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Ann L. Carlson
Valparaiso University

Megan Telligman
Valparaiso University

Rob Swanson
Valparaiso University, Rob.Swanson@Valpo.edu

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ORIGINAL ARTICLE

Incidence and post-pollination mechanisms of nonrandom mating in *Arabidopsis thaliana*

Ann L. Carlson · Megan Telligman · Robert J. Swanson

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Abstract Compatible pollinations from many different taxa display nonrandom mating. Here we describe a system for examining questions of nonrandom mating in *Arabidopsis thaliana*. Using this system, we demonstrate that *Arabidopsis thaliana* displays nonrandom mating between distinct accessions. Statistical analysis of these data demonstrates aspects of both pollen competition and male–female complementarity in these matings. Cytological experiments implicate pollen germination and pollen tube growth rates as possible causal factors in these nonrandom mating efficiencies.

Keywords *Arabidopsis thaliana* · Nonrandom mating · Pollen competition · Pollen tube growth

Introduction

Because of their lack of mobility, flowering plants are sometimes thought to be passive mates, accepting all sperm indiscriminately. The pollen dusted across the female flower, however, is a mixture, whose proportions do not often match proportions within progeny. In other words, some pollen have greater mating success—a phenomenon called nonrandom mating. Although incompatible pollen can be blocked because it is from a different species or because it is from self in an out-breeding flower [these blocks are heavily studied (deNettancourt 1997; Hiscock and Tabah 2003; Hua et al. 2008; Kay and Schemske 2008; Kermicle and Evans 2005; Nasrallah 2002; Takayama and Isogai 2005; Wheeler et al. 2001)], our focus is upon variation among *compatible* mates, about which far less is known at the molecular and cellular scales. Nonrandom mating at this level is of particular interest because of the potential outcomes such as sexual selection (Charlesworth et al. 1987; Marshall and Folsom 1991; Mulcahy 1979; Skogsmyr and Lankinen 2002; Stephenson and Bertin 1983; Willson and Burley 1983) and/or mitigating the fitness cost of inbreeding depression (Armbruster and Rogers 2004).

Although nonrandom mating between compatible mates, which has been likened to animal mate choice (Marshall and Folsom 1991; Snow 1994; Stephenson and Bertin 1983), is often discussed for obligate outcrossing species, nonrandom mating is quite common in self-compatible species (Bowman 1987; Cruzan 1993; Cruzan and Barrett 1993, 1996; Haileselassie et al. 2005; Quesada et al. 1991; Sarigorla et al. 1992; Skogsmyr and Lankinen 1999; Snow 1991; Snow and Spira 1991a, 1996). Because plants with diverse reproductive strategies and from a variety of different environments display nonrandom mating, no single species is ideal for study of all its aspects and consequences. We have chosen *Arabidopsis thaliana* to investigate this process because of the powerful genetic resources and cell labeling tools available to facilitate the genetic and cell biological dissection of this process.

In this research update, we present tools and approaches we are utilizing to examine questions of nonrandom mating in *A. thaliana*. We performed nonrandom mating experiments to explore two questions: How widespread and strong is nonrandom mating in *A. thaliana*? Is nonrandom mating due primarily to male factors or female factors? Further, we have performed cytological experiments to explore the question: What pollen behaviors are key to

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A. L. Carlson · M. Telligman · R. J. Swanson (✉)
Department of Biology, Valparaiso University,
Valparaiso, IN 46383-6493, USA
e-mail: Rob.Swanson@Valpo.edu

mating success, and can we quantify performance of these behaviors?

Materials and methods

Arabidopsis strains and growth conditions

We obtained ecotypes CS57923 (Bay-0), CS57924 (Shahdara) (Loudet et al. 2002), CS37007 (Van-0), CS39287 (Est-1) (Balasubramanian et al. 2009), CS933 (Col-4), CS20 (Ler-0) (Lister and Dean 1993) and Col-0 from the Arabidopsis Biological Resource Center (ABRC). We grew plants in Shultz premium potting soil with daily watering, and fertilized (18–18–21 at 200 ppm) twice per week. Plants grew under 12 h 130 μE fluorescent lighting at 22°C.

Nonrandom mating assays

To obtain virgin females for nonrandom mating assays, we emasculated buds during stage 11–12 of development (Smyth et al. 1990). We then allowed pistils to develop to stage 14 before we performed assays. For competitions, we harvested anthers from stage 14 flowers and visually inspected them for levels of dehiscence. We chose two anthers from each potential father, and readied them on forceps. We used a stereomicroscope (Leica ZOOM2000) to better visualize the stigma when we applied pollen from Col-NPTII on half the available surface area of the virgin stigma. We then applied pollen from the competing father on the remaining stigma surface area. We completed each assay within 1 min. Mature siliques were collected and seed paternity was assayed by growing seeds on Murashige and Skoog media containing 50 μg/ml kanamycin (Murashige and Skoog 1962).

In order to test whether we can consistently deliver equal amounts of pollen in a competition, we dusted equal amounts of Col-NPTII and Col-4 pollen on virgin Col-4 pistils. If the NPTII insertion does not change the Col pollen competitive ability, and we are consistently delivering roughly equal amounts of pollen, we would expect a 1:1 ratio of progeny from these competitions. In 10 control crosses, we observed 207 Col-NPTII progeny and 227 Col-4 progeny. These control competitive pollinations show no statistical difference in the competitive ability of the two pollen types, and do not differ substantially from the expected 1:1 ratio in progeny: $\chi^2(1, N = 434) = 0.92$, $P = 0.33$ (1:1 null hypothesis).

Pollen germination assays

We plated pollen from 5 stage 14–15 anthers (Smyth et al. 1990) on solidified pollen growth medium (18% sucrose, 0.01% boric acid, 9 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.5% agar). Pollen germinated over the course of 24 h. We then scored pollen as germinated or ungerminated based on the presence of a pollen tube using a Nikon SMZ1000 stereo microscope. For each trial, we scored over 500 pollen grains.

Semi in vitro pollen tube growth assay

We performed semi in vitro pollen tube growth assays as described (Palanivelu and Preuss 2006). To obtain virgin females, we emasculated buds during stage 10 of development (Smyth et al. 1990). We then allowed females to develop to stage 14 before we performed assays. We excised whole mature pistils from flowers, then pollinated. We then cut pistils with surgical scissors (World Precision Instruments, Sarasota, USA) in the style, between the stigma and ovary, and placed the stigma portion on solidified pollen growth medium (18% sucrose, 0.01% boric acid, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.5% agar). We allowed pollen tubes to elongate for 3 h and 45 min, before we took digital pictures on a Zeiss Stemi SV11 APO. We measured pollen tube length from the tip of the growing pollen tube to the stigma-style interface using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). This measurement is a relative length, rather than the absolute length of the pollen tube, as it does not include the length of the pollen tube that traverses the stigma tissue.

Results and discussion

A. thaliana displays nonrandom mating between accessions

In order to efficiently test seed paternity in nonrandom mating experiments, we substitute wild-type Columbia accession (Col-4) pollen for Col pollen containing an integrated, intergenic kanamycin antibiotic resistance marker (Col-NPTII). Thus, when we place Col-NPTII pollen in competition with pollen from other accessions, we can quantify the progeny of these competitive pollinations via a simple plate germination assay that tests for the presence of the antibiotic resistance marker gene. Col-NPTII is an F2 homozygous T-DNA insertion mutant obtained from the SIGnAL project (Genbank BZ377762) (Alonso et al. 2003). The intergenic T-DNA lies between At1g28440 and At1g28450. This strain displays a 4:0 segregation of NPTII-mediated kanamycin resistance, demonstrating homozygosity (data not shown). When this strain is crossed to Col-0, and the F1 is allowed to self

fertilize, the resulting progeny display 3:1 segregation of NPTII-mediated kanamycin resistance: $\chi^2(1, N = 535) = 3.50$, $P = 0.061$ (3:1 null hypothesis). This demonstrates that there is only a single T-DNA insertion in the Col-NPTII line. Also, since Col-NPTII shows the expected 3:1 ratio of segregation, this is fairly strong evidence that the presence of the antibiotic resistance marker insertion does not change the competitive capacity of the pollen. This conclusion is supported by control competitions between Col-NPTII and Col-4 pollen on Col-4 pistils (see "Materials and methods").

To gauge how widespread and strong the phenomenon of nonrandom mating is in *A. thaliana,* we performed pollen competitions between Col-NPTII pollen and pollen from the common laboratory accession Landsberg *erecta* (Ler-0) as well as accessions collected from Canada (Van-0, collected at a field border in Vancouver), Russia (Est-1, collected at a railway slope near Pinsa), East Asia (Shahdara, collected from the Pamiro-Alay mountains near Tadjikistan) and Germany (Bay-0, collected from fallow-land habitat near Bayreuth, Germany). We dusted equal amounts of pollen from Col-NPTII and pollen from each accession on virgin Col-4 pistils, as well as virgin pistils from each accession (9–32 competitions each). We subsequently collected seeds for kanamycin resistance paternity testing. We conducted chi-square tests on the sum of progeny from each set of competitions to determine if there is a statistically significant difference in the competitive abilities of pollen from different accessions (Table 1). Without nonrandom mating, we expect 50% of the seeds to be fathered by each accession; instead, in all cases, we find statistically significant differences in paternity. In some cases, such as competitions between Col-NPTII and Ler-0, and Col-NPTII and Shahdara, the differences between the observed seed paternities are extreme (89 and 11%, and 92 and 8%, respectively).

Both male and female factors contribute to nonrandom mating success

Such nonrandom mating could be due to one pollen donor being of superior quality; pollen competition. Alternatively, such nonrandom mating could be due to differences in male–female complementarity; pistil favoritism towards some pollen donors, either actively or inactively, or handicapping of some pollen donors, either actively or inactively. Evidence of differential male–female complementarity is found in a number of plant systems

Table 1 Nonrandom mating survey of six accessions of *Arabidopsis thaliana*

Pistil	Proportion Col-NPTII progeny	Proportion Ler-0 progeny	# Comp., # progeny	Chi-square (*df*, test statistic)	*P* value
Col-4	0.889	0.111	28, 1026	1, 620.67	5.4×10^{-137}*
Ler-0	0.923	0.077	32, 1363	1, 975.30	4.1×10^{-214}*
Pistil	**Proportion Col-NPTII Progeny**	**Proportion Bay-0 Progeny**	**# Comp., # progeny**	**Chi-square (*df*, test statistic)**	***P* value**
Col-4	0.361	0.638	12, 562	1, 42.82	6.0×10^{-11}*
Bay-0	0.422	0.578	12, 384	1, 9.37	0.002*
Pistil	**Proportion Col-NPTII Progeny**	**Proportion Shahdara Progeny**	**# Comp., # progeny**	**Chi-square (*df*, test statistic)**	***P* value**
Col-4	0.917	0.083	11, 434	1, 301.94	1.2×10^{-67}*
Shahdara	0.855	0.145	14, 325	1, 164.19	1.4×10^{-37}*
Pistil	**Proportion Col-NPTII Progeny**	**Proportion Van-0 Progeny**	**# Comp., # progeny**	**Chi-square (*df*, test statistic)**	***P* value**
Col-4	0.427	0.573	12, 396	1, 8.49	0.003*
Van-0	0.675	0.325	13, 338	1, 41.19	1.4×10^{-10}*
Pistil	**Proportion Col-NPTII Progeny**	**Proportion Est-1 Progeny**	**# Comp., # progeny**	**Chi-square (*df*, test statistic)**	***P* value**
Col-4	0.650	0.350	9, 346	1, 31.26	3.3×10^{-8}*
Est-1	0.410	0.590	11, 300	1, 9.72	0.002*

We show proportions for ease of comparison, but we performed statistical tests on numbers of seeds. We compared observed seed paternity numbers to an expectation of equal seed paternity (1:1 model) using a chi-square test

Comp. indicates the number of competitions done. # Progeny indicates the total number of seeds counted across all competitions

df Degrees of freedom

* Denotes statistical significance in competitive ability of the two pollen donors on pistils of indicated accessions

(Haileselassie et al. 2005; Marshall and Diggle 2001; Marshall and Fuller 1994; Pfahler 1967; Stephenson and Bertin 1983), but see (Shaner and Marshall 2003). If competitive differences between pollen donors were only due to pollen competition, one would predict competitive abilities of pollen grains to remain constant on pistils from different accessions. We explored this by examining reciprocal competitions, where we kept the pollen donors constant, while varying the pistil accessions. Independent measures *t* tests were conducted on the data to determine if there were statistically significant changes in competitive ability of pollen between pistils (Table 2). Although the mean competitive ability of every pollen donor changed when the pistil accession was changed, only in one case, Van-0 and Col-4, did this change lead to a statistically significant difference in seed paternity. In this case, our data demonstrates nonself pollen performing better than self-pollen. We are currently obtaining more accessions of *A. thaliana* to determine whether there are other instances of differential male–female complementarity, and whether they consistently favor nonself pollen.

Thus, in the few accessions we have sampled, we observe pollen competition leading to nonrandom mating, and one instance of differential male–female complementarity. The existence of nonrandom mating between different *A. thaliana* accessions provides us the opportunity to use unique genetic and cell biological resources to examine different aspects of nonrandom mating. For example, we are currently utilizing recombinant inbred lines between different accessions of *A. thaliana* to map both male and female gene loci involved in nonrandom mating. We are also utilizing different cell biological techniques to examine different stages of fertilization with the goal of correlating pollen tube cell behaviors and nonrandom mating efficiencies. Two techniques we are utilizing provide insight into *pollen germination* and *pollen tube elongation*.

Pollen germination: quantifying pollen germination efficiency

Although pollen germination is relatively accessible both in vivo (on the stigma papillae) and in vitro (in pollen growth media), few studies separate early germination events from later elongation, or report quantifiable germination data (Marshall and Diggle 2001; Marshall and Folsom 1991). Shortly after pollen deposition, pollen grains hydrate and the delicate pollen tube must escape the durable exine shell of the pollen grain. Not all pollen grains are viable on dehiscence, thus pollen germination efficiencies can have a major impact on levels of nonrandom mating.

We use a simple plate assay to quantify pollen germination efficiencies. We plate mature pollen grains on pollen growth media. We then quantify pollen tube germination via stereo microscopy (Table 3). We have currently quantified germination efficiency for three *A. thaliana* accessions; Col-4, Bay-0 and Shahdara. We performed a one way independent measures ANOVA that reveals significant differences in germination efficiency, F (2, 19) = 5.54, $P = 0.013$. We performed Tukey HSD post hoc tests to follow up this analysis. We observe significant differences between plants Bay-0 and Shahdara. Bay-0 had a higher germination rate ($M = 58.2$, SD = 14.0) than Shahdara ($M = 36.4$, SD = 9.0). Although we use this technique to reveal inherent differences in germination potential of different pollen, these values may change when pollen germinate on a pistil. We are currently developing in vivo germination tests to complement these in vitro studies.

Tube elongation: quantifying pollen tube growth rates

Pollen germination and pollen tube elongation speeds are among the most studied factors in nonrandom mating. Theoretically, faster pollen tubes are more likely to be

Table 2 Pollen competition on pistils from varied accessions

Pollen used in competition	Proportion of Col-NPTII sired seeds on indicated pistil	Proportion of Col-NPTII sired seeds on indicated pistil	Independent measures *t* test (*df*, *t* stat)	*P* value
Ler-0/Col-NPTII	Col-4 = 0.889	Ler-0 = 0.923	56, −0.97	0.33
Bay-0/Col-NPTII	Col-4 = 0.361	Bay-0 = 0.422	20, −0.95	0.35
Shahdara/Col-NPTII	Col-4 = 0.917	Shahdara = 0.855	21, 1.44	0.16
Van-0/Col-NPTII	Col-4 = 0.427	Van-0 = 0.675	21, −3.56	0.002*
Est-1/Col-NPTII	Col-4 = 0.650	Est-1 = 0.410	16, 1.47	0.16

We show proportions of seeds sired by Col-NPTII on indicated pistils for ease of comparison. For statistical testing, we transformed the proportion of seeds sired by Col-NPTII in each individual cross using an arcsine square root transformation. We then used these numbers to perform an independent *t* test

* Denotes statistical significance in differences of competitive ability of pollen donors on pistils from indicated accessions

Table 3 *Arabidopsis thaliana* in vitro pollen germination efficiency

Pollen accession	# Trials	Total pollen germinated/ pollen counted	Mean % germination and standard deviation
Col-0	8	2,169/4,303	50.5 ± 7.1
Bay-0	10	3,021/5,181	58.2 ± 14.0*
Shahdara	4	732/2,012	36.4 ± 9.0*

* Denotes statistical significance in differences of germination efficiency between these two accessions

sires, an idea supported by quite a few studies (Levin 1975; Pasonen et al. 1999; Skogsmyr and Lankinen 1999; Snow and Spira 1991a, b, 1996). Many pollen tube growth studies are performed in vitro, in pollen growth medium. We have utilized a semi-in vitro technique to examine rates of pollen tube growth. We dust virgin pistils with pollen then cut the pistils transversely, placing the pollinated stigma and a portion of the style on solidified pollen growth medium. We allow pollen tubes to elongate through the style and out onto the medium before measuring the length from the tip of the pollen tube to the stigma-style interface. This technique has two advantages. First, we can set up pollen tube races to measure the relative growth rates of pollen from different accessions. Second, we can use this technique to investigate the impact pistils from different accessions have on pollen tube growth rates. For example, we dusted virgin Col-0 and Ler-0 pistils with Col-0 pollen. We then cut these pistils transversely and placed them on solidified pollen growth medium. We measured pollen tube lengths after 3 h and 45 min. We performed an independent measures t test on this data and show a statistically significant change in pollen tube growth rate of Col-0 pollen tubes on different accession pistils $t(14) = 5.16$, $P = 0.0001$ (Fig. 1). As demonstrated here, we can use this technique to measure the effect of pistil identity on pollen tube growth rates. This technique may provide us insight into aspects of differential male–female complementarity.

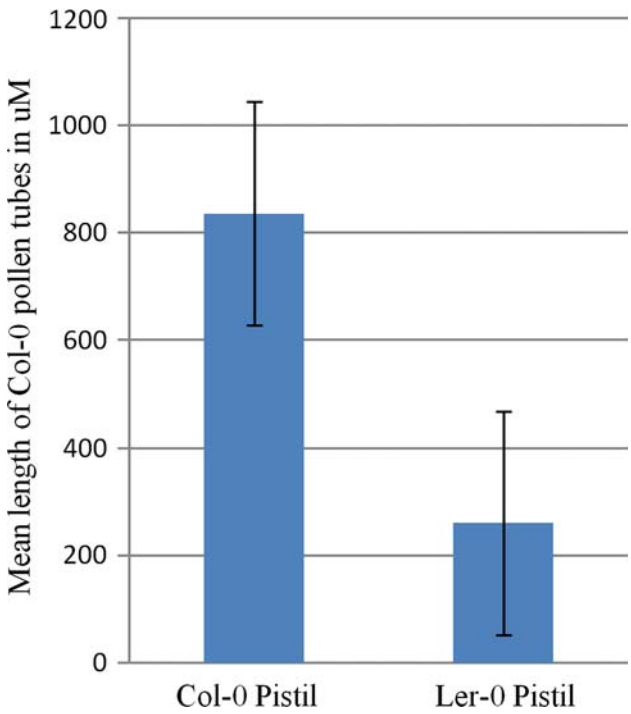

Fig. 1 Semi in vitro pollen tube growth. Mean length of Col-0 pollen tubes grown on a Col-0 pistil after 3′45″ is 836 ± 208 μM. Mean length of Col-0 pollen tubes grown on Ler-0 pistil after 3′45″ is 261 ± 236 μM. Lengths were measured from the tip of the growing pollen tube to the stigma-style interface

Conclusion

The weight of evidence from many plants with varied ecological strategies is that nonrandom mating occurs among compatible donors. Nonrandom mating can be due to factors that affect: (1) pollen delivery (for which self-compatible *A. thaliana* would be a poor system), (2) the stages leading to fertilization, or (3) differential seed abortion (which we have not observed in *A. thaliana*). We propose to exploit genetic and cell biological tools available to the *A. thaliana* model system to concentrate upon those middle stages between pollen deposition and fertilization. Our investigations reveal the presence of nonrandom mating in *A. thaliana* and provide the opportunity to define precise cellular mechanisms, as well as eventually genes, that different accessions utilize in nonrandom mating. These types of studies cannot tell us whether these mechanisms evolved for increased choice among mates, or whether they are a passive consequence of incongruity due to local selection or genetic drift. It is our hope, however, that they will yield specific predictions that will allow these questions to be explored at a genic level in more ecologically complex but experimentally less tractable systems.

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