Chapter 8

Workflow to Characterize Mutants with Reproductive Defects

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Abstract

Reverse genetics approaches for characterizing phenotypes of mutants in a gene of interest (GOI) require thorough genotyping and phenotypic analysis. However, special challenges are encountered when a GOI is expressed in reproductive tissues: a variety of assays are required to characterize the phenotype and a mutant may show sporophytic and/or gametophytic defects in male and/or female reproductive tissues, which are structurally and functionally intertwined. Here, we present a streamlined workflow to characterize mutants with reproductive defects, primarily using Arabidopsis as a model, which can also be adapted to characterize mutants in other flowering plants. Procedures described here can be used to distinguish different kinds of reproductive defects and pinpoint the defective reproductive step(s) in a mutant. Although our procedures emphasize the characterization of mutants with male reproductive defects, they can nevertheless be used to identify female reproductive defects, as those defects could manifest alongside, and sometimes require, male reproductive tissues.

Key words Seed set, Male gametophyte and sporophyte, Transmission efficiency, Pollen, Pollen tube, Pistil, Ovules, Pollen tube–pistil interactions, Aniline blue staining, Transmission efficiency, Fertility

1 Introduction

The rise of comparative genomics and next-generation sequencing technologies have increased the need for reverse genetics approaches to characterize the function of a gene of interest (GOI). In angiosperms, reproductive mutants can be challenging to characterize, as a flower is comprised of four different types of tissues: female sporophyte, female gametophyte, male sporophyte, and male gametophyte [1–3]. Resolving the function of a GOI in reproduction requires multiple analyses, including expression and mutant analysis. Cell-specific expression datasets are beginning to be beneficial to confirm expression of a GOI [4–8]; however, mutant analysis is still required to determine the role of the gene in those cells during sexual reproduction of angiosperms.

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Here, we created a pipeline of experiments that can be used to begin analyzing mutants with reproductive defects. We start with identification of GOIs functioning in reproduction and obtaining mutants in Subheading 3.1. In Subheading 3.2, we describe how to score seed set in *Arabidopsis thaliana* siliques and explain in Subheading 3.3, how that assay can be used to examine a series of crosses to determine if the mutation is primarily affecting the male and/or female sporophyte and male and/or female gametophyte. In the final two sections, we describe assays that could be used to follow-up on the results from Subheading 3.3. In Subheading 3.4, we describe using transmission efficiency, where the transmission of the transgene is analyzed in the progeny, to confirm the gametophytic defects caused by the mutation. In the final Subheading 3.5, we describe using aniline blue staining to identify which post-pollination step is defective in the mutant of a GOI. In summary, the methods described here can be used to characterize reproductive mutants and gain insights into the reproductive function of a GOI.

2 Materials

2.1 Seed Set Analysis

Materials and Equipment

1. Microscope slides (75 × 25 × 1 mm).
2. Forceps.
3. Scissors.
4. Stereoscope with a bright light source.
5. Double-sided tape (¾”).
6. Syringe needles (27 G).
7. 1 mL syringe.

2.2 Transmission efficiency

Materials and equipment

1. Microscope slides (75 × 25 × 1 mm).
2. Forceps.
3. Scissors.
4. Stereoscope with a bright light source.
5. Double-sided tape (¾″).
6. 1.5 mL microcentrifuge tubes and microcentrifuge tube rack.
7. Gas sterilization chamber.
8. 100 mL graduated cylinder.
9. 250 mL beaker.
10. Petri dishes (100 × 15 mm).

11. Growth chamber for growing Arabidopsis seedlings on plates.

Solutions

1. Gas sterilization solution (to be prepared right before sterilization and can be reused for 2 weeks):
   (a) To gas sterilize seeds, place open tubes of seeds labeled with pencil (not with a permanent marker) in a glass sterilization chamber.
   (b) Place a beaker with 95 mL of bleach in the chamber.
   (c) Carefully add 5 mL of 75% hydrochloric acid to the beaker, then immediately close the lid of the sterilization chamber.
   (d) Remove seeds from sterilization chamber after 3–5 h. Seeds are now ready to be plated. Left over seeds can be stored in a refrigerator for future use.
2. Murashige and Skoog (MS) plates with appropriate antibiotic:
   (a) In a flask, at least twice the volume of the media, add the following: 1/2× strength MS Salt Bases, 2% sucrose, 0.9% Bacto Agar, and 0.05% MES hydrate.
   (b) Dissolve in dH_2O, then adjust pH to 5.8.
   (c) Autoclave solution.
   (d) Before pouring solution into petri dish plates, add appropriate amount of antibiotic to the MS agar and swirl to mix.
   (e) Plates could be used immediately or stored up to 3–6 months at 4 °C.

2.3 Aniline Blue Staining

Materials and Equipment

1. Forceps.
2. Stereoscope with a bright light source.
3. Scissors.
4. 96-well plates, flat bottomed, ≥200 μL.
5. Plastic wrap or parafilm.
6. Resealable plastic bag (quart-sized).
7. Multi-channel pipette or single channel pipette.
8. 200 μL pipette tips.
9. Microscope slides (75 × 25 × 1 mm).
10. Coverslips (18 × 18 mm, #1.5).
11. Syringe needles (27 G).
12. 1 mL syringe.

13. Clear nail polish; we recommend "Hard As Nails" by "Sally Hansen," which does not react with mounting solution, form precipitates, or interfere with observation or staining.

Solutions

1. Fixative solution: glacial acetic acid and 100% ethanol (1:3 volume ratio).
2. Ethanol series: separate bottles of 70%, 50%, and 30% ethanol in dH_2O.
3. dH_2O.
4. Alkaline treatment solution (ATS): 8 M NaOH.
5. Decolorized aniline blue solution (DABS):
   (a) Prepare the 0.1% (w/v) aniline blue in 108 mM K_3PO_4 (pH 11) and store at 4 °C overnight.
   (b) Filter the solution using decolorizing active carbon powder and filter paper. Collect the filtrate in a dark container to avoid exposure to light.
   (c) Repeat step "b" with clean filter paper and decolorizing active carbon powder until the filtrate color is clear/faint light blue.
   (d) Once filtered, add glycerol to a final concentration of 2% glycerol.
   (e) Store the solution in the dark at 4 °C.
   (f) DABS solution can last at least 3 years, but its color may turn yellow over time. Color change does not reflect poor staining ability.
6. Mounting solution: DABS with a total concentration of 15% glycerol.

3 Methods

3.1 Obtain or Generate Mutants of a Gene of Interest with Decreased Expression in Reproductive Tissues

1. Confirm expression of GOI in flowers (i.e., anthers, pollen, pistil, or ovules) (*see* **Note 1**).
2. Obtain mutants from stock center or generate mutants using antisense or CRISPR technology in the GOI; genotype mutation in GOI using PCR or sequencing (*see* **Note 2**).
3. Perform RT-qPCR to confirm that there is decreased or no expression of the GOI in the mutant reproductive organ(s), compared to that in wild type, in which the GOI is typically expressed.

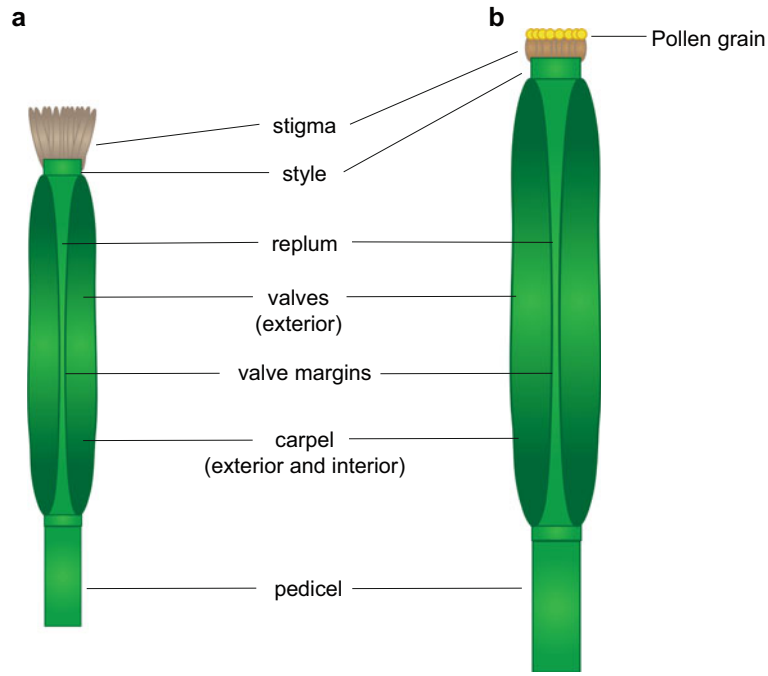

Fig. 1 Diagrams of *Arabidopsis thaliana* pistils. (**a**) An emasculated pistil, which is ready to receive pollen in a cross. (**b**) A pollinated pistil is relatively larger and has elongated to accommodate the developing seeds

3.2 Seed Set Analysis in a GOI Mutant Silique

1. Place a piece of double-sided tape on a clean microscope slide.
2. Excise a silique from the main stem of GOI mutants using scissors and/or forceps (*see* **Note 3**). Make sure to excise the silique with the pedicel (*see* Fig. 1), so that it can be used to handle and/or hold the sample during microsurgery in subsequent steps.
3. Place the silique on the double-sided tape on the microscope slide, ensuring that both carpel valves are visible with the replum facing upwards (*see* Fig. 1) and the pedicel is not on the tape. Press the silique down on the tape with forceps, once the silique is in the correct position.
4. Place the prepared sample under the stereoscope. The silique should be positioned horizontally with the majority of the silique within the field of view and in focus.
5. Using a needle attached to a 1 mL syringe, make four incisions (two at the ends of each carpel valve) perpendicular to the replum (*see* Fig. 1) [9].
6. Keeping the smooth slide of the syringe needle down (the suction hole in the needle facing you), use the syringe needle to cut open the carpel that is closest toward you, by making a horizontal incision just below the replum and continue to make

incision by moving from one end of the carpel valve to the other end, taking care to avoid the septum (*see* Fig. 1, *see* **Note 4**). Gently use the syringe needle to peel the carpel wall back and stick the removed carpel wall to the double-sided tape, so that you can return to the opened pistil rapidly, without letting it [9].

7. Rotate the slide 180°, so that now the unopened carpel is close to your dissecting hand.
8. Score ovules in the exposed carpel as unfertilized, viable, and aborted (*see* Fig. 2, *see* **Note 5**). Make sure to look for any unfertilized ovules, as they are smaller and may be obscured or hidden behind larger fertilized ovules. To avoid risking exposed ovules from drying, it is important to score the ovules in the opened carpel before opening the second carpel.
9. Repeat **step 6** for the unopened carpel. Rotate slide 180° and repeat **step 8**.
10. Repeat **steps 2–9** for a total of three siliques per plant. A seed set noticeably different than wild type might indicate one or more fertility defects (*see* Fig. 2, *see* **Note 6**).

3.3 Pinpointing Source of Reproductive Defects Using Crosses

1. To determine if fertility defects are caused by sporophytic or gametophytic defects, perform each of the following crosses at least three times and score seed set (Subheading 3.2) in crossed pistils (10–14 days after pollination) (*see* **Note 7**). Although determining the source of fertility defects is the first goal of these crosses, they can be used to perform additional assays, such as aniline blue staining described in Subheading 3.5, to further characterize the reproductive phenotype.
2. Choosing the right bud for emasculation and flower to supply pollen in a cross are essential to maintain consistency and to compare seed set from different crosses (*see* Fig. 3). Emasculate a "stage -1" bud, wait for 24 h, and pollinate it with pollen preferably from a flower at "stage 2" and/or "stage 3" (*see* Fig. 3). Perform crosses using buds in inflorescences in the primary and/or first two secondary branches. Avoid performing crosses on young (just bolted) or older (with too many branches and about to cease flowering in a few days) plants. Label each cross by listing the female and male parents on a piece of labeling tape that is placed immediately below the crossed pistil, after removing older flowers and siliques to make room for the tape label. When multiple genotypes are involved in a crossing experiment, errors can be avoided by designating a distinct colored tape for each genotype.
3. A day after performing the crosses, inspect each cross, and remove older flowers above and younger buds below the crossed pistil, to prevent them from being mistaken for a

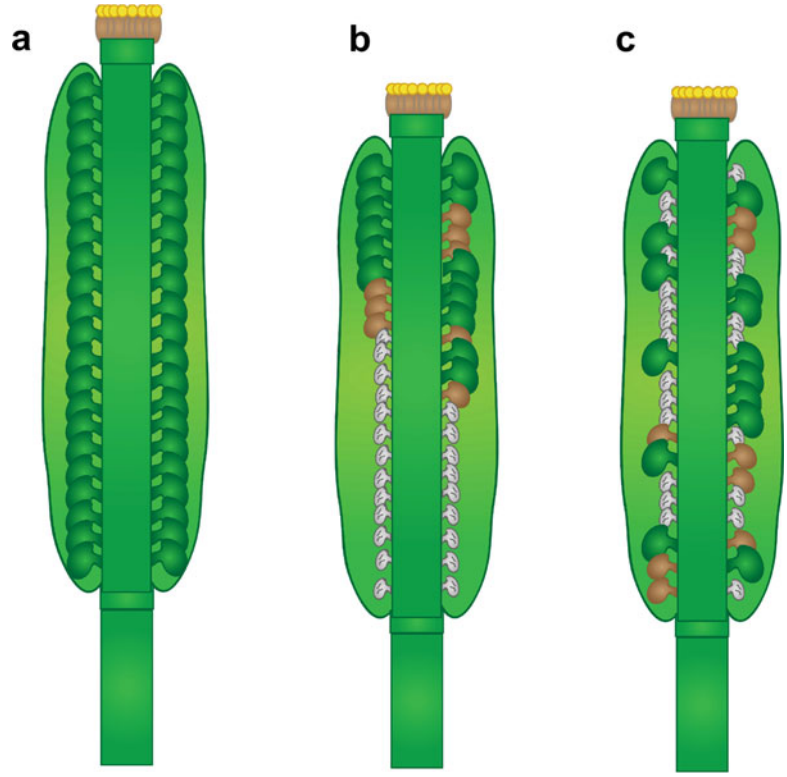

Fig. 2 Scoring seed set in *Arabidopsis thaliana* siliques. (**a**) Wild-type siliques typically show full seed set and each developing seed is relatively big, healthy, plump, and green. (**b**) Mutant siliques exhibiting male reproductive defects tend to show siliques with unfertilized ovules (shown as shriveled and gray) toward the bottom of the silique, as mutant pollen tubes are unable to grow the full length of the pistil and fail to fertilize the ovules in the bottom of a pistil. However, the seed set pattern alone may not be sufficient to distinguish the male gametophytic defect from the male sporophytic defect. (**c**) Mutant siliques exhibiting female gametophytic defects tend to have unfertilized ovules randomly in a silique, as ovules containing a wild-type or mutant female gametophyte are randomly distributed and fail to be fertilized in a heterozygous mutant pistil or randomly fail to be fertilized in a homozygous mutant pistil. (**b**, **c**) Post-fertilization defects can cause aborted seeds (shown as brown and flat) and point to recessive sporophytic defect(s) in embryo and/or endosperm development when homozygous seeds are produced by the fertilization of mutant ovule by a mutant pollen tube. Siliques with decreased seed set caused by the presence of unfertilized ovules and/or aborted seeds may be smaller than wild-type siliques, which is reflected in the diagram

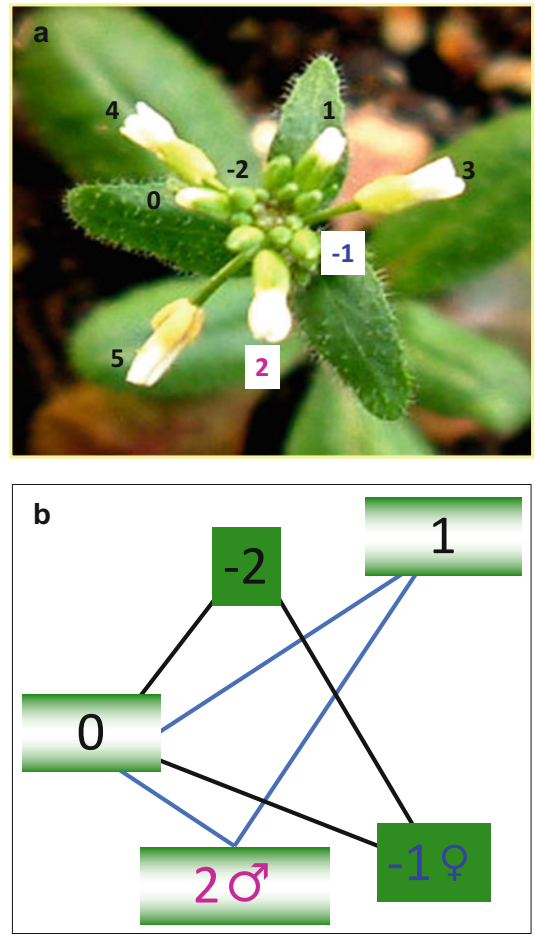

Fig. 3 Selecting buds and flowers to serve as female and male parents in a cross, respectively. (**a**) Bird's eye view of an *Arabidopsis thaliana* inflorescence (Landsberg ecotype). The youngest bud with white petals protruding beyond the green calyx, is designated as "stage 0" and the oldest flower with white petals protruding beyond the yellow calyx is designated as "stage 5." The oldest bud that is entirely green is designated as "stage -1" (blue) should be chosen for emasculation. Twenty-four hours after emasculation, this emasculated pistil should be pollinated with pollen from a donor flower that is at "stage 2" (pink) and/or at "stage 3," at the time of pollination. As the flower matures, there is a change in the color of the calyx from green to yellow and an increase in the size of the flower. (**b**) A diagram showing *Arabidopsis thaliana* flowers and buds that tend to be in the corners of an imaginary triangle. The buds to be chosen for emasculation (stage -1, blue) and the flower that can serve as a male donor (stage 2, pink) are indicated

crossed pistil at the time of harvest. About 24–36 h after pollination, a successfully crossed pistil with fertilized ovules tends to accumulate anthocyanin pigments in the ovary wall.

4. The suggested crosses below will help characterize the fertility defect(s), if the mutant allele is recessive. If not, *see* **Note 8**. In the crosses below, the mutant allele is designated as "*m*," and the wild-type allele is indicated as "+." The pistil parent is listed first and denoted by the symbol (♀), while the pollen donor is listed second and denoted by the symbol (♂):
   (a) *m/m* ♀ × +/+ ♂.
   (b) +/+ ♀ × *m/m* ♂.
   (c) *m*/+ ♀ × +/+ ♂.
   (d) +/+ ♀ × *m*/+ ♂.
   (e) *m/m* ♀ × *m*/+ ♂.
   (f) *m*/+ ♀ × *m/m* ♂.
   (g) *m*/+ ♀ × *m*/+ ♂.
5. If a, c, e, f, and g show the phenotype, it indicates that the female reproductive organ is defective.
6. If b, d, e, f, and g show the phenotype, it indicates that the male reproductive organ is defective.
7. If only "a" shows the phenotype, it indicates that the female sporophyte is defective.
8. If only "b" shows the phenotype, it indicates that the male sporophyte is defective.
9. If "c" shows the phenotype, then it indicates that the female gametophyte is defective (*see* **Note 9**).
10. If "d" shows the phenotype, then it indicates that the male gametophyte is defective (*see* **Note 10**). In the event "d" does not show a phenotype, it should not be immediately assumed that there is no male gametophytic defect. Since such crosses are performed with unlimited pollen, the number of wild-type pollen from a heterozygous anther is still in vast excess of wild-type ovules in the pistil and they can fertilize all of the ovules and thus mask the defective mutant pollen tube growth. To rule out this possibility, limited pollination experiments need to be performed by depositing 20–30 pollen grains from a heterozygous mutant anther. If the seed set phenotype manifests in limited pollination experiments as well, then the phenotype is not attributable to mutant pollen being outcompeted by wild-type pollen; instead, these results point to inherent defects in mutant pollen function.

11. If selfed mutant pistils, but none of the reciprocal crosses (a–d), show the phenotype, then the mutant can be designated "self-sterile," which can be characterized by performing crosses "e" and "f".
12. Of all the listed crosses, if "e" alone shows the phenotype, it indicates that the interactions between male gametophyte and female sporophyte are defective [10].
13. Of all the listed crosses, if "f" alone shows the phenotype, it indicates that interactions between male sporophyte and female gametophyte are defective (*see* **Note 11**).
14. If e, f, and g show the phenotype along with selfed crosses but not in a, b, c, and d, it indicates that interactions between the male gametophyte and the female gametophyte are defective.
15. If no phenotype is observed in any of these crosses or selfed mutant pistils, it indicates that the single mutation in the GOI is not sufficient to cause the phenotype (*see* **Note 12**).

3.4 Transmission Efficiency to Examine Gametophyte Function

Measuring transmission efficiency (TE) of a mutation in the progeny, after the mutation is given an opportunity to be transmitted to the seed through one or both gametophytes, can help confirm gametophytic defects revealed by crosses listed in Subheading 3.3. If one or both gametophytes is defective in function due to the mutation, then the gametophyte will be defective in fertilization and will not produce a seed or generate seeds at a reduced rate. Consequently, frequency of mutation in the progeny is significantly decreased compared to the expected frequency.

1. A rapid way to measure TE is to use the resistance marker that is only associated with the mutation in GOI, this method is listed below. If no resistance marker is available *see* **Note 13**.
2. Gametophyte function is being tested in this assay. Hence, it is imperative to perform the following set of reciprocal crosses from the list of crosses mentioned in Subheading 3.3 (Fig. 4):
   - *m*/+ ♀ × +/+ ♂,
   - +/+ ♀ × *m*/+ ♂.
3. Perform four replicates of each crosses or enough replicates to score at least 200 seedlings from each type of cross.
4. Harvest the seeds from these crosses individually (typically 21–31 days after crossing, when siliques are brown and senesced). Harvesting individual siliques will come handy in tracking down errors in crossing, such as accidental crosses with a different parent or auto-selfing by the plant.
5. Desiccate siliques for 7 days after harvesting.
6. Surface sterilize seeds, then plate on 1/2× strength MS media plates with the appropriate antibiotic.

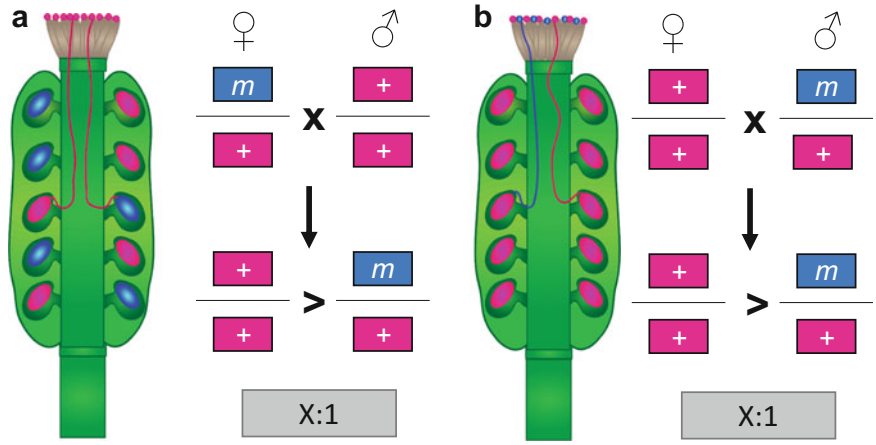

Fig. 4 Reciprocal crosses between wild type and heterozygous mutant to identify male or female gametophytic defects. (**a**) A heterozygous pistil pollinated with wild-type pollen (magenta) results in fertilization of either the wild-type (magenta) or the mutant (blue) female gametophyte. If the mutant female gametophyte is defective, then it is less likely to be fertilized by the wild-type pollen tube and results in significantly reduced frequency of heterozygous individuals in the progeny compared to wild-type siblings (*X*:1, where X is significantly greater than 1). (**b**) A wild-type pistil pollinated with heterozygous pollen (magenta) results in growth of either wild-type (magenta) or mutant (blue) pollen tubes. If the mutant pollen tube is defective, then most wild-type ovules are fertilized by wild-type pollen tubes. This will result in a significantly reduced frequency of heterozygous individuals in the progeny compared to wild-type siblings (*X*:1, where *X* is significantly greater than 1)

7. Stratify the seeds on plates in dark at 4 °C for 3–4 days.
8. Place in a plate growth chamber (example, Percival growth chamber) with the desired light condition (*see* **Note 14**).
9. 10–12 days later, score seedlings for antibiotic resistance (*see* **Note 15**).
10. To calculate transmission efficiency, divide the number of resistant seedlings by the number of sensitive seedlings. Significant reduction from the expected TE of 1 point to defective gametophyte function (Fig. 4).
11. In case of male gametophytic defects (Fig. 4b), a second round of TE experiments involving limited pollination crosses between the same parents should be performed. If the transmission of the mutation through the male gametophyte could not be restored even after limited pollination of mutant pollen on wild-type pistils, then it points to inherent defects in mutant pollen function.

3.5 Aniline Blue Staining of Pistils to Observe PT–Pistil Interactions

Aniline blue staining of selfed and manually pollinated pistils can pinpoint the reproductive step(s) that is (are) defective in the mutant (*see* **Note 16**). A key advantage of this protocol is that it does not rely on transgenic marker expression (such as YFP or GUS) in pollen tubes to visualize their growth in pistils. Thus, it can also be used to study pollen tube growth in any species or

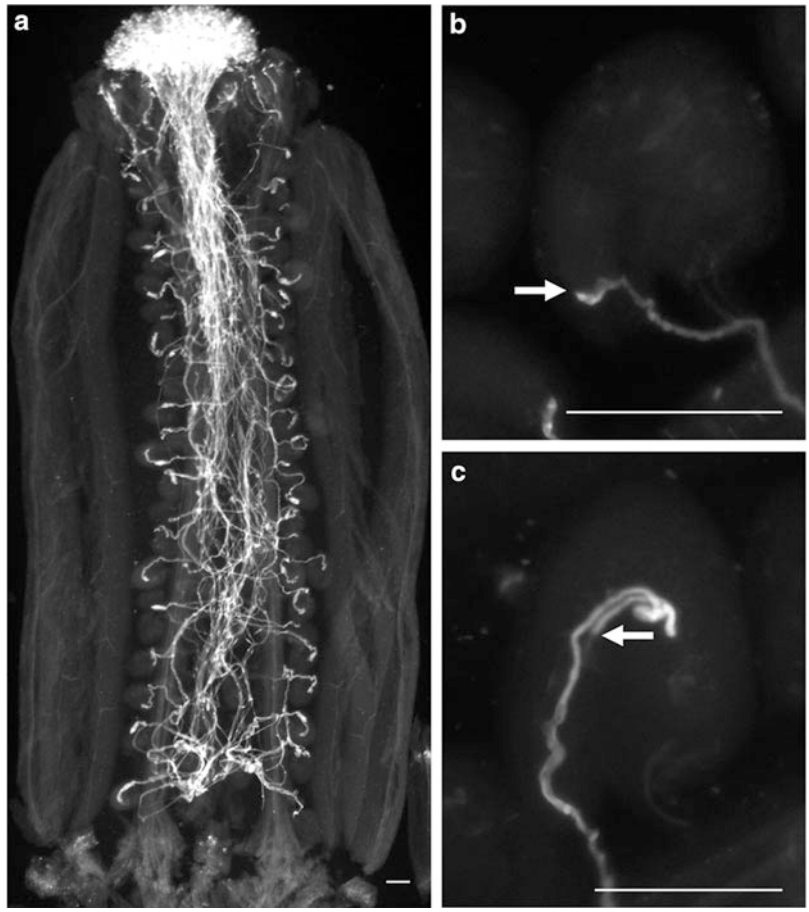

Fig. 5 Aniline blue staining assay to observe pollen tube–pistil interactions, including interspecific crosses. (**a**) Pollen tube growth in an *Arabidopsis thaliana* pistil pollinated with *Sisymbrium irio* pollen. Fluorescent staining shows that pollen tubes have germinated on the stigma, travelled down the transmitting tract, and entered the ovary chamber. Once there, some pollen tubes have entered the ovules and underwent normal pollen tube reception, while a majority either failed to enter the ovule or showed pollen tube reception defects inside the ovule. (**b**) A close-up view of an *A. thaliana* ovule interacting with an *A. thaliana* pollen tube showing successful pollen tube reception (burst of the tube), which is manifested as pollen tube growth arrest in ovules in the micropylar end of the ovule, where the synergid cells are located (white arrow). Pollen tube burst is not visible in such instances because aniline blue stains the callose in pollen tube cell walls and not the cytoplasm. After pollen tube burst, the wall disintegrates and only the cytoplasm is released into the synergid cell, which is not stained by aniline blue. (**c**) A close-up view of an ovule showing pollen tube reception defect, which is visualized as pollen tube overgrowth in the micropylar end of the ovule. White arrow marks the tip of the pollen tube showing pollen tube reception defect. Scale bar (**a**–**c**) = 10 mm

interspecific crosses (*see* Fig. 5a). This protocol is a modified version of the standard *A. thaliana* aniline blue protocol [11]. Modifications made include instructions for high-throughput processing of pistils, mounting modifications to ensure samples are positioned to score PT–ovule interaction defects, and incorporation of crosses for our recommended workflow.

1. Based on the results from Subheading 3.2 and/or Subheading 3.4, perform corresponding crosses from those listed in Subheading 3.3. Summarized below are the crosses from Subheading 3.3 that need to be performed for identifying the pollen tube growth defects underlying the corresponding reproductive defects. To quantify the pollen tube behaviors in pistils, perform indicated crosses in at least three pistils per plant that is used as a female parent in the cross:
   (a) If the mutation is male sporophytic, perform cross "b."
   (b) If the mutation is male gametophytic, perform crosses "b" and "d."
   (c) If the mutation is female sporophytic, perform cross "a."
   (d) If the mutation is female gametophytic, perform crosses "a" and "c."
   (e) In self-sterile mutants, perform crosses of selfed mutant pistils.
   (f) In cases where your mutant is defective in the interactions between male gametophyte and female sporophyte, perform crosses of selfed mutant pistils and "e."
   (g) In the rare case of a mutant with failed interactions between the male sporophyte and female gametophyte, perform crosses of selfed mutant pistils and "f."
   (h) If the mutant shows a failed interaction between the male gametophyte and the female gametophyte then perform crosses of selfed mutant pistils and e, f, and g.
2. Collect crosses 24 h after pollination in a 96-well plate for high-throughput processing. (If you have a small number of pistils (<12) to process, *see* **Note 17**).
3. Add 200 μL of fixative solution to each well, seal the plate lid with plastic wrap or parafilm and place in a resealable plastic bag to prevent evaporation. Keep pistils in solution at room temperature until pistils turn colorless (minimum 2 h minimum to a maximum of 48 h). Fixed pistils can be stored at 4 °C at this step while awaiting further processing.
4. Discard fixative solution via multi-channel or single channel pipette, taking care to avoid damaging or picking up pistils accidentally that get stuck to pipette tips. Having no more than three pistils per Eppendorf tube or two pistils per well in a 96-well plate also will help in not damaging the pistils during processing.
5. Pipette 200 μL of 70% ethanol to wash pistils, wait 10 min, and discard the solution.
6. Add 200 μL of 50% ethanol to wash pistils, wait 10 min, and discard the solution.

7. Add 200 μL of 30% ethanol to wash pistils, wait 10 min, and discard the solution.
8. Add 200 μL of dH_2O to wash pistils, wait 10 min and discard the solution.
9. Add 200 μL of ATS (NaOH) solution, ensure pistils are submerged in solution, cover with plastic wrap to avoid evaporation, and incubate at room temperature overnight (*see***Note 18**).
10. Discard NaOH solution. Add 200 μL of dH_2O to pistils. Care must be taken in handling of pistils, as by this step they have become softened from NaOH treatment. Wait 10 min, then discard the solution.
11. Add 200 μL of DABS to each well. Ensure pistils are submerged. Cover plate with plastic wrap to avoid evaporation and then place at room temperature in the dark for 24 h.
12. After 24 h, samples can be mounted on slides using DABS with 15% glycerol or can be stored in a parafilm sealed plate in the dark at 4 °C.
13. To mount pistils on slides, clean slides and coverslips with 75% ethanol. Using forceps, pick the pistil by its pedicel from the 96-well plate. Remove excess DABS by gently sliding pistil up the side of the 96-well plate during removal.
14. Quickly place the pistil on a clean microscope slide under a stereoscope, so that both ovary valves are visible, with the replum in the middle.
15. Rapidly add 10 μL of mounting solution around the pistil, avoiding air bubbles. Position the pistil with a needle attached to a 1 mL syringe and pop air bubbles, if necessary.
16. Using forceps, quickly place a coverslip on top of the pistil and gently press. Applying slight pressure will separate the valves away from the replum, allowing ovules to be exposed even as most of them remain attached to the pistil and pollen tube growth in the pistil to the ovule is preserved. If too much pressure is applied, most of the tissues will be crushed. If enough pressure is not applied, the ovules are not exposed and continue to remain behind the ovary wall and the pollen tube growth and interactions with ovules are obscured. Therefore, care and practice should be taken to master this step, as it is what will allow consistent scoring of pollen tube–ovule interactions in the pistil.
17. Seal the slides with a clear nail polish in the following manner:
    (a) Place a dot of nail polish in each of the four corners of the coverslip and let it dry.
    (b) Seal edges with nail polish and let it dry.

(c) Repeat sealing the edges with another coat of nail polish and let it dry.

18. Keep samples in a dark microscope slide box until ready to view. If mounted correctly and maintained properly, samples can last at least 2 years.

19. To visualize aniline blue staining, observe in an epi-fluorescence microscope after filtering the UV light through recommended filter sets (UV-1A, UV-2A, and UV-2B), which have an excitation and emission spectrum, of 370 and 509 nm, respectively (*see* Fig. 5, *see* **Note 19**):

    (a) Selfed crosses of wild type typically show normal pollen grain germination and pollen tube growth through the stigma, style, and transmitting tract [12]. Upon entering the ovary chamber, the pollen tube locates an ovule, enters through the micropylar end, arrests growth, and bursts to release the two sperm cells to complete double fertilization (Fig. 5b) [12]. All of these steps in pollen tube navigation could be observed in aniline blue-stained pistils, except for sperm release.

    (b) If pollen grains do not germinate on the stigma, the mutant is defective in pollen tube germination in crosses between those parents.

    (c) If pollen tubes fail to grow on the stigma or through the transmitting tract, the mutant shows failed interactions between pollen tubes and sporophytic pistil tissues.

    (d) If pollen tubes do not exit the transmitting tract, then the mutant shows failed interactions in long-distance pollen tube attraction and/or failed interactions between pollen tubes and sporophytic pistil tissues.

    (e) If the pollen tube enters the ovary chamber but does not grow toward an ovule, there may be failed interactions between female sporophytic and/or gametophytic tissues.

    (f) If the pollen tube grows along the funiculus of an ovule but does not enter the micropyle end of the ovule, then pollen tube attraction is defective.

    (g) If the pollen tube reception is defective, pollen tube will enter an ovule but fail to arrest growth and continue to coil within the female gametophyte (Fig. 5c).

    (h) If multiple pollen tubes enter a single ovule, then the mutant shows a polytubey phenotype [13, 14].

    (i) Additional experiments, involving marking the sperm with fluorescent markers, are required to determine if this phenotype is due to failure to release sperm cells [15] (*see* **Note 20**).

4 Notes

1. An excellent resource to check gene expression in *A. thaliana* and a variety of other plant species is available through The Bio-Analytic Resource (BAR) for Plant Biology https://www.bar.utoronto.ca/.
2. The Arabidopsis Information Resource (TAIR, https://www.arabidopsis.org/) and the Arabidopsis Biological Resource Center (ABRC, https://abrc.osu.edu) contain seed stocks of T-DNA insertion lines in *A. thaliana*. For studying male reproductive defects, Blue SAIL lines, if available in the GOI, are preferable, as these T-DNA insertion lines also carry the pollen and pollen tube expressed *pLAT52::GUS* transgene in the *quartet (qrt)* mutant background, which allows all meiotic products to remain as a single tetrad [16].
3. For seed set analysis of selfed siliques, we prefer to select siliques at positions between 6 and 10 on the main stem of plants with at least 20 siliques [17].
4. Take caution when cutting below the replum. Too deep of an incision will damage the ovules or dislodge the funiculus from the septum. Too shallow of an incision will not open the carpel.
5. Unfertilized ovules will remain small, shriveled, and remain light in color (*see* Fig. 2). Viable ovules will be green with mature embryos. Aborted ovules will be flat or misshaped, larger than unfertilized ovules and be brownish or white in color. If all ovules look white, select older siliques or wait until the plant is older before selecting more siliques to score, it is most likely due to selecting a pistil too young to score. Suitable siliques for scoring should have viable ovules at mid- to late-bent cotyledon stage [18, 19].
6. It is imperative to include wild-type plants that were grown besides the mutant plants as a control in all seed set scoring experiments.
7. Emasculate buds at 12c stage [20], wait 24 h for the pistil to mature, and then pollinate them manually. If the mutant is associated with an antibiotic resistance marker, determining whether fertility defects are sporophytic or gametophytic can be confirmed by analyzing transmission efficiency of the mutation (Subheading 3.4).
8. If the mutant allele is dominant negative and shows sporophytic defects, phenotyping in heterozygous individuals and recapitulating the phenotype by introducing the transgene into wild-type plants may be required.

9. This is the key cross to make this conclusion. Such a mutation will also cause a phenotype in crosses a, e, f, g listed in Subheading 3.3.
10. This is the key cross to make this conclusion. Such a mutation will also cause a phenotype in b, e, f, g listed in Subheading 3.3.
11. We want to note this type of self-sterile mutant is highly unlikely because by the time pollen tube (the male gametophyte) extends and interacts with the female gametophyte, there is no physical or other known influence of the male sporophyte on the pollen tube. The last derivative of the male sporophyte on the male gametophyte is in the pollen coat, which too gets left behind on top of the stigma as part of the "foot" formed when the pollen grain makes initial contact with the stigma. If seed phenotypes instead of merely seed set are being scored, then "e" and "f" crosses in conjunction with "c" and "d" crosses can be useful in testing the possibility that seed defect is dependent on the genotype of the zygote in the seed. For example, if neither "c" or "f" cross show a seed phenotype but the seed phenotype is only seen in "a" cross, then it indicates that the seed defect is caused by the mutant maternal sporophyte [21].
12. In such instances, perform other cytological or molecular analysis to uncover subtle defects [22] or examine if GOI is a member of a gene family to rule out redundancy (either for shared function or compensatory function) as the reason for lack of a phenotype [23, 24].
13. TE analysis should be performed only after confirming that the T-DNA insertion (and by extension, the resistance marker in the T-DNA) is only in the GOI and that the T-DNA insertion in the GOI is tightly linked with the reproductive phenotype. The former can be achieved by back crossing mutant to wild type and the latter can be established by performing co-segregation analysis, in which the T-DNA insertion in the GOI confirmed by genotyping is correlated with the reproductive phenotype. Any reproductive phenotype or genotype can be used to measure TE; however, it is important to ensure that what is being used to assess TE is tightly linked with the mutation in the GOI.
14. Constant light will encourage faster growth and early flowering; however, the plants may be stressed under these conditions. If stress is observed in wild-type plants grown on regular MS plates, check the amount of light that plants are exposed to using a light meter. The following conditions are sufficient to raise healthy plants: plates are incubated in 75–100 μmol m^{-2} s^{-1} of light in 24 h light at 21 °C until 10–14 days old, when they are scored; then resistant plants are

immediately transplanted into soil and grown at 16 h light (100–120 μmol m^{-2} s^{-1}) at 21 °C and 8 h dark at 18 °C as described [25].

15. Resistant seedlings will typically have true leaves, longer roots, and remain green. Sensitive seedlings will turn white, have little to no root growth, and not develop true leaves. We often see that SALK lines may have resistant plants that can be extremely sensitive to Kanamycin; in such instances, delay scoring resistance until 15–20 days after plating. In cases where antibiotic resistance is silenced (often seen with Kanamycin resistance in SALK lines from ABRC), genotyping will be required (*see* **Note 13**).
16. Aniline blue stains callose-rich tissues—in pollinated pistils, this includes pollen grains, pollen tubes, pistil vascular tissue, funiculi from ovules, and damaged tissues [26].
17. Processing steps can be adjusted for small sample sizes; instead of placing into a 96 well plate, add each pistil to a 1.0 mL microcentrifuge tube. Reduce volumes of all solutions in **steps 3–11** from 200 to 150 μL in Subheading 3.5.
18. When adding ATS, due to difference in viscosity of solutions, there is an increased chance of introducing air bubbles. It is important to remove any air bubble in the tube/well, so that pistils do not dry after accidentally getting trapped in the air bubble.
19. Slides should be scored within 24 h after mounting. Occasionally, slides that have been overexposed to UV light tend to become cloudy and obfuscate scoring. Longer DABS staining time (at least 24 h) may help increase the durability of a sample.
20. Crosses to a dual pollen tube and sperm cell marker such as *pLAT52::GFP, HTR10::RFP* [15] can be used to visualize defects in sperm cell release.

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