

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Sites of Self-Pollen Tube Inhibition in Papaveraceae (*sensu lato*)

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Science

in

Biology

by

Paul Bilinski

Committee in Charge:

Professor Joshua R. Kohn, Chair
Professor Elsa Cleland
Professor David Holway

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2010

DEDICATION

This thesis is dedicated to

Babcia

Helena Labuda

whose commitment to her children and grandchildren forever changed the history of my family. And to whose green thumb inspired my passion for all things green.

EPIGRAPH

Plant and your spouse plants with you; weed and you weed alone.

Jean-Jacques Rousseau

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ABSTRACT OF THESIS

Sites of Self Pollen Tube Inhibition in Papaveraceae (*sensu lato*)

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Paul Bilinski

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Angiosperms prevent inbreeding with a number of molecular mechanisms. *Papaver rheoas* (Papaveraceae) has a gametophytic self-incompatibility system that is well-characterized at the molecular level. In *Papaver*, self pollen is arrested immediately upon contact with the stigma. Immediate pollen arrest may be required because *Papaver* flowers have stigmatic rays directly on top of the ovary and no style. There is much variation in floral structures among Papaveraceae and self-incompatibility is widespread. However, except for *Papaver*, no documentation of the site of self-pollen tube inhibition within the family exists. We examined the site of self-pollen tube inhibition in four species of Papaveraceae representing a broad phylogenetic and morphological sample.

We used two types of tissue preparation. Squash preparation was used for species with soft stigmas while woody tissue was sectioned with a cryostat and panels were stitched into a mosaic to visualize whole stigmas. In three species, self-pollen tube inhibition appeared similar to that described for *P. rheoas*, despite variation in the architecture of female tissues. Self-tubes were arrested early and usually did not grow longer than 100 μm . In *Argemone munita*, self pollen tubes progressed further, growing up to 500 μm . However, growth appeared to occur along the stigmatic hairs and ceased once tubes contacted the stigma surface. Despite variation in floral architecture, rapid death of self-pollen tubes occurs before or just after penetration of female tissue in all species examined. This is consistent with the hypothesis that members of the family share the same incompatibility mechanism.

INTRODUCTION

Self-incompatibility, the ability of many plants to recognize and reject their own pollen in order to avoid the deleterious effects of self-fertilization, is found in many plant families (Igic et al. 2008; Weller et al. 1995). Many different forms of self-incompatibility are known and, in a few cases, the molecular mechanisms involved have been at least partially elucidated (Franklin-Tong 2008). Nevertheless, our knowledge of even the basic phenomenology of incompatibility remains incomplete for many plant groups. In single locus gametophytic self-incompatibility (GSI), haploid pollen is rejected if the allele it carries at the self-incompatibility (S-) locus, matches either allele present in the diploid female reproductive tissue (deNettancourt 1977). Two systems of GSI have been characterized at the molecular level. In the Rosaceae, Plantaginaceae and Solanaceae, the stylar product of the S-locus responsible for self-pollen rejection is an RNase (S-RNase; McClure et al. 1990) while the pollen expressed gene it interacts with is thought to encode an F-box protein known as SLF or SFB (Entani et al. 2003; Ushijima et al. 2003; Ikeda et al. 2004; Qiao et al. 2004; Sijacic et al. 2004; Sassa et al. 2007). In S-RNase based incompatibility, self-pollen grains germinate, their tubes extend into the stigma, and growth is typically retarded in the distal third of the style. A different molecular mechanism of GSI, known as the S-glycoprotein model, is documented from *Papaver rhoeas* (Papaveraceae, Foote et al. 1994; Franklin-Tong 2008; Wheeler et al. 2009). Both pistil and pollen S-locus recognition proteins have been isolated and these bear no homology to the S-RNase/SLF-SFB system. The female component of the system is a small (~15 kDa) secreted peptide and that binds to a recently characterized (Wheeler et al. 2009) pollen-expressed receptor. In *P. rhoeas*, self-pollen tubes cease

growth very soon after hydration. Some self-pollen tubes never emerge from the colpal aperture while most are arrested immediately upon contact with the stigma (Franklin-Tong and Franklin 1993; Franklin-Tong 2008).

Self-pollen tube inhibition in *P. rhoeas* is thought to result from initiation of a signaling cascade that leads rapidly to programmed cell death (PCD; Thomas and Franklin-Tong 2004). It is clear that in *P. rhoeas*, self-pollen identification occurs directly on the surface of the stigma prior to penetration of the pollen tube into the female tissue. Once pollen is identified as self, the cascade of inhibition begins with a change in the Ca^{2+} gradient within the shaft of the pollen tube. This is followed by reactions that lead to the breakdown of the F-actin cytoskeleton of pollen tube. Equalization of the calcium gradient inhibits the function of a 26kDa enzyme named p26 (McClure and Franklin-Tong 2006). This enzyme is an sPPase which plays a vital role in pollen tube elongation. P26 is phosphorylated and its function is halted and therefore the pollen tube will not elongate. This occurs early in the pollen tube extension and therefore only very short pollen tubes are observed. While the inhibition of p26 stops the pollen tube from getting far, the change of the Ca^{2+} gradient begins the pathway leading to PCD (Thomas and Franklin-Tong 2006). The process of PCD is accomplished through the fragmentation of the DNA of the incompatible pollen. This affect of the change of the calcium gradient finalizes the process of incompatible pollen inhibition at the surface of the stigmatic tissue.

P. rhoeas has a very unusual floral structure with stigma rays sitting directly atop of the rather hard ovary (Fig. 1). The immediate stigmatic inhibition of pollen tube growth observed in *P. rhoeas* may result from the species' lack of a style or alternative

tissues for self-pollen arrest. Among Papaveraceae (*sensu lato*, including Fumariaceae), many of whom are known to be self-incompatible, there is wide variation in the structure of female floral tissues. However, there are no reports on the site of inhibition of self-pollen outside of *Papaver*. For instance, self-incompatible *Platystemon californicus* (Hannan 1981) has several long soft stigmas that extend upwards from the ovary (Fig. 1). Another self-incompatible species, *Argemone munita* (Paape 2009) has woody stigmas fused into a central mass. *Romneya coulteri*, has a woody and hairy stigmatic head formed from the fusion of many individual stigmas. Flowers of self-incompatible California poppy *Eschscholzia californica* (Beatty 1936; Cook 1962) have four thin linear stigmas extending out of the ovary. Self-incompatible *Dicentra spectabilis* (Schemske 1978) has a style subtending its arrowhead-shaped stigma. These species encompass a wide phylogenetic sample of Papaveraceae (Hoot et al. 1997) and possess a variety of floral morphologies and potential sites for pollen inhibition. Here we examine the site of self-pollen tube inhibition using microscopy.

MATERIALS AND METHODS

Plant Material

Seeds of *P. californicus* were collected from: Lake Cuyamaca State Park east of San Diego (N 32°58' 59.1", W 116° 33' 45.4"), Hastings Natural History Reserve (N 36° 23'07.1", W 121° 33' 16.6"), and along the side of Carmel Valley Road near Carmel, CA (N 36° 26'34.5", W 121° 38' 53.7"). Individuals of the *Argemone munita* were originally collected as seeds from Valentine Eastern Sierra Reserve (N 37°56' 52.1" W 118°49' 31.7"), Mammoth Lakes, CA and Emerson Oaks UC Reserve (N 33°28' 22.9" W 117°19' 34.9") near Temecula, CA. These seeds were used for greenhouse crosses. All seeds for *A. munita* and *P. californicus* were placed in 1" plug trays in moist Sungro professional mix for germination. Seeds in trays were exposed to a cold treatment at 4°C for 45-90 days. Very low rates of germination were observed. Seedlings of *A. munita* were grown in natural light conditions in a glasshouse while *P. californicus* seedlings were grown under fluorescent lights in 14h/10h day/night cycles.

Seeds of *Eschscholzia californica* were collected from Antelope Valley, Los Angeles, CA (N 34°43' 29 W 118°23' 54.6") and from commercial seed sources. Seeds were placed in a sealed Petri dish on top of moist filter paper and kept in the dark for 72 hours. All seeds showing a radical (>90%) were then transferred into wet soil in small pots. Seedlings were allowed to grow under fluorescent light in 14h/10h day/night cycles in a growth chamber. Juvenile plants were then transferred to potting soil and moved to the glasshouse and natural light conditions to support flowering. Individuals of *Romneya coulteri* were all perennial plants growing on the UCSD campus (N 32°52'37.74" W

117°14'1.11"). Individuals of *Dicentra cucullaria* were purchased as potted plants from a local nursery.

Controlled Pollinations to Confirm Self-Incompatibility

To confirm the incompatibility phenotype of study plants, fruit set and seed production were compared in hand self- and cross-pollinated flowers. Pollen was collected from flowers that were bagged to prevent contamination several days prior to opening. These plants were revisited for the remainder of the flowering season and unopened flowers were emasculated before pollen dehiscence. Stigmas were then hand pollinated using self or outcross-pollen. A sufficient volume of pollen was applied to visually cover portions of the stigmatic tissue. After pollination, flowers were re-covered with bags to prevent insect visitation. Fruits were allowed to develop for several weeks and were collected prior to seed release. Seed set was recorded and observations were made to ensure that self pollinations did not produce seed.

Pollination Protocol for Stigmatic Imaging

Flowers were bagged prior to opening and emasculated prior to anthesis. Anthers containing pollen were then taken from another open flower on the same plant for self-pollinations, or from another plant for cross-pollinations, and brushed against the stigmatic tissue of the flower until pollen was clearly visible on stigmas. Bags were then replaced over the flowers.

Slide Preparation

For species having soft stigmatic tissue (*P. californicus*, *D. spectabilis*, *E. californica*), squash preparations were used while those with woody stigmas (*A. munita* and *R. coulteri*) were prepared with cryostat sectioning or a combination of both

methods. For squash preparations, stigmatic tissue was removed from the plant 24 hours after pollination and placed in a -75°C freezer for storage. For use, tissue was thawed at room temperature for 30 minutes and then submerged in $500\mu\text{m}$ of aniline blue stain (0.2g aniline blue, 20g tripotassium orthophosphate, 1L distilled water). Stigmas were then placed on slides, surrounded in petroleum to prevent loss of stain, squashed using the cover-slip, and sealed with clear nail polish.

For cryostat sectioning, whole gynoecia were trimmed from flowers twenty-four hours after pollination. Sagittal or transverse sections were cut through the stigmatic tissue using a razor blade. Stigma halves were embedded in Tissue-Tek® O.C.T.™ (Sakura) for 10-20 minutes to allow for penetration. Samples were then frozen in 2-Methylbutane pre-cooled in liquid nitrogen. Samples were then placed into a cryostat with a chamber temperature of -14°C . After being allowed to warm to this temperature, $100\mu\text{m}$ sections were cut. These were placed on ionized slides, samples were encompassed with liquid plastic, and allowed to dry in air for approximately twenty four hours. Slides were then rehydrated in $50\mu\text{l}$ of aniline blue solution for 20 minutes. The solution was removed and samples were then washed in Phosphate buffered saline (.145 M NaCl, .01 M phosphate, pH 7.1) twice to remove any unbound fluorophores. A final treatment of gelvatol (1:2 polyvinyl alcohol:glycerol) was applied to preserve fluorescence before the sample was sealed with a cover-slip and clear nail polish.

Image Gathering

Images from squash preparations were captured using an excitation filter of 510-560nm depending on the clarity of the images. These were captured using a Nikon Eclipse E600 fluorescent microscope with a 10X Nikon objective. This allowed pollen

tube illumination (509nm) as well as some auto-fluorescence (approximately 520nm). Stitched mosaic images from cryostat sections were gathered using an Olympus DSU confocal microscope with dapi and alexafluor filters (transmittance: 365 and 488, respectively), Hamamatsu EMCCD camera, and automated XYZ stage. Panels were stitched together using Neurolucida® software.

RESULTS

Controlled Pollinations for SI Measure

In order to confirm self-incompatibility in the individuals used for this study, crosses were performed to examine fruit set. Due to a limited number of individuals and a limited amount of flowers per individuals, only a few flowers were used for these tests in most species. In every individual examined, at least 2 self pollinations were performed and no seeds were set from any of these pollinations. Cross pollinations performed on these same individuals produced seed. Again, at least two outcrosses were performed.

Pollen Tube Length Analysis

Three species, *D. spectabilis* (Figure 2), *E. californica* (Figure 3), and *P. californicus* (Figure 4) showed a phenotype consistent with the pollen behavior previously observed in *P. rhoeas*. In *D. spectabilis*, self-pollen grains extended tubes in many directions at the surface of the stigma, but growth was never seen to extend more than a millimeter into the stigmatic tissue in the direction of the style (Figure 2a). In contrast, out-cross-pollen grains that were deposited on all sides of the stigmatic surface extended toward a central position on the stigmatic tissue and grew through it into the style (Fig. 2b).

In *E. californica*, self-pollen grains did not extend tubes into the soft long linear stigma whether deposited near the proximal (Fig. 3a) or distal (Fig. 3b) regions. After cross-pollinations, pollen tubes are clearly present at the base of the stigmatic tissue. Cross-pollen tubes in *E. californica* are able to penetrate into the transmitting tract at any location, a clear difference from *D. spectabilis*.

The final species that exhibits a phenotype very similar to *Papaver rhoeas* is *Platystemon californicus*. Self-pollen in this species extends short tubes toward the interior of the stigmatic tissue but fail to elongate further (Figure 4). Inhibition occurs before any substantial penetration of the stigma. While Figures 4a and 4b appear similar, outcross-pollen tubes (Fig. 4b) extend into the stigma and then towards the distal end of the linear stigma (Fig. 4d). Figures 4c and 4d show that following self-pollination, no tubes extend to the distal end of the stigma and pollen deposited at the distal end is also inhibited. In contrast, many cross-pollen tubes are observed at the distal end of the stigma.

A. munita shows a phenotype that appears somewhat different from other species (Figs. 5, 6). Initial pollen tube growth appears similar in both self and outcross-pollen. The squash preparations seen in Figures 5a and 5b shows similar pollen tube growth following self- and cross-pollination with self-pollen tubes extending several hundred microns, much further than seen in other species. Using the method of cryostat sectioning and mosaic stitching, differences following self- and cross-pollinations are visible. Using this method, pollen tubes now appear blue, while green is auto-fluorescence of other tissues. In Figure 5c, no pollen tube illumination is present beyond a thin layer at the surface of the stigma while tubes from cross-pollen (Fig. 5d) extend further.

Further information can be extracted when examining the full stitched mosaics seen in Figure 6. These images show that pollen tube inhibition following self-pollination occurs prior to entry into stigmatic tissue below the surface hairs. The tubes from outcross-pollen continue to penetrate the stigmatic tissue past this barrier (Fig. 6b). These images provide evidence of cross-pollen tubes extending to the distal end of the stigma

and down the transmission tract. The mass of pollen tubes in blue follow a common path toward the ovaries, a phenotype that was not observed in following self-pollination (Figs. 6a, c).

Images from the woodiest species examined, *R. coulteri* (not shown), did not provide conclusive evidence about the site of inhibition of pollen tubes in the stigma. The stigma of *R. coulteri* was several times larger, thicker, and more pubescent than any of the other species examined. The external hairs growing from the stigmatic tissue were brittle after freezing and did not maintain shape during sectioning. This prevented us from being able to obtain images of either self- or cross-pollen tubes growing from the pollen grain source. No image gathered captured any pollen grains with elongating pollen tubes from *R. coulteri*.

In summary, in three of the four species from which sufficient images were obtained, incompatible pollen tubes ceased to grow upon contact with female tissue and self-pollen tubes were extremely short in comparison to outcross-pollen tubes. However, in *A. munita*, self-pollen tubes extended approximately 300 μ m along the stigmatic hairs before encountering the stigmatic surface and then ceased growth.

DISCUSSION

Despite stark differences in floral morphology among self-incompatible members of the Papaveraceae, self-pollen tube inhibition occurs shortly after contact with female tissue, consistent with pollen tube behavior previously observed in *Papaver rhoeas* (Franklin-Tong 2008). In *D. spectabilis*, *E. californica*, and *P. californicus*, self-pollen tubes were arrested at the surface of the stigma and did not extend more than 100 μ m. The only exception found occurred in *A. munita* which had a woodier stigma covered in a dense mat of hairs. self-pollen tubes extended through the hairy layer, apparently between the hairs, before being arrested at an auto-fluorescent layer representing the stigma surface. Therefore, in no case were self-pollen tubes able to grow within female tissue.

The variable flower morphologies among species examined formed two different groupings: species with, thin, soft stigmas (*E. californica*, *P. californicus*, and *D. spectabilis*) and species with hairy, woody female tissue that lacks a true style (*A. munita*, *R. coulteri*), the differences seen in pollen tube growth could be divided along these differences. Squash preparations were sufficient to observe the site of pollen tube inhibition in all species with soft female tissue (*E. californica*, *P. californicus* and *D. spectabilis*). Cryostat preparations and stitched mosaic imaging allowed us to observe the site of self-pollen tube inhibition in *A. munita*, but could not resolve the site of inhibition in *R. coulteri* because the outer layer of hairy stigmatic tissue did not remain intact in upon sectioning despite attempts using several variations of the freezing procedure. The consistency observed in the site of inhibition of pollen tubes suggests the maintenance of the SI mechanism across Papaveraceae (*sensu lato*). From previous studies, it has become clear that self-incompatibility has arisen independently several times in angiosperms.

Some closely related families display different types of homomorphic self-incompatibility (e.g. GSI in the Solanaceae and SSI in the Convolvulaceae; Matton *et al.* 1994). Within families, however, only one mechanism of homomorphic self-incompatibility is generally found. The only exception is the family Polemoniaceae, where *Linanthus parviflorus* and *Phlox drummondii* are reported to maintain gametophytic and sporophytic self-incompatibility systems, respectively (Levin 1993; Goodwillie 1997). It was noted that these two species both displayed pollen tube growth patterns similar to one another, suggesting microscopical evidence may not be definitive for assessing common molecular mechanisms. However, in the Papaveraceae, recent work (Paape *et al.* in review) found homologues of the S-alleles of *Papaver* expressed in female tissue of *A. munita*, *P. californicus*, and *R. coulteri*. Crossing studies with the first two of these species found that putative S-locus genotype predicted incompatibility phenotype, suggesting these homologues were functional S-alleles (Paape *et al.* in review). The molecular basis of self-incompatibility appears homologous with *Papaver*. However, no sequence information has yet been obtained from *E. californica* or *D. spetabilis*. Such sequence information, when obtained, would strengthen inference of a single basis of GSI among Papaveraceae (*sensu lato*).

Beyond serving as an analysis of pollen tube growth, this experiment attempted to test the usefulness of methods other than squash preparation for pollen tube observation. The squash preparation causes tissue to shift and fails to generate images in which a barrier to pollen tube growth can be easily visualized. This was especially true in *A. munita* where squash preparation shows that pollen tubes were not inhibited early because pollen tube length was much longer than recorded in other species (Fig.5a, c).

Squash preparations show that self-pollen tube inhibition appears to occur at random locations in the stigma rather than at the stigmatic surface. When examining cross-pollinations, squash preparation showed that tubes growing in any portion of the female tissue, in contrast to what is seen in *P. rhoeas*, where cross-pollen tubes funnel down a central transmission tract (Franklin-Tong and Franklin 1993).

In contrast, cryostat preparation offered a technique that maintained the integrity of the spatial relationship between pollen tubes, stigmatic hairs, and the stigma surface allowing for better imaging of the thicker tissues. Such structures as the transmitting tract and stigmatic surface that are lost in squash preparation were visible through cryostat sectioning. The large, thick nature of these tissues in *A. munita* supported the implementation of mosaics rather than single panels. Since all cross-pollen tubes and some self-pollen tubes extended longer than the field of view, stitched mosaics provide the benefit of being able to trace pollen tubes progress through the entirety of the tissue by using automated image capture techniques. Stitched mosaics, by preserving the orientation of tubes across the entire stigma allowed us to see that self-pollen tubes have a common site of inhibition (Fig 6). These techniques also allowed us to visualize cross-pollen tubes extending into the transmission tract of *A. munita*, another piece of information lost in squash preparation. However, cryostat sectioning had some limitations, as when we were unable to maintain the integrity of the stigma when sectioning the species with the woodiest stigma, *R. coulteri*.

In conclusion, this study extended our knowledge of the sites of self-pollen tube inhibition among Papaveraceae (*sensu lato*). Images comparing self to outcross pollinations in *A. munita*, *D. spectabilis*, *E. californica*, and *P. californicus* found that in

all species except *A. munita*, self-pollen tube arrest occurred immediately upon contact with female tissue. In *A. munita*, contact with the long stigmatic hairs did not arrest self-tubes but tubes-growth did halt immediately upon contact with the stigmatic surface at the base of the hairs. Cryostat sectioning proved essential for visualizing the site of inhibition in *A. munita*, where squash preparations failed to maintain tissue integrity and pollen tube orientation, making it difficult to observe the site of inhibition. This technique will likely be useful in other taxa, particularly those with large masses of stigmatic tissue. The ongoing development of new tools in microscopy will continue to improve our ability to image thick tissue without compromising structure.



Figure 1. Floral Diversity in Papaveraceae and Fumariaceae. Top row left to right: *Papaver rhoeas*, *Platystemon californicus*, *Argemone munita*. Bottom row left to right: *Eschscholzia californica*, *Romneya coulteri*, *Dicentra spectabilis*

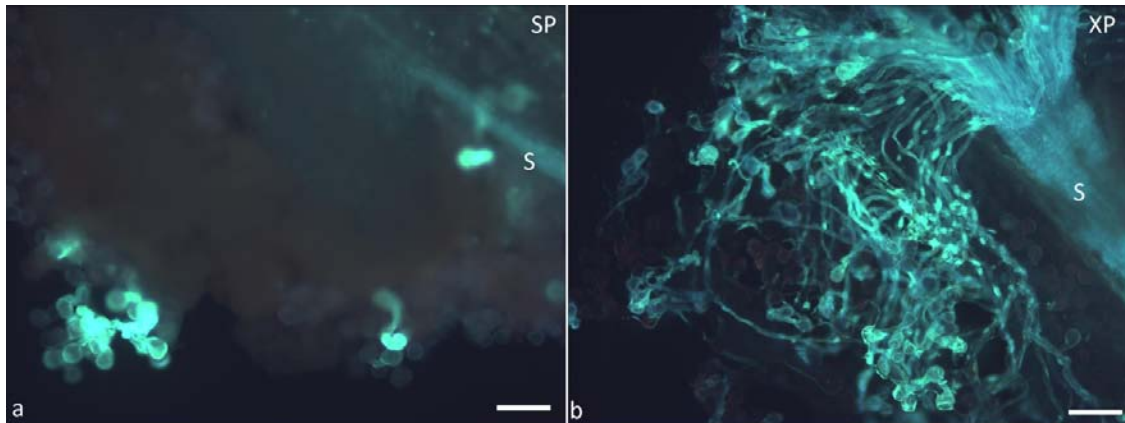


Figure 2. Fluorescent light microscopy images of stigmatic tissue from *Dicentra spectabilis* after exposure to outcross pollen (XP) and self-pollen (SP). Specimens were fixed using squash preparation. Scale bar = 100 μ m. **a.** Self-pollen tubes do not elongate into the female tissue. **b.** Cross-pollen tubes grow in large numbers toward the style (marked with the letter S).

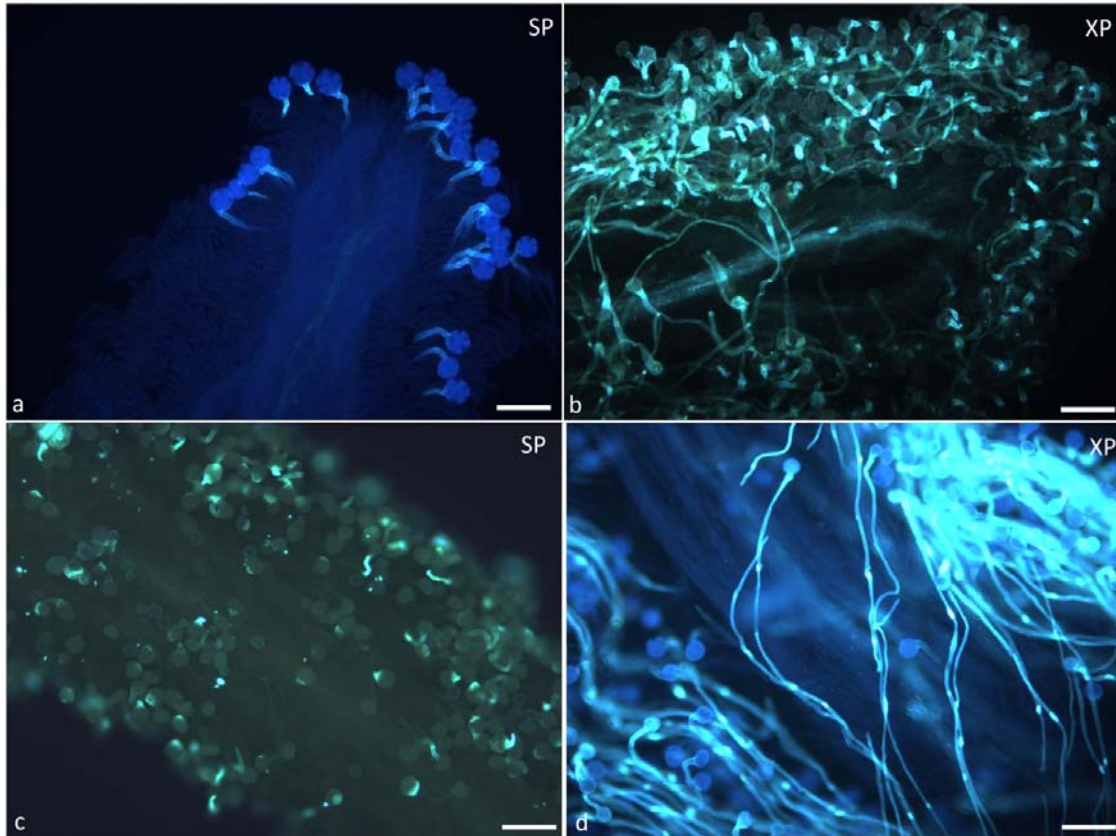


Figure 3. Fluorescent light microscopy images of stigmatic tissue from *Eschscholzia californica* after exposure to outcross-pollen (XP) and self-pollen (SP). Specimens were fixed using squash preparation. Scale bar = 100 μ m. **a, b.** Images of the proximal ends of stigmatic tissue exposed to different pollen treatments. Pollen tube growth into the stigmatic tissue is only present in cross-pollination **c, d.** Images of the distal ends of stigmatic tissue exposed to different pollen treatments. Pollen tube penetration continues in the direction of the ovaries after cross-pollination.

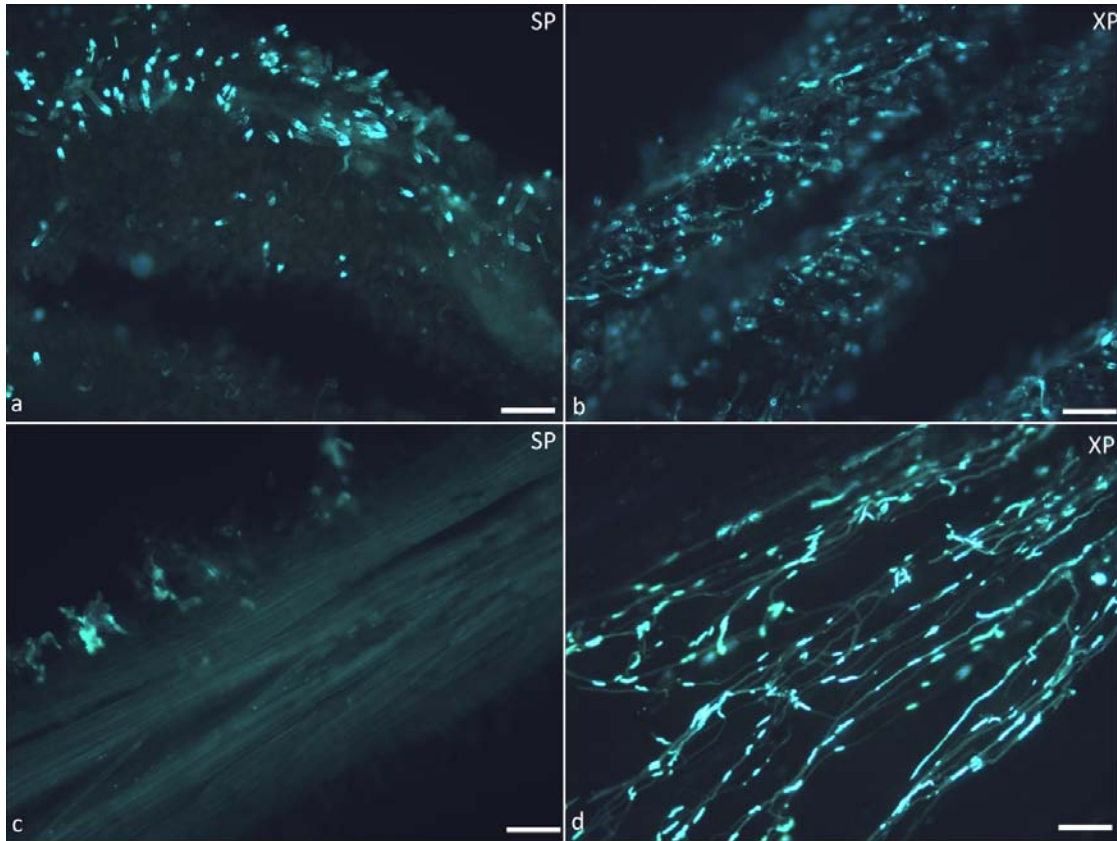


Figure 4. Fluorescent light microscopy images of stigmatic tissue from *Platystemon californicus* after exposure to outcross-pollen (XP) and self-pollen (SP). Tissue was fixed using squash preparation. Scale bar = 100 μ m. **a, b.** Images of the proximal end of stigmas. Pollen tube growth in both treatments appears fragmented, although fragments appear longer in the cross-pollination treatment. **c, d.** Images of the distal end of stigmas. Pollen tubes are only present and elongating after cross-pollination.

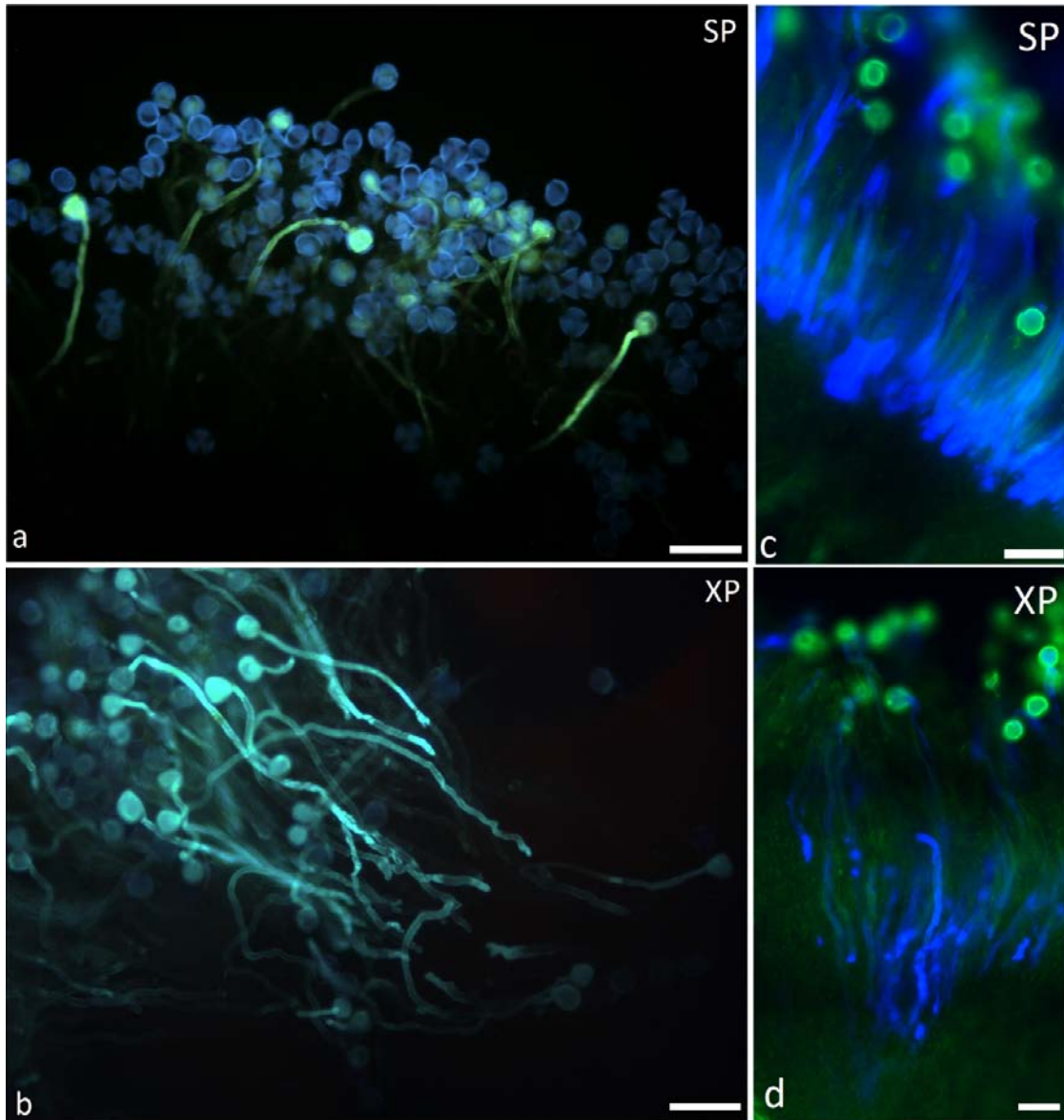


Figure 5. Fluorescent light microscopy images of stigmatic tissue from *Argemone munita* after exposure to outcross-pollen (XP) and self-pollen (SP). Scale bar = 100 μ m. **a, c.** Panels were prepared with squash preparation. **b, d.** Panels are zoomed-in images of stitched mosaics prepared with cryostat sectioning. In stitched mosaics, wavelength coloration was added so that pollen tubes (emitting at 509nm) and appear blue. Another image was captured to show plant tissue auto-fluorescence which emitted at 520nm. This appears green.

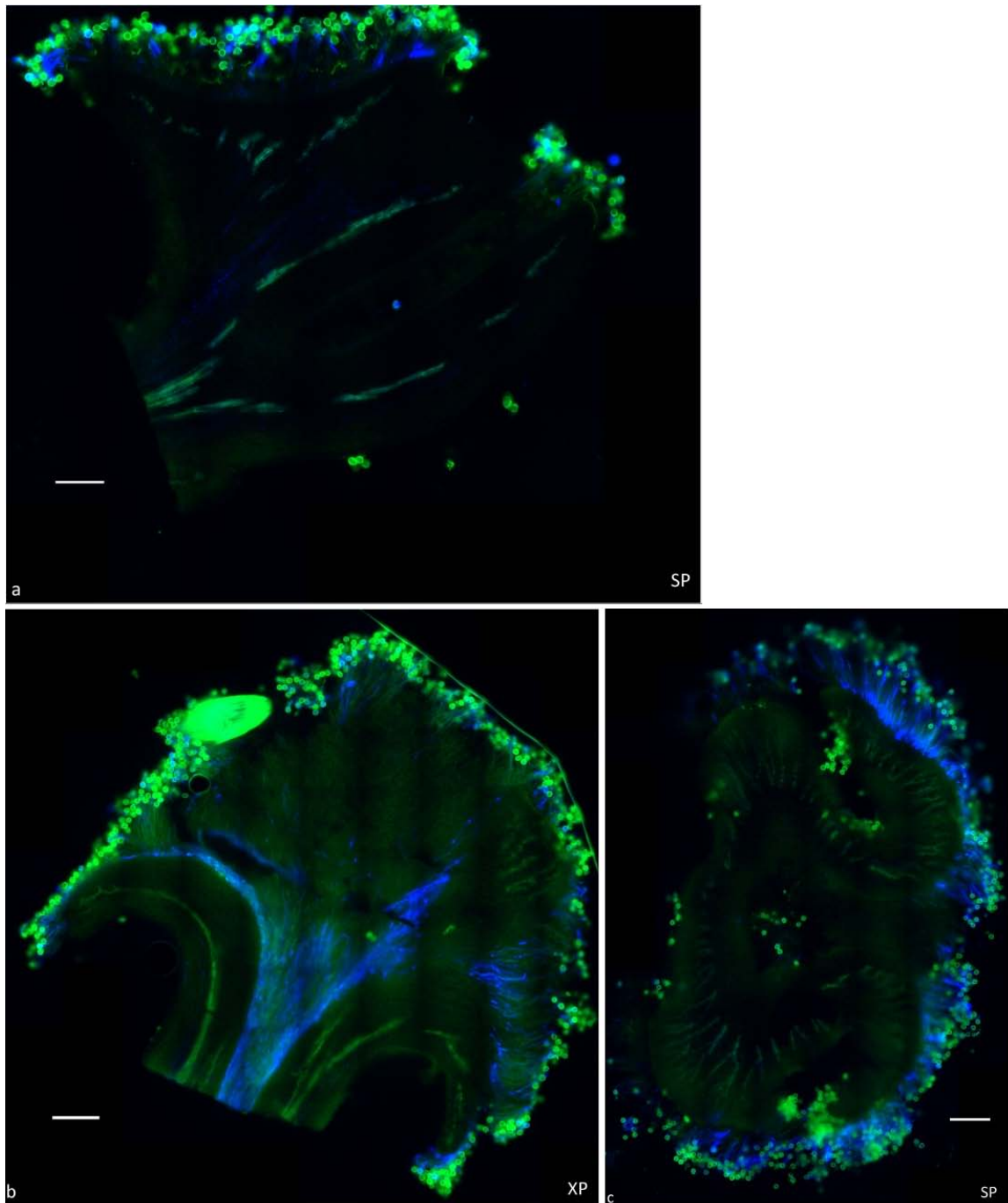


Figure 6. Fluorescent light microscopy images of sectioned stigmatic tissue from *Argemone munita* after exposure to outcross pollen (XP) and self-pollen (SP). Images are stitched mosaics. Scale bar = 500 μ m. **a, b.** Sagittal sections of stigmatic tissue. Penetration of pollen tubes (blue) into style occurs only following cross-pollination. **c.** Transverse section of stigmatic tissue following self-pollination. Pollen tubes (blue) end before penetrating through the stigma surface.

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