (~50% dead pollen) after crossing into the Columbia *quartet1* background (Chen and McCormick, 1996). It might therefore be advisable to perform crosses to *quartet* in different ecotypes if the mutant background ecotype is different from the ecotypes available for *quartet* stocks. The *quartet1* mutant is available from the *Arabidopsis* stock center in the Landsberg *erecta* and Columbia ecotypes; the recently described *quartet3* mutant (Rhee *et al.*, 2003) might prove useful for crosses to mutants in the WS or RLD ecotypes.

As an alternative to crosses with tetraploid plants, pollen mutants can be crossed (Figure 5) with a sporophytically

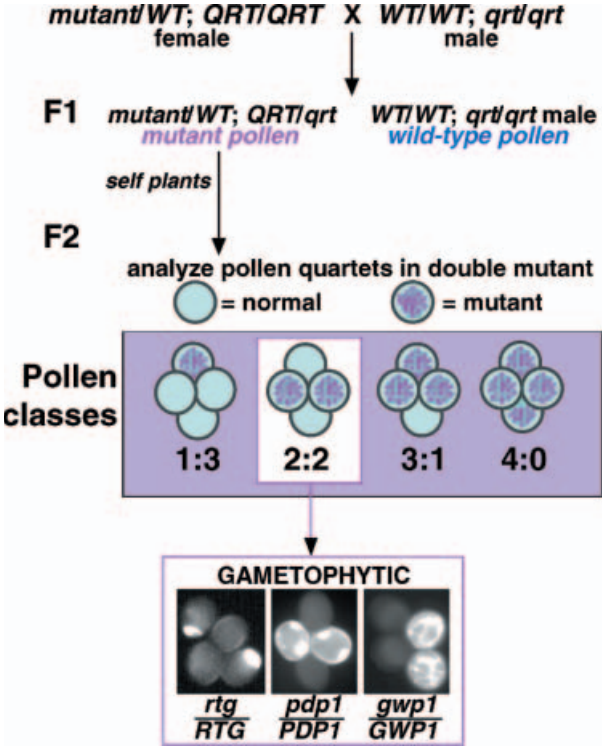


Figure 4. Crossing scheme used to determine if a pollen mutant is gametophytic.
Plants heterozygous for a pollen mutation are crossed as female to plants homozygous for *quartet*. F1 progeny exhibiting mutant pollen are self-pollinated and the F2 progeny are screened for double mutants. The pollen mutation is gametophytic if most quartets in the double mutant show a ratio of two affected: two normal pollen grains. Three examples of gametophytic mutants in the *quartet* background are shown: *rtg* = *raring-to-go*; *pdp1* = *polka dot pollen 1*; *gwp1* = *gift-wrapped pollen 1*. If, by contrast, the double mutant plants exhibit variable distributions of affected pollen (as shown within the purple box), the mutant likely represents a sporophytic mutant. If the pollen mutant can be maintained as a homozygote and the mutant exhibits strong expressivity, crosses to *quartet* will yield 4:0 ratios (*mutant*/*mutant*;*qrt*/*qrt*; all pollen grains affected). *mutant* = mutant gene affecting pollen, *WT* = wild-type gene, *QRT* = wild-type, *qrt* = *quartet* mutant.

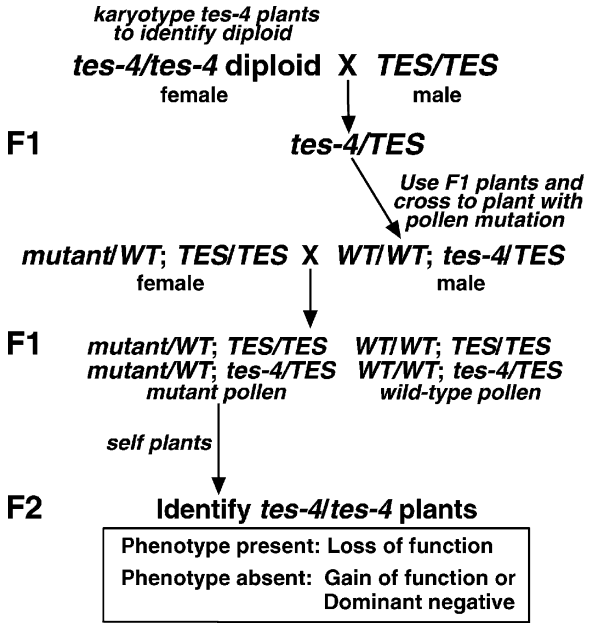


Figure 5. Crossing scheme used to determine if a pollen mutant is a loss-of-function mutant, or a gain-of-function or dominant negative mutant.
Karyotype *tes-4/tes-4* plants to identify diploid plants for use in crosses. Cross *tes-4/tes-4* diploids as female to wild-type *Arabidopsis* to generate F1 progeny that are diploid *tes-4/TES*. These *tes-4/TES* heterozygotes are then crossed, as male, to plants heterozygous for a given pollen mutation. From this second cross, identify those F1 progeny that have mutant pollen and self all of these plants. Identify the *tes-4/tes-4* plants (large pollen, low seed set) among the F2 progeny. Score these *tes-4/tes-4* plants for presence of the pollen mutant phenotype. If the pollen mutant phenotype is observable in the large pollen, then the pollen mutation is likely a gain-of-function or dominant negative, because the presence of the wild-type allele in the common cytoplasm is not able to block the phenotype. If no *tes-4/tes-4* plants exhibit the pollen mutation, then the wild-type allele present in the common cytoplasm rescued the pollen defect and the pollen mutation is likely a loss-of-function mutant. *tes-4* = *tetraspore-4* allele, large pollen that lacks internal cell walls, *TES* = wild-type, *mutant* = mutant gene affecting pollen, *WT* = wild-type.

acting, recessive mutant called *tetraspore* (*tes*) (Spielman *et al.*, 1997). The *stud std)* mutant (Hülskamp *et al.*, 1997) has the same phenotype as *tes* and was later shown to be allelic to *tes* (Yang *et al.*, 2003). Because *tes* and *stud* meiocytes do not undergo cytokinesis after pollen meiosis II, all the nuclei remain in a common cytoplasm. In *mutant*/+; *tes*/*tes* plants, both the mutant pollen allele and the wild-type allele will be present in large pollen grains. If there is no pollen phenotype, then the pollen mutant being tested is a loss-of-function allele. However, if the mutant pollen phenotype is observed, then the wild-type allele was not able to rescue the mutant phenotype, and the mutation is either a dominant negative or gain-of-function allele. The weak *tes-2* allele produces incomplete callose intersporal walls within the large pollen grain (Spielman *et al.*, 1997) and therefore is not suitable for these crosses. In *tes* pollen, nuclear fusion sometimes occurs, resulting in aneuploid sperm and reduced fertility (Spielman *et al.*, 1997). For this reason, *tes* should be karyotyped in order to identify diploid plants for crosses. To karyotype plants, fix anther filaments (Ross *et al.*, 1996), stain with DAPI, gently squash tissue on a microscope slide, and count chromosomes in the elongated cells of the filaments.

Before using transformation to confirm whether a lesion in a candidate gene is responsible for the mutant phenotype, it is useful to determine whether the pollen mutant of interest is a loss-of-function, dominant negative, or gain-of-function mutant. Candidate genes are transformed into either wild-type or mutant plants via *Agrobacterium tumefaciens* (Clough and Bent, 1998). For loss-of-function mutants, wild-type is transformed into the mutant background. If the phenotype is rescued in these transgenic plants, it confirms that the identified gene is responsible for the mutation. For gain-of-function or dominant negative mutants, the mutant allele is transformed into wild-type. Transgenic plants surviving selection would be expected to show the mutant phenotype in pollen. Some transformation constructs use genomic DNA and therefore the endogenous promoter drives gene expression; if cDNAs are being tested for complementation, it is advisable to use a pollen-specific promoter, such as Lat52p (Twell *et al.*, 1990) to drive expression, because the commonly used 35S promoter is poorly expressed in pollen (McCormick *et al.*, 1991).

Obtaining homozygous lines of gametophytic mutants

Homozygous lines for gametophytic mutants are desirable. Detailed microscopic analysis and pollen germination assays are easier when the pollen genotype of a plant is uniform. Homozygous lines are needed to determine whether a gametophytic mutation also confers a sporophytic phenotype. Screens for suppressors of a gametophytic mutation are feasible if a homozygous line is available, but such screens are difficult in a heterozygous background, because it is not straightforward to distinguish pollen grains carrying a putative suppressor of a phenotype from the wild-type pollen grains already present in the heterozygous plant.

Although pollen mutations generally exhibit segregation distortion, some pollen mutations (e.g. *gum* and *mud*; Lalanne and Twell, 2002) are relatively easy to obtain as homozygous lines, while a few others transmit through the male, albeit at a low frequency. For example, the gametophytic mutant *sidecar pollen* transmits through the male around 5% of the time (Chen and McCormick, 1996). To determine whether a pollen mutation can transmit through the male at a rare frequency, score several hundred progeny from a selfed mutant plant. If the pollen mutant has a variable phenotype, it may be possible to identify plants that, on average, produce mutant pollen with a weaker phenotype. Such plants can be selfed and progeny examined for possible homozygotes.

If mutant pollen is viable and can grow a pollen tube, but cannot compete with wild-type pollen to fertilize ovules, it may still be possible to recover homozygotes from sparse pollinations, by controlling the amount of wild-type pollen that is present in the cross. Using the *quartet* mutant can be useful in this respect, because each tetrad has only two wild-type pollen grains. Cross an individual or very few pollen quartets from a *mutant*/+; *qrt*/*qrt* plant onto a female that is heterozygous for the pollen mutant (*mutant*/+). To isolate individual quartets for crosses, dab dehiscent pollen onto a glass slide. To lift individual quartets, briefly dip the end of a forceps or hypodermic needle into water, blot to remove excess, then touch the forceps or needle to individual quartets and transfer to the stigma surface of an emasculated mutant flower.

Phenotypic characterizations

Histochemical analysis of mutant phenotypes

Microgametogenesis is characterized by distinct developmental stages (Figure 3; reviewed in McCormick, 2004). A general method to determine the stage first affected in a mutant is to sort flower buds by size (Piffanelli *et al.*, 1998), dissect anthers from the flowers and examine different developmental stages, using histochemical staining. A table in Regan and Moffatt (1990) lists the appropriate concentrations for histochemical stains frequently used to analyze the cellular components of pollen grains. Two of the most widely used stains are 4′,6-diamino-2-phenylindole (DAPI) (Molecular Probes, Portland, OR, USA), which stains DNA (Coleman and Goff, 1985), and decolorized aniline blue (DAB), which contains a fluorochrome (Sirofluor) that specifically binds to β-1,3-glucan (Evans and Hoyne, 1984), a major component of the pollen tube wall. Visualization of these dyes requires a fluorescence microscope. Solutions of DAPI and DAB should be stored in the dark.

A typical DAPI stock solution at 1 mg ml^{-1} in distilled water is made and stored at 4°C. A DAPI working solution at 1 μg ml^{-1} (in distilled water) will stain nuclei; at a lower concentration (0.25–5.0 μg ml^{-1} in distilled water) DAPI can be added to pollen germination medium to stain nuclei during pollen tube growth (Coleman and Goff, 1985). A stock solution of 0.1% DAB is prepared by decolorizing water-soluble aniline blue (Fisher Scientific) in aqueous phosphate (0.067–0.1 M K_3PO_4, or K_2HPO_4, or a mixture of both) (Currier and Strugger, 1956; Martin, 1958). The solution is initially dark blue or purple but will eventually turn pale yellow as it decolorizes. If a blue precipitate forms after addition to the sample, the pH of DAB can be adjusted (more basic): a small amount (a microliter at a time) of phosphate buffer at a higher pH can be applied to the sample; this dilution will not substantially affect the fluorescence intensity. The pH of the phosphate buffer used varies in the literature, but in general the higher the pH (up to 11.5), the more intense the fluorescence (Smith and McCully, 1978). Make a working solution of DAB within the range of 0.005–0.1% by diluting the stock with 0.067–0.1 M phosphate buffer. If the fluorescence is too intense, the DAB concentration can be reduced *in situ* by flooding the sample with phosphate buffer and carefully wicking away with a tissue along the edge of the well. This is easily performed with fixed sections as they can be completely rinsed and the aniline blue reapplied. Sirofluor can be used for vital staining at neutral pH (Hough *et al.*, 1985).

For light microscopy, Alexander's (1969) stain is a reliable way to score pollen viability; grains that are viable will stain a dark blue or purple, grains that are dead will stain pale turquoise blue. The stock solution for Alexander's stain is: 10 ml 95% ethanol, 5 ml 1% malachite green in 95% ethanol, 5 g of phenol, 5 ml 1% acid fuschin in H_2O, 0.5 ml 1% orange G in H_2O, 2 ml glacial acetic acid, 25 ml glycerol and 50 ml H_2O; the stock solution should be stored in the dark at room temperature. For a working solution, dilute 1:50 in H_2O. Note that the original recipe for this stain includes chloral hydrate (a controlled substance in the USA). For viability staining, chloral hydrate can be omitted. Alexander's stain is not a vital stain. For fluorescence microscopy, fluorescent proteins are increasingly being used to image pollen and pollen germination (Cheung, 2001; Faure *et al.*, 2002). Note that *Arabidopsis* pollen that is not fully hydrated has substantial autofluorescence in the GFP channel, and transgenic pollen will not easily be distinguished from wild-type pollen, unless the promoter used to drive GFP is strong (e.g. LAT52; Twell *et al.*, 1990).

The penetration of some histochemical stains into pollen can be inhibited by the anther wall or the pollen exine, and longer incubation times may be required for certain stains at certain developmental stages. For example, penetration of DAPI into mature dehiscent pollen typically takes longer than penetration into pollen grains from earlier developmental stages; in that event, slides can be prepared and held at 4°C (to maintain humidity and prevent drying out) until the staining intensity is sufficient. The most consistent results are obtained with pollen from mature but not yet dehiscent anthers (from flowers with white petals). DAPI fluorescence is sometimes obscured during the transition from unicellular to bicellular development, by autofluorescence from the newly forming microspore wall. If DAPI staining is faint, it is not a good idea to increase the concentration, because higher concentrations result in intense yellow fluorescence. This yellow fluorescence can also occur if flower samples are repeatedly screened, because the DAPI concentration builds up; to rehydrate samples that have dried out, add H_2O rather than extra aliquots of DAPI. Some water-soluble chemicals can be delivered to developing inflorescences, as described in detail in Magnard *et al.* (2001) and in Johnson and McCormick (2001). Meiosis and pollen development proceed relatively normally, and seeds set.

Some problems associated with penetration of histochemical stains into pollen can be overcome by fixing and sectioning anthers. Fixation of anthers or flowers (Ross *et al.*, 1996) is improved by applying a vacuum during fixation to ensure penetration through the anther wall. Vacuum infiltration is also useful for infiltrating resin, because incomplete penetration often results in sections that lack or have few pollen grains. For a thorough discussion on how to prepare flower tissues for sectioning, consult other sources, such as Ruzin (1999).

Phenotypic variability and statistical analysis

Pollen mutants that are the outcome of a lesion in a single gene can exhibit variable phenotypes, in the same anther, the same flower, or on different flowers of one plant. For example, the *raring-to-go* pollen mutant has three classes of pollen grains: aborted, *rtg* (premature pollen tubes) and normal pollen (Johnson and McCormick, 2001). Analysis of the mutant plants throughout pollen development, as well as tetrad analysis, revealed that some pollen grains first showed signs of the *rtg* phenotype and then aborted later during development. Thus in *rtg*/+ plants, the aborted and *rtg* pollen grains represented the phenotypic range for the affected pollen, and together comprised about 50% of the pollen grains.

To determine the range of phenotypes of affected pollen as well as to determine the percentage of affected and normal pollen, it is important to confirm that variability is not related to additional mutations present in the mutant background. Although analysis of primary mutants (T-DNA insert lines or M1 plants) is useful, mutant lines should be crossed to wild-type, in order to clean up the background, before carrying out detailed characterizations of possible phenotypic variability. To rule out developmental differences caused by anther location within the flower, the four

tall anthers can be separated from the two short anthers during dissection and observed separately.

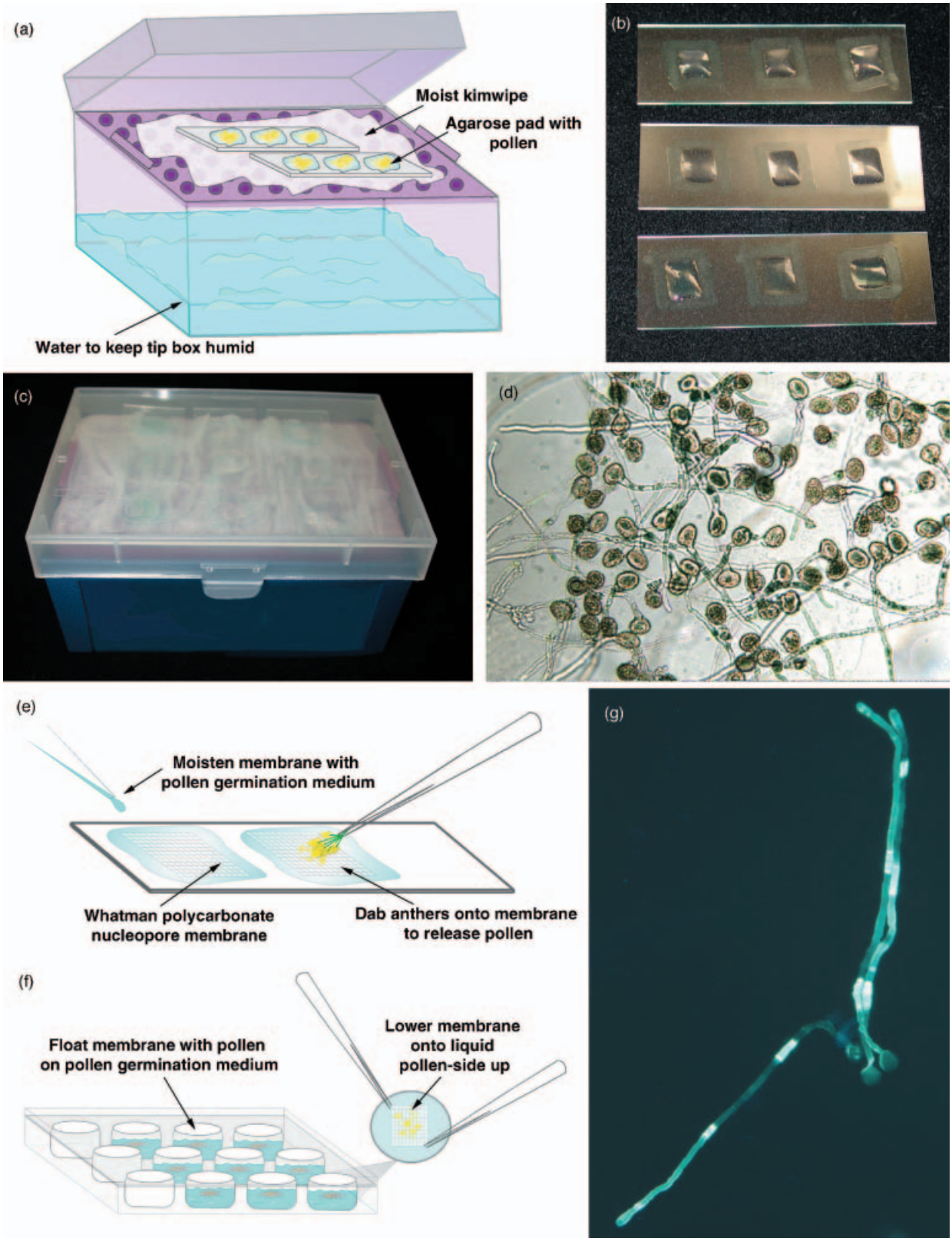
Pollen counts are used to determine the percentages of affected pollen and normal pollen. To conduct pollen counts, individual pollen grains from a given flower or anther are scored as affected or normal. Some pollen mutants (Chen and McCormick, 1996; Park *et al.*, 1998) have been shown to have reduced expressivity of the mutant phenotype: that is, pollen grains carrying the mutant allele do not exhibit the mutant phenotype. Accordingly, a large sample size is needed for statistical analysis. Conducting pollen counts from one mutant plant or from just a few flowers will not provide adequate data. Pollen counts for multiple flowers from an individual plant will give a single plant mean; for the population mean pollen from many mutant plants should be counted. To reduce the time on the microscope required for pollen counts from large numbers of flowers, images of multiple pollen fields from each collected flower can be captured digitally and scored later, to identify affected and normal pollen.

Pollen germination

Arabidopsis has dry stigma-type flowers. *In vivo*, it is known that the pollen coat is important in adhesion (Zinkl *et al.*, 1999) and that it facilitates pollen hydration for germination (Hülskamp *et al.*, 1995a; Mariani and Wolters-Arts, 2000; Preuss *et al.*, 1993). *In vitro* germination presumably does not fully mimic the *in vivo* pollen–pistil interaction. Mutations that cause defects in pollen germination are some of the most desired, but for the novice, *in vitro* pollen germination assays are frustrating and problematic. Sometimes the picture is optimistic – *in vitro* pollen germination ranged from 60 to 75% in the ecotypes Ler and WS (Fan *et al.*, 2001; Golovkin and Reddy, 2003; Mouline *et al.*, 2002) to nearly 100% in ecotype Columbia (Thorsness *et al.*, 1993). Azarov *et al.* (1990) tested 10 *Arabidopsis* ecotypes, including Ler and Columbia; germination ranged from 76.6 to 95.8%, depending on the ecotype. However, Scholz-Starke *et al.* (2003) used the same medium as Fan *et al.* (2001) and reported only 7% germination with the Colombia ecotype, whereas the C24 ecotype showed 70–85% germination. Some days nothing will germinate, even if it appears that conditions are identical to the day before, when germination was excellent. As a result of such potential variations in ecotype or the conditions used for plant growth, germination medium will need to be optimized. For example, Fan *et al.* (2001) tested many parameters and found that, for them, optimizing the potassium concentration in the medium, and correcting the pH was important – the pH of water can vary and as a first step can be checked and buffered, if germination percentages are suboptimal. We advise starting with a simple medium (e.g. Li *et al.*, 1999; Thorsness *et al.*, 1993), and modifying it as necessary. Each experimenter should optimize the protocol for themselves; although initial success rates differ widely, even in our laboratory, they improve with practice.

We recommend germination on solid medium or on nylon membranes that are floated on liquid medium, following the protocols illustrated in Figure 6, because we think maintaining humidity is critical; for example, we noticed that pollen does not hydrate fully if the agarose pad is too firm. With these protocols (Figure 6), using the Thorsness *et al.* (1993) germination medium and Columbia pollen, we routinely obtain ~70% germination, and occasionally nearly 100% germination (Figure 6d). Li *et al.* (1999) recommended drying flowers for 2 h before dabbing pollen onto 0.5% agarose germination pads. We tried this and their medium recipe with Columbia pollen and obtained ~75% germination. Other protocols have been used; for example, Derksen *et al.* (2002) achieved reasonable germination percentages (~50%) when pollen was applied to dialysis membranes resting on top of semi-solid medium. Hicks *et al.* (2004) germinated pollen in 30 μl drops of liquid germination medium on coated microscope slides. It is important to experiment with pollen from multiple flowers and to test a protocol on several different days. We tested different pollen from different flowers on agarose pads prepared with either the Thorsness *et al.* (1993) or the Li *et al.* (1999) recipes; on occasion pollen from a given flower burst instead of germinating while pollen from other flowers tested at the same time with the same medium germinated well. Prehydrating the pollen before applying it to germination medium may prevent bursting and also was shown to be critical for germinating pollen previously stored at −20°C (Pickert, 1988). We have found that it is better to use a dissecting microscope, rather than magnifying eyeglasses or the naked eye, when applying pollen onto agarose germination pads or membrane filters. By looking through the microscope, you can avoid inadvertent problems. For example, we noticed that pressing the anther too far into the agarose germination pad reduced germination efficiency. Also, if the pollen is applied in a clump (pollen on top of other pollen) rather than a monolayer, access to the germination medium or liquid might be restricted and this could result in reduced germination efficiency. Placing other portions of the flower in the germination medium can sometimes improve pollen germination percentages (Derksen *et al.*, 2002; Ryan *et al.*, 1998). To our knowledge this anecdotal effect has not been experimentally analyzed, but might be because of slight changes in the ion concentration or pH of the medium, or to the release of chemoattractants, as recently described in lily (Kim *et al.*, 2003).

Low germination efficiency can be problematic if the desire is to assess whether a mutant gene is affecting pollen germination. *Arabidopsis* flowers have two types of anthers (Figure 1a), so it is possible that slight maturity differences could fool a researcher into thinking that 50% of the pollen is affected, if pooled pollen is collected and then germinated. If


(a)
Moist kimwipe
Agarose pad with pollen
Water to keep tip box humid
(b)
(c)
(d)
(e)
Moisten membrane with pollen germination medium
Whatman polycarbonate nucleopore membrane
Dab anthers onto membrane to release pollen
(f)
Float membrane with pollen on pollen germination medium
Lower membrane onto liquid pollen-side up
(g)

using pollen that was harvested in bulk, first assay a subset of the pollen for viability. Pollen viability was found to closely correlate with the % of pollen germinated *in vitro* (Pickert, 1988), but the amount of viable pollen was always slightly higher than that which germinated. Hülskamp *et al.* (1995a) reported that flowers from the same inflorescence varied in germination percentage with at least one flower producing pollen that had a significant level of germination (10–30%) while the other flowers had none or barely germinated. We have found that pollen germination frequency can vary widely, even among anthers from a single flower. A possible explanation for these observations is that trinucleate pollen loses viability quickly and that some flowers in an inflorescence are past their prime; *Arabidopsis* pollen reportedly has longevity of less than 3 days (Pickert, 1988). Yamamoto *et al.* (2003) postulated that dehiscent pollen may autolyze storage compounds necessary for pollen germination, so that older pollen would be less likely to germinate. Pickert (1988) reported that the best germination rates were obtained with pollen from flowers where the long anthers extend above the stigma (Muller's B4 stage; comparable with stage 14, Smyth *et al.*, 1990). Figure 5 of Kandasamy *et al.* (1994) illustrates subtle differences in bud size that correlate with slight maturation differences in tricellular pollen grains. To ensure that fresh pollen is harvested, remove open flowers from the plants to be assayed a day before, and collect pollen from freshly opened flowers the next day. Alternatively, harvest mature flowers with non-dehiscent anthers and allow the flowers to dry on a glass slide until the anthers dehisce.

Given the variability in pollen germination, the best option to evaluate whether a mutant gene is affecting pollen germination is to examine pollen germination of the mutant in the *quartet* background. Even if the overall germination percentages are poor, if tubes from two pollen grains of each tetrad appear normal while two appear aberrant, then the conclusion that the mutant gene affects pollen germination is robust. For example, Golovkin and Reddy (2003) showed that in *qrt1/qrt1* plants that were otherwise wild-type, three or four of the pollen grains in a quartet had tubes (although the overall pollen germination rate was poor – only one in five quartets formed pollen tubes). However, in the mutant they were characterizing (*no pollen germination 1*), only one or two pollen grains in a quartet germinated. Testing germination percentages of a pollen mutant in the *quartet* background requires two rounds of crossing. An alternative approach is to examine pollen germination frequency of pollen released from a single anther. This is easily performed by splaying flowers with dehiscent anthers (Figure 1) and dabbing the anthers onto pollen germination medium; the pollen from each anther will be deposited in separate areas of the medium. A valid assessment of germination frequency can be obtained in those anthers whose pollen shows a high percentage of germination. Even from bulked pollen, it is possible to evaluate whether a mutation significantly affects pollen tube growth, but independent experiments (different days) and assays of large numbers of pollen grains are required to demonstrate reproducibility. Programs such as NIH image (http://rsb.info.nih.gov/nih-image) can be used to measure pollen tube lengths and facilitate statistical analyses.

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Supplementary materials

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2147/TPJ2147sm.htm:

Figure S1. Assembly of pollen 'wand'.

Drape an 80-micron mesh filter over the left opening of part (2). Secure filter by threading on part (1) to part (2). Thread part (3) to the right-side of part (2). Once part (3) has been threaded, drape the 35-micron mesh filter over the left opening of part (4). Secure the filter by inserting the filter covered left-side of part (4) into the right opening of part (2) until firmly in place. Remove the black rubber

Figure 6. Pollen germination protocols.

(a) Cartoon showing set-up for pollen germination. Pipette tip box with water in the bottom and a moistened kimwipe on the platform maintains humidity. Glass slides with agarose pads are dabbed with anthers to release pollen, and the slides are placed on top of the moistened kimwipes. The lid is closed and the box is incubated at 28°C.

(b) Glass slides with pollen germination medium solidified with 1% low-melt agarose.

(c) Humid chamber set-up.

(d) Pollen (Columbia-O) after 4 h germination on an agarose pad prepared with the Thorsness *et al.* (1993) germination medium.

(e) Cartoon showing preparation of Whatman™ polycarbonate nucleopore membrane (pore size 0.4 micron) for pollen germination.

(f) Cartoon showing placement of polycarbonate nucleopore membranes with pollen onto liquid pollen germination medium in 12-well microtiter plates.

(g) Columbia pollen tubes after germination on a black polycarbonate nucleopore membrane (0.4 micron pore), using Thorsness *et al.* (1993) germination medium. Pollen tubes were stained with decolorized aniline blue (DAB). Black membranes do not interfere with fluorescence microscopy. Note that the tubes grow in one plane. To eliminate air bubbles, the membrane was lifted off the surface of the germination medium and inverted (pollen side down) onto a 5-μl droplet of DAB on a cover glass. Then the cover glass was turned over and, using forceps to control the speed, was lowered at an angle onto a standard glass slide onto which a 10-μl drop of DAB was dotted. It is important to rest the edge of the cover glass in the DAB drop before lowering the cover glass.

gasket from part (5) and slide part (6) over part (5) as shown. Drape the 6-micron mesh filter over the left opening of part (5), secure with the black rubber gasket. Insert the mesh-covered part (5) into the right opening of part (4) until firmly in place. Make sure the black rubber gasket is at the right edge of part (4). Slide part (6) onto the right-side on part (4) and align the pins of part (4) to the locking screw of part (6). Twist part (6) to lock. Note: Duct tape was used to secure part (5) to the vacuum hose. Both parts (4) and (5) were cut to the length dimensions shown.

Figure S2. Schematic illustrating transmission of a *mutant* allele through the male or female gametes.

(a–c) Potential segregation patterns for selfed progeny from a heterozygous mutant plant. (a) Normal transmission of the mutant allele through the female and male parent. (b) Example of impaired transmission of the mutant allele through the male parent. To test whether impaired transmission is through the male or female or both, perform reciprocal crosses (see below). Even if impaired transmission through the male (or female) is confirmed, the mutant allele may transmit rarely through that parent. Large numbers (maybe on the order of hundreds) of F_2 progeny can be scored to identify rare homozygotes. (c) The homozygous mutant is lethal.

(d–g) Reciprocal crosses to determine if transmission is affected in either parent. (d) Unaffected transmission of the mutant allele through the female parent yields the expected ratio of one wild-type:one mutant in the F_1 progeny. (e) Lack of transmission of the mutant allele through the male parent yields 100% wild-type F_1 progeny. (f, g) Impaired transmission of the mutant allele through the female parent (f) or the male parent (g) yields a larger number of wild-type than mutant F_1 progeny. +, Wild-type allele; m, mutant allele; ♀, female parent; ♂, male parent; ⊠, failed transmission of mutant allele.

Table S1 Detailed supplier information for tools and reagents

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