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Sexual Plant Reproduction

Analysis of pollen-style interactions in *Petunia hybrida*; the determination of variance in male reproductive success

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Summary. A factorial cross design was used to evaluate the influence of differences among pollen donors and recipients on variation in stylar attrition of pollen tubes in self-fertile plants of *Petunia hybrida*. Pollinations to flower buds were used to reduce the inhibitory ability of the style and these crosses were compared with flower pollinations to assess the degree of stylar influence on pollen fertilization ability. There was less pollen tube attrition after bud pollinations than after flower pollinations, indicating that styles of buds were less able to inhibit pollen tubes. The variance component for plants acting as pollen donors tended to be greater after flower pollinations than after bud pollinations. The lower variance in male success after bud pollinations indicates that differences among pollen donors after flower pollinations were due to stylar inhibition of pollen rather than differences in pollen vigor. Since the level of variation in pollen growth after pollination to flowers was greater among clones than among ramets within clones, the differences in pollen fertilization ability are probably genetically based.

Key words: Pollen-style interactions – Male reproductive success – *Petunia hybrida* – Bud pollinations – Variance components

Introduction

The process of reproduction in plants occurs in a series of stages from pollen transfer to receptive stigmas, pollen germination and growth to the ovary, and ovule fertilization followed by seed maturation. Variation in the reproductive success of individuals acting as pollen donors and recipients could occur at any of these stages; however, the potential for donors and recipients to influence the success of the pollen they produce or receive differs among stages (Stephenson and Bertin 1983; Lyons et al. 1989). There would be little or no opportunity for recipients to influence the composition of their stigma loads, but pollen-style interactions may lead to differential success of pollen from different sources (Waser et al. 1987). Pollen tube growth to the ovary may be a forum for the determination of differential reproductive success among pollen donors.

Variation in the ability of pollen to grow to the ovary is often ascribed to the action of *S*-locus incompatibility systems, which are considered to have one locus and tend to produce discrete inhibition reactions. The usual interpretation of *S*-locus incompatibility is that pollen tube failure is the result of the inhibitory action of the style (Nettancourt 1977). Less is known about incompatibility reactions that are quantitative in expression and in which only a portion of the pollen tubes reach the ovary. These have been termed pseudo-incompatibility reactions for species that are normally self-incompatible (Nettancourt 1977), but quantitative variation in pollen growth can dominate some breeding systems (Sayers and Murphy 1966; Waser et al. 1987; Cruzan 1989). The avoidance of selfing has been proposed as the primary selective force maintaining *S*-locus incompatibility systems (Barrett 1988; Olmstead 1989). In addition to reducing selfing, incompatibility systems may allow plants to modify the levels of bi-parental inbreeding they experience (Waser et al. 1987). Hence, pollen-style interactions may provide a mechanism for plants to control the paternity of their seeds.

The potential for pollen-style interactions to determine the fertilization success of pollen has prompted several authors to suggest that processes analogous to mate choice in animals may occur during this stage of a plant's reproductive cycle (Willson and Burley 1983; Stephenson and Bertin 1983; Lyons et al. 1989; Marshall and Folsom 1991). However, there are no widely accepted operational definitions of concepts such as "male competition" and "female choice" in plants. Indeed, the ad hoc application terminology designed for animal social behavior to plant mating systems may obscure

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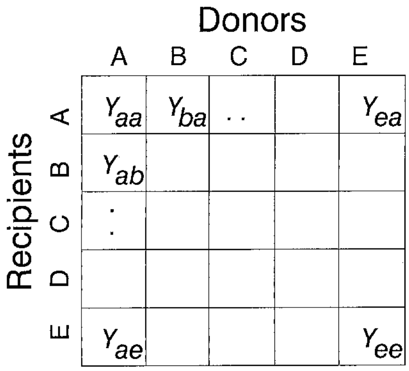

Recipients \ Donors	A	B	C	D	E
A	Y_{aa}	Y_{ba}	..		Y_{ea}
B	Y_{ab}				
C	⋮				
D					
E	Y_{ae}				Y_{ee}

Fig. 1. The factorial cross design used to analyze variance in the reproductive success of *Petunia hybrida* plants acting as pollen donors and recipients. The amount of stylar attrition of pollen tubes was measured for each cross (Y_{ee}) after flower or bud pollinations. The same five plants were used as both donors and recipients

important evolutionary issues unique to plants (Charlesworth et al. 1987; but see Marshall and Folsom 1991). One approach for delimiting the role of male (pollen) and female (stylar) characteristics in the fertilization process uses the components of variation for pollen performance after crosses among plants as indicators of variance in male and female reproductive success (Lyons et al. 1989). Lyons et al. suggest that the size of the variance components obtained from factorial crossing designs (as in Fig. 1) indicate the potential for evolution of characters affecting reproductive success. They also point out that variance in the fertilization ability of a donor's pollen could have one of two possible causes. When pollen from different donors is tested across several recipients, variance in fertilization ability could be due either to characteristics associated with pollen ("male competitive ability," hereafter *pollen vigor*) or with stylar control ("female choice," hereafter *stylar inhibition*). Such models are now just beginning to be applied, and few data exist from other designs that examine variance in cross performance and its proximate causes (Lyons et al. 1989).

Here I use a factorial cross design to determine the amount of variation in pollen fertilization ability in *Petunia hybrida* (Solanaceae). I further determine the degree to which mediation by recipient styles controls pollen fertilization ability by using bud pollinations to render styles physiologically passive. In this study I assess the fertilization ability of pollen as the amount of stylar attrition (i.e., the proportion of pollen tubes in the stigma that do not reach the ovary because pollen tube growth has ceased; Cruzan 1989).

Since the plants I use are derived from strains that have been under cultivation for some time, it is difficult to make evolutionary interpretations that apply to this hybrid species or its progenitors. However, the primary purposes of this exercise are (1) to test the feasibility of applying models designed to delimit sources of variation in reproductive success, and (2) to assess whether factors not associated with *S*-locus incompatibility can be important in determining the fertilization success of pollen from different sources. Furthermore, the information available on pollen-style interactions in *Petunia* can be used to facilitate the interpretation of the results obtained. Application of the techniques discussed in this study to plant populations will help identify the factors that determine the reproductive success of individuals and may allow the assessment of the potential for evolution of these characters.

Materials and methods

Single factorial

I obtained *Petunia hybrida* Hort. seeds from a commercial distributor (Northrup King Seeds, Fresno, Calif.). Commercially available *Petunia* seeds are produced by the hybridization of inbred strains (Ewart 1984). I grew plants in the greenhouse and used stem cuttings to clone plants into several pots each. From these plants I chose five separate hybrid lines (with different flower colors) and crossed them in a complete 5 × 5 factorial design where each individual acted as both a pollen donor and recipient (Fig. 1). Using several different ramets of each plant, I replicated each cross between three and five times. I made all pollinations on several consecutive mornings in the greenhouse by rubbing a freshly dehisced anther across the stigma of a recipient flower to produce an even coat of pollen. Flowers last approximately 4 days and styles naturally abscise after 5–6 days. I excised styles at the top of the ovary 5 days after pollination and collected the fruit when it was mature. Within 5 days of pollination all pollen tube growth has ceased, and fruit set is apparently unaffected by style removal after 5 days (unpublished data).

After fixing styles in 70% ethanol, I cleared them in 8 *N* NaOH for 1 h at 60° C, then stained them for 24 h in 0.1% aniline blue in 1 M K_3PO_4. Using a razor blade, I removed 0.5-mm-thick sections from the base near the cut end and from just below the stigma of each style, stained these sections further by adding one drop of 0.1% calcofluor white, and then macerated each on a slide. Using an epi-illumination fluorescence microscope, I counted all pollen tubes present for each section at 100 ×. Tubes were easily distinguished, although clumping of tubes, which occurred to varying degrees on all slides, made counts less accurate. I counted the seeds from each fruit and weighed them as a group.

Flower vs bud factorials

From the progeny of the above crosses I chose five individuals (one plant was from a self-pollination, while the other four consisted of two pairs of reciprocal full sibs) for a pair of factorial crosses in which each cross was made to both a mature flower and a bud of each recipient. Flower buds were pollinated approximately 1 day before opening (Shivanna and Rangaswamy 1969) and were nearly the same size as flowers. For each cross, I used pollen from a single flower of the donor and made both the bud and flower pollination to the same ramet of a recipient clone at the same time. I performed each cross-pollination once and replicated the self-pollinations twice in a complete 5 × 5 factorial design (Fig. 1). I analyzed pollen tube growth and seed set as described above.

Data analysis

I analyzed data from the three factorials as the proportion of pollen tubes present in the stigma that did not reach the ovary ("stylar attrition"), and as the proportion of pollen tubes reaching the base of the style that did not result in seeds ("ovarian attrition"; Cruzan 1989). In an analysis of covariance using the GLM procedure of SAS (SAS Institute 1985), I entered the donor and recipient identity and pollination date as random factors, and the number of pollen tubes present in the stigma as a covariate. For the single factorial, I entered the ramet identity as a random factor nested within the donor or recipient. I tested the main effects (donor and recipient) in the single factorial using in an ANOVA that combined flower pollinations from both experiments over the interaction mean square and the mean square for variation among ramets. Since all variables approximated normal distributions, I left them untransformed.

Results

Single factorial

There was quantitative variation in the amount of pollen tube attrition after both self- and cross-pollinations of *Petunia* flowers (Fig. 2). The distribution of attrition values for self pollinations did not differ from the distribution obtained for cross pollinations (Komolgorov-Smirnov $D=0.13$, $P>0.05$). Self-pollinations did not consistently have high attrition values as would be expected for a self-incompatible species. In only one case did the amount of attrition for a cross rise above 90% (Fig. 2).

Stylar attrition of pollen tubes had consequences for variation in the number of seeds set (Table 1). The number of pollen tubes reaching the base of the style was a better predictor of the number of seeds produced in a fruit than was the number of tubes present in the stigma (Table 1). Failure of pollen tubes in the ovary (ovarian attrition) also affected seed set, but since ovule number was not determined it is unclear whether ovarian attrition is due to excess pollen tubes reaching the ovary or to post-fertilization ovule abortion.

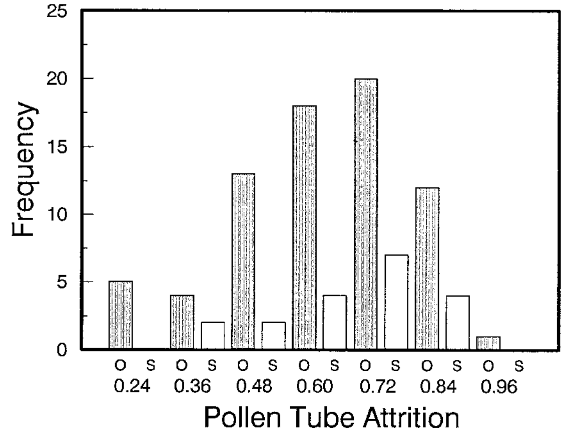

Fig. 2. The amount of stylar attrition occurring after self- and cross-pollinations to flowers in *Petunia hybrida.* Stylar attrition was measured as the fraction of pollen tubes in the stigma that did not reach the ovary. The *dark bars* represent cross-pollinations and the *empty bars*, self-pollinations. Data are untransformed stylar attrition scores from the single factorial experiment

Table 1. The predictive value of pollen tube numbers in the stigma and at the base of the style, and the amount of ovarian attrition for the numbers of seed set in *Petunia hybrida.* *t*-Tests indicate whether each partial regression coefficient is equal to zero. Standardized partial regression coefficients (slopes) are from the REG procedure of SAS (SAS Inst. 1985). Data are from the single factorial experiment

Variable	Slope	*t*-Value	Probability
Tubes in stigma	0.127	1.99	0.0496
Style base	0.835	11.27	0.0001
Ovarian attrition	−0.844	12.49	0.0001

Table 2. Analyses of variance for stylar attrition of pollen tubes after pollination of flowers in *Petunia hybrida.* Separate analyses are given for the crosses among five hybrid lines (single factorial) and five of their progeny (bud-flower factorials; only data for flowers), and for all crosses combined. Tests for donor and recipient effects were made using the interaction mean square and the mean square for variation among ramets within a clone (Ram) as error terms

Source	Error MS	Single		Bud-Flower		Combined	
		df	*F*	*df*	*F*	*df*	*F*
Donor (D)	D × R	4	1.53	4	1.87	8	2.23+
Donor (D)	Ram(D)	4	1.97	–	–	–	–
Recipient (R)	D × R	4	1.08	4	1.64	8	1.22
Recipient (R)	Ram(R)	4	1.17	–	–	–	–
D × R	Error	14	1.59	14	0.50	28	1.17
Ram(D)	Error	21	1.24	–	–	–	–
Ram(R)	Error	21	1.46	–	–	–	–
Date	Error	2	0.35	4	0.30	6	0.74
Tubes	Error	1	15.20**	1	4.20	1	18.64***

+, $P<0.06$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$

Variation among pollen donors was greater than among recipients in both generations after pollinations to flowers (Table 2). However, differences among donors were not significant at the 0.05 level in either generation and were only marginally significant when both generations were combined (Table 2). The number of pollen tubes present in the stigma had a strong influence on the number reaching the base of the style (Table 2, Fig. 3), but none of the other factors examined had large effects on variation in pollen tube attrition after flower pollinations (Table 2).

Flower vs bud factorials

Pollen tube attrition was lower for bud pollinations than for flower pollinations in 25 of the 30 pairs of pollinations (Table 3) and there was no difference in the reduction in attrition for self compared to cross pollinations ($F=0.00$, $P>0.97$). Styles in buds tended to be shorter than styles in flowers, but for a sub-sample of the styles analyzed there was only a weak relationship between style length and the amount of attrition ($r=0.23$, $P>0.40$, $N=15$), suggesting that style length alone does not account for the differences in the level of attrition observed. More pollen tubes reached the base of the style after bud pollinations than after flower pollinations, even though the former typically had fewer pollen tubes present in their stigmas (Table 3). Flowers and buds were both pollinated with equivalent amounts of pollen so

Table 3. Means and standard deviations (in parentheses) for parameters measured after pollinations to buds and flowers in *Petunia hybrida*. Seed weights are in micrograms. Probabilities are from t-tests for the difference between each pair of means

Cross	Pollen tubes		Attrition		Seed	
	Stigma	Base	Stylar	Ovarian	Number	Weight
Flowers	709.4	359.5	0.434	0.266	244.0	104.5
	(275.4)	(148.9)	(0.273)	(0.469)	(150.0)	(28.6)
Buds	521.4	389.4	0.214	0.331	234.5	102.8
	(245.1)	(221.2)	(0.288)	(0.395)	(133.1)	(18.1)
Probability	*	ns	*	*	ns	ns

* $P<0.05$

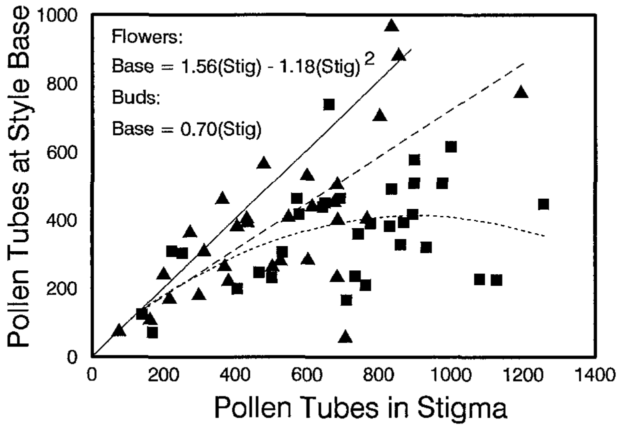

Fig. 3. The number of pollen tubes reaching the base of the style as a function of the number of pollen tubes in the stigma for flower (*square*) and bud (*triangle*) pollinations in *Petunia hybrida*. The predicted line for flower pollinations (*small dash*) is from a quadratic regression analysis, and for bud pollinations (*large dash*) is from a linear regression analysis. The *solid line* represents the expected relationship for a slope of one

the lower number of pollen tubes in the stigmas of buds was probably the result of lower germination rates. For flower pollinations the number of pollen tubes at the base of the style was a decelerating function of the number of pollen tubes in the stigma (Fig. 3) with both linear ($P=0.021$) and quadratic ($P=0.076$) components. After bud pollinations, on the other hand, the slope of the predicted line for the same relationship was closer to a one-to-one correspondence between the number of tubes in the stigma and at the base of the style and was adequately explained by a linear model (linear $P<0.001$, quadratic $p>0.72$; Fig. 3). Ovarian attrition was greater for bud pollinations than for flower pollinations and, as a consequence, seed set did not differ between the two treatments (Table 3). Since ovule number was not known, it is not clear whether ovarian attrition is due to excess pollen tubes reaching the ovary or to post-fertilization ovule abortion.

Bud pollinations had a larger effect on donor than recipient performance (Table 4). The mean square for the interaction of donor identity and pollination type (bud vs flower) was greater than the interaction between recipient identity and pollination type, but the difference was not significant ($F=3.05$, $df=4/4$, $P<0.25$; Table 4).

Table 4. Analysis of variance for the effect of bud and flower pollinations on the amount of stylar attrition in *Petunia hybrida*. In this analysis all effects were treated as fixed and tested over the error mean square

Source	*df*	Mean square	F-Value	P-Value
Bud vs flower (T)	1	0.245	5.03	0.035
Donor (D)	4	0.033	0.68	0.612
Recipient (R)	4	0.043	0.90	0.479
D × R	14	0.044	0.90	0.566
T × D	4	0.116	2.38	0.080
T × R	4	0.038	0.78	0.546
Date	4	0.026	0.54	0.711
Tubes in stigma	1	0.322	6.60	0.017
Error	24	0.049		

Table 5. Variance components for pollen tube attrition after pollination to flowers and buds in *Petunia hybrida*. Data were analyzed in model II analyses of covariance with donor and recipient identities treated as random factors and the number of pollen tubes in the stigma entered as a covariate using the GLM procedure of SAS (1985). Type IV sums of squares were used to estimate the variance components. The error term was pooled across bud and flower crosses

Source	Flowers		Buds	
	S^2	Explained	S^2	Explained
Donor (D)	0.0307	11.3%	−0.0190	0.0%
Recipient (R)	0.0100	3.7%	0.0099	4.9%
D × R	−0.0364	0.0%	0.0719	35.3%*
Date	−0.0205	0.0%	0.0313	15.4%+
Tubes in stigma	0.1875	68.9%**	0.0466	22.9%*
Pooled error	0.0440	16.2%	0.0440	21.6%

+, $P<0.06$; * $P<0.05$; ** $P<0.01$; *** $P<0.001$

The larger effect of the donor-by-pollination type interaction was apparently because of the differences in both the level of variation (Table 5) and the rank order performance of donors (Fig. 4).

Differences among pollen donors after flower pollinations explained 11.3% of the variation in stylar attrition (Table 5, Fig. 4). The number of pollen tubes present in the stigma explained most of the variation in stylar attrition, and once this effect is removed from the model,

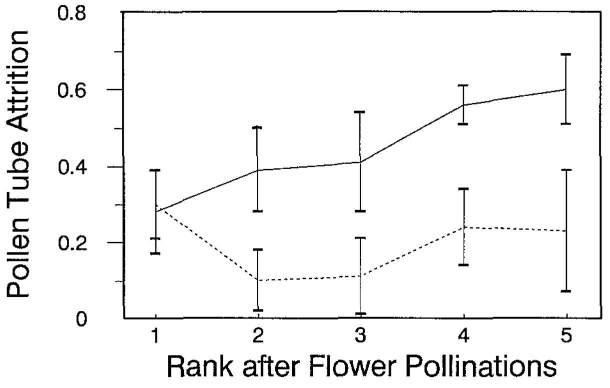

Fig. 4. The rank performance in stylar attrition for five pollen donors after flower pollinations (*solid line*) and bud pollinations (*dashed line*) in *Petunia hybrida*. Each donor was tested on the five plants ranked (including itself) in a complete factorial cross design

the amount of remaining variance due to donor genotype increases to 36.3%. Recipient identity, on the other hand, explained only 3.7% of the total variation and 11.8% when the effect of the number of pollen tubes present in the stigma is removed. Neither recipient genotype nor the donor-by-recipient interactions had significant effects. After bud pollinations, the variation among males was negligible, and the variation among recipients was similar to the level of variation obtained for flower pollinations.

The rank order of donor performance for flower pollinations differed from the order obtained after bud pollinations (Fig. 4). The donor-by-recipient interaction was greater after bud pollinations than after flower pollinations (Table 5). The increase in the interaction term may be due to variation in the developmental stage at which buds were pollinated; flowers open synchronously over the course of the morning, while pollinations were always performed at the same time of day. Pollination of buds that were closer to anthesis could result in partial inhibition of pollen tubes by the style, which would increase the level of variation due to the donor-by-recipient interaction.

Discussion

Variance in male reproductive success can occur because of differences in male competitive ability or because of a preference by the majority of females for a particular male phenotype (Bateman 1948). These mechanisms of selection have been proposed to explain characteristics of plant reproduction (Willson and Burley 1983; Stephenson and Bertin 1983; Lyons et al. 1989; Marshall and Folsom 1991), with much speculation about their importance in plants (Charlesworth et al. 1987). Lyons et al. (1989) highlighted pollen tube growth as the stage in a plant's life cycle where pollen characters may affect a plant's fertilization success. My work with *Petunia* has shown that there can be variance in the fertilization ability of pollen and that its performance is mediated by recipient styles. For the evolution of pollen fertilization ability to occur, the characters controlling variance in male reproductive success must be heritable (Lande 1981).

Results obtained here with *Petunia hybrida* suggest that genetically determined variation in pollen fertilization ability may occur in plants; however, this analysis suffers from several faults, including relatively small sample sizes and unknown genetic origins of the material used. The small number of plants used in this type of analysis is difficult to remedy, since, as pointed out by Lyons et al. (1989), the amount of work required increases as the square of the sample size. This problem may be alleviated by redistributing the effort made to include a greater number of pollen donors and fewer recipients. Alternatively, if there were a large amount of variation among recipients, a better assessment of variation among a few donors could be obtained by testing them across a larger number of recipients. The application of this experimental protocol in natural populations should also be done with a degree of ecological realism; comparison of plants should be made among individuals that are within close pollen dispersal range of each other. The evaluation of pollen-style interactions with factorial designs coupled with manipulations that reduce the inhibitory ability of styles will help provide a better understanding of the evolution of plant reproductive characters (Uyenoyama 1988).

The genetic relationship among the strains used in this study may have contributed to the level of variation observed among donors. The five different hybrid color morphs used in the single factorial experiment were presumably derived from different strains, which may have higher levels of genetic differentiation than plants obtained from a single population. However, it is interesting to note that the level of variation among donors was similar in both generations; there were still differences among donors when full sibs were compared. Analyses of donor reproductive success in populations displaying spatial genetic differentiation would also be likely to include closely related individuals.

Although some strains of *Petunia* do have single locus self-incompatibility (Mather 1943; Ascher 1984), commercial strains, such as those used in this study, are self- and cross-compatible to varying degrees. Compatibility responses due to one or two loci would fall into discrete classes, but crosses among these *Petunia* plants produced a continuous and unimodal distribution of attrition values, indicating that more than a few factors contribute to variation in stylar attrition. Moreover, the amount of pollen tube attrition observed after self pollinations did not differ from the amount observed after outcross pollinations, indicating that, if *S*-alleles are segregating in these plants, they are not being expressed. Previous work on incompatibility responses in *Petunia* indicates that there can be polygenic control of pollen-style interactions, and that pollen and stylar characters can be affected by different sets of loci (Takahashi 1973; Ascher 1984).

Pollen tube growth rate is often assumed to be the largest determinant of pollen fertilization success, but several studies have found that many pollen tubes fail to reach the ovary (Sayers and Murphy 1966; Waser et al. 1987; Cruzan 1989). These observations suggest stylar control; plants should be under strong selection to produce pollen with high fertilization ability. In *Petunia*, recipient styles appear to be controlling the fertilization ability of pollen since stylar attrition is reduced after bud pollinations (Shivanna and Rangaswamy 1969). If the variation in fertilization ability among donors had persisted after bud pollinations, the control of fertilization ability could have been attributed to intrinsic differences in pollen (i.e., due to differences in pollen vigor).

The mechanism controlling pollen-style interactions in self-fertile *Petunia* strains is not known, but it is probable that mature styles produce inhibition substances that are lacking in flower buds. Inhibition substances responsible for *S*-locus incompatibility have been identified in the related species *Nicotiana alata*, and these compounds are known to be produced upon anthesis (Anderson et al. 1986). The inhibition substances found in *Nicotiana* were not present in self-compatible plants (Anderson et al. 1986), so pollen-style interactions in *Petunia* plants may be controlled by compounds that are not associated with *S*-locus incompatibility.

The differences in fertilization ability observed among donors after flower pollinations would contribute to variation in male reproductive success. The amount of stylar attrition was reduced after bud pollinations and the variation among plants acting as pollen donors became negligible, indicating that variance in pollen growth ability was largely mediated by the style. Since there was little variation among donors after bud pollinations, there appears to be little difference among plants in their pollen vigor, but stylar inhibition of pollen tubes does occur and accounts for the majority of variation in male reproductive success during this stage of the life cycle. The patterns of stylar attrition observed for these crosses may represent differences due to the environmental conditions to which different plants were exposed; however, this is unlikely since crosses were made using pollen from a single flower, and flower and bud pollinations were made at the same time. Moreover, the amount of variation for pollen performance among ramets within a clone was much less than among clones, indicating that the differences observed in pollen siring ability are probably genetically based.

The plants used in the present study are fully self-compatible, but variation in pollen tube attrition can also be produced by the action of *S*-loci. Whether pollen inhibition is due to incompatibility loci or to separate pollen and stylar loci will determine the potential for evolution of pollen fertilization ability. In the *S*-locus incompatibility model, the same gene (or parts of a supergene) is transcribed in both pollen and styles. Pollen carying an allele that matches one of the two stylar alleles will tend to be inhibited, while non-matching pollen will grow normally. This type of interaction leads to (1) higher levels of pollen tube attrition after self-pollinations, and (2) frequency-dependent selection that favors the maintenance of a number of incompatibility alleles at equal frequency in populations (Wright 1965; Yokoyama and Hetherington 1982). If *S*-loci actions are the major determinant of variation in pollen fertilization ability then all plants in the population should have equal levels of fertilization success (if all *S*-alleles are at equal frequency – the equilibrium condition for a large population), and the evolution of pollen fertilization ability as an independent character could not occur (Charlesworth et al. 1987). In this system models that have been proposed for the evolution of sexual systems (e.g., Lande 1981) would not apply.

Pollen- and style-specific characters affecting pollen fertilization ability that are inherited independently could produce variation in pollen fertilization ability. Such independent characters have previously been found in *Petunia* (Ascher 1984) and could be responsible for the attrition observed. In this case, pollen and stylar characters could evolve separately from one another. Evolution of pollen fertilization ability may be possible in plant species for which a significant component of pollen fertilization ability is determined by independent loci. At present, however, the degree to which pollen fertilization ability in natural populations is determined by independent loci is largely unknown. As more information on pollen-style interactions in natural population is accumulated we will be better able to assess the importance of *S*-loci and independent loci in the determination of the reproductive success of plants.

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