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Induced mutations: A novel tool to study phenotypic integration and evolutionary constraints in *Arabidopsis thaliana*

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ABSTRACT

Adaptive phenotypic evolution is constrained by the availability of genetic variation and the patterns of phenotypic integration and genetic architecture among characters. Phenotypic and genotypic variance/covariance matrices provide a convenient quantitative summary of such patterns and are widely used in evolutionary studies. These matrices are, for mathematical tractability, assumed to remain constant or change only proportionally in the course of evolution. However, this assumption is tenuous over the medium and long term and cannot possibly hold at all taxonomic scales. One important source of genetic change is mutations, but low natural rates of mutation make them difficult to study at the population level. We used ethyl-methane-sulphonate to induce mutations in a flowering plant and assess their impact on the means and phenotypic variances of seven morphological and life-history traits as well as the covariances among these characters. We found that only one trait mean changed in response to mutagenesis, but that mutations generated new variance in all traits in a roughly dose-dependent fashion. Common principal components analyses of the matrices showed that the degree of divergence from a non-mutagenized control population is roughly dose-dependent. This overall divergence of covariance structure is the product of idiosyncratic mutation-induced changes in particular pairwise correlations among traits. One well-studied correlation between time to flowering and the number of rosette leaves at flowering persists in all mutagenic treatments. We discuss these findings and their implications for quantitative genetic models that assume constant variance/covariance matrices.

Keywords: *Arabidopsis*, canalization, genetic constraints, mutation, phenotypic covariance, pleiotropy.

INTRODUCTION

Modern views of evolution incorporate the idea that natural selection is constrained by biases in the underlying genetic and epigenetic systems that produce phenotypic variation. Responses to selection, for example, may be limited in magnitude or constrained in direction

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by a lack of sufficient genetic variation within populations, by developmental contingencies that bias the probabilities of producing some phenotypes over others, or by covariation among characters (Lovtrop, 1974; Lande, 1976, 1980; Maynard Smith *et al.*, 1985; Clark, 1987; Schluter, 1996). Studies of phenotypic integration and genetic architecture and their role in adaptive evolution are, as a result, becoming more common (Roff, 1997), although we still have much to learn about the forces that influence genetic architecture (Roff, 2000).

Quantitative analyses and theoretical models of constraints typically employ an elegant statistical summary of the relationships among traits: the phenotypic or genetic variance/covariance matrix, **P** and **G** respectively (Lande, 1979; Cheverud, 1984, 1988). This statistical approach quantifies the variation in populations as a matrix whose diagonal elements are the variances of individual traits and whose off-diagonal elements are all pairwise covariances among traits. In conjunction with estimates of a vector of selection coefficients acting on the same traits, quantitative genetic models can predict evolutionary changes in the short term, taking into account both direct responses to selection acting on each trait and indirect responses via correlations with other traits.

Predicting medium- and long-term responses to selection, however, is more problematic and requires the assumption that the currently observable patterns of (co)variation remain relatively constant (or at least proportional) during the course of evolutionary change (Lande, 1976, 1979). There is no theoretical justification for this assumption (Turelli, 1988; Arnold, 1992) and, unless new mutations precisely restore the (co)variation lost in the course of adaptive evolution, it is unlikely to hold true over extended periods. Houle (1991) has also argued that the typical mutation/selection balance models used to study the dynamics of these matrices are mathematically tractable at the expense of ignoring the underlying functional mechanisms that produce patterns of covariation. These mechanisms, he also demonstrated, matter, and the same correlations can be produced in a variety of functional ways that have different evolutionary implications (Deng *et al.*, 1999). Statistical summaries such as **P** and **G**, therefore, fail to capture important temporal and functional information about evolutionary constraints.

Several researchers have studied how extant patterns of covariation change among populations or taxa. In general, genetic and phenotypic covariance matrices are very similar within species, but the conclusions differ among groups at higher taxonomic levels (recently summarized in Paulsen, 1996; Steppan, 1997; Roff, 1999). Taking a novel approach, Schluter (1996) found that divergence among populations of three spine sticklebacks is biased towards 'evolutionary paths of least resistance' as estimated by the first principal component of these matrices, supporting the hypothesis that genetic architecture can have a long-term influence. However, he also found that this influence decays over time, and a similar study of greenfinch populations found divergence in directions other than the evolutionary path of least resistance (Merilä and Björklund, 1999). Therefore, the common assumption that the covariances among quantitative characters are effectively constant and their use to reconstruct past selection or to predict future evolutionary trajectories beyond the short term seems questionable (Lande, 1979; Lande and Arnold, 1983; Turelli, 1988; Leroi *et al.*, 1994; Van Tienderen and Koelewijn, 1994; Schlichting and Pigliucci, 1995; Shaw *et al.*, 1995; Pigliucci, 1996; Roff, 1996).

Furthermore, Wagner and colleagues (Wagner, 1995a; Wagner and Altenberg, 1996), extending the earlier ideas of Olson and Miller (1958) and of Berg (1959, 1960), argued that genetic architecture itself may be moulded by natural selection and advocated that the term 'variation' be restricted to *static* descriptions of the relationships among traits such as

the variance/covariance matrices. This they differentiate from 'variability', referring to *potential* variation due to recombination of current alleles and mutations that generate new ones. They argue that evolutionarily persistent patterns in variability (i.e. biases in the impacts of mutation and recombination on phenotypes) are more important long-term constraints than current patterns of variation because they determine the ways in which the **G**-matrix can change. Therefore, if we want to understand long-term evolutionary constraints, we must address the dynamics of covariation (i.e. covariability) rather than limiting our inquiries to currently available covariation. Another matrix, **M**, summarizing the mutational covariation is a convenient way to summarize these effects (Camara and Pigliucci, 1999). In so far as **M** is a multivariate summary of the functional architecture (*sensu* Houle, 1991) among traits, it represents an important influence on the dynamics of phenotypic integration. Changes in **G** from generation to generation, therefore, may be predictable from knowledge of the influences of selection, drift and mutation, extending the predictive power of quantitative genetic models into longer time-frames, and improving our understanding of evolutionary constraints.

Along these lines, Carrière and Roff (1995) have developed models of how selection acting to increase the frequency of a single allele with known pleoitropic effects alters genetic architecture. They do so by adding a term for pleiotropic effects at a single locus to standard quantitative genetic models and derive expectations for the covariance among traits as a function of allele frequencies at that locus. Deng *et al.* (1999) used **M** as a multivariate summary of such pleoitropic effects across all loci influencing the traits under study, and incorporated it into standard quantitative genetic models and concluded that both selection and pleoitropic mutations are important determinants of **G**.

Both theoretical and empirical studies of the pleiotropic impact of mutations on fitness have been carried out in *Drosophila* (see Lynch *et al.*, 1999) and more recently in *Arabidopsis* (Schultz *et al.*, 1999). Most of these studies, however, were primarily concerned with accumulation of genetic load within populations, few of them addressing traits other than fitness or the correlations among traits other than fitness components. In contrast, most studies of mutations in plants typically focus on identifying loci of special interest through reverse genetic methods rather than on the impact of mutations on population-level parameters (e.g. Winkler *et al.*, 1998).

In this paper, we take two novel approaches. First, we address the impact of mutations on morphological and phenological covariation in plants rather than on fitness *per se*. Different combinations of phenotypic traits may be adaptive in novel or changing environments and mutations considered detrimental in one set of conditions may well be advantageous in others. Second, we used ethyl-methane-sulphonate to induce mutations in a laboratory population of the model plant *Arabidopsis thaliana* (Brassicaceae) derived from a large, naturally variable wild population. Although it is beyond the immediate scope of the current study, we anticipate that the background knowledge of the molecular and developmental biology of *A. thaliana* and the availability of a wide range of molecular techniques for mapping and identifying loci of interest will allow mechanistic interpretations of our population-level results in future studies.

MATERIALS AND METHODS

We generated mutations in wild type seeds of the Kendalville ecotype (obtained from Lehle seeds, catalogue # WT-16; http://www.arabidopsis.com) by exposing them to a distilled

water control or one of four concentrations (0.1, 1, 3 or 10 $mmol \cdot l^{-1}$) of ethyl-methane-sulphonate (EMS) for 24 h at 23°C as seed. We refer to these seeds and the plants they grow into as the M_1 generation and their selfed progeny as the M_2 generation. Ethyl-methane-sulphonate is known to induce point mutations, mostly GC → AT transitions (Lightner and Casper, 1998), at a rate of approximately 3.7×10^{-6} $locus^{-1} \cdot cell^{-1} \cdot mmol^{-1} \cdot h^{-1}$ in seeds thus exposed (Koornneef *et al.*, 1982). Furthermore, these mutations influence a variety of quantitative characters (Brock, 1976). Using Reidi and Koncz's (1992) estimate that the *A. thaliana* genome consists of about 28,000 loci, our highest EMS concentration is expected to produce approximately 25 mutations per cell. However, we assayed the relative efficacy of our EMS treatments using the standard technique of scoring the siliques of M_1 plants for the frequency of albino embryo mutations (Table 1) (Ivanov, 1973; Mednik, 1988). For clarity, we sometimes present and discuss our results in terms of the frequency of these visible mutations rather than theoretical calculations from EMS exposures.

We collected seeds from each M_1 plant in bulk and used the M_2 seeds they produced in our experiment. Since each cell in the exposed M_1 embryo is mutagenized independently, M_1 plants are chimeras for induced mutations with sectors derived from different embryonic cell lineages carrying different mutations, although individual siliques are generally derived from single embryonic cell lineages (Ivanov, 1973). As a result, the M_2 progeny of a single M_1 plant, especially those from different siliques, may carry a variety of mutations segregating in Mendelian fashion within lineages derived from single embryonic cells. M_2 sibships collected from entire plants, therefore, are not true families in a quantitative genetic sense.

To synchronize germination, we stratified the M_2 seeds on agar plates containing standard cell culture medium (Valvekens *et al.*, 1988) for 1 week at 4°C in complete darkness. Each plate held seeds from a single self-pollinated M_1 parent. After stratification, we placed the agar plates containing the seeds under high-intensity artificial lights for 1 week. At the end of this period, most seedlings had initiated the development of four rosette leaves, but they were still very small.

We then transplanted three seedlings from each of approximately 40 M_1 parents for each of the five treatment lines individually into 5 cm pots containing Fafard # 2™ potting mix (~115 individual plants from each of the four EMS-treated lines and the control, for a

Table 1. Frequency of albino embryo mutations produced by the EMS treatments

EMS treatment ($mmol \cdot l^{-1}$)	M_1 siliques examined (*n*)	Siliques with segregating albino embryo mutations	
		n	%
0.0	223	5	2.2
0.1	222	6	2.7
1.0	116	11	9.5
3.0	133	11	8.3
10.0	130	58	44.6

total of 575 plants). Low germination in some dishes resulted in slight variation in sample sizes among treatments. Each pot was fertilized with three pellets of Osmocote™ 14-14-14 N-P-K time-released fertilizer at the time of transplanting. Osmocote pellets were first sieved, and we used only the largest pellets to reduce pot-to-pot variation in the amount of fertilizer. For each plant we recorded the following seven traits:

1. The duration of the vegetative growth period, beginning on the date of emergence of the first true leaf and ending on the day that the first inflorescence began elongation (bolting or the age at sexual maturity).
2. The duration of the flowering period, beginning at bolting and ending when the first silique dehisced (age of first reproduction).
3. The number of rosette leaves at bolting, a measure of meristem allocation during the vegetative phase.
4. The height of the main inflorescence when the first silique dehisced, an architectural character.
5. The summed length of all inflorescences, gauging overall plant size.
6. The total number of siliques at first reproduction, an estimate of reproductive fitness, since seed number and germination rates are highly correlated with fruit production (Westerman, 1970).
7. The number of non-elongated inflorescences when the first silique dehisced, a measure of potential yet not realized allocation to reproductive meristems.

Statistical methods

We transformed the raw data by expressing each observation as a proportion of the maximum observed value for that character in the entire experiment. This transformation expresses fitness components as relative fitness as well as providing a common scale for all characters, an important consideration since principal components estimation is sensitive to scale differences between the original variables. Although subjecting these proportions to the angular transformation improved the normality of the data, the pattern of statistical significance for tests done on the proportions and their angular transformations were identical. Therefore, for ease of interpretation, we present the data as untransformed proportions. Because M_2 offspring are not true families, we only performed phenotypic analyses using all the observations rather than using family means or analyses of variance to partition genetic and environmental contributions.

We tested four kinds of null hypotheses: (1) equality of means; (2) equality of variances between each level of EMS treatment and the control; (3) common phenotypic covariance structure among the EMS treatments; and (4) homogeneity of the phenotypic correlation between leaf number and the length of the vegetative growth period, for which we had an *a priori* interest because these two traits are known to be highly genetically correlated in *A. thaliana* (Mitchell-Olds, 1996; Alonso-Blanco *et al.*, 1998).

Tests of equality of means. We tested hypotheses about the means of each trait by first using multivariate analyses of variance to protect the experiment-wise error rate and then using univariate analyses of the seven traits to dissect out their contributions to the multivariate effects. The statistical model was a one-way design in which the only factor was the EMS treatment, a fixed effect. These analyses were performed using SAS version 7.0 (SAS, 1990).

Tests of equality of variances. We tested the null hypothesis that the phenotypic variance of each trait in each of the EMS-treated lines was equal to that of the control line using simple *F*-ratios of the variances of untransformed data. We used PROC UNIVARIATE in SAS version 7.0 (SAS, 1990) to estimate the variances and calculated the *F*-ratios and associated *P*-values using Microsoft Excel version 7.

Tests of common covariance structure. We tested hypotheses about the overall similarity of the variance/covariance matrices using the method of common principal components analysis (CPCA) (Flury, 1987; Airoldi and Flury, 1988; Steppan, 1997). All CPC analyses used a program written and kindly provided by Patrick Phillips (http://wbar.uta.edu/software/cpc.htm). Since the original Kendalville population harbours a good deal of phenotypic and genetic variation (Pigliucci, 1997), these tests are not direct comparisons of the contributions of new mutations, but rather of matrices containing both pre-existing and mutationally induced (co)variation. For these overall matrices, we used separate CPC analyses to first compare the matrices for the five lines simultaneously and then to test the four comparisons of each EMS-treated line to the 0 $\text{mmol} \cdot \text{l}^{-1}$ EMS control. These tests evaluate the degree to which novel mutations resulted in divergence in matrix structure from the pre-mutagenesis population. Because we applied the EMS treatments to a bulk collection of seeds from the control population, we cannot logically pair observations (e.g. by family) to subtract the pre-mutagenesis trait values from their post-mutagenesis values to estimate **M**, the (co)variance matrix among these differences. Instead, we subtracted the entire variance/covariance matrix of the control population from each EMS-treated population. This produces estimates of mutational matrices, but the appropriate degrees of freedom are unknown, precluding tests of differences among them using CPCA (P. Phillips, personal communication). We therefore used Mantel's tests of matrix correlation (Mantel, 1967; Dietz, 1983) among all pairs of mutational matrices. For these analyses, we used a program written by Adam Liedloff (http://www.sci.qut.edu.au/nrs/mantel.htm).

Common principal components analysis tests for similarity among covariance matrices based on the asymptotic theory developed by Flury (1987, 1988) using a hierarchical series of hypotheses (Flury, 1987; Airoldi and Flury, 1988; Steppan, 1997; Phillips and Arnold, 1999). Detailed descriptions of these analyses are available elsewhere (Phillips and Arnold, 1999). Briefly, however, CPCA tests for differences in covariance structure using log-likelihood ratios between models that constrain subsets of the parameters of principal components structure to values estimated from data pooled across classes (Flury, 1987, 1988). Such tests of significance check whether or not the constraints on the parameters significantly alter the fit of the model and, therefore, whether the assumption of common parameters is justifiable. Constraining eigenvectors to pooled values provides tests for common orientation of principal components, whereas constraining eigenvalues to proportional or equal values tests for matrix proportionality or equality, respectively.

The hierarchy of similarity among matrices developed by Flury ranges from unrelated matrices assumed to share neither eigenvectors nor eigenvalues, through a series of partial CPC models that share subsets of their eigenvector structure and then to matrix proportionality and matrix equality. These tests are hierarchical because adding further constraints to the model presumes that earlier constraints are justified (i.e. the constrained models are nested). Models may be tested in either a 'step-up' procedure in which each

model is tested against the next one in the hierarchy, or a 'jump-up' procedure that tests all constrained models against the completely unconstrained model. We used the 'jump-up' procedure because it is more appropriate for detecting overall matrix divergence; the 'step-up' procedure is more appropriate for tests of specific axes of variation (Phillips and Arnold, 1999). Based on this procedure, the best fitting model in the hierarchy is the one below the first significant P-value starting from the bottom of the hierarchy (Table 3). Furthermore, we used the program's default procedure of constraining eigenvectors in order of their ranked eigenvalues.

Tests of the leaf number vs vegetative period correlation. We examined the effects of induced mutations on the well-studied correlation between leaf number at bolting and the length of the vegetative period. Specifically, we tested two null hypotheses: (1) that the correlation coefficients between the number of leaves at bolting and the length of the vegetative period are homogeneous among EMS treatments using the test described in Sokal and Rohlf (1981); (2) that the slope of the relationship between the two traits is homogeneous among EMS treatments using a CPC analysis on these two traits only. This technique is preferable to standard regression analysis or analysis of covariance because a two-dimensional test of common principal components effectively tests for homogeneity of slopes (eigenvectors) without the arbitrary assignment of independent and dependent variables.

RESULTS

Multivariate analysis of variance on all seven traits simultaneously revealed significant overall differences among the treatments (Wilks' $\lambda = 0.88$, $F = 2.34$, $P < 0.001$). However, univariate analyses on each of the characters separately showed no significant effects of EMS treatment on the means of six of the seven traits we measured (Fig. 1a,b); the only exception was the duration of the flowering period ($F = 6.12$, $P < 0.001$; Fig. 1b).

In contrast, induced mutations did alter the phenotypic variance of most traits (Table 2, Fig. 2). The responses were roughly but not strictly dose-dependent for fitness correlates (Fig. 2a), but not for morphometric and phenological traits (Fig. 2b), which showed an increase in variance at lower levels of mutagenesis and a drop off at the highest level.

Common principal components analyses of the overall pattern of covariation among the seven traits we measured showed significant heterogeneity of principal components structures when all five treatment levels were analysed simultaneously, rejecting even the hypothesis of a single common principal component (Table 3a). Comparisons of each level of EMS mutagenesis to the control (Table 3b–e) showed that the 0.1 and 3 $mmol \cdot l^{-1}$ treatments shared two common principal components with the non-mutagenized control, the 1.0 $mmol \cdot l^{-1}$ treatment shared none, and the 10 $mmol \cdot l^{-1}$ treatment shared a single common principal component with the control. Mantel's tests of matrix correlation for all possible pairings of mutationally induced (co)variance matrices showed significant matrix correlations between all EMS treatments, but the strength of the relationship and deviations from the 1 : 1 isometric line varied among comparisons (Fig. 3). The complete variance/covariance and correlation matrices and the details of the principal component structures for each matrix are included as Appendices 1 and 2 respectively.

Tests of whether or not the correlation coefficients between the number of leaves at bolting and the length of the vegetative period varied among the treatment levels found no evidence to reject the null hypothesis that they are equal ($\chi^2 = 7.83$, d.f. = 4, $P = 0.0980$).

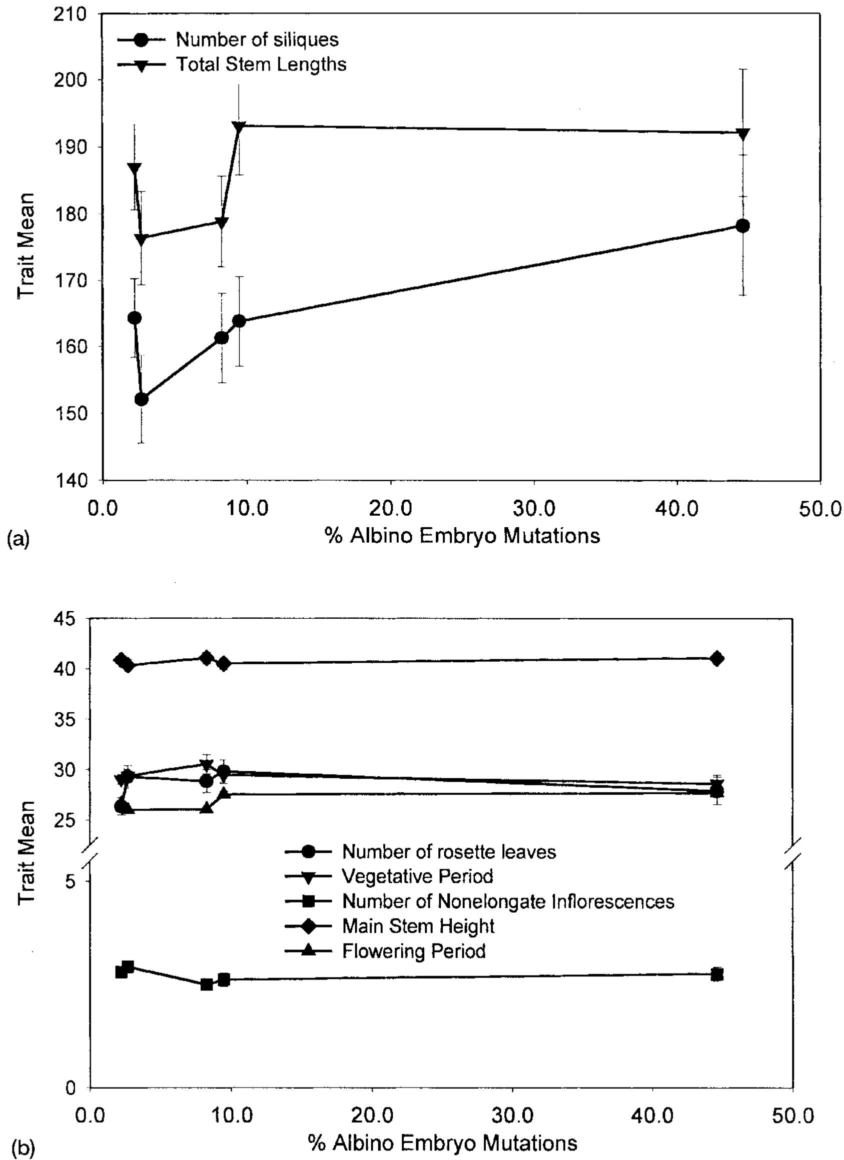

Fig. 1. Means of all traits as a function of the number of albino embryo mutations observed. (a) Fitness correlates; (b) morphology and timing traits. Data are untransformed to facilitate interpretation. Error bars are standard deviations. See text for associated statistical tests.

Furthermore, a CPC analysis on these two characters only in all five treatment levels failed to reject the null hypothesis that the five 2×2 variance/covariance matrices were proportional ($\chi^2 = 13.354$, d.f. = 8, $P = 0.1002$). This indicates that the slopes of the relationship between these two characters were homogeneous and that these two traits show proportional increases in variance in response to mutagenesis.

Table 2. Comparisons of the variance for each trait to the control (0 mmol · l^{-1}) using the original scales of measurement (significant tests are indicated by **bold** type)

	Leaves	Main stem	All stems	Fruits	Veg. per.	Flow. per.	NEI
Control (0 mmol · l^{-1})							
Variance	92.49	19.64	4961.13	4354.88	28.68	9.31	2.67
d.f.	125	125	125	125	125	125	125
0.1 mmol · l^{-1}							
Variance	**142.82**	**34.84**	5305.61	4639.80	**49.91**	7.49	2.60
d.f.	**111**	**111**	111	111	**111**	111	111
F	**1.54**	**1.77**	1.07	1.07	**1.74**	1.24	1.03
P	**0.009**	**0.001**	0.357	0.364	**0.001**	0.121	0.444
1 mmol · l^{-1}							
Variance	**184.46**	**41.45**	**7030.62**	**5987.31**	**58.83**	**13.10**	3.48
d.f.	**135**	**135**	**135**	**135**	**135**	**135**	135
F	**1.99**	**2.11**	**1.42**	**1.37**	**2.05**	**1.41**	1.30
P	**0.000**	**0.000**	**0.024**	**0.036**	**0.000**	**0.027**	0.067
3 mmol · l^{-1}							
Variance	**141.72**	**34.02**	4849.25	4803.51	**99.30**	9.50	2.41
d.f.	**111**	**111**	111	111	**111**	111	111
F	**1.53**	**1.73**	1.02	1.10	**3.46**	1.02	1.11
P	**0.010**	**0.001**	0.452	0.296	**0.000**	0.455	0.291
10 mmol · l^{-1}							
Variance	**171.14**	24.49	**7520.91**	**9305.01**	**67.79**	11.26	2.45
d.f.	**93**	93	**93**	**93**	**93**	93	93
F	**1.85**	1.25	**1.52**	**2.14**	**2.36**	1.21	1.09
P	**0.001**	0.125	**0.015**	**0.000**	**0.000**	0.160	0.333

Note: Leaves = number of rosette leaves at bolting; Main stem = height of main inflorescence; All stems = summed length of all inflorescences; Fruits = number of siliques at first reproduction; Veg. per. = duration of vegetative period; Flow. per. = duration of flowering period; NEI = number of non-elongated inflorescences.

DISCUSSION

Previous work addressing the stability of the genetic architecture has relied upon comparisons among populations, species and genera (reviewed by Roff, 1999). Taken together, these studies demonstrate that the covariance structure changes over time, implying that changes occur mostly at taxonomic levels above species (Roff, 2000). Roff and Mousseau (1999) argue that the small differences within species are consistent with divergence through genetic drift. Lynch (1988) and Houle *et al.* (1996) reviewed the literature on spontaneous rates of polygenic mutation in a number of species, emphasizing the importance of mutational variance in models of phenotypic evolution. However, manipulative studies such as this one can directly test hypotheses about the impact of mutation and/or selection on patterns of covariation among quantitative traits.

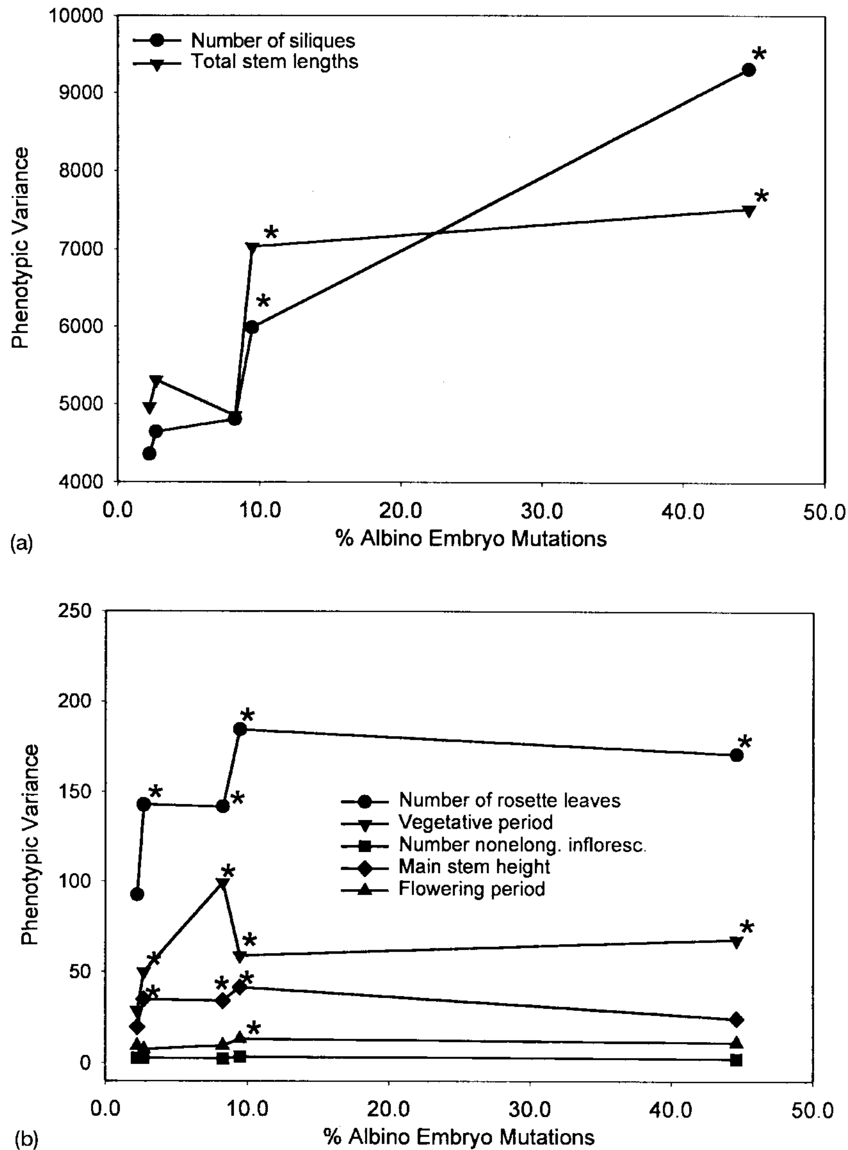

Fig. 2. Variances of each trait as a function of the number of albino embryo mutations induced by EMS treatment. (a) Fitness correlates; (b) morphology and timing traits. Asterisks indicate variances that differ significantly from the 0 $mmol \cdot l^{-1}$ EMS control. See Table 2 for associated statistical tests.

Mutation accumulation approaches have a long history in *Drosophila*, beginning with the work of Mukai and colleagues (Mukai, 1964; Mukai *et al.*, 1965, 1972; Mukai and Yamazaki, 1968; see also Lynch *et al.*, 1999). These studies, however, emphasize the pleiotropic effects of novel mutations on fitness components and the correlations among

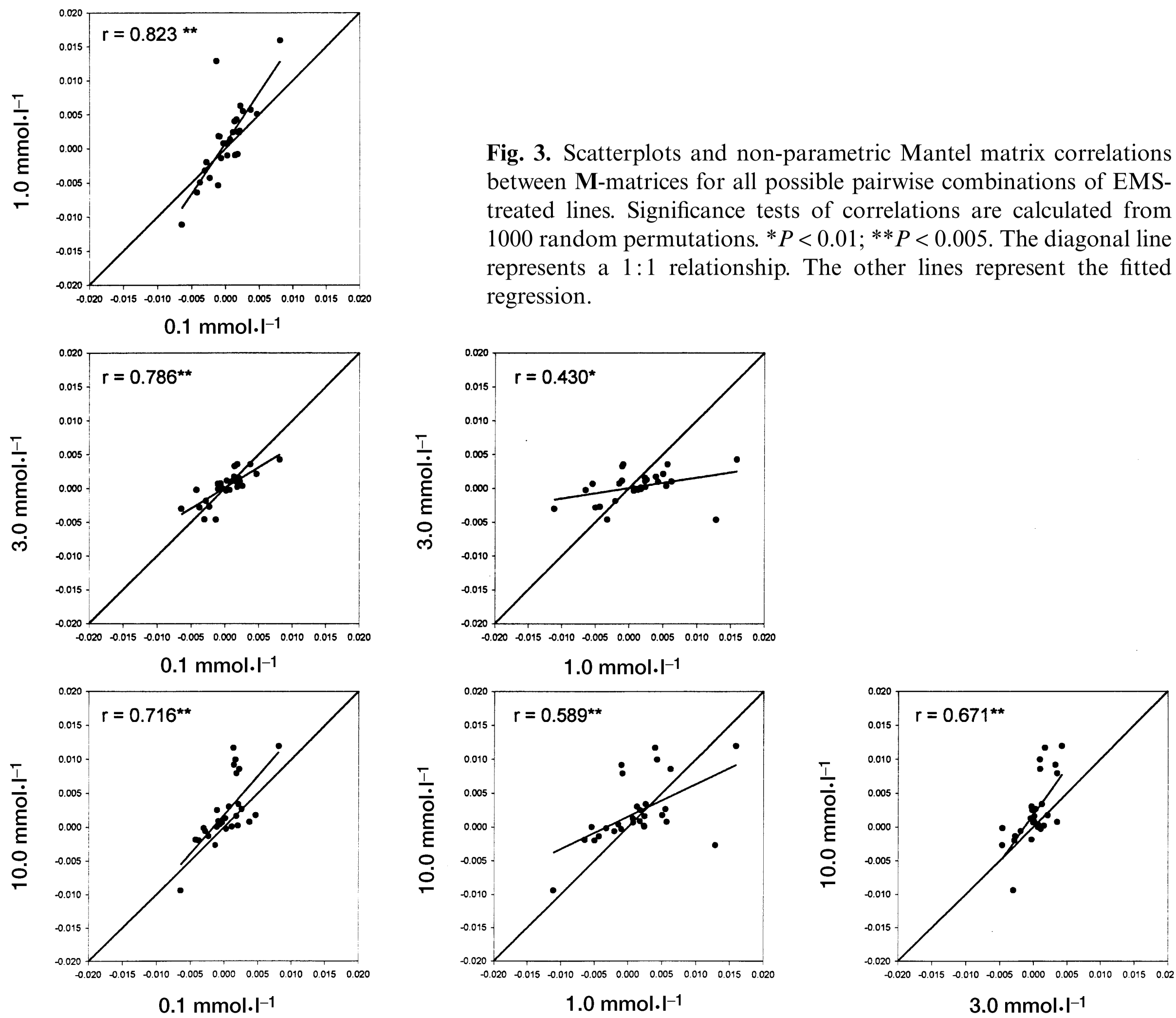

Fig. 3. Scatterplots and non-parametric Mantel matrix correlations between **M**-matrices for all possible pairwise combinations of EMS-treated lines. Significance tests of correlations are calculated from 1000 random permutations. $*P < 0.01$; $**P < 0.005$. The diagonal line represents a 1:1 relationship. The other lines represent the fitted regression.

Table 3. Results of common principal components analyses comparing all EMS treatments simultaneously (a) or each EMS treated population to the control (b–e) (the best fitting model is indicated by **bold** type)

Model				
Higher	Lower	χ^2	d.f.	P
(a) All 5 treatments				
Equality	Unrelated	319.1	112	0
Proportionality	Unrelated	274.4	108	0
CPC	Unrelated	217.0	84	0
CPC(5)	Unrelated	211.0	80	0
CPC(4)	Unrelated	169.2	72	0
CPC(3)	Unrelated	135.6	60	0
CPC(2)	Unrelated	83.0	44	0.0003
CPC(1)	Unrelated	45.2	24	0.0055
Unrelated				
(b) Control *vs* 0.1 mmol·l^{-1}				
Equality	Unrelated	112.9	28	0
Proportionality	Unrelated	106.0	27	0
CPC	Unrelated	80.5	21	0
CPC(5)	Unrelated	78.7	20	0
CPC(4)	Unrelated	55.1	18	0
CPC(3)	Unrelated	27.1	15	0.0276
CPC(2)	**Unrelated**	**9.0**	**11**	**0.6217**
CPC(1)	Unrelated	4.8	6	0.5676
Unrelated				
(c) Control *vs* 1 mmol·l^{-1}				
Equality	Unrelated	133.9	28	0
Proportionality	Unrelated	91.3	27	0
CPC	Unrelated	76.3	21	0
CPC(5)	Unrelated	76.3	20	0
CPC(4)	Unrelated	46.9	18	0.0002
CPC(3)	Unrelated	35.9	15	0.0018
CPC(2)	Unrelated	22.5	11	0.0211
Unrelated				
(d) Control *vs* 3 mmol·l^{-1}				
Equality	Unrelated	92.5	28	0
Proportionality	Unrelated	86.1	27	0
CPC	Unrelated	77.7	21	0
CPC(5)	Unrelated	75.4	20	0
CPC(4)	Unrelated	46.8	18	0.0002
CPC(3)	Unrelated	39.7	15	0.0005
CPC(2)	**Unrelated**	**13.9**	**11**	**0.2384**
CPC(1)	Unrelated	10.4	6	0.1079
Unrelated				

(e) Control *vs* 10 mmol·l^{-1}				
Equality	Unrelated	85.6	28	0
Proportionality	Unrelated	64.2	27	0.0001
CPC	Unrelated	45.1	21	0.0017
CPC(5)	Unrelated	44.7	20	0.0012
CPC(4)	Unrelated	43.3	18	0.0007
CPC(3)	Unrelated	41.5	15	0.0003
CPC(2)	Unrelated	25.3	11	0.0082
CPC(1)	**Unrelated**	**5.6**	**6**	**0.4724**
Unrelated				

them (Houle *et al*., 1994; Fernandez and Lopez-Fanjul, 1997; Keightley and Caballero, 1997). A few recent studies have addressed bristle number (Mackay *et al.*, 1992, 1994, 1995; Fry *et al*., 1995) and host use (Fry *et al.*, 1996). Schultz *et al.* (1999) studied mutation accumulation in *Arabidopsis* and estimated their deleterious impact on fitness and on the divergence among lines propagated by single-seed descent. Similarly, R. Shaw and D. Byers (personal communication) have used such lines to monitor divergence via mutation accumulation.

Another approach, the one we have adopted here, is to generate mutations in the laboratory. EMS mutagenesis has been used extensively in studies of *Drosophila* (Mukai, 1970; Ohnishi, 1977a,b,c; Keightley and Ohnishi, 1998). This manipulative approach may be less easy to apply to specific natural populations because we cannot be sure that the types (and certainly the frequencies) of mutations are comparable to those in nature. On the other hand, our approach may allow us to assess the consequences of the introduction of novel mutations in a more general fashion, beyond what is observable in natural or laboratory populations. Furthermore, there is no reason to think that accelerating the mutation accumulation process should cause qualitative differences in the results any more than artificial selection should be considered as fundamentally different from natural selection.

The Kendalville ecotype used in this experiment harboured substantial genetic variation for a number of traits before EMS treatment (Pigliucci, 1997); we used it for this reason. Most of the ecotypes of *A. thaliana* available from collections have been propagated by single-seed descent to produce genetically uniform material for molecular research. In this study, we wished to determine how novel mutations affect a natural population of *A. thaliana.* With the exception of a single character, flowering period, EMS mutagenesis had no significant impact on the means of these traits, including two components of fitness, a result consistent with a previous study of the impact of EMS-induced mutations into several genetically homogeneous lines (Camara and Pigliucci, 1999). This is surprising in light of the commonly held assumption that most mutations are deleterious. Clearly, at least some of the mutations we produced using EMS enhance rather than detract from fitness in this species.

The different result for flowering period may be due to the peculiarities of the phenology of *Arabidopsis* in North America. This species can be a spring ephemeral in harsher northern regions (M. Pigliucci, personal observation), such as the Michigan site where this population originates (F. Lehle, personal communication), or a winter annual in milder climates, such as the southeastern United States (M.D. Camara and M. Pigliucci,

personal observations; H. Callahan, personal communication). Both of these phenological 'strategies', however, rely on rapid development and flowering (after overwintering in the case of winter annuals). Apparently, there is natural selection to either complete reproduction before competing vegetation develops or before the onset of harsh summer conditions. H.S. Callahan and M. Pigliucci (submitted) have detected significant directional selection for rapid development in naturalized populations of *A. thaliana* in eastern Tennessee. The asymmetrical effects of novel mutations on mean duration of the flowering period, therefore, is consistent with the hypothesis that a history of directional selection for a short life-cycle has driven the Kendalville population close to the minimum physiologically possible value of this trait (Pigliucci *et al.*, 1998), such that most mutations increase the duration of the flowering period. Since our controlled conditions relaxed natural selection pressure, plants with later flowering than usual could complete their life-cycle and produce offspring.

The increase in phenotypic variance of morphological and timing traits as well as of components of fitness due to novel mutations was not strictly dose-dependent either in terms of the EMS concentrations used or of the numbers of mutations they induced. One explanation of these results may be an increase in the number of lethal mutations at higher doses of EMS, with the survivors retaining less overall variation due to mortality. Similarly, the degree of divergence in the phenotypic covariance structure of the five treatments as measured using CPCA was broadly but not strictly dose-dependent, with more induced mutations producing less common structure with the control treatment except, again, at the highest dose used.

Mantel matrix correlation tests revealed significant correlations between all possible pairs of **M**-matrices, but the slopes of the relationships were variable among comparisons (Fig. 3). In particular, correlations involving the 1.0 and 3.0 $mmol \cdot l^{-1}$ EMS treatments tended to show a greater departure from the 1 : 1 line than other correlations. These two treatments, therefore, not only have less common covariance structure with the controls as indicated by the CPCA analyses, but they are also less similar to the other EMS treatments. Noting that even our high mutation dose produced a number of mutations that could accumulate naturally within a relatively short evolutionary time, our results show that mutation accumulation can alter the **G**-matrix in *Arabidopsis* significantly.

Therefore, these results argue that the common assumption that the genetic architecture is effectively constant, or at least proportional, among populations or through time is not likely to hold in natural populations if mutations are accumulating at a significant pace. Even our moderate doses of EMS had a detectable impact on the covariance structure relative to the non-mutagenized control population. Although the mutations studied in this experiment were artificially generated, both their mild nature (point mutations, as opposed to chromosome breakage) and the fact that they impact the covariance structure even at low to moderate frequencies, argue that these results have implications for natural populations experiencing normal rates of mutation.

Although the overall structure of the covariance matrix among these traits was altered by induced mutations, some elements of the matrix were more resistant to change than others (see Appendix 1). In particular, the well-studied genetic correlation between the time to flowering (length of the vegetative period) and the number of rosette leaves in *Arabidopsis thaliana* seemed highly resistant to change and may be effectively constant (although late flowering ecotypes provide an interesting exception to this constraint:

Pigliucci and Schlichting, 1998). There is no evidence that either the strength or the slope of this relationship was affected by induced mutations in our experiment, and this correlation may constrain medium- and long-term responses to natural and artificial selection. In fact, in a separate experiment, M.D. Camara and M. Pigliucci (in prep.) found that this correlation dominated the direction of responses to bivariate artificial selection along and orthogonal to the major axis of variation of the correlation itself. Highly persistent correlations that are unaltered by novel mutations such as the one between leaf number at bolting and flowering time may therefore represent more fundamental and quasi-inevitable constraints on phenotypic evolution.

Houle (1991) has argued that the mathematical tractability of mutation–selection balance models with pleiotropic effects of new mutations being allele- rather than locus-specific has made them attractive for evolutionary biologists, but our emerging understanding of the functional mechanisms of gene action makes them unrealistic. We found that the impact of new mutations on specific correlations among traits was idiosyncratic. The high correlation between the duration of the vegetative period and the number of rosette leaves at bolting persisted in the mutagenized populations, as did the correlation between the summed lengths of stems and the number of fruits, the latter reflecting the inability of this plant to alter inter-node length on the reproductive stem. Similarly, the moderate correlations between the height of the main stem and the summed length of all stems and the number of fruits were present in all treatments. Other correlations, though, were more labile. The weak correlation between the duration of the vegetative period and the height of the main inflorescence, for example, reversed sign in the 0.1, 1.0 and 3.0 $\text{mmol} \cdot \text{l}^{-1}$ treatments and was absent in the 10 $\text{mmol} \cdot \text{l}^{-1}$ treatment. Moreover, mutations produced significant correlations on the order of 0.5 between the duration of the vegetative period and the duration of flowering in the same treatments when there was no such correlation in the unmutagenized control. Although our results do not directly address the biochemical or developmental mechanisms underlying patterns of covariance, the heterogeneity in how specific correlations behave in response to mutagenesis implies that information on the underlying genetic mechanisms (Schultz and Haughn, 1993) will be helpful in providing a context in which to interpret these statistical relationships.

Olson and Miller (1958), Berg (1959, 1960) and, more recently, Wagner and co-workers (Wagner, 1995a,b; Wagner and Altenberg, 1996) have argued that selection should adaptively mould patterns of covariation among phenotypic traits to reflect selective requirements for the functional integration of phenotypes. Suites of traits that must function in a coordinated fashion to produce viable organisms, they hypothesize, should be tightly correlated with each other, but not with other suites or 'modules' of traits that serve other functions. In a study designed to test this hypothesis using field data, Armbruster *et al.* (1999) found no support for the *a priori* prediction of consistent patterns of coupling among floral traits of nine tropical plant species. Despite our lack of *a priori* predictions for dividing the traits we measured into functional modules, inspection of the correlation matrices among the traits measured in this experiment (see Appendix 1) provides little evidence in support of this idea. Future studies focusing on groups of phenotypic traits for which there are *a priori* expectations of integration based on either their ecological functions or genetic architecture would be most rewarding.

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APPENDIX 1

Phenotypic variance/covariance matrices (diagonal and above; in *italics*) and correlation matrices (below diagonal) calculated from relativized data. Correlations with $P < 0.1$ are indicated by **bold** type

	Leaves	Main stem	All stems	Fruits	Veg. per.	Flow. per.	NEI
Control ($n = 119$)							
Leaves	*0.0152*	*+0.0019*	*–0.0005*	*–0.0002*	*+0.0041*	*–0.0006*	*+0.0028*
Main stem	**+0.21**	*0.0050*	*+0.0035*	*+0.0029*	*+0.0008*	*–0.0007*	*+0.0009*
All stems	–0.04	**+0.46**	*0.0116*	*+0.0091*	*–0.0009*	*+0.0005*	*+0.0032*
Fruits	–0.02	**+0.45**	**+0.92**	*0.0084*	*–0.0008*	*–0.0005*	*+0.0035*
Veg. per.	**+0.70**	**+0.24**	–0.18	**–0.19**	*0.0022*	-2.2×10^{-5}	*+0.0009*
Flow. per.	–0.07	**–0.16**	+0.07	–0.09	–0.01	*0.0044*	*–0.0006*
NEI	+0.11	+0.06	+0.15	**0.18**	+0.10	–0.04	*0.0423*
0.1 mmol·l^{-1} ($n = 107$)							
Leaves	*0.0233*	*–0.0012*	*+0.0009*	*+0.0016*	*+0.0067*	*+0.0041*	*–0.0037*
Main stem	+0.08	*0.0088*	*+0.0047*	*+0.0026*	*–0.0020*	*–0.0006*	*–0.0002*
All stems	+0.05	**+0.42**	*0.0138*	*+0.0107*	*–0.0015*	*+0.0012*	*–0.0010*
Fruits	+0.11	**+0.28**	**+0.92**	*0.0098*	*–0.0006*	*+0.0016*	*–0.0003*
Veg. per.	**+0.69**	**–0.34**	**–0.21**	–0.09	*0.0040*	*+0.0020*	*–0.0014*
Flow. per.	**+0.45**	–0.10	**+0.17**	**+0.27**	**+0.53**	*0.0036*	*–0.0016*
NEI	–0.12	–0.01	–0.04	–0.01	–0.11	–0.14	*0.0409*

Appendix 1—*continued*

	Leaves	Main stem	All stems	Fruits	Veg. per.	Flow. per.	NEI
1 mmol·l^{-1}(*n* = 126)							
Leaves	*0.0311*	*–0.0013*	*–0.0015*	*–0.0010*	*+0.0010*	*+0.0045*	*–0.0084*
Main stem	–0.07	*0.0107*	*+0.0056*	*+0.0037*	*–0.0012*	-5.4×10^{-6}	*0.0027*
All stems	–0.06	**+0.43**	*0.0180*	*+0.0134*	*–0.0023*	*+0.0019*	*–0.0032*
Fruits	–0.05	**+0.32**	**+0.90**	*0.0124*	*–0.0018*	*+0.0021*	*–0.0015*
Veg. per.	**+0.80**	**–0.17**	**–0.25**	**–0.24**	*0.0047*	*+0.0023*	*–0.0034*
Flow. per.	**+0.32**	–0.00	**+0.18**	**+0.24**	**+0.43**	*0.0062*	*–0.0060*
NEI	**–0.20**	+0.11	–0.10	–0.06	**–0.21**	**–0.32**	*0.0551*
3 mmol·l^{-1} (*n* = 104)							
Leaves	*0.0194*	*–0.0027*	*+0.0027*	*+0.0034*	*+0.0044*	*+0.0015*	*–0.0003*
Main stem	**–0.21**	*0.0086*	*+0.0046*	*+0.0029*	*–0.0011*	*–0.0011*	*+0.0007*
All stems	**+0.17**	**+0.44**	*0.0126*	*+0.0101*	*–0.0002*	*+0.0003*	*+0.0030*
Fruits	**+0.24**	**+0.31**	**+0.89**	*0.0101*	*+0.0003*	*+0.0007*	*+0.0006*
Veg. per.	**+0.65**	**–0.24**	–0.04	+0.06	*0.0024*	*+0.0015*	*–0.0018*
Flow. per.	**+0.16**	**–0.18**	+0.04	+0.10	**+0.46**	*0.0045*	$+9.4 \times 10^{-5}$
NEI	–0.01	+0.04	+0.14	+0.03	**–0.19**	+0.01	*0.0376*
10 mmol·l^{-1} (*n* = 80)							
Leaves	*0.0271*	*+0.0017*	*+0.0086*	*+0.0077*	*+0.0067*	*+0.0012*	*–0.0067*
Main stem	+0.14	*0.0058*	*+0.0036*	*+0.0036*	*+0.0002*	*+0.0005*	*+0.0033*
All stems	**+0.37**	**+0.33**	*0.0202*	*+0.0190*	*–0.0005*	*+0.0035*	*+0.0013*
Fruits	**+0.33**	**+0.33**	**+0.95**	*0.0201*	*–0.0011*	*+0.0028*	*+0.0015*
Veg. per.	**+0.66**	+0.04	–0.06	–0.13	*0.0038*	*+0.0002*	*–0.0004*
Flow. per.	+0.10	+0.09	**+0.34**	**+0.27**	+0.04	*0.0053*	*–0.0006*
NEI	**–0.20**	**+0.22**	+0.05	+0.05	–0.03	–0.04	*0.0395*

Note: Leaves = number of rosette leaves at bolting; Main stem = height of main inflorescence; All stems = summed length of all inflorescences; Fruits = number of siliques at first reproduction; Veg. per. = duration of vegetative period; Flow. per. = duration of flowering period; NEI = number of non-elongated inflorescences.

APPENDIX 2

Eigenvector structure of each of the five treatments: Loadings of original variables on principal components

	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Control							
Leaves	+0.010	–0.21	+0.92	–0.06	–0.23	–0.20	+0.12
Main stem	+0.05	+0.24	+0.22	+0.02	–0.22	+0.84	–0.36
All stems	+0.14	+0.71	+0.11	–0.61	+0.20	–0.10	+0.19
Fruits	+0.14	+0.59	+0.10	+0.74	–0.06	–0.26	–0.05
Veg. per.	+0.03	–0.11	+0.26	+0.19	+0.92	+0.20	+0.01
Flow. per.	–0.02	+0.01	–0.05	+0.18	–0.12	+0.36	+0.91
NEI	+0.97	–0.17	–0.14	–0.01	–0.01	+0.03	+0.00
Variance explained	0.0436	0.0198	0.0163	0.0006	0.0009	0.0032	0.0048

0.1 mmol·l^{-1}							
Leaves	–0.24	–0.79	+0.43	+0.20	–0.27	–0.03	–0.16
Main stem	–0.01	–0.06	–0.34	+0.89	+0.26	–0.10	+0.11
All stems	–0.06	–0.35	–0.64	–0.16	–0.13	+0.65	–0.00
Fruits	–0.04	–0.33	–0.49	–0.31	+0.01	–0.73	+0.14
Veg. per.	–0.08	–0.20	+0.23	–0.13	+0.40	+0.16	+0.84
Flow. per.	–0.07	–0.19	+0.04	–0.17	+0.83	+0.05	–0.49
NEI	+0.96	–0.26	+0.07	+0.01	+0.02	+0.01	–0.00
Variance explained	0.0421	0.0258	0.0245	0.0072	0.0028	0.0007	0.0012
1 mmol·l^{-1}							
Leaves	–0.33	–0.61	–0.64	+0.03	+0.22	–0.10	+0.23
Main stem	+0.05	+0.22	–0.24	+0.93	–0.12	–0.11	–0.04
All stems	–0.06	+0.54	–0.49	–0.15	+0.15	+0.63	+0.14
Fruits	–0.04	+0.42	–0.41	–0.30	+0.00	–0.68	–0.32
Veg. per.	–0.12	–0.25	–0.14	+0.00	–0.22	+0.34	–0.86
Flow. per.	–0.14	–0.01	–0.14	–0.13	–0.93	+0.01	+0.28
NEI	+0.92	–0.21	–0.30	–0.08	–0.07	+0.03	+0.01
Variance explained	0.0598	0.0347	0.0280	0.0083	0.0049	0.0014	0.0010
3 mmol·l^{-1}							
Leaves	+0.00	–0.61	+0.69	+0.33	+0.04	–0.21	+0.03
Main stem	+0.06	–0.12	–0.44	+0.75	–0.46	–0.05	–0.12
All stems	+0.16	–0.55	–0.41	–0.16	+0.11	+0.14	+0.67
Fruits	+0.08	–0.52	–0.31	–0.31	+0.13	–0.06	–0.72
Veg. per.	–0.05	–0.13	+0.20	–0.03	–0.30	+0.92	–0.11
Flow. per.	+0.00	–0.07	+0.10	–0.46	–0.82	–0.31	–0.09
NEI	+0.98	+0.14	+0.13	+0.01	–0.02	+0.04	–0.04
Variance explained	0.0383	0.0254	0.0196	0.0060	0.0043	0.0008	0.0010
10 mmol·l^{-1}							
Leaves	–0.55	+0.15	+0.76	+0.30	–0.08	+0.06	–0.01
Main stem	–0.10	–0.13	+0.00	+0.03	–0.01	–0.93	–0.33
All stems	–0.57	–0.24	–0.30	–0.33	–0.64	+0.07	+0.04
Fruits	–0.55	–0.25	–0.34	+0.12	+0.68	+0.14	–0.14
Veg. per.	–0.07	+0.05	+0.33	–0.87	+0.33	–0.07	+0.12
Flow. per.	–0.10	–0.02	–0.08	+0.16	+0.09	–0.31	+0.92
NEI	+0.22	–0.92	+0.31	+0.05	–0.01	+0.09	+0.05
Variance explained	0.0478	0.0418	0.0203	0.0014	0.0009	0.0047	0.0048

Note: Leaves = number of rosette leaves at bolting; Main stem = height of main inflorescence; All stems = summed length of all inflorescences; Fruits = number of siliques at first reproduction; Veg. per. = duration of vegetative period; Flow. per. = duration of flowering period; NEI = number of non-elongated inflorescences.