A Quantitative Model of In-Vivo Pollen Tube Growth in Arabidopsis

A senior thesis presented

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Abstract

In seed plants, pollen tubes are cellular extensions of pollen grains that form during plant pollination, and which carry the sperm nuclei carried in the pollen through the female reproductive tissue to an unfertilized ovule. In flowering plants, pollen tubes grow through the style of the female, gradually finding their way to the ovules in a pattern of growth that ensures each ovule is almost always fertilized by exactly one pollen grain. The system of guidance and control which directs pollen tube growth both attracts pollen tubes to all ovules and prevents multiple-fertilization, likely through a series of guidance cues that have been only partially identified and understood. Here, we examine proposed mechanisms of pollen tube guidance in the model angiosperm *Arabidopsis thaliana*, where the problem has been extensively studied, and build a computational approximation of the space in which pollen tubes can grow, tracking them from pollination to fertilization. Through simulated "trials" of pollen tube growth under various hypothetical systems proposed to factor into pollen tube guidance, we demonstrate that within the space of our approximations and tested parameters only the possibility of a quickly-released post-fertilization repellent signal appears to be a necessary and sufficient condition to largely eliminate polytubey and lead to most ovules being fertilized. We also demonstrate here how a computational model of the system can be used to simulate mutant phenotypes—both theoretical and observed—and how it may thus be able to shed light into other aspects of the system as well.

Introduction

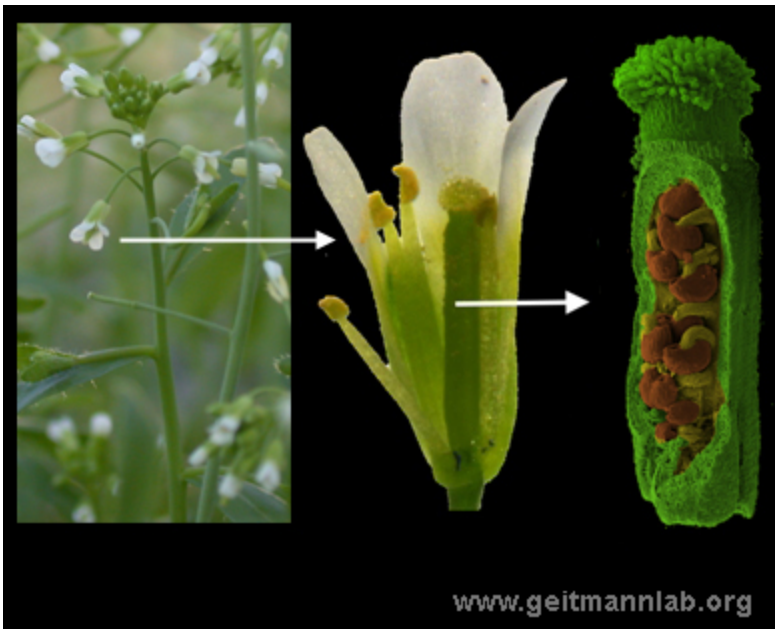
The process of reproductive cell fertilization in seed plants involves the growth of pollen tubes—cellular extensions of pollen grains that digest their way through the pistil and into a female ovule, where they release their two sperm nuclei to create a zygote and triploid primary endosperm cell. Pollen tube growth—the ability both of pollen tubes to grow through female tissue and to find their way to an unfertilized ovule—has been studied in a number of model organisms, including *Arabidopsis thaliana*, on the reproductive systems of which this paper's simulations were modeled.

For the purpose of modeling the process, we break down the growth of pollen tubes in *Arabidopsis* into four major of stages. In the first, pollen tubes grow into and through the style of the flower. In the second, pollen grow through the "transmitting tract," a tube within the pistil that contains stacks of cells aligned vertically, divided by a basal-to-apical barrier that effectively divides the transmitting tract into two semi-cylinders (reference paper, image? not sure which to use here necessarily). In this stage, the pollen grow in a roughly straight direction towards the basal end of the flower, breaking down the cellular walls of the transmitting tract cells they grow through. Eventually, most pollen tubes will wander out of the side of the transmitting tract and through the septum, a sticky layer of cells that surrounds the transmitting tract, forming its outside surface. In the third stage, pollen tubes grow along the surface of the septum (which, due to the pattern of cellular development in the flower, is surrounded by air), eventually following guidance signals to the base of a funiculus.

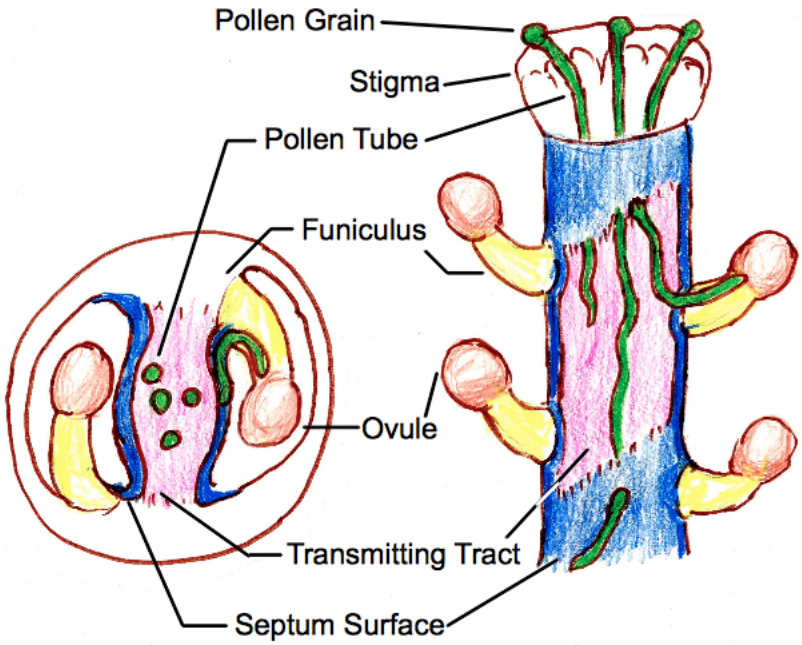
In the fourth stage of growth, the pollen tube grows up the funiculus, a long thin bundle of cells leading to the base of an ovule, which the pollen tube enters by growing through one of the micropylar cells, followed by fertilizing the ovule (Hulskamp et al., 1995). Figures 1 shows images representative of pollen growth.

Figure 1:

A.



A. Several photographs of an *Arabidopsis* flower. Left: Location and appearance of *Arabidopsis* flowers growing on the plant. Center: A cut-away of the flower shows the location and shape of of the pistil, the female part of the flower. Right: A cut-away of the pistil shows the ovules and attached funiculi as they naturally appear, curled up within the outer layers of the pistil.



B. **C.**

In parts B and C of this figure, we are looking at a cartoon representation of the physiology of the female parts of a flower.

A. A horizontal section throught the flower shows the transmitting tract tissue, in pink, with pollen tubes (green) growing downwards through it. One pollen tube has emerged onto the surface of the septum (blue), and is growing up the surface of the funiculus, in pink, towards the micropylar region of an ovule (ovule is light brown). Surrounding tissues are not colored.

B. Looking at a separated and distorted transmitting tract and surrounding tissues, we see pollen landing on the stigma, growing through the style and into the transmitting tract, eventually exit to grow on the surface of the septum before climing up a funiculus to an ovule.

There are a few remarkable things to note about the pollen tube growth and guidance system, a testament to its evolutionary success. The first is a lack of multiple- targeting; that is, the same ovule is almost never fertilized by two separate pollen grains, a defect which would lead to a triploid and thus non-viable zygote. While this could to some extent be the result of a rapid block to polyspermy following fertilization (such as a physical change in the cell wall of the egg cell),

in vitro studies (Shimizu and Okada, 2000) have shown that pollen tubes growing in a mostly straight path will turn away from recently fertilized ovules, suggesting a change of signals responsible for attracting pollen tubes to an ovule. The second interesting feature of the system is the ability in any given flower to have every or nearly every ovule fertilized—in an *Arabidopsis* flower bearing approximately 50 ovules, the seed count will almost always be as high as the ovule count, even with less than 100 grains of pollen (reference? this seemed anecdotal when we talked about it).

A number of past studies have made inroads towards understanding the workings of the pollen tube guidance system. A relatively recent paper (Okuda et al., 2009) identified several proteins, termed "LUREs," released from synergid cells and capable of attracting pollen tube growth. Antisense inhibition of LURE proteins in vivo interfered with pollen tubes' abilities to target ovules, and the identified LURE proteins are cysteine-rich, suggesting that they might form structurally important cysteine bonds and could be easily deactivated by cleavage of these bonds by a specific enzyme.

A further study (Stewman et al., 2010) demonstrated the ability of mathematical modeling to gain insights into the factors controlling directional change in pollen tube growth. The group used a semi-in vitro method to examine ovules' ability to attract pollen tubes, recording the positions of each tube tip at each time point. Leaving ovules to "incubate" on an artificial medium, cut styles were pollinated and placed on the same medium, allowing pollen tubes to grow roughly in the direction of the ovules. By measuring the degree to which pollen tubes

turned based on their distance from ovules and the time of said ovules' incubation, the group was able to quantitatively analyze ovules' attractive effects on pollen tubes, finding data that agreed with a model where pollen tubes' rate of turning is proportional to the change of concentration of an attraction across their tip, where the attractant is constantly secreted from the ovule. Factoring into account ovule incubation times, Stewman et al. were able to approximate the rate at which the attractant diffused, at 66.72 μm2/min—a very similar rate to that of ubiquitin, a protein predicted to be roughly the same size as the 8-9 kDa LURE proteins a previous study had identified.

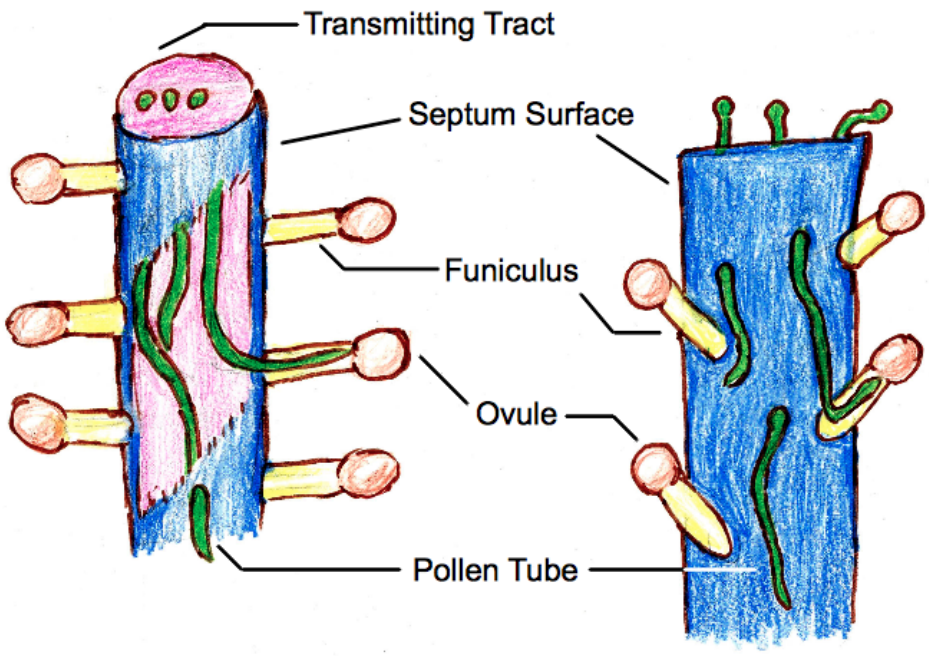
Preliminary implications have suggested a number of other factors exist that may potentially recruit or turning away the growth of pollen tubes, although most of the studies detailing these examine only their effect on in vitro growth and not the extent to which they play a part in in vivo guidance (Palanivelu, 2006). Another suggested point of the system has been that some sort of basal attractant signal may exist in the transmitting tract and/or on the surface of the septum, drawing pollen tubes downwards through the pistil through an increasing concentration gradient, an idea suggested by analyses of pollen tube defects (Crawford et al., 2007). For the purposes of this study, we simplify these possible signals to a generic attractant, and potentially a generic repellent and generic basal attractant, as will be further discussed in the Methods section of this paper.

Methods

The Model Itself

Modeling the process of pollen growth required certain geometric simplifications of the transmitting tract, septum, funiculi and ovules. First, we treated the transmitting tract as a simple cylinder. Pollen grains landed randomly on the top, with their growth-start times distributed uniformly over a 30 minute interval, producing a pattern of pollen reaching the top of the transmitting tract we consider similar to that resulting from pollen grains first growing through the stigma and style and into the tract. The pollen grew downwards through the transmitting tract towards the basal end, moving in random steps "outwards" through the bundled cells and closer to the surface of the septum. Upon emerging onto the surface of one side of the septum, which we modeled as a flat plane, the pollen was then allowed to move two-dimensionally until it reached the base of a funiculus, at which point it could choose, based on the concentration gradient at the base of the funiculus, whether or not to grow up the funiculus; upon reaching the top of a funiculus, it could again choose, based on concentration gradient and any physical block established by the ovule, whether or not to grow into the ovule and release its sperm nuclei. Figure 2 shows the translation of the physical flower structures into the geometric representations used in our model.

Figure 2:



A. **B.**

In this figure, we are looking at cartoons of increasingly abstracted models of the female parts of a flower.

A. Looking at a separated and distorted transmitting tract and surrounding tissues, we approximate the transmitting tract as a cylinder. We bypass the style and stigma, instead choosing to start monitoring pollen tube growth from reaching the top of the transmitting tract. The funiculi are treated as tubes, growing straight out, whose surface pollen climb if they reach one and are not repelled.
B. "Unrolling" the septum, we can model pollen tube growth on this surface. In this case, we treat the septum as a flat, two-dimensional surface on which pollen tubes begin growing once they have reached the surface of the cylindrical transmitting tract.

The pollen's growth on the surface of the septum was influenced by the concentration gradient of an attractant signal (and possibly other signals) released by ovules. The signal molecule was modeled as diffusing in one dimension down the length of the funiculus, and then two-dimensionally across the surface of the septum, allowing the funicular bases to effectively act as point-sources that set up a gradient to attract pollen tube growth.

Equations used to model diffusion of attractant

To model attractant concentration, we first assume that each untargeted ovule releases an attractant in a single pulse of concentration C, which diffuses out down the funiculus and across the septum; the base of the funiculus is thus treated as a point source for release of the attractant, most of which diffuses laterally along the septum's surface as if it were a 2-dimensional surface. The protein must diffuse a certain distance to get to the base of the funiculus, and we can approximate this distance as L+R, where L is the length of the funiculus from septum to ovule and R is the radius of an ovule, representing the amount of diffusion the signal must undergo to escape from the ovule. The attractant, we assume, is a signaling molecule that diffuses with diffusion constant D and breaks down with half-life h.

Then, on the surface of the septum, at a distance r from the point where the funiculus meets the septum, we model the attractant concentration as following the two-dimensional form of Fick's law of diffusion, the classical solution to the diffusion equation. This gives the concentration at time t as $(0.5)^{t/h}(\frac{C}{4\pi Dt}e^{-(r+R+L)^2/4Dt})$ for a single, instantaneously released pulse of attractant protein with concentration equal to C.

If we assume continuous release at a rate of k/time units and beginning at time T0, the concentration at time T becomes $\int_{t=T_0}^{t=T}(0.5)^{t/h}(\frac{C}{4\pi Dt}e^{-(r+R+L)^2/4Dt})dt$ with a gradient in the radial

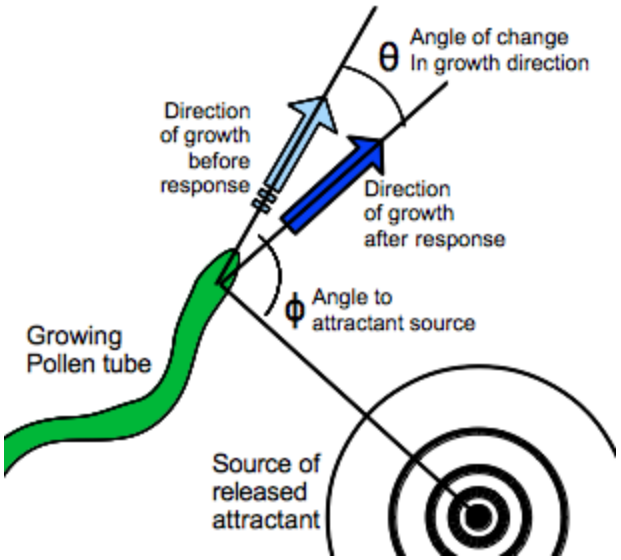
direction of $k\frac{d\int_{t=T_0}^{t=T}(0.5)^{t/h}(\frac{C}{4\pi Dt}e^{-(r+R+L)^2/4Dt})dt}{dr}$, where k is the rate of attractant release. However, the actual concentration of the attractant is not by itself important, as the effect of the protein is equal to k*s, where k is the rate at which the attractant is released and s is the chemoattractive strength of the attractant (in units of [angle]*[concentration]$^{-1}$*[distance]$^{-1}$); then the total chemoattractive effect on a growing pollen tube is equal to $\theta(r,t) = k * s\frac{d\int_{t=T_0}^{t=T}(1-\frac{0.693}{h})(\frac{C}{4\pi Dt}e^{-(r+L)^2/4Dt})dt}{dr}$, with θ(r,t) measuring turning rate in radians per one second step. Using values for k*s (the strength of the chemotactic effect) and D (the diffusion constant) derived from the cited modelling paper (Stewman et al., 2010), as well as some approximations about the size of various components in Arabidopsis, we can generate approximate values for the attractant strength of the gradient at a distance r from the ovule, at time t.

Pollen tube response to attractant

Following equations used in earlier modelling work again (Stewman et al., 2010), we assumed a model in which each pollen tube grows at a certain roughly constant rate V, and at each time step has its angle of growth altered by the gradient of attractant it grows through equal to $\theta(r,t) = G * sin(\phi) + \sigma N(0,1)$, where ϕ is the angle between a vector pointing in the direction of growth and a vector pointing towards the ovule and G is the strength of the attractive gradient (Figure 3 illustrates these angles and vectors). σ is a variable describing the average random variation of the angle per time-step (as the pollen tube sort of "wobbles around" looking for ovules), and N(0,1) is a standard normal distribution.

To model chemotactic response in this paper, we assume that each pollen tube tip grows at a constant velocity and changes its angle in a discrete response step every minute. Pollen tube tips are assumed to have negligible width. Concentration gradient is calculated as the difference between concentration at the nearest calculated distance from the ovule and at the second closest calculated distance, as, to save computational time, concentrations are computed over an array of distances with a preset resolution.

Figure 3:



(this figure could function as an inset of a more zoomed-out diagram)

In this figure, we present a cartoon showing the relevant angles mentioned in calculating a pollen tube's change in angle; specifically, the angle of directional change itself as well as the angle formed by the pollen tube's growth vector and the vector pointing from the center of a modeled point-source of attractant (*Adapted from Stewman et al. 2010)*

Anatomical dimensions used in models

Some generalizations were made in order to estimate physical parameters used in the model. In the Stewman paper, the attractant signal had a diffusion constant of approximately $6.67x10^{1}$ μm^2/min, or $6.7x10^{-5}$ mm^2/min. While this is an estimated diffusion constant for in vitro medium, we used it as a starting approximation for the rate of in vivo diffusion in the model. The Stewman data also predicts such an attractant in vitro setting up a gradient with power*concentration (k*s) of approximately 0.045 radian/min after 240 minutes of continuous release; if we assume a base radial offset for attractant leaving ovules of 0.12 mm, this gives us a power*concentration constant of about 0.00000285.

Other approximations of constants were made based on observed dimensions of *Arabidopsis* physiology, from a combination of microscope photographs and videos. The funiculus was treated as a linear path for growth and diffusion with length of approximately 80 nm, and a collision distance—the range within which pollen tubes passing by the funiculus were considered to have come in contact with it—of 20 nm. The one half of the surface of the septum on which pollen grew was treated as a flat plane with a length of 3 mm and a width of 0.2 mm. Each half-pistil modeled contained 24 ovules, staggered over two parallel rows.

Pollen were assumed to grow at a base rate of 2 nm/min, in paths with a random wobble modeled as 0.05 times a standard normal distribution. Approximately 100 pollen were assumed to be present in each pollination event, leading to 50 pollen in each model run (as the septum is effectively divided in two, and the model represents only one of these two halfs). In the case of multiple pollen phenotypes, the proportion of pollen bearing each phenotype was treated as

constant rather than probabilistic. The time at which pollen finished growing through the stigma was uniformly distributed over a 30 minute period for most simulations, and the simulation was run for a total of 1500 one-minute time-steps. Gradients established by released chemical signals were assumed to equilibrate after 500 minutes, and the gradient was pre-calculated at various distances from ovules with a resolution of 1500 points per millimeter.

Results

1. General features of a model for pollen distribution within the pistil

We simplified the growth of pollen tubes in *Arabidopsis* down into four distinct stages. In the first, pollen tubes grow into and through the stigma of the flower. In the second stage, pollen tubes grow through the transmitting tract within the style. Here, we represent the transmitting tract as a cylindrical tube: pollen tubes enter at a random point in the top of the style and begin to grow down towards the basal end, following a roughly straight line with random variation and possibly sensitive to an attractant gradient set up by distant ovules.

Most pollen tubes will eventually leave the transmitting tract to begin growth on the two-dimensional surface of the septum. While it is likely that some quantity of ovule-released attractant signal diffuses into the transmitting tract, both attracting pollen to the surface of the septum and causing it to emerge on the septum closer to the base of funiculi, such simulations proved more difficult to compute and showed little to no difference in effective pollen targeting

from simple random growth within the transmitting tract. In our simplified model, pollen tubes are treated as growing randomly within the transmitting tract but with a slight tendency at each time step to grow towards the surface of the septum more often than away from it.

In the third stage, pollen tubes grow along the surface of the septum (which, due to the pattern of cellular development in the flower, is surrounded by air); we model this by treating the septum surface as an effectively flat plane along which pollen tubes can travel, responding to guidance signals. These guidance signals result from signaling molecules released by ovules, signals which diffuse down the funiculi and across the sticky surface of the septum, setting up an idealized concentration gradient.

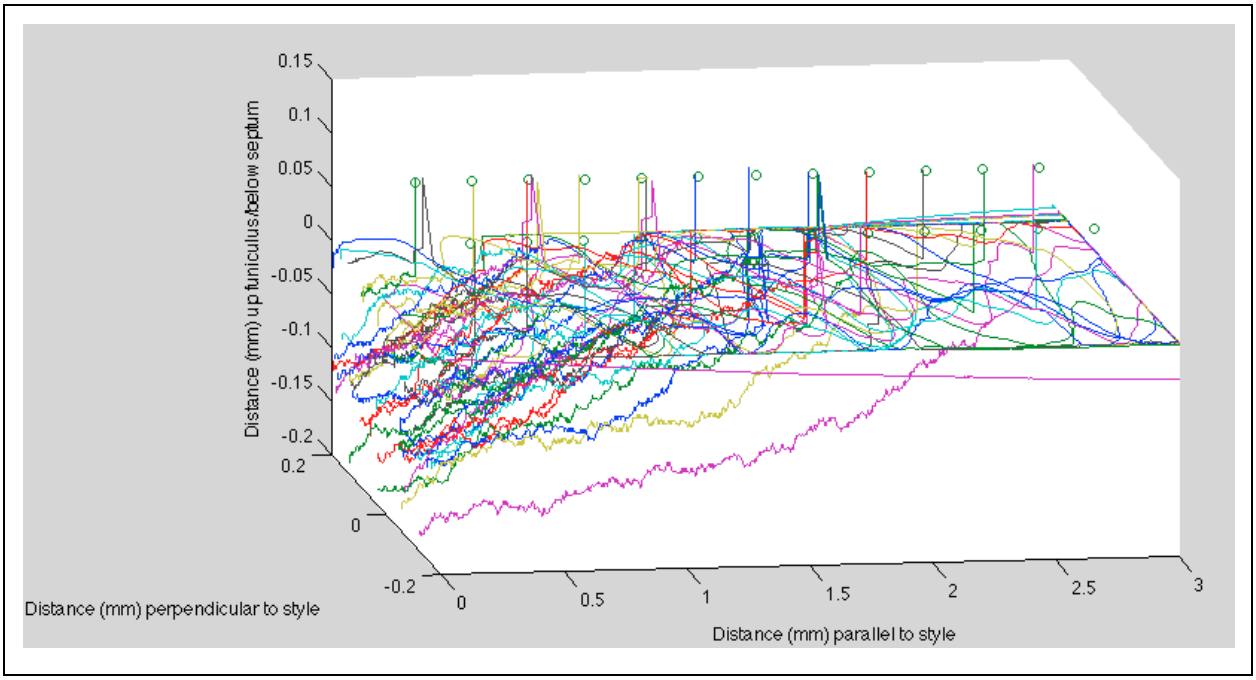
In the fourth stage of growth, the pollen tube grows up the funiculus, a long thin bundle of cells leading to the base of an ovule. We treat the funiculus as a one-dimensional path, with pollen tubes growing up a funiculus if the signaling gradient attracts them up it, checking at the end whether the ovule will allow entry of a pollen tube, and if so fertilizing that ovule. A pollen tube will grow up the base of a funiculus if it collides with the funiculus' base, and if the attractant gradient shows higher concentrations of attractive signals higher up the funiculus; a change from attractant to repellent (or attractant pointing in the opposite direction) gradient signaling will cause the pollen tube to grow back down the funiculus. We recognize this may not be the exact pattern of growth that occurs in such cases in nature, it is likely that some sort of gradient-strength based decision-making process determines whether pollen tubes grow all the way up a funiculus or just skirt the base after possibly growing slightly up the funiculus, and our

model's allowance of pollen tubes to grow back down funiculi is a simplification of such a process.

Lastly, in the model, pollen tubes will release their sperm nulei into ovules only in the event that there is still a higher or approximately equal concentration of attractant within the ovule, which would direct continued pollen growth into the micropylar region of the ovule; additionally, in certain simulation conditions a fertilized ovule undergoes a chemical or physical change that prevents the entry of additional pollen tubes. Various parameters we tested included such a rapid block to multiple fertilization a certain amount of time after the initial fertilization event, as well as the possibility of a repellent signal released rapidly after fertilization, which would create an effective block to second-fertilization.

Figure 4 shows an example of a trial simulation. The pollen tubes grow for some time in the transmitting tract, emerging one by one onto the septum, where they continue to grow until they hit a funiculus, which they climb up.

Figure 4:
The following figure provides an example of a pollen growth simulation, depicting the growth patterns themselves on the simulated "unrolled" septum. Pollen tubes enter the top of the transmitting tract to the left, and grow in the basal (right) direction, climbing upwards when they hit a funiculus. Ovule locations are represented by circles.



2. Analysis of data

All graphs bearing error bars represent trials of fifty growth trials, with bars indicating standard deviation. These data are presented in two styles of graph, the first showing the average percentage of ovules that were untargeted, single-targeted, double-targeted, et cetera; the second showing the average number of ovules targeting each ovule position among the twenty-four spaced-out ovules.

3. Results of simple model, and predictions about half-life and diffusion rate of attractant

In the simplest model, we assume that the ovules release a polypeptide signal which attracts pollen tubes, based on the assumptions discussed in the Methods section. Three factors control

the strength and distribution of this signal: 1) The strength of the signal's effect; 2) The rate at which the signal diffuses; and 3) The rate at which the signal is broken down in relevant tissues—that is, its half-life.

A single constant, based on attractant strength and signal release rate, can be estimated from data in the Stewman et al. paper (see Methods). While diffusion rate of the signal is likely different in plant tissue than in vitro, variations within an order of magnitude had only marginal effects on gradient distribution and pollen guidance, and so the parameters of diffusion were approximated as those determined in the previous work.

Making various guesses for the half-life of the signal affects the effective "range" of the attractant gradient; if it is too small, the attractant is mostly broken down by the time it reaches the base of a funiculus, and if it is too large, the attractant persists for a long period of time, weakening the gradient to the point of ineffectiveness. We generated a number of sample trials with various attractant half-lives, and can assume a reasonable attractant half-life in our simplified model to be somewhere between around 1 and 16 hours, all of which performed very similarly in the event of a simple attractant signal; 2 hours was chosen as a reasonable half-life time, as this led to marginally better pollen tube targeting in the simple case where ovule targeting only led to a cessation of attractant release.

4. Eliminating polytubey and distributing pollen tubes

A number of scenarios for increased complexity in the model could potentially address the two major features of pollen targeting—targeting all ovules once, and targeting ovules only once—that the simple attractant model fails to address in our system.

A. Cessation of the attractant

The simplest possible modification to the system would be cessation of the attractant upon fertilization; in this model, as soon as ovules are fertilized, they cease to release attractant. However, making such a modification seems to have little impact on polytubey (Figure 5A).

A second possibility is that some sort of attractant-disabling signal is released upon fertilization, destroying the effectiveness of the attractant gradient. We simplify this and model a similar scenario, wherein the attractant released by a specific ovule rapidly breaks down after that ovule is fertilized, and this is the case for each ovule—while an attractant-disabling signal or enzyme would be more difficult to model, the simplification of rapidly "turning off" the attractant created by one ovule should have a similar effect, depleting the attractant by a greater amount closer to the ovule. As the same figure demonstrates, this also had a relatively minor effect towards reducing polytubey. However, causing ovules to rapidly lose their attractant gradient after fertilization led to already-fertilized ovules snaring fewer passing pollen. The small decrease in polytubey caused by assuming rapid attractant breakdown was accompanied by a significantly greater percentage of ovules being targeted by single pollen tubes and fewer untargeted ovules, probably due to the this effect..

B. Some sort of signal or physiological factor may act as a basal attractant to ensure targeting of most ovules

Another model for the growth of pollen tubes suggests that some sort of physiological substrate or gradient set up along the length of the transmitting tract acts as a "basal attractant," compelling pollen tubes with some force to grow towards the base of the style by adding a second attractant gradient term, this one based on an attractant of even slope from the apex to the base of the pistil. There is evidence of this existing in the literature within the transmitting tract (Crawford et al., 2007). Testing its existence in the septum is a little difficult, and it is possible that a basal attractant based solely in the transmitting tract would lead to a similar effect to two basal attractants, one of them septum-localized. We note, however, that the typically observed septum emergence pattern has pollen tubes emerging preferentially near the apical end of the style (Hulskamp et al., 1995), while pollen tubes tend to have little difficulty targeting ovules anywhere along the length of the septum.

We observed that pollen tubes seemed to more consistently fertilize basal ovules when the model included a basal attractant. We tested various strengths of this potential basal attractant signal, and found that in the absence of a repellent, this had a marginal effect on targeting, although it did appear to affect ovule distribution (Figure 5C); however, with a repellent signal present (to be mentioned later), we were able to manipulate the parameters of semi-random transmitting tract growth, which included a tract-localized basal attractant, to eliminate the need

for a septum-localized basal attraction gradient.

C. Gamete fusion as a possible trigger for the block to polytubey

Another hypothesis for preventing multiple-targeting of ovules is that ovules, when fertilized, rapidly change, creating a physical or chemical barrier to further fertilization within seven minutes (still not sure what reference we have for this, or if this is unpublished results?). Investigating this possibility with various simple attractant systems shows a marked reduction in polytubey; however, in modeled growth, a large number of pollen tubes still climb funiculi of ovules that have already been fertilized, only to flounder around when they reach the ovule, unable to fertilize it. Combined with the features already tested, this still appeared unsatisfactory at blocking polytubey (Figure 5A).

One behavior observed in the model in such cases was that pollen tubes often reached a funiculus less than ten minutes apart, and thus the second of a pair of pollen tubes targeting the same ovule would also enter the ovule and release its sperm nuclei before the rapid block to fertilization had time to occur.

D. A repellent signal under various conditions

We next investigated the idea that, following fertilization, ovules might release a signaling molecule that functions similarly to the attractant, causing an opposite effect—that is, inducing

pollen tubes to turn towards decreasing concentrations of a signal. This was modeled as simply adding a negative component to the attractant gradient, causing it to point growth away from ovules once sufficient repellent was released. While our model allowed for various diffusion rates and half-lives of such a repellent signal, we chose to run most simulations with a signal of identical diffusion constant and half-life to the attractant, as would be close to the case if a released repellent molecule were similar to the attractant.

Our results (Figure 5B) show that a repellent signal released with 10x the power*release rate of the attractant can be a significant block to polytubey, in combination with several other factors. Essentially, it appears important that the repellent is able to set up a negative gradient that pollen turn away from.

However, adding a repellent signal alone did not have a satisfactory effect. Several other suggested possibilities were tested for their ability to aid in the prevention of polytubey. Most significantly the idea that pollen tubes could turn down a funiculus and return to the septum, should a repulsive gradient form, led to a significant increase in single-targeting, as well as a decrease in the number of untargeted ovules, likely due to pollen returned to the septum in this manner seeking out new ovules (Figure 5B). As the modelled scenario in which pollen tubes grew back down funiculi required the establishment of a gradient pointing away from ovules, allowing this feature without a repellent signal had no effect on targeting in any other cases.

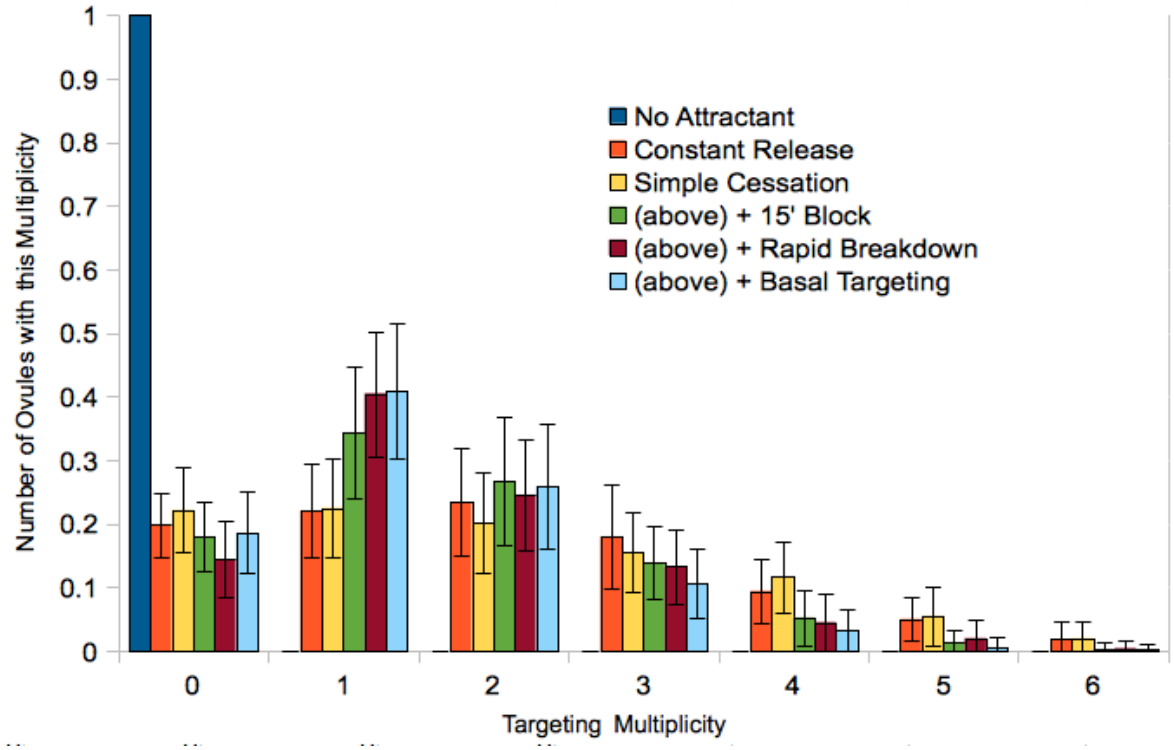
E. Attractant-moderated growth slowdown

Existing work points to the possibility that pollen tubes slow down their growth as they near ovules, potentially due to high concentrations of an ovule-released attractant also serving as a retardation signal. While previous work has demonstrated through simulation that such a slowdown would increase the accuracy of pollen tubes had in targeting specific sources of attractant (Stewman et al., 2010), the effect had no statistically significant bearing on the frequencies of single-targeting or polytubey in our model.

Figure 5: Effects of Various Guidance Hypotheses on Ovule Targeting

In this and subsequent figures, bars represent the average rate of targeting over fifty trials; error bars represent standard deviation, showing not the expected error but rather the average variation from one trial to the next.

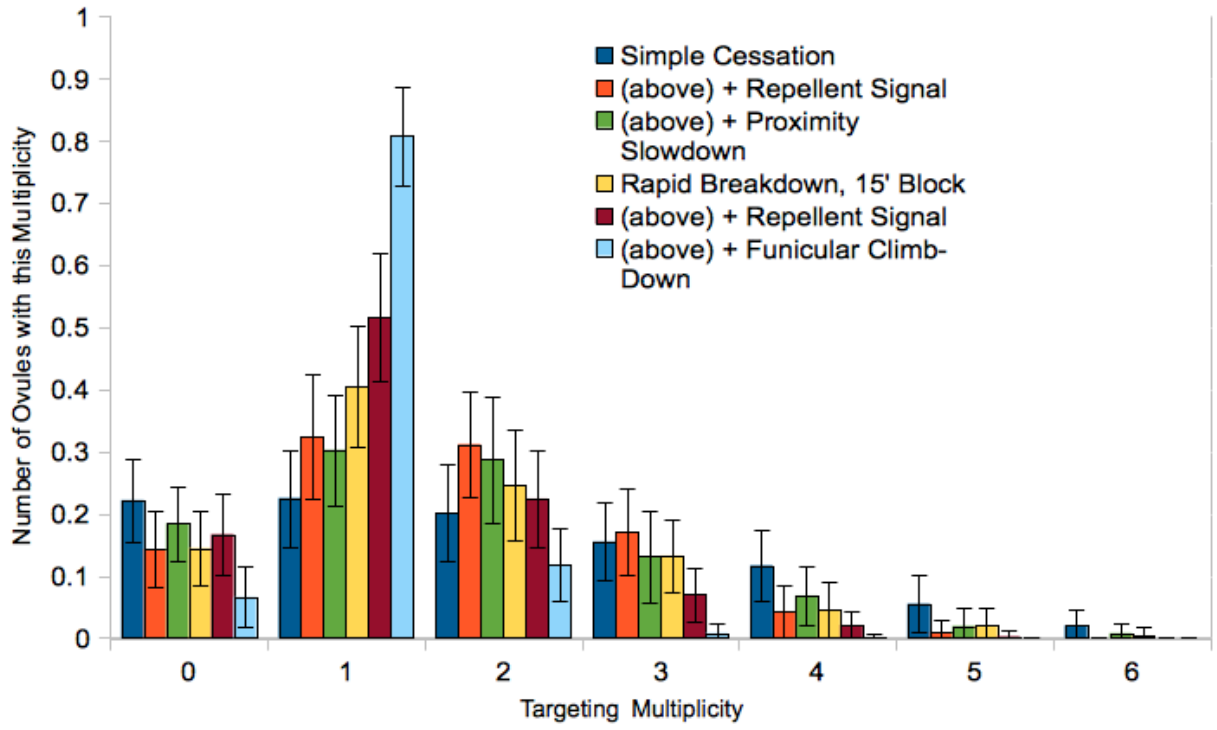
A.



In the absence of an attractant, all ovules in the simulation were targeted zero times. In the presence of a constantly-released attractant, the rate of single-targeting was approximately

equal to the rate of zero-targeting, and most ovules were targeted by multiple pollen tubes. Allowing fertilization to simply cause ovules to cease releasing attractant had little effect on the rate of single-targeting. Adding a physical block that prevented pollen tube entry 15 minutes after ovule fertilization increased the rate of single targeting. Adding to the above factors a reaction that caused attractant to break down 100x as fast in the vicinity of a fertilized ovule led to slightly more single targeting. A basal attractant that acted similarly to the ovule attractant but weakly directed growing pollen tubes towards the basal end of the stigma had no noticeable effect on targeting when added to the above factors.

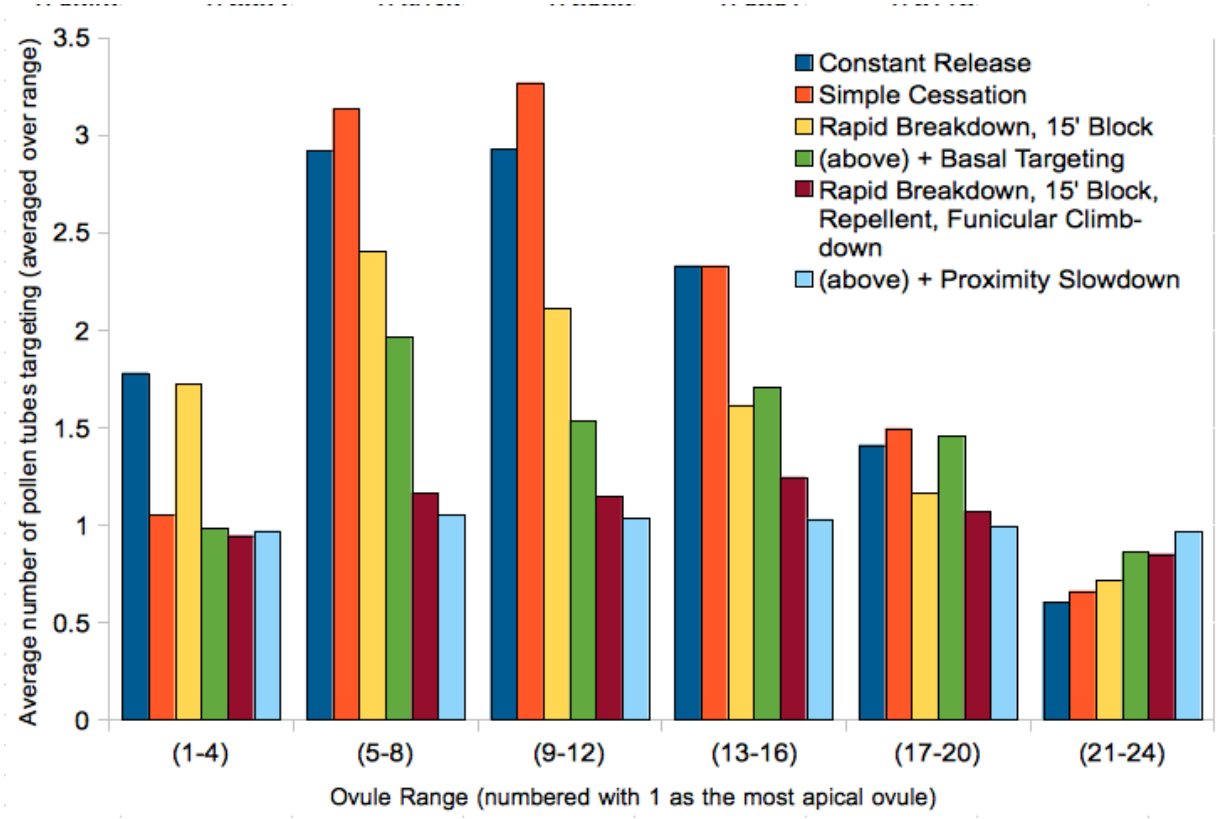
B.



Adding a “repellent” signal which was continuously released from an ovule for the hour following its fertilization, and which had 10x the chemotactic strength of the attractant,

increased single-targeting rates significantly. All scenarios described in a) showed substantial gains in single-targeting with the addition of a repellent signal, and these gains appeared to stack with those gained from other potential barriers to polytubey. Causing pollen tubes to slow down when in near proximity to a funicular base had no significant effect on single-targeting rates. Allowing pollen to climb back down to the surface of the septum in the event that they had begun to grow onto the funiculus when a change of signals suggested reversing course significantly increased the single-targeting gains presented by the repellent, leading to the highest single-targeting rate of any set of trials.

C.



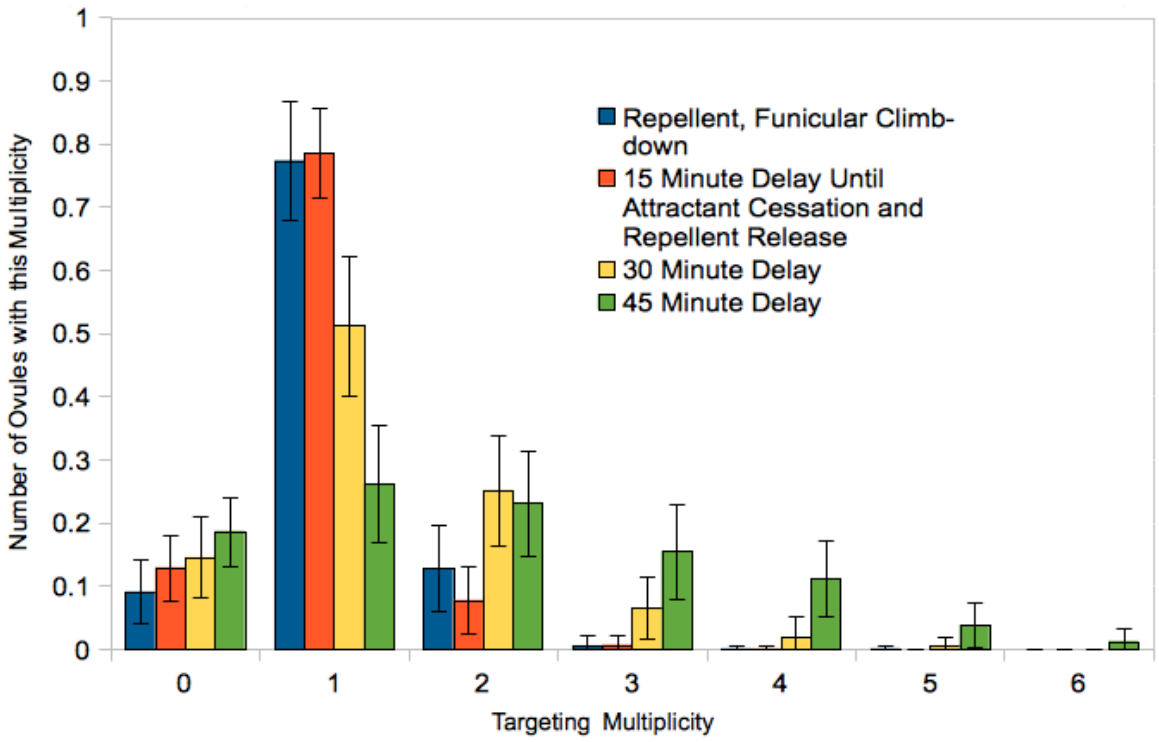
Here, we show the effect various trial scenarios have on which ovules are targeted (out of a virtual pistil with 24 ovules). In general, adding features to the model that reduced polytubey led to a more even distribution in pollen across all ovules, while trials with fewer of these features led to higher targeting of ovules located towards the middle of the pistil. Adding a basal attractant that targeted pollen tubes to the lower end of the septum also appeared to decrease targeting of apically-located ovules and increase basal targeting.

5. Testing the possibility of a delay between fertilization and ovule response

Next, we considered the possibility that a delay of some extent exists between the time a pollen tube enters its ovule and the system's response time, a delay that may incorporate time required for gamete fusion or for other downstream chemical changes. The simulation was run with a repellent present of 10x the attractant strength*release rate (and with pollen allowed to climb back down funiculi) as well as a rapid block to fertilization, both of which began to take effect some time interval after the instant of fertilization (Figure 6). Adding a short delay between fertilization and release of repulsion signal had a small effect on predicted fertilization phenotype between immediate response and 15-minute delay, but this delay caused significant problems as it lengthened to 30 and then 45 minutes (Figure 6). This means that the best model established in section 3 would still function effectively given a time delay of approximately fifteen minutes or less between pollen tube-egg fertilization and the actual chemical changes brought on by fertilization.

Figure 6:

The important chemical changes that occur following ovule fertilization may not be instantaneous, as growing through the micropylar cells of an ovule and initiating gamete fusion are likely to take time. By inserting a delay between the moment of fertilization and change in ovule state with regards to release of an attractant and repellent, we tested what might be a reasonable range of time this response could take without disrupting the system. There appears to be little difference in the targeting profile when the delay is only 15 minutes long, but significantly longer delays appear to lead to markedly lower rates of single-targeting.

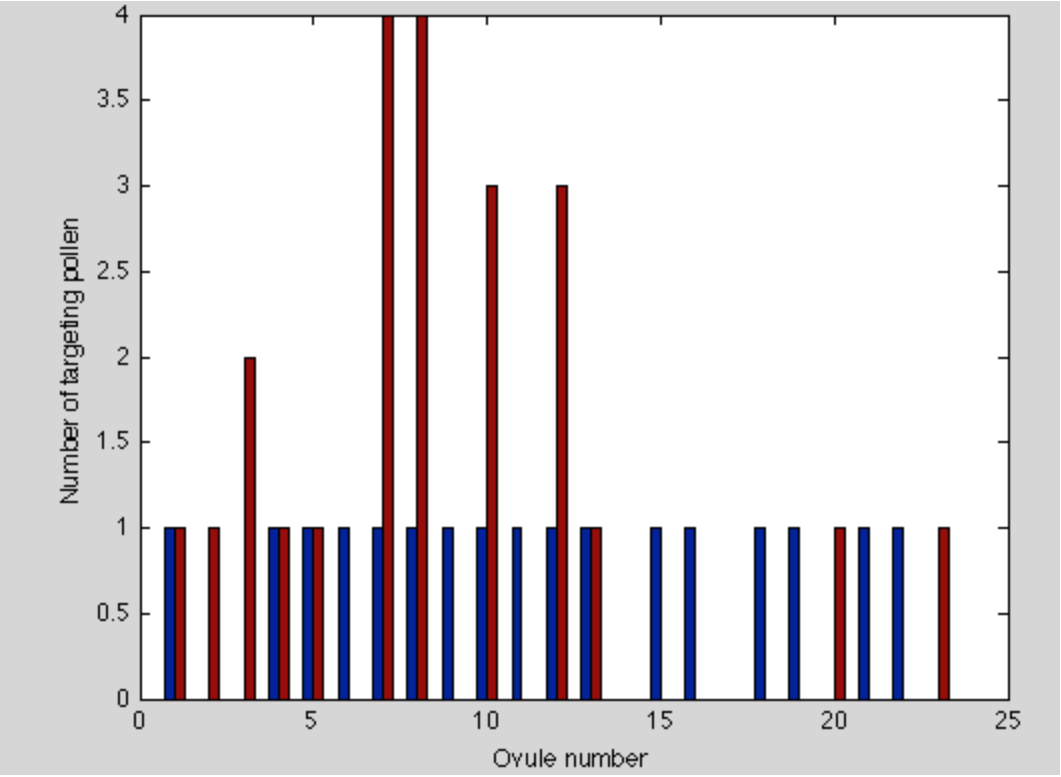


6. Phenotype Test: Modelling polytubey observed in hap2 heterozygous mutants.

The *Arabidopsis* hap2 mutant is unable to perform gamete fusion with its sperm, and mixtures of hap2 and normal pollen tubes lead to a phenotype in which only half of all pollen tube-ovule targeting events—those between ovules and wild-type sperm—lead to ovule fertilization (Johnson et al., 2004). Other experimental work (reference? this is the stuff Kristen's been doing,

right?) demonstrated that this leads to frequent multiple targeting of ovules by hap2 sperm, while most ovules in such experiments were only fertilized by at most one wild-type pollen tube. Experiments with this condition (50% faulty sperm) programmed into the model matched real observations, fitting especially well in the model containing a repellent signal, where ovules were frequently multiply-targeted by *hap2* pollen but usually at most single-targeted by wild-type pollen (an example of one such run can be seen in Figure 7).

Figure 7:
An example of a *hap2* pollination simulation (red represents *hap2* pollen; blue is WT). While similar average numbers of each pollen phenotype targeted ovules over many runs, individual run profiles consistently showed differences between the two. Specifically, ovules were often targeted by multiple hap2 pollen or by none at all, while ovules were almost always targeted by at most a single wild-type pollen.



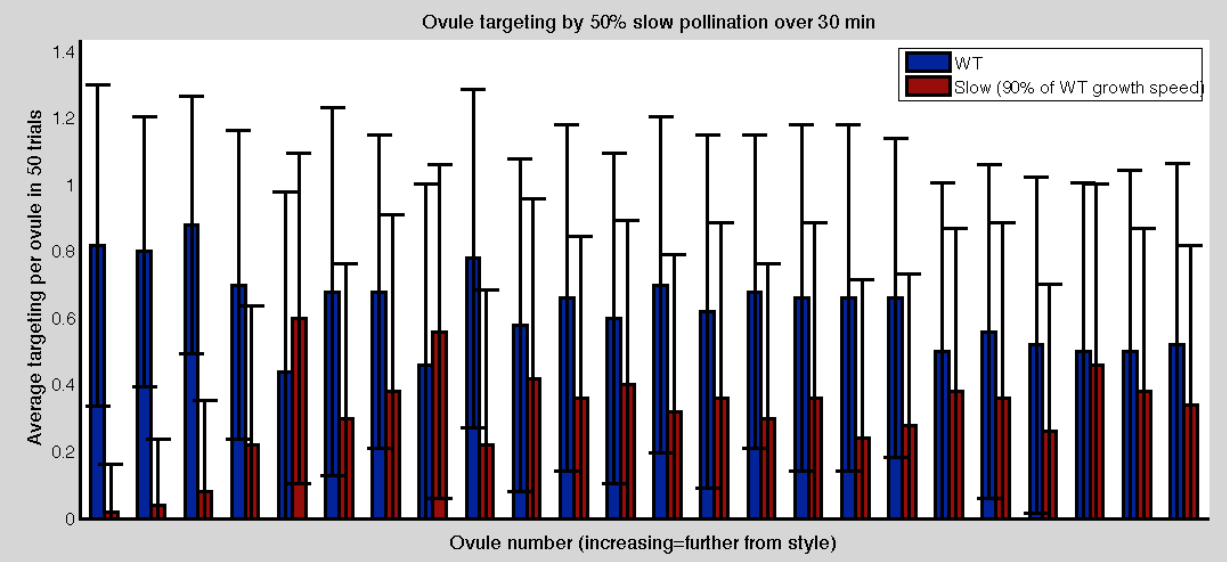
7. Phenotype Test: Predicting a hypothetical "slow pollen" mutant

We also tested the model's ability to predict the phenotype of a novel mutant. In this experiment, we considered a hypothetical "Slow" mutant, whose pollen grew at a rate approximately 90% of the wild-type pollen growth rate. In a fertilization with equal parts slow and WT pollen, wild-type significantly outperformed slow pollen, with the slow pollen only responsible for fertilizing a fraction of the ovules, with most of these located towards the middle of the pistil or at the basal end (Figure 8A). The severity of this phenotypic variation also appeared to correlate with the difference in pollen growth speeds.

In contrast, allowing pollen to land on the style over in a random uniform distribution over 24 hours significantly diminished the phenotype, as temporal spacing decreased the degree to which small variations in speed affected pollen competitiveness (Figure 8B). These data suggest a possible novel phenotype that should result in, and possibly be predicted from, a specific pattern of growth, where concurrent pollination leads to far less mutant competitiveness than a pollination spaced out over a longer interval of time.

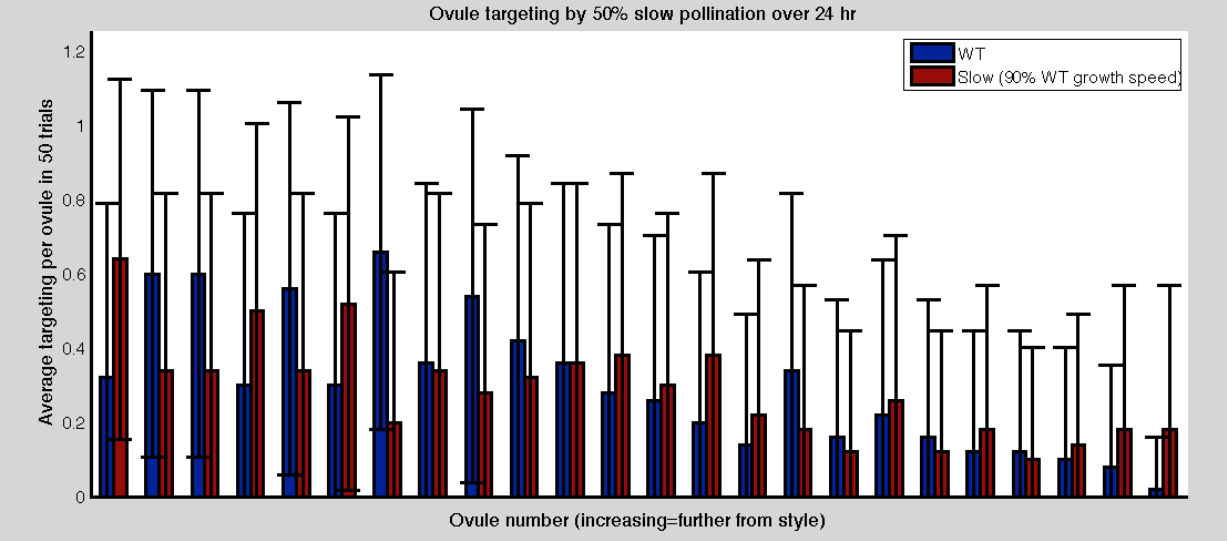
Figure 8

A. An example of a pollination with half pollen of the "10% slower" phenotype, with pollen emerging from the basal end of the style and into the transmitting tract over a 30 minute interval, approximating the timing resulting from a single multiple-pollen lab pollination.



B.

The same scenario, assuming more distributed pollination times, with pollen growing through the style over a 24-hour interval. The distribution of slow and wild-type pollen in fertilizations appears much more even.



Discussion

1. The Limits of Mathematical Modeling

Many of the hypotheses addressed in this study have been previously proposed, either as observations on qualitative analyses of pollen tube growth or as hypothetical answers to the problem of eliminating polytubey. Still others are evaluations of ideas for patterns governing growth that common sense dictates should or should not most likely be effective. While computational analysis does not definitively answer any of these questions or tell us the most likely scenario, it does provide us with a means to gauge the actual feasibility of various proposals and explanations for the full system governing ovule targeting.

These simulations are limited by the set of constraints, simplifications and approximations made to allow a reasonable scope for testing possible system models. While most of these simplifications appear to be valid shortcuts, the fact is that actual pollen tube guidance will always be more complex and subtle than any model we build, likely taking into account multiple ovule-released guidance signals and possibly other, less powerful physiological and chemical guidance factors located at various points inside the flower. Moreover, it is likely that the signals in question diffuse and are broken down at different rates in different tissues, a set of possibilities too complex to effectively analyze with a limited parameter space. Even the best simulated runs only approached 90% single-targeting of ovules, while real *Arabidopsis* pollination has higher

healthy seed counts than this; and the paths that our simulated pollen take are probably more jagged and edge collision-prone than those of actual pollen tubes.

Nevertheless, simulation has a valuable place in guiding analyses of the feasibility of various possible workings of a system. Most specifically, we demonstrate here how a potential rule-bound system wherein fertilized ovules release a signal that turns away growing pollen tubes is sufficient to establish a high rate of single-targeting in ovule fertilization. Significantly, we also demonstrate the shortcomings of other explanations under the set of parameters and simplifications used in this model, and in combination with experimental data make a robust set of assertions that we believe our data present strong evidence for a specific model fitting closely to the reality of the system.

2. Ensuring high rates of ovule targeting while preventing polytubey

It is clear that a continuous release of attractant from the ovules is not sufficient to generate full ovule targeting. Continuous release leads to many pollen tubes climbing the same funiculus, creating crowding that leads to increased polytubey, wherein many ovules would be targeted by multiple tubes, leading to fertilized ovules with extra chromosomes and nonviable seeds. The two major features of pollen tube targeting—a lack of untargeted ovules as well as a lack of multi-targeted ovules—are actually related, as all simulations with high rates of polytubey led to certain ovules capturing a large enough fraction of the pollen to reduce the population of unattracted pollen that had a chance to target other ovules, leading to higher rates of unfertilized

ovules. A number of scenarios for increased complexity were examined to address the necessary features of a guidance system.

A. Tested model features that appear to be insufficient in preventing polytubey

Simply assuming that ovules cease releasing attractant after fertilization is unlikely to have a significant effect on decreasing polytubey, as much of the attractant released by ovules persisted for some time afterwards, causing the attractive gradient associated with these ovules and funiculi to persist at a slowly weakening strength. Alternately, a rapid breakdown of attractant after fertilization also proved insufficient, as with the attractant gradient reduced or gone, pollen tubes tended to continue growth along a straight path, continuing growth up funiculi or continuing growth towards just-targeted funiculi, allowing some degree of multiple-targeting as well as causing many pollen tubes to "wander off," becoming associated with already-fertilized ovules and unable to fertilize remaining ovules.

A block to additional tube entry into ovules occurring fifteen minutes after initial fertilization also proved insufficient, with a similar effect. It was observed in the model in such cases that pollen tubes often reached a funiculus less than ten minutes apart, and thus the second of a pair of pollen tubes targeting the same ovule would fuse with the ovule before the rapid block to fertilization had time to occur. While two pollen tubes traveling so closely together would likely be a rare occurrence in nature, where pollen land on a stigma one grain at a time over a matter of days, the closely-timed pollen emergence from the stigma used in these instances of the model

was based on the single mass-pollination events that tend to occur in *Arabidopsis* lab settings. Even when a hundred grains of pollen are placed on a pistil at the same time, leading to pollen entering the transmitting tract within a short time window of each other, multiple-fertilization is rare, and thus the fact that there is little increase in multiple-targeting in lab pollinations serves to help discount the idea that a ten- or fifteen-minute-after-fertilization block to polytubey is alone enough to prevent it.

Basal attraction and high-attractant-concentration-driven growth slowdowns also had little effect on the incidence of single- or multiple-targeting. While these effects may or may not exist in the real system, their effects are probably more significantly tied to other aspects of the process, such as insuring basal ovules are equally targeted and helping pollen tubes to find ovules at the end of funiculi, respectively.

B. A repellent signal

Several experiments have documented certain chemicals which direct pollen tube growth away from the source of chemical signal, suggesting the possibility of a "repellent" signal that the plant could use to deter pollen tubes from already-fertilized ovules (Palanivelu and Preuss, 2006). Videos of simulated semi-in vivo mass pollination also suggest that pollen tubes may turn away from an ovule with increased frequency a short time after that ovule has been fertilized.

Our results demonstrated that, when pollen tubes were allowed to grow back down funiculi, a

strong repellent signal was the most effective feature that could be added to the model to prevent polytubey and encourage targeting of all ovules. There are many potential factors which could govern such a repellent signal, such as whether the signal diffuses quickly or slowly, its half-life on the relevant tissues, and whether it was released continuously or in one large pulse.

Still, the key features of the repellent signal appeared to be that it must be strong enough to overcome the force of attraction after a short period of release, as simply matching the repellent in strength times release rate to the attractant did not set up a gradient sufficiently strong sufficiently quickly to prevent multiple-targeting, even when the attractant was rapidly degraded. The repellent also must be able to set up a response fast enough to turn away pollen tubes soon after fertilization in order to keep the ovule from "trapping" too many other pollen tubes that can no longer fertilize it.

3. Possible alternative hypotheses

More possible theories, naturally, arise to explain what is observed in the system. One such theory is that pollen tube quorum-sense to some extent; in this case, pollen tubes could release a compound that repels other pollen tubes, leading tubes to take different paths and better distribute them between the ovules of a flower (Shimizu and Okada, 2000). However, calculating the effect of each pollen tube on each other tube proved computationally complex enough to necessitate a differently-programmed model, in which other simplifications were made.

Simulations using a pollen tube-released pollen tube repellent in place of an ovule-released repellent gradient demonstrated that in order to have a significant effect on pollen tube distribution, the pollen tube-released repellent must be extremely strong, enough to effectively set up a gradient as strong as a fertilized ovule; in a limited simulation of semi-in vitro growth, this pollen tube-pollen tube repulsion was powerful enough distort the paths of pollen tubes away from each other far more than normally observed in nature.

Moreover, modeling the *hap2* test along with a pollen-pollen repulsion theory lead to identical targeting by *hap2* and non-*hap2* pollen tubes, a phenomenon clearly not observed. Significantly, the *hap2* data support the claim that an ovule-released repellent signal is more important than one the tubes might release in preventing polytubey.

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