

PLEIOTROPIC EFFECTS OF AN ALLELE PRODUCING WHITE FLOWERS IN *IPOMOEA PURPUREA*

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Although it is generally believed that pollinators are the primary selective agents driving flower-color evolution, it has recently been suggested that pleiotropic effects of mutations affecting flower color may serve as important constraints on floral evolution. We examined this hypothesis using white-flowered variants of the common morning glory, *Ipomoea purpurea*. Previous experiments indicate that the white-flowered *a* allele has a transmission advantage because of increased selfing and no detectable pollen discounting. We confirm this transmission advantage using a large field experiment in which both selfing rate and outcross success were measured for all three genotypes at the *A* locus. We also demonstrate that this transmission advantage is opposed by apparent pleiotropic effects in *aa* individuals manifested as reduced survival from germination to flowering. The magnitude of this effect, in combination with the known magnitude of inbreeding depression, more than compensates for the transmission advantage. Our results thus support the notion that deleterious pleiotropy may influence the evolutionary trajectory of flower-color mutants.

KEY WORDS: Anthocyanins, flower color, *Ipomoea*, loss-of-function, pleiotropy.

The idea that differences in flower color among species are due primarily to divergent selection imposed by different pollinators can be traced back to the 18th century (Kölreuter 1761; Sprengel 1793, 1996) and was elaborated by Darwin (1862) and others (see Fenster et al. 2004 for a review). Over the past 50 years, it has been championed by a number of evolutionary biologists (Baker 1963; Stebbins 1970; Grant 1993). The recognition of “pollination syndromes,” of which floral color is an integral component (Fægri and van der Pijl 1966), provided indirect evidence in support of this idea, despite the fact that existence and importance of pollination syndromes has been questioned (Fenster et al. 2004). In addition, numerous demonstrations of pollinator discrimination among flowers of different colors suggest the potential for pollinators to commonly act as selective agents, although direct demonstrations of pollinators exerting selection on flower color

variation are few (Rausher 2008). More recently, it has been argued that in addition to direct selection on flower color due to pollinators, indirect selection on pleiotropic effects of genetic variants affecting floral hue may also influence flower-color evolution (Rausher and Fry 1993; Simms and Bucher 1996; Fineblum and Rausher 1997; Armbruster 2002; Irwin et al. 2003; Strauss and Whittall 2006). However, a recent literature review (Rausher 2008) found only two convincing demonstrations that selection acts on pleiotropic effects of genetic variants for flower color (Levin and Brack 1995; Schemske and Bierzychudek 2001, 2007). In neither of these cases does indirect selection through pleiotropy oppose direct selection mediated through pollinators. Consequently, evidence that pleiotropy can counteract selection imposed by pollinators, and thus constrain floral-color evolution, is virtually nonexistent.

Here we examine whether pleiotropy is associated with white-flowered variants at the *A*-locus of the common morning glory, *Ipomoea purpurea*, and if so, whether selection on

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pleiotropic effects opposes selection due to pollinators. A previous investigation using artificial arrays of potted plants indicated that variation at the *A* locus influences the mating system (Fehr and Rausher 2004). Specifically, we demonstrated that selfing rates are higher for the white-flowered *aa* genotype than for the purple-flowered *AA* genotype, but that male outcross success was similar for the two genotypes. We have also found a similar pattern of selection on white- versus purple-flowered variants at the *W* locus in the same species that appears to arise from pollinator behavior (Rausher et al. 1993; Fry and Rausher 1997). This combination of increased selfing without pollen discounting implies that natural selection acting directly on features associated with the mating system favors an increase in the white (*a*) allele (Fisher 1941).

Despite this apparent advantage, the *a* allele remains very rare in natural populations: among 22 populations from North Carolina, Virginia and Georgia, the average frequency of *aa* individuals is less than 0.01 (Coberly 2003). This rarity suggests that some deleterious pleiotropic effect associated with that allele opposes the benefit associated with increased selfing. Here we address this issue by quantifying fitness components of the three genotypes at the *A* locus in an experimental population of *I. purpurea*. In addition, we ask whether the mating advantage associated with increased selfing found in a previous study also occurs in a more realistic setting.

Materials and Methods

EXPERIMENTAL ORGANISMS

The tall morning glory, *I. purpurea* (L.) Roth (Convolvulaceae) is a weedy annual with a mixed mating system that is common in agricultural fields and other disturbed sites throughout southeastern North America. Plants typically germinate between late May and August, depending on the timing of soil disturbance and rainfall, and are killed by the first frost, which ranges from late September to early December in the piedmont of North Carolina. The showy flowers open at dawn, then wither and close by late morning of the same day. The primary pollinators in this region are bumblebees (*Bombus pennsylvanicus*).

The *a* allele is one of two known mutants causing white flowers in *I. purpurea* (Barker 1917). The *a* allele is a partially recessive, with white, light, or darkly pigmented phenotypes (*aa*, *Aa* and *AA* genotypes, respectively). The *a* allele is caused by a transposable element insertion into the CHS-D gene that codes for chalcone synthase, the first enzyme of the anthocyanin pathway (Habu et al. 1998).

EXPERIMENTAL DESIGN

For the main experiment conducted in 2000, experimental seeds of known genotype at the *A* locus, and with the background genotype randomized at unlinked loci, were generated by a modification of the crossing design described by Rausher and Fry (1993). Initial

crosses were conducted using eight inbred lines established from seed collected at a single site in Durham County, North Carolina and propagated by 13 generations of self-fertilization (selfing). Each of four white-flowered (*aa*) inbred lines was initially crossed with one of four pigmented (*AA*) inbred lines (great-grandparental pairs). One heterozygous offspring (*Aa*) of each pair (grandparent) was selfed, and a single heterozygous offspring was chosen and selfed again. For three more generations, a single heterozygous parent was chosen and selfed to create an inbred line heterozygous at the *A*-locus (for a total of four generations of inbreeding). In the final "parental" generation, the heterozygotes were discarded and the *AA* and *aa* individuals from each line were outcrossed to both *AA* and *aa* individuals descended from each of the other lines to generate experimental seeds of all three genotypes. Experimental seed of each genotype was thus descended from one of six grandparental lines, which we designate as "units" in our statistical analyses. This design ensured that seeds of the three *A*-locus genotypes had similar histories of inbreeding. For a pilot experiment conducted in 1999, experimental seeds were generated in a similar manner, except that only three pairs of grandparental lines were used (producing seed belonging to one of three units), only homozygote seeds were used in the experiment, and only three generations of selfing were performed.

EXPERIMENTAL METHODS

For the 2000 main experiment, experimental seeds were planted in a disked agricultural field in Durham County, North Carolina in June. For the 1999 pilot experiment, in which we only measured viability, experimental seeds were planted in a disked old-field in Oxford County, North Carolina. A randomized block design was used, with 10 blocks and an average of 4.7 seeds per genotype per unit per block (840 seeds) in the main experiment and 5 blocks with an average of 4.4 seeds per genotype per unit per block in the pilot experiment. In the main experiment, seedlings were watered once one week after planting and wild *I. purpurea* were removed by hand from the experimental plot and surrounding area. No known *I. purpurea* populations existed within 2 miles of either study site.

In the pilot experiment, we measured early survival (to 2 weeks after planting). In the main experiment, we measured survival to flowering; plants that grew but never flowered were counted as not surviving (flower counts were conducted until the first killing frost in October. No plants survived the frost to set seed. Only 13 of the 526 surviving plants did not flower including 5, 3, and 5 of the *aa*, *Aa* and *AA* genotypes, respectively). In addition, we collected and counted all seeds produced (for methods, see Rausher and Simms 1989). The total number of seeds produced was used as our estimate of the female component of fitness. Differences among genotypes in survival were assessed using a three-way *G*-test (Bishop et al. 1991), with color genotype,

unit, and survival (yes or no) as the main effects. Because the order of entry of main effects in this type of analysis can sometimes affect the outcome, we performed the analysis for all six possible orders of entry. Differences among genotypes in seed production for plants that produced at least one seed were tested using a three-way analysis of variance implemented by the GLM procedure in SAS (SAS Institute 1990). The main effects of color genotype, block, and unit were all considered fixed. A full model with all interactions was examined, but nonsignificant higher-order interactions are not reported.

To estimate relative male fitness of the three color genotypes, we quantified two aspects of the mating system, selfing rate and male outcross success, using a modified version of Chang and Rausher's (1999) pollen trapping approach. Twelve pollen trap plants were randomly assigned to a position within each of 10 experimental blocks (120 pollen trap plants, with an equal frequency of each line and color genotype, within 840 experimental plants). The pollen trap plants were treated in exactly the same manner as the experimental plants except that two flowers were emasculated on each plant each evening. Flowers were emasculated between 1700 and 1900 h, prior to anther dehiscence, to prevent self-fertilization. A small vertical slit was cut in the developing corolla and anthers were removed with forceps. A previous study has shown no detrimental effects of flower emasculation on seed set (Chang and Rausher 1999), and pollinators approaching emasculated flowers all subsequently entered the flower in a normal fashion (L. C. Coberly, personal observation). Seed capsules from emasculated and un-emasculated flowers on each trap plant were collected and packaged individually. A total of 557 offspring were selected at random from pollen trap plants and reared to flower-

ing in the greenhouse where they were scored for flower color to determine paternal allele type (*a* or *A*). Because no sampled seeds were from the same capsule, each scored offspring represents an independent pollination event.

The selfing rate, *s*, for each genotype at the *A* locus, as well as the overall proportion, α , of *a* alleles in the outcross pollen pool, were determined using a standard likelihood approach (e.g., Fry and Rausher 1997; Chang and Rausher 1998, 1999) described in detail in Appendix I. Hypotheses were tested comparing pairs of nested models that differ in the types of constraints imposed on the models (see Table 3). The statistical significance of the constraint, and thus of the corresponding hypothesis, was assessed in standard fashion with the statistic $\Lambda = -2 \ln(L_c/L_u)$, where L_c is the likelihood of the more constrained model and L_u is the likelihood of the less constrained model (Hocking 1985). Λ is distributed approximately as χ^2 with degrees of freedom equal to the difference in number of constrained and unconstrained parameters. A significant result indicates that there is a significant difference between the fit of the models, and the one with fewer constraints is preferred.

Results

SURVIVAL

White-flowered plants had significantly lower survival rates than pigmented plants during both the 1999 and 2000 field season (Table 1). In the 1999 experiment (which did not examine survival of the light-flowered heterozygotes), the survival probability of white-flowered (*aa*) plants was only 90% that of the dark-flowered plants (*AA*) (Table 1B, *S* × *G* effect). In 2000, there was an overall effect of genotype on survival (Table 1B, *S* × *G* effect). When this

Table 1. Number of individuals surviving and dying, by genotype, in the 1999 and 2000 experiments. **A.** Numbers and percent survival. **B.** Three-way *G*-test results. *G*, genotype; *U*, unit; *S*, survival. *W/P* × *S* tests whether survival differs for white versus pigmented (*AA* and *Aa* pooled) genotypes. *D/L* × *S* tests whether survival differs for Dark (*AA*) versus Light (*Aa*) genotypes. ** *P* < 0.01. *** *P* < 0.001.

A. Genotype	Year 1999			Year 2000		
	Survived	Died	% Survival	Survived	Died	% Survival
White (<i>aa</i>)	99	15	0.86	143	130	0.52
Light (<i>Aa</i>)	–	–	–	184	100	0.65
Dark (<i>AA</i>)	110	4	0.96	176	107	0.62

B. Contrast	Year 1999		Year 2000	
	df	G	df	G
<i>G</i> × <i>U</i> × <i>S</i>	2	2.3	10	5.8
<i>G</i> × <i>U</i>	2	0	10	1
<i>U</i> × <i>S</i>	2	3.5	5	3.9
<i>G</i> × <i>S</i>	1	7.5**	2	9.9**
<i>W/P</i> × <i>S</i>	–	–	1	9.4***
<i>D/L</i> × <i>S</i>	–	–	1	0.4

overall effect is broken down into two contrasts, dark and light genotypes did not differ significantly in probability of survival (Table 1B, D/L \times S effect), although survival of whites was only 82% as high as survival of pigmented genotypes (Table 1B, W/P \times S effect). None of the effects involving unit were significant in either year, suggesting that there is little effect of background genotype on survival.

SEED PRODUCTION

There was no significant difference in the mean number of seeds produced per plant among white-, light- and dark-flowered plants (Genotype effect, Table 2). The genotype \times block effect was significant, indicating that there was an effect of genotype in at least some blocks. However, plots of seed set by genotype and block indicate that there is no consistent trend: in some blocks, whites produce more seeds; in other blocks darks produce more seeds; in other blocks light-flowered plants produce more seed; and in yet other blocks there is little difference among the genotypes (online Supplementary Fig. S1). In effect, these trends cancel each other and result in the absence of a significant main effect of genotype.

MALE FITNESS

We first tested the hypothesis that selfing rates were equal for all three flower color genotypes by comparing two pairs of the likelihood models: model 3 versus model 1 and model 4 versus model 2 (Table 3A). Within each pair, the two models differ only by the constraint imposed on selfing rates, although across the models, the two pairs differ in that the first assumes that α (proportion of outcross pollen received that carried the *a* allele) is unequal for the three genotypes, whereas in the second pair it is assumed equal for all three genotypes. In both cases, the difference in likelihood of the two models is of borderline significance, suggesting that genotypes may differ in selfing rate (Table 3B).

Next, we tested the hypothesis that α (the amount of *a* pollen received by the female parent) is the same for all three flower-color genotypes, by comparing two pairs of the likelihood models: model 2 versus model 1 and model 4 versus model 3. In each

Table 2. Analysis of variance for seed production in the 2000 experiment. df: degrees of freedom; MS: mean square; *F*: *F*-statistic; *P*: probability of observed *F*. Higher-order interaction terms were not significant and thus are not reported.

Source	df	MS	<i>F</i>	<i>P</i>
Genotype (G)	2	0.1	0.15	0.86
Unit (U)	5	0.29	0.43	0.83
Block (B)	9	9.73	14.13	<0.0001
G \times U	10	1.18	1.72	0.07
G \times B	18	1.28	1.86	<0.018
U \times B	45	1.09	1.59	<0.012
Error	393	0.69	–	–

pair, the two models differ only by the constraint imposed on the α 's, although across the models, the two pairs differed in that the first assumes that selfing rates are unequal for the three genotypes, whereas the second assumes they were equal. In neither case was the difference between the models significant (Table 3B), indicating that there is no reason to believe that α differs among genotypes.

The results of these tests suggest that model 2, with similar α 's but different selfing rates for the three genotypes, is better than models 1, 3, or 4, although support for its difference from model 4, with similar α 's and selfing rates, is marginal. We further explored whether selfing rates differed among genotypes by comparing model 4 with each of the models 5 and 6 (Table 3B). These comparisons were preplanned because bumblebees, the primary pollinators of *I. purpurea*, do not appear to distinguish darkly pigmented (AA) from lightly pigmented (*Aa*) flowers (Brown and Clegg 1984; Fry and Rausher 1997), and therefore we believed a priori that if two genotypes had similar selfing rates they would be AA and *Aa*. Model 5 is significantly better than model 4 ($P = 0.03$), indicating that selfing rate of *aa* differs from that of AA and *Aa*. By contrast, model 6 is not significantly better than model 4, indicating that a model in which the selfing rates of *aa* and *Aa* are equal, but allows that of AA to differ is not better than a model with all three selfing rates equal. In a final comparison, model 2 is not significantly better than model 5, which has one fewer parameter, indicating that allowing selfing rates of AA and *Aa* to differ from each other, as well as from that of *aa*, is no better than the model that constrains these two selfing rates to be equal. Together, these results imply that the best model considered so far is model 5, with all three genotypes receiving the same frequency of the *a* allele from the outcross pollen pool, and with equal selfing rates for AA and *Aa* (but not *aa*) plants.

Finally, we tested the hypothesis that the frequency of the *a* allele in outcross pollen differs from that expected if all three genotypes contribute equally to the outcross pollen pool. This expectation is equivalent to asking whether the common α differs from the gene frequency among individuals that survived to flower, which, from the data in Table 1, is 0.468. The appropriate comparison, which is between models 5 and 7, is not significant (Table 3B), indicating that there is no reason to believe that the two alleles were transmitted differentially through outcross pollen among genotypes.

This comparison also indicates that model 5 is not significantly better than model 7, which has one fewer parameter. Both models give essentially the same estimates for selfing rates and the proportion of the *a* allele in the outcross pollen (a single α ; Table 3C): selfing rates are almost three times higher for *aa* than for AA or *Aa* individuals, and the proportion of the *a* allele in outcross pollen reflects the frequency of the *a* allele in the plants in the experimental population. The latter result indicates that

Table 3. Model constraints and tests of differences among genotypes in selfing rate and proportion of a allele in seeds that are outcross pollinated. A. Description of models and constraints. L: Likelihood of model ($\times 10^{168}$). B. Statistical comparisons of nested models. C. Maximum likelihood estimates of parameters from models 5 and 7.

A.			
Model	L	Constraints	Description
1	369	α 's unconstrained s 's unconstrained	Selfing rates and proportion of a allele in outcross pollen allowed differ among genotypes.
2	59.3	$\alpha_{AA} = \alpha_{Aa} = \alpha_{aa}$ s 's unconstrained	Proportion a in outcross pollen equal for all genotypes; selfing rates allowed to differ.
3	18.8	α 's unconstrained $s_{AA} = s_{Aa} = s_{aa}$	Selfing rates equal for all genotypes; proportion a in outcross pollen allowed to differ.
4	3.24	$\alpha_{AA} = \alpha_{Aa} = \alpha_{aa}$ $s_{AA} = s_{Aa} = s_{aa}$	Selfing rates equal for all genotypes; proportion a in outcross pollen equal for all genotypes.
5	31.0	$\alpha_{AA} = \alpha_{Aa} = \alpha_{aa}$ $s_{AA} = s_{Aa}$	Proportion a in outcross pollen equal for all genotypes; selfing rates of AA and Aa equal.
6	5.87	$\alpha_{AA} = \alpha_{Aa} = \alpha_{aa}$ $s_{Aa} = s_{aa}$	Proportion a in outcross pollen equal for all genotypes; selfing rates of Aa and aa equal.
7	28.6	$\alpha_{AA} = \alpha_{Aa} = \alpha_{aa} = 0.468$ $s_{AA} = s_{Aa}$	Proportion a in outcross pollen pool equal to frequency in population; selfing rates of AA and aa equal.

B.						
Constrained Model	Unconstrained Model	Null hypothesis	Λ	df	P	
2	1	α equal for all genotypes	3.66	2	0.16	
3	1	s equal for all genotypes	5.95	2	0.051	
4	2	s equal for all genotypes	5.82	2	0.055	
4	3	α equal for all genotypes	3.53	2	0.17	
4	5	$s_{AA} = s_{Aa} \neq s_{aa}$	4.52	1	0.03	
4	6	$s_{AA} \neq s_{Aa} = s_{aa}$	1.19	1	0.27	
7	5	$\alpha =$ frequency of a in population	0.16	1	0.68	
5	2	$s_{AA} \neq s_{Aa}$	1.30	1	0.24	

C.			
Model	Parameter		
	α	$s_{AA} = s_{Aa}$	s_{aa}
5	0.479	0.2	0.53
7	0.468	0.19	0.54

there is no detectable pollen discounting (reduced male outcross fitness) associated with the higher selfing rate of the aa homozygotes. These patterns are very similar to those found previously in a small-array experiment (Fehr and Rausher 2004), and to those found for the W locus in *I. purpurea*, for which white-flowered individuals (ww) have higher selfing rates than pigmented individuals (WW and Ww) but do not suffer from pollen discounting (Rausher et al. 1993; Fry and Rausher 1997).

Discussion

TRANSMISSION ADVANTAGE OF WHITE ALLELE

In a previous investigation, using AA (pigmented flowers) and aa (white flowers) plants, Fehr and Rausher (2004) found that white-flowered individuals had a higher selfing rate than plants with pig-

mented flowers. Despite this advantage, white-flowered individuals were just as successful at transmitting pollen to other plants. The results reported here confirm this pattern. In particular, although male outcross success was similar for the three genotypes, the selfing rate of the aa (white) individuals was approximately 2.5 times that of the AA and Aa (pigmented) individuals. Given the absence of pollen discounting and relatively weak inbreeding depression in *I. purpurea* (approximately 12% and 24% for male and female fitness, respectively; Chang and Rausher 1999), the increased selfing rate of white-flowered plants is expected to provide a strong transmission advantage to the white allele (Fisher 1941; Fehr and Rausher 2004). The direct effect of variation at the A locus on mating system parameters thus appears to favor the a allele. This transmission advantage is similar to that found for

variation at the *W* locus in *I. purpurea* (Rausher et al. 1993; Fry and Rausher 1997).

At the *W* locus, the higher selfing rate of white-flowered plants appears to be due to a pollinator preference for plants with pigmented flowers (Brown and Clegg 1984; Rausher et al. 1993; Fry and Rausher 1997). At the *A* locus, the situation is less clear. Fehr and Rausher (2004) failed to detect a pollinator preference for pigmented flowers. However, because they observed substantially fewer pollinator visits than either Rausher et al. (1993) or Fry and Rausher (1997), their failure to observe a pollinator preference may be due to small sample size. Regardless of mechanism, the effect of flower color on selfing rate would tend to favor an increase in the frequency of the *a* allele. Population censuses, however, indicate that this allele remains at very low frequencies in populations throughout the southeastern United States (Coberly 2003), despite its apparent presence in this region as early as 1814 (Sims 1814).

PLEIOTROPIC EFFECTS OF FLOWER COLOR

Our results suggest that significant deleterious pleiotropy associated with the *aa* genotype contributes to preventing an increase in the frequency of the *a* allele. In particular, in two separate field experiments we found that white-flowered (*aa*) genotypes had substantially reduced survival. Moreover, a crude analysis based on the estimated magnitudes of this effect and of inbreeding depression suggest that together these two effects are likely sufficient to more than offset the transmission advantage of the *a* allele.

In Appendix II, we show that when the *a* allele is rare, the expected relative fitnesses of genotypes *A*- and *aa* are

$$W_{A-} = 2[s_{A-}(1 - \delta) + (1 - s_{A-})]$$

$$W_{aa} = l_{aa}[2s_{aa}(1 - \delta) + (1 - s_{aa}) + (1 - s_{A-})],$$

where s_i is the selfing rate of genotype *i*, δ is the magnitude of inbreeding depression, and l_{aa} is the survival to reproduction of *aa* seeds relative to *A*-seeds. From the results reported here, we take l_{aa} to be 0.857, which is the average survival of *aa* (relative to a survival rate of one for *A*-individuals) for the two years of our study (e.g., $l_{aa} = \frac{1}{2}(0.86/0.96 + 0.52/0.635)$, Table 1). We also take $s_{A-} = 0.2$ and $s_{aa} = 0.53$ (Table 3C). Finally, from Chang and Rausher (1999), we estimate δ as the average of inbreeding depression for male and female fitness, i.e., $\delta = \frac{1}{2}(0.12 + 0.24) = 0.18$. Substituting these values into the above equations yields the relative fitness of the *aa* plants to be $W_{aa}/W_{A-} = 1.833/1.928 = 0.95$, which indicates that when both beneficial and detrimental effects are taken into account, the *a* allele is disadvantageous. Even without inbreeding depression (i.e., setting $\delta = 0$), the relative fitness $W_{aa}/W_{A-} = 0.999$, suggesting that by itself the reduction

in survival of *aa* individuals is roughly of a magnitude that can offset the pollen transmission advantage by itself. Thus, although we have no estimates of the degree to which the parameters in the above equations vary from site to site or from year to year, this analysis is at least consistent with the hypothesis that deleterious pleiotropy associated with the *a* allele keeps its frequency from increasing in natural populations.

Although our experiments do not provide information on the nature of the deleterious pleiotropy we detected, some reasonable guesses can be made. The *a* allele results from an insertion of a transposable element into the exon of the structural gene for chalcone synthase D (Habu et al. 1998). Because chalcone synthase is the first enzyme of the flavonoid pathway (Holton and Cornish 1995), its inactivation is expected to prevent the production of flavonoids in vegetative as well as floral tissue. This expectation is corroborated by our observation that pigmentation is dramatically reduced in the vegetative tissues throughout *aa* plants.

Flavonoids have been shown to perform a number of important physiologically and ecologically important functions in plants, including mediating interactions with mycorrhizal fungi and thus water uptake, conferring protection from UV radiation, and providing defense against herbivores and pathogens (Shirley 1996). In the southeastern United States, *I. purpurea* plants are subject to periods of intense heat and drought during the growing season (Coberly and Rausher 2003). They are also subject to substantial herbivory (Simms and Rausher 1987, 1989). It seems likely that failure of *aa* individuals to produce flavonoids reduces the ability of plants of this genotype to cope with these stressors, leading to greater juvenile mortality. Another possibility is that blockage of the flavonoid pathway may increase flux down the competing lignin pathway (Bessaue et al. 2007), producing a detrimental overproduction of lignin.

Although our results are consistent with the hypothesis that deleterious pleiotropy is associated with the *a* allele in *I. purpurea*, one limitation of our study must be kept in mind: it is possible that the adverse effects on survival we detected are not due to true pleiotropy, but to variation at a closely linked gene. Although our breeding design should have effectively broken up associations between the flower-color locus and other unlinked loci, it would not have been effective at breaking up initial associations between the flower-color locus and tightly linked loci. Definitively ruling out this possibility would require transgenic manipulation, independently derived *a* alleles, or numerous generations of marker-assisted backcrossing to yield truly isogenic lines. Unfortunately, this approach is seldom an option in evolutionary studies on plants. The best that is typically achieved is a comparison of nearly isogenic lines (NILs) (e.g., Bradshaw and Schemske 2003), which may still be differentiated at hundreds of genes. Our approach, involving several generations of single-seed-descent selfing, is essentially equivalent to the NIL approach

in reducing the size of the differentiated block of genes in linkage disequilibrium around the target floral-color gene, and thus should yield a similar level of confidence regarding whether effects are truly due to pleiotropy.

VARIATION IN DEGREE OF PLEIOTROPY AMONG GENES

The evolution of white from pigmented flowers has occurred repeatedly throughout the angiosperms (Rausher 2006, 2008). Spontaneous mutations eliminating floral pigment production have been identified in both structural and regulatory sequences in a variety of species (Holton and Cornish 1995), indicating that either type of mutation has the potential to serve as the raw material for adaptive loss of floral pigmentation. Nevertheless, limited evidence suggests that evolutionary elimination of floral pigments typically involves inactivation of regulatory rather than structural sequences (Rausher 2006, 2008). As has been suggested by several authors (Quattrocchio et al. 1999; Coberly 2003; Rausher 2006; Whittall et al. 2006), such a pattern is expected a priori because knockouts of anthocyanin structural genes (those coding for pathway enzymes) are expected to have greater deleterious pleiotropic effects than knockouts of anthocyanin regulatory sequences. Because of the manifold uses of flavonoids by plants (see above), knockout mutations of structural genes produce white flowers, but they also potentially reduce plant fitness by eliminating the production of beneficial flavonoids throughout the plant. By contrast, most known mutations in anthocyanin regulatory sequences tend to be very tissue-specific in their effects, reducing pigmentation in flowers but not other tissues (Ludwig and Wessler 1990; Cone et al. 1993; Quattrocchio et al. 1993; Huits et al. 1994). Because these mutations do not generally block flavonoid production in vegetative tissues, the expected magnitude of deleterious pleiotropy is expected to be less than for structural gene mutations. In turn, this implies that the net benefit of a white-flower mutant, which is the difference between the fitness benefit of producing white flowers and the fitness reduction due to pleiotropy, is expected to be greater for regulatory sequence mutants. Because the probability of fixation of a mutation is proportional to its net selective benefit, a knockout of an anthocyanin regulatory sequence is more likely to become fixed than a knockout of a structural gene. To date, however, it has not been possible to evaluate this hypothesis because there has been no comparative data on the relative pleiotropy of anthocyanin structural and regulatory genes.

The data presented here, along with similar information from previous studies on the *W* locus in *I. purpurea*, allow a preliminary comparison of this type. As described above, the *a* allele produces white flowers by inactivating a structural gene (chalcone synthase) in the anthocyanin pathway. By contrast, the naturally occurring *w* allele produces white flowers by inactivating the gene *Ipmyb1*, which is a transcription factor (Chang et al. 2005). Consistent with

expectations, vegetative tissues of *ww* individuals contain anthocyanins and other flavonoids (Schoen et al. 1984). Moreover, in a series of previous field experiments, we have repeatedly failed to detect any deleterious pleiotropic effects associated with the *w* allele. Survival, seed production, seed size, and male reproductive success of the *W*-locus white genotype is either equal to or higher than that of the pigmented genotypes (Rausher et al. 1993; Rausher and Fry 1993; Fry and Rausher 1997; Mojonier and Rausher 1997). These results, together with the evidence presented here for the *a* locus, are consistent with the interpretation that greater deleterious pleiotropy is associated with structural gene knockouts in the anthocyanin pathway than with regulatory knockouts. To the extent that this result may be generalized, it provides an explanation for why regulatory sequences may be more commonly involved in floral-pigment evolution than structural sequences.

One potential limitation of this comparison is that the magnitude of deleterious pleiotropy was not estimated simultaneously for the *a* and *w* alleles. It is therefore conceivable that the observed differences in the degree of measured pleiotropy are due to differences in environmental conditions under which the experiments were conducted. Although we cannot rule out this possibility, we believe that failure to find deleterious pleiotropy associated with *w*-white alleles in several experiments performed in different years (Rausher et al. 1993; Rausher and Fry 1993; Fry and Rausher 1997; Mojonier and Rausher 1997) can be taken as reasonable evidence of its general absence. In spite of these caveats, our results at the very least indicate that the hypothesis that regulatory sequences are preferentially involved in the evolutionary loss of floral pigments deserves further examination.

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Appendix I

To estimate genotype-specific mating parameters, we employed likelihood-estimation procedures, as is commonly done when genetic information on parents and offspring are available. Specifically, we estimated six parameters: α_{AA} , α_{Aa} , and α_{aa} , the proportion of outcross pollen carrying the *a* allele received by each of the maternal genotypes *AA* (darkly pigmented flowers), *Aa* (lightly pigmented flowers), and *aa* (white flowers) respectively; and s_{AA} , s_{Aa} , and s_{aa} , the selfing rates for each of the same three genotypes. The data consist of the number of seeds of a particular genotype *i*, where *i* = *AA*, *Aa*, or *aa*, that were produced by a maternal parent of genotype *j*, where *j* = *AA*, *Aa*, or *aa*, which is designated by N_{ij} .

The assumed probabilities of obtaining an offspring of genotype *i* from a maternal plant of genotype *j*, P_{ij} , are given in Table A1 for seeds from both emasculated and unemasculated flowers. To illustrate how these probabilities are obtained, we consider two examples. The probability that an *aa* offspring will be produced by an emasculated mother of genotype *aa* is simply the proportion of the *a* allele in the outcross pollen that lands on *aa* stigmas, so $P_{aa,aa} = \alpha_{aa}$. For unemasculated maternal flowers of the same genotype, offspring can be produced by either selfing, with probability s_{aa} , or outcrossing, with probability $(1 - s_{aa})$. If produced by selfing, the probability that the offspring will be *aa* is 1.0. If produced by outcrossing, the probability that the offspring will be *aa* is the frequency of the *a* allele in the outcross pollen that lands on *aa* stigmas, α_{aa} . Consequently,

Table A1. Assumed probabilities, P_{ij} , and number, N_{ij} , of seeds having genotype *i* given that it was produced by a maternal parent of genotype *j*

Maternal Genotype	Offspring Genotype	P_{ij}	N_{ij}
Emasculated			
<i>aa</i>	<i>aa</i>	α_{aa}	37
<i>aa</i>	<i>Aa</i>	$1 - \alpha_{aa}$	37
<i>Aa</i>	<i>aa</i>	$\frac{1}{2} \alpha_{Aa}$	20
<i>Aa</i>	<i>Aa</i>	$\frac{1}{2}$	39
<i>Aa</i>	<i>AA</i>	$\frac{1}{2}(1 - \alpha_{Aa})$	19
<i>AA</i>	<i>Aa</i>	α_{AA}	46
<i>AA</i>	<i>AA</i>	$1 - \alpha_{AA}$	67
Unemasculated			
<i>aa</i>	<i>aa</i>	$\omega_{aa} + (1 - \omega_{aa}) \alpha_{aa}$	56
<i>aa</i>	<i>Aa</i>	$(1 - \omega_{aa})(1 - \alpha_{aa})$	18
<i>Aa</i>	<i>aa</i>	$\frac{1}{4}\omega_{Aa} + \frac{1}{2}(1 - \omega_{Aa}) \alpha_{Aa}$	34
<i>Aa</i>	<i>Aa</i>	$\frac{1}{2}$	41
<i>Aa</i>	<i>AA</i>	$\frac{1}{4}\omega_{Aa} + \frac{1}{2}(1 - \omega_{Aa})(1 - \alpha_{Aa})$	23
<i>AA</i>	<i>Aa</i>	$(1 - \omega_{AA}) \alpha_{AA}$	46
<i>AA</i>	<i>AA</i>	$\omega_{AA} + (1 - \omega_{AA})(1 - \alpha_{AA})$	74

the overall probability that the offspring will be *aa* is $P_{aa,aa} = s_{aa} + (1 - s_{aa}) \alpha_{aa}$. Other probabilities are calculated in similar fashion.

As described in the text, likelihoods were calculated for each of several models, which differed in the number and type of constraints imposed on the parameters. For any given model, the likelihood of the data given the model, *L*, is given by

$$L = C \prod_{i=1}^{14} P_{ij}^{N_{ij}}, \tag{A.1}$$

where *C* is a constant that does not depend on the parameters being estimated, and may thus be ignored. The maximum likelihood, as well as the values of the parameters associated with this likelihood, were obtained by finding the minimum of $-\ln(L)$ with the FindMin procedure in Mathematica® (Wolfram 1991). We used the Random function to generate 10 random number seeds for each parameter and ran the FindMin function over 150 iterations for each parameter estimate.

Appendix II

In this appendix, we derive the equations for the relative fitnesses of genotypes *A*- and *aa*. Because all estimates of survival, seed production, and mating system parameters were indistinguishable between *AA* and *Aa* individuals, we assume that their fitnesses are equal.

Our approach is to count the number of copies of a gene an individual transmits to the next generation through both ovules and pollen, as in Chang and Rausher (1998). Because we seek to understand whether a recent mutation for white flowers will increase in a population, we assume that *a* is sufficiently rare that there is only one *aa* individual in a large population of *N* individuals.

For *A*-individuals, the number of gene copies transmitted through ovules by selfing is $Fs_{A-}(1 - \delta)$, where *F* is the number of seeds produced per plant (assumed the same for all genotypes), s_{A-} is the selfing rate of *A*-individuals, and δ is the magnitude of inbreeding depression. Because one gene copy is transmitted through the same individual's pollen for every selfed ovule, the total number of copies transmitted through selfing is $2Fs_{A-}(1 - \delta)$. The number of copies transmitted through ovules for outcrossed seeds is simply $F(1 - s_{A-})$.

The number of copies transmitted through outcross pollen is slightly more complicated and is based on the assumption, verified in this study, that all genotypes have equal outcross male success. In a population of *N* pigmented (*A*-) individuals, the total number of outcrossed seeds produced is $NF(1 - s_{A-})$. In the single *aa* individual, the number of outcrossed seeds produced is $F(1 - s_{aa})$. The total number of outcrossed seeds in the population is

thus $NF(1 - s_{A-}) + F(1 - s_{aa})$. Because these are shared equally by the outcross pollen of $N + 1$ individuals, the total number of gene copies transmitted per individual through outcross pollen is $[NF(1 - s_{A-}) + F(1 - s_{aa})] / (N + 1)$. With N large, this is approximately equal to $F(1 - s_{A-})$. Consequently, the fitness of $A-$, which is just the sum of the numbers of gene copies transmitted, is

$$W_{A-} = 2F[s_{A-}(1 - \delta) + (1 - s_{A-})].$$

In a similar fashion, the total transmission success of aa individuals is derived to be

$$T_{aa} = F[2s_{aa}(1 - \delta) + (1 - s_{aa}) + (1 - s_{A-})].$$

However, this is not fitness, because it does not incorporate the reduced survival of aa . Doing so yields,

$$W_{aa} = l_{aa}T_{aa} = l_{aa}F[2s_{aa}(1 - \delta) + (1 - s_{aa}) + (1 - s_{A-})].$$

These equations for fitness differ from those given in the text only by the constant F . Because we are interested only in relative fitness of the different genotypes, F can be dropped from both equations without consequence.

Supplementary Material

The following supplementary material is available for this article:

Figure S1. Relationship between mean seed number and flower-color genotype, by experimental block.

This material is available as part of the online article from:

<http://www.blackwell-synergy.com/doi/abs/10.1111/j.1558-5646.2008.00355.x>

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