THE CONTRIBUTION OF SPONTANEOUS MUTATION TO VARIATION IN ENVIRONMENTAL RESPONSE IN Arabidopsis thaliana: RESPONSES TO NUTRIENTS

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Abstract.—Although the evolutionary importance of spontaneous mutation is evident, its contribution to the evolution of ecological specificity remains unclear, because the environmental sensitivity of effects of new mutations has received little empirical attention. To address this issue, we report a greenhouse in which we grew plants from 20 mutation-accumulation (MA) lines, advanced by selfing and single-seed descent from a single common founder to generation 17, as well as plants from five lines representing the founder, in high and low nutrient conditions. We examined 11 traits throughout life history, including germination, survivorship, bolting date, flowering date, leaf number, leaf size, early and late height, mean fruit size, total seed weight, and reproductive biomass. Comparison of trait means between the two generations did not support the commonly held view that new mutations affecting fitness in these MA lines are strongly biased toward deleterious effects. We detected significant variance among MA lines for one fitness component, mean fruit size, but we did not detect a significant contribution of mutations accumulated in these MA lines to genotype by environment interaction (GEI). These results suggest that other evolutionary mechanisms play a more important role than spontaneous mutation alone in establishing the GEI found for wild collections and lab accessions of Arabidopsis thaliana in previous studies.

Key words.—Arabidopsis thaliana, cross-environment correlation, genotype by environment interaction, mutation accumulation, nutrient.

Received May 9, 2002. Accepted December 12, 2002.

Among the many manifestations of genetic variability, genetic variation in responses to environmental conditions (genotype by environment interaction; GEI) has attracted particular attention. Theoretical studies have demonstrated that when different genotypes are selectively favored in different environments, GEI can play an important role in adaptation to heterogeneous environments (Via and Lande 1985, 1987), adaptive divergence that may culminate in speciation (Maynard Smith 1966; Dickinson and Antonovics 1973), and the maintenance of genetic variation (Levene 1953; Hedrick 1986; Gillespie and Turelli 1989). When GEI is largely due to differences among environments in the magnitude of genetic variance, the rate of response to selection is expected to differ among populations subject to different environmental conditions.

These considerations have motivated studies of GEI of natural populations (e.g., Futuyama and Peterson 1985; Rawson and Hilblish 1991; Via 1991; Shaw et al. 1995), which have in some cases demonstrated that the ranking of genotypes with respect to fitness differs among environments, that is, reaction norms for fitness cross. This may be interpreted as a consequence of environment-dependent selection favoring alternative alleles in different environments, thus maintaining them at substantial frequencies, such that studies of genetic variation in reaction norms detect genetic trade-offs in performance in different environments. Recently, an alternative mechanism has been proposed to account for the evolution of crossing reaction norms. Fry et al. (1996) have pointed out that a steady influx of spontaneous mutations that are generally mildly deleterious but that differ among environments in the severity of their effects would also generate crossing reaction norms (see Fig. 1 of Fry et al. 1996). Such mutations are expected to persist if their effects on fitness, particularly in the most common environment, are slight. Even in the absence of new mutations that enhance fitness in some environments, ecological specialization could evolve due to mutations that are deleterious in all environments, but to differing degrees in different environments. Fry et al. (1996) have pointed out that if such mutations contribute substantially to GEI observed in natural populations, similar patterns of GEI should be evident for lines that differ from each other only through recent spontaneous mutation.

To date, empirical work to establish the properties of spontaneous mutation has largely emphasized inferences of the genomic mutation rate, \( U \), for diverse organisms (reviewed in Lynch et al. 1999). There is growing interest in the effects of newly arising mutations because the contribution of a new mutation to a population’s standing genetic variation and future evolution depends on its phenotypic effect (Caballero and Keightley 1994). Evidence of how the effects of new mutations depend on the environmental conditions in which the organisms are grown will contribute to understanding the role of mutation in the evolution of phenotypic plasticity and ecological specialization (Whitlock 1996, 2000; Kawecki et al. 1997). Moreover, clarification of the range of mutational effects and their environmental sensitivity is crucial to the development of approaches to estimate mutational properties, including \( U \), reliably (Kondrashov and Houle 1994; Shaw et al. 2002).

Several recent mutation-accumulation (MA) studies of Drosophila melanogaster have assessed variation in the environmental dependence of the effects of new mutations on organismal fitness (reviewed in García-Dorado et al. 1999). Mackay et al. (1992) developed MA lines from a highly in-
bred strain by randomly choosing 10 males and 10 females to advance each generation. MA lines are advanced at small population size so that fixation of newly arising mutations is expected to be dominated by drift, rather than selection. In a study of 18 of these MA lines advanced to generation 200, Fry et al. (1996) documented striking variation among lines in fitness responses to five treatments differing in substrate, temperature, and adult density. The MA line-by-environment interaction was overwhelmingly attributable to differences in the ranks of lines in different environments, suggesting that the steady influx of new mutations contributes directly to genetically based fitness trade-offs among environments found for natural populations. This finding is consistent with the model assumption by Kawecki et al. (1997) of generally deleterious mutations varying in the fitness decrements they impose in different environments. In a further study of these lines, Wayne and Mackay (1998) found significant interaction between MA lines and temperature for ovariole number and body size.

In a study of more than 100 MA lines advanced by single pair, brother-sister mating for more than 100 generations, Fernández and López-Fanjul (1997) found no systematic trends in standardized estimates of the variance introduced by mutation for four assay environments ranging in mean fecundity over a factor of four. Mutation-accumulation lines were found to vary substantially in their responses to certain environment pairs; across-environment mutational correlations were typically between $-0.1$ and $0.3$, but between two particular environments, standard and high saline, the line correlation for fecundity of $-0.97$ evidenced extreme trade-offs.

Thus, evidence from Drosophila documents condition dependence of effects of new mutations. Yet, because none of these studies directly compared advanced generation MA lines to founder generations, they do not definitively distinguish between two hypotheses that differ importantly in their evolutionary consequences: (1) new mutations tend to be deleterious in all environments, but the degree of deleterious effect may differ among environments, and (2) new mutations commonly differ in the sign as well as the magnitudes of their effects in different environments.

One further study of D. melanogaster directly compared MA lines with controls that had been subject to natural selection and were thus considered to have accumulated few mutations. Kondrashov and Houle (1994) found that fitness differences between two MA lines and a control line varied strikingly among 50 environments tested. Nevertheless, both MA lines exhibited fitness lower than the control in nearly all the environments. The most extreme decrements of MA line fitness, relative to control, were found for the harshest environments; that is, those in which mean absolute fitness, obtained as an average over all lines, was lowest. The difference between the overall mean fitness across MA lines and mean fitness of control lines is a key statistic in a commonly used method for inferring genomic mutation rates, $U$ (Bate man 1959). Accordingly, Kondrashov and Houle (1994) concluded that MA studies conducted under relatively benign conditions could yield extreme underestimates of $U$.

In studies of mutational effects in the nematode Caenorhabditis elegans, it is feasible to compare founder stocks (before mutation) directly with advanced generation MA lines, because the worms are readily revived from freezing. Vassilieva et al. (2000), studying environment dependence of mutational effects, found greater rates of mutational decline in mean fitness in 12°C compared to 20°C, yet correspondingly greater mutational increases in variance, such that the estimate of $U$ for the intrinsic rate of increase was an order of magnitude lower in the harsher environment. For two components of fitness, as well as a composite measure of fitness, the correlations among lines across environments were consistently and significantly positive.

Here we extend consideration of the mutational contribution to GEI to a plant, Arabidopsis thaliana. As part of a larger study of spontaneous mutation in A. thaliana, we evaluated variation among a subset of MA lines in their responses to soil nutrient conditions. Previous studies of wild acces sions of A. thaliana (Pigliucci and Schlichting 1995, 1996; Pigliucci and Byrd 1998), as well as of lines in which mutations have been induced by ethyl methanesulfonate (van Tienderen et al. 1996; Pigliucci and Byrd 1998), have documented GEI in responses to nutrient concentration. We grew MA lines advanced by single seed descent from a single highly inbred founder to generation 17. Together with these, we grew plants representing the founder generation to allow direct comparison of MA lines with control lines in the two nutrient environments. We addressed the following questions about the composite effects of mutations that have accumulated in these lines: To what extent do spontaneous mutations that influence life history and morphological characters in A. thaliana express different effects in different nutrient environments? What is the pattern of the mutational GEI in A. thaliana?

**Materials and Methods**

Arabidopsis thaliana is a tractable subject of study of spontaneous mutation for several reasons. Its flowers exhibit a high degree of autogamous selfing (Abbot and Gomes 1989). As a result, advancement of MA lines by selfing does not constitute an unusual mode of reproduction for this species. In addition, A. thaliana’s annual habit makes assessment of lifetime fitness feasible within a relatively short period of time.

The MA lines were derived from a single founder individual drawn from material ascribed to the Columbia accession of A. thaliana that had been maintained by selfing and single-seed descent for several generations. As a result, the plants were expected to be in mutation-drift equilibrium, such that most of the loci in the founder are homozygous. A total of 120 progeny obtained from the founder by selfing were grown in a growth chamber, each establishing one MA line. Thereafter, the MA lines were advanced by single-seed descent (for more details see Shaw et al. 2000). This method, which minimizes selection within lines, ensures that the variation among lines observed in advanced generations is due to the composite effects of distinct mutations fixed primarily by genetic drift. Seeds collected from each generation were kept in a 4°C cold room. By generation 17, only three of the 120 lines had been lost.

To establish sublines from each MA line of generation 0
and generation 17, four plants from each line were grown simultaneously in random positions in a growth chamber. Generation 0 lines were established from full sibs of the founder individual. All of the selfed seeds from these sublines were collected and kept in a 4°C cold room prior to this study (see further details in Shaw et al. 2000). By establishing sublines of both generations simultaneously, we synchronized the age of seeds, and randomized over MA lines the environmental conditions in which the experimental seeds were produced.

For this experiment, we chose 20 MA lines from generation 17 and five lines from generation 0. Each MA line was represented by a randomly chosen pair of the four available sublines. The design included more lines from generation 17 than from generation 0, because a primary objective of this study was to assess the variation in environmental sensitivity attributable to mutations accumulated in the MA lines over 17 generations. The lines in generation 0 are expected to be genetically nearly identical, because these lines had been isolated from each other and the founder for only one generation. The distribution of traits in generation 0 thus reflects variation primarily due to microenvironment. Use of two sublines for each MA line allows for direct estimation of maternal effects. A total of 10 seeds from each of the sublines was planted in each environmental condition. This design resulted in a total of 1000 individuals (25 lines × 2 sublines/line × 10 individuals/subline × 2 nutrient levels) initially included in this experiment.

The growth medium used in this experiment consisted of half Sunshine mix number 5 (Sun Gro Horticulture, Seneca, IL) and half coarse vermiculite. Prior to the experiment, we carried out a pilot study to determine differences in nutrient conditions that induce detectable differences in the mean fitness of A. thaliana plants. Based on results of this study, we chose two nutrient levels for this experiment; for the low-nutrient treatment, no supplemental nutrients were added to the soil mixture, whereas the high-nutrient treatment received 20 medium-sized pellets of Osmocote (14-14-14; Scotts Co., Marysville, OH) fertilizer per pot at the beginning of the experiment. Pellets of roughly similar size were collected by passing the Osmocote through sieves of two different mesh sizes. Only the pellets that passed through the large mesh (about 3 mm) but not the small mesh (about 2 mm) were used. A total of 20-Osmocote pellets were added to each 2.5 × 2.5 × 3-inch pot. These Osmocote pellets, which release nutrients gradually, were evenly mixed with the soil.

We carried out the planting in two temporal batches. The first temporal batch was planted on 8 August 1999, and the second batch planted two weeks later. Two seeds were planted in each pot and were germinated on a laboratory bench exposed to natural light and photoperiod through adjacent east-facing windows. At this stage, water could only be provided from the bottom of the pots; hence it was necessary to separate pots with different nutrient levels into different germination trays. The number of seeds that germinated in each pot was recorded daily. Two weeks after planting, pots were moved into a greenhouse and were fully randomized (see below). The number of seedlings that germinated in each pot but died before this time was recorded and seedlings were thinned to one per pot. Among the 1000 pots originally planned for the experiment, 86 pots had no seedlings. Three seeds were newly planted for each of these empty pots, and these replacement pots were included as the third batch. After the replacement, an additional 19 plants from the original 1000 plants and 25 plants from the replants died, resulting in total of 956 plants included in the experiment.

Plants in the first two temporal batches were distributed to three benches (spatial blocks) in the greenhouse that held 300, 400, and 300 pots, respectively. The last temporal batch (the replacements) was placed at the end of the third spatial block. Mutation-accumulation lines and nutrient conditions were fully randomized within the batch and the block. The temperature in the greenhouse was set at 23°C throughout the experiment. No supplemental lighting was provided at the beginning of the experiment. However, because of shortening day length, lights were used beginning 24 September and maintained at 16:8 light:dark cycles. The experimental plants were supported with wooden sticks to prevent entangling. Water was applied to the soil surface when it appeared dry.

In addition to germination and survivorship, three types of characters were recorded during the course of this experiment. Two phenological characteristics, the number of days between germination and bolting and the number of days between bolting and flowering were recorded for each plant. In addition, four morphological traits were measured; the number of the rosette leaves, the length of the largest rosette leaf at anthesis, the infructescence height at anthesis, and the final height of the infructescence at the end of the experiment. Finally, three reproductive characters were measured at the time of or after the harvesting of the plants. We harvested the infructescences when plants had clearly senesced and most of the fruits had turned yellow or brown. At this time, the length of the fifth, seventh, and ninth fruits (counting from the base of the plant) was measured with digital calipers. The collected material was oven-dried for at least 24 hours at 35°C. The dried material was gently pressed between two pieces of paper to split the fruits and then passed through a fine-mesh sieve to separate the seeds from the rest of the structural tissues. The dry mass of the infructescence and the total seed weight were then obtained using a Mettler (Columbus, OH) digital balance.

**Analyses**

Each pot was scored for the germination rate of the planted seeds and the survival rate of the germinated seedlings. Germination rate was defined as the proportion of seeds planted that germinated in each pot and survival rate as the proportion of seedlings germinated that survived for at least two weeks till thinning. To infer the effects of the experimental factors on germination and survival rates of the seeds planted, analyses of variance were carried out on the arcsine square-root transformed rates with the following predictor variables: temporal batch, nutrient levels, germination trays (nutrient [batch]), generation, MA lines(gen), subline (line[gen]), nutrient × line[gen] and nutrient × subline (line[gen]). Germination trays, MA lines, nutrient × line, subline (line[gen]) and nutrient × subline (line[gen]) are treated as random factors, the others as fixed. Significance tests appropriate for
mixed models were obtained from the SAS software package (SAS Institute 1989).

Analyses of the remaining traits were initially carried out jointly by multivariate analysis of covariance (MANCOVA) to account for intercorrelations among traits within a category, phenological, morphological, and reproductive fitness in inferring the overall influence of the main effects on the study plants. In a MANCOVA to assess effects of generation, nutrient treatment, and their interaction, all experimental plants were included. In addition to these factors: MA lines nested within generation, maternal sublines nested within MA line, and their interaction with nutrient treatment; three environmental covariates were also included in these and all of the following analyses: the spatial blocks in the greenhouse (block), the time of planting (batch), and location on the bench (position). The first two covariates were categorical and the last one, position, was treated as continuous, ranging from 1 (the easternmost plant position) to 37 (the westernmost). In addition, we conducted MANCOVs for each nutrient environment separately with predictor variables generation, MA lines, and sublines nested within MA line. Similar MANCOVs were carried out for each generation separately.

To identify influences of the factors on individual traits, univariate analyses of covariance (ANCOVAs) were carried out for all traits measured. We also plotted the residuals from the linear models and inspected their distributions to detect outliers. Once identified, outliers were removed from the analyses. The number of outliers removed for analyses of a particular trait ranged from 0 to 5. Following removal of these outliers, the distribution of the residuals more nearly conformed to the normality assumption for analyses of variance. In no cases did it change the conclusion of the analyses. No transformation was needed for these analyses.

Variation among lines within each environment and the cross-environment covariance among lines were estimated by restricted maximum likelihood methods (REML) using Quercus software (Shaw and Shaw 1994). Cross-environment genetic correlations for each trait were calculated from these estimates. These analyses included variance and covariance due to maternal effects, as well as the random environmental variances for each trait. Significance tests were conducted using asymptotic parametric bootstrap (APB; Shaw and Geyer 1997).

For the three morphological traits that showed significant MA line variance in at least one of the environments (final height and mean fruit size) or covariance between environments (leaf number), we calculated the contribution of mutation (\(V_M\)) to the observed phenotypic variance using the following equation: \(V_M = V_{MA}/(2t)\) (Lynch and Hill 1986), where \(V_{MA}\) represents the variance among MA lines and \(t\) represents the number of generations that the MA lines have diverged, here 17. Mutational heritability (\(h^2\)) and coefficient of variation due to mutation \(CV_M\) were also calculated for these three traits. Because our data indicate that the assumption of the Bateman method (i.e., mutations are unidirectional in their effects) is not satisfied, we did not apply this method to obtain estimates of genomic mutation rate or the mean mutational effect. The limited size of the dataset also precluded application of maximum likelihood to estimate mutational parameters.

To examine the magnitude of composite mutational effects in different nutrient conditions, we plotted line means of characters measured in high- and low-nutrient conditions. The least square mean (LSM) for each of the MA lines was first obtained from the univariate analyses described above. An overall generation 0 least square mean for a particular trait was obtained for each of the nutrient conditions. This value was used as the reference value and was subtracted from the LSMs of the 25 MA lines to obtain their adjusted LSMs in that particular nutrient condition. The adjusted LSMs in the two nutrient conditions are presented in Figure 1.

Multiple regression was used to quantify both phenotypic and genetic selection on the characters under the greenhouse conditions in which they were measured. Dry seed weight as a measure of fitness was used as the response variable and the phenological and morphological characters were used as predictor variables. Partial regression coefficients estimate the direct selection on each character (the selection gradient, \(\beta\); Lande and Arnold 1983) under our experimental conditions. For the phenotypic selection analysis, the observations comprised all plants of generation 17; the environmental covariates were included in the model. For the genetic selection analysis, which accounts for environmentally induced correlations between traits (Rausher 1992), least square means of both response and predictors for each MA line were used in the analyses. For a subset of the traits, selection within each treatment was also estimated by REML as the genetic covariance between fitness and traits.

**Results**

**Germination and Survivorship**

Germination rate was high in this experiment; in most of the pots at least one seed germinated. In 907 of the 1086 pots planted every seed germinated (i.e., germination rate = 1); in 161 pots one seed germinated and in only 18 pots did no seed germinate. The germination rate of the seeds planted in each pot did not differ significantly between generations, among MA lines or between nutrient treatments. However, germination rates varied significantly among batches (\(F_{2,68.3} = 15.88, P < 0.0005\); specifically, far less germination occurred in batch 3 than in batches 1 and 2. Germination rates also varied among trays holding pots (\(F_{33,944} = 2.87, P < 0.0005\)). Sublines also differed in germination rates (\(F_{25,25} = 6.02, P < 0.0005\)). Finally, a slight generation-by-nutrient effect was found (\(F_{1,21.08} = 4.9, P < 0.05\); generation 17 plants had similar germination rates in both high- and low-nutrient conditions, but generation 0 plants had higher germination rates in low-nutrient conditions (87%) than in high-nutrient conditions (81%). These findings should be interpreted with caution because the residuals were not normally distributed.

As with germination rates, survival rates of the germinated seedlings did not vary significantly between generations or nutrient levels, or among lines. Survival rates varied significantly only among germination trays and sublines (\(F_{33,933} = 2.8, P < 0.0005\); and \(F_{25,25} = 3.3, P < 0.0005\), respectively).

**Joint Analyses of Three Kinds of Characters**

The overall analysis demonstrated highly significant effects of the nutrient treatment for all groups of traits (phe-
a. Leaf number
(14.8, 15.5)

b. Reproductive biomass
(0.20, 0.93) g

c. Seed weight
(0.10, 0.50) g

d. Mean fruit size
(1.55, 1.63) cm

**Fig. 1.** The adjusted least square means for the mutation-accumulation lines in the two nutrient conditions. The 95% confidence intervals for the means of the generation 0 lines are represented by the shaded area. The areas that are darkly shaded indicate the parameter space that falls in the 95% confidence intervals for both environments. The open and filled squares indicate the generation 17 lines that fall outside or inside the darkly shaded area, respectively. Standard errors (SE) for the means of generation 17 lines are all very similar within an environment. Average SE for the generation 17 lines in each environment is represented by the bars (vertical, high nutrient; horizontal, low nutrient) at the right bottom corner of the figures. Values in the parentheses are the overall means for the generation 0 lines in the low- (first number) and high- (second number) nutrient environments.

*Low Nutrient*

nological: $F_{2,24} = 30.47$; morphological: $F_{4,22} = 461.08$; and reproductive: $F_{3,23} = 1092.95$; $P < 0.005$ for all traits). No main effect of generation was detectable, yet for phenological traits, the interaction between generation and treatment was significant ($F_{2,24} = 3.75$, $P < 0.05$). Together, these results suggest that for two of the three types of characters measured differences between generations were slight. As the exception, phenological traits differed between generations in their response to the treatments (Table 1). Generation 17 MA lines grown in high-nutrient conditions tended to flower more rapidly than plants from generation 0 ($\text{LSM}_{\text{gen17}} = 6.93$, $\text{LSM}_{\text{gen0}} = 7.21$); whereas in low-nutrient conditions, mean time from bolting to flowering was nearly the same for both generations.

Analysis of the treatments separately demonstrated significant variation among MA lines for both morphological characters and reproductive characters in the high-nutrient environment and the reproductive characters in the low-nutrient environment (Table 1), demonstrating mutational divergence among the lines used in this experiment. In addition,
maternal effects were detected for phenological characters in both environments and for the reproductive characters in the low-nutrient environment (Table 1).

Because all lines from generation 0 are just two generations removed from the single common ancestor of the MA lines, it was expected that variation among generation 0 lines would be slight. This expectation was supported by further analyses of the generation 0 separate from the generation 17 plants (analysis not shown). In contrast, plants from generation 17 grown in the high-nutrient environment exhibited significant line variance for both morphological \((F_{76,69.4} = 1.63, P < 0.05)\) and reproductive \((F_{114,93.7} = 1.56, P < 0.05)\) characters. Similarly, the line effect found in the low-nutrient environment for the reproductive traits was also mainly among lines of the generation 17 plants \((F_{35,54.5} = 1.71, P < 0.05); \) also see Table 2.

**Within-Environment Analysis of MA Lines**

Analyses of variance for individual characters revealed significant line effects in three cases: final height of generation 17 plants in the high-nutrient environment and the mean fruit size for generation 17 lines in both high- and low-nutrient environments (Table 2). Consistent with the ANOVAs, REML estimates for the genetic variance among MA lines were significant for the mean fruit size in both the high- \((V_{MA} = 4.91 \times 10^{-2} \text{mm}^2)\) and low-nutrient \((V_{MA} = 5.76 \times 10^{-2} \text{mm}^2)\) environments and marginally significant for the final height in the high-nutrient environment \((V_{MA} = 60.27 \text{mm}^2)\), APB test \(P < 0.09\).

For these two traits expressing significant variation among lines, the contribution of mutation to the observed phenotypic variance, \(V_M\), was obtained, and from this, estimates of mutational heritability \((h_M^2)\) and the mutational coefficient of variance \((CV_M)\) (Table 3). The standardized values are roughly comparable to our previous estimates for these lines (Shaw et al. 2000) and to estimates for diverse traits in other organisms (Houle et al. 1996).

**Cross-Environment Analyses**

To understand how cumulative effects of mutations varied in response to different nutrient environments, we analyzed all of the generation 17 plants grown in both high- and low-nutrient conditions together (Table 4). Analysis of covariance revealed that nutrient level significantly and consistently affected most of the characters measured. Plants grown in high-nutrient conditions tended to take longer between bolting and flowering, were taller at the time of first flowering, had more and larger leaves, showed a greater final height, and produced greater total seed weight, higher biomass, and larger fruits than ones in low-nutrient conditions (Table 2). The magnitudes of differences in the morphological measurements between the low- and high-nutrient environments range from 0–50%. Both reproductive biomass and seed weight are 3.6 times greater in the high- than in the low-nutrient environment (Table 2). For no trait did the line-by-treatment interaction approach significance, providing no evidence that lines responded differently to the nutrient treatments.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Low nutrient</th>
<th>High nutrient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(F)</td>
<td>(df)</td>
</tr>
<tr>
<td>Gen</td>
<td>0.84</td>
<td>2.10</td>
</tr>
<tr>
<td>Line (gen)</td>
<td>0.28</td>
<td>2.17</td>
</tr>
<tr>
<td>Subline (gen)</td>
<td>0.79</td>
<td>2.17</td>
</tr>
</tbody>
</table>

* \(P < 0.005, ** P < 0.001, *** P < 0.0005\).

Just as few traits displayed significant variation among MA
lines, there also were few traits for which the cross-environment genetic covariance among lines approached statistical significance. In the case of mean fruit size, which showed significant genetic variance in both environments, the associated covariance differed significantly from zero and yielded a cross-environment genetic correlation of 0.88. This value is significantly different from zero ($P < 0.01$), but not from one. Also, for leaf number, REML revealed a significant covariance across nutrient environments (COV = 0.0358, APB test, $P < 0.05$), corresponding to a correlation among lines of 1.0. This trait is also included in Table 3. For two other characters, flowering time and final height, estimates of the genetic correlations across nutrient environments were very close to one, yet these were not significantly different from zero. Genetic correlations for bolting date, plant weight, and seed weight were not estimated because of the genetic variances in one or both environments were zero.

These findings provide no evidence for trade-offs between performance in the two environments with respect to any of the traits, as Figure 1 illustrates. Considering first the trait for which this experiment detected significant variance among lines within each environment as well as significant cross-environment genetic correlation, the positive correlation detected between the mean fruit sizes in high- and low-nutrient conditions is evident (Fig. 1d). The most strikingly distinct line produced larger fruits in both environments. Apart from this, lines showing differences from generation 0 in one environment differed in the same direction or hardly at all in the other environment, although there are exceptions. The remaining reproductive traits, reproductive biomass and seed weight, varied widely among lines from positive to negative when grown in high nutrients but varied very little in low-nutrient conditions (Fig. 1b,c).

Considering the morphological traits, nearly all possible combinations of mutational effects in the two environments are represented in our lines. In addition, some MA lines showed neutral effects in the low-nutrient conditions, indicated by means close to zero, with positive or negative effects in the high-nutrient conditions. We present results for leaf number (Fig. 1a), as an example of the diverse array of environmental responses of the MA lines.

### Selection on Mutational Effects

The phenotypic selection analysis suggested that selection in both environments consistently promoted earlier bolting, and increased size of plants due to longer leaves and greater final height (Table 5). The genotypic selection analysis identified selection at the genetic level toward earlier flowering. Specifically, fewer days between bolting and flowering increased the total seed weight produced, as indicated by the negative and significant or marginally significant ($P = 0.04$ and 0.08) partial regression coefficients in both environments (Table 5). The genotypic analysis failed to detect selection on other traits, perhaps because of the substantially reduced power of this analysis, but several traits, particularly leaf number, appeared to be subject to divergent, albeit weak, selection in the two environments. Estimates obtained from REML estimation of the mutational covariance (COV$_M$) between fitness and the remaining traits (not shown) confirmed the interpretation of the regression analysis of MA line means. Confidence intervals on the estimates were wide, however.

**Discussion**

This study does not support either of two recently advanced hypotheses concerning environment dependence of muta-

### Table 2. Univariate ANOVAs for nine characters of plants from generation 17 grown in two nutrient environments. Values listed are the $F$-ratios for significance tests. Degrees of freedom are the numerator dfs. The denominator dfs obtained by Satterthwaite method were used for the analyses but are not listed here.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Days to bolting</th>
<th>Bolting to flowering (days)</th>
<th>Height at flowering (cm)</th>
<th>Leaf number</th>
<th>Largest leaf length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(LSM ± SE)</td>
<td>Low</td>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>df</td>
<td>(24.6 ± 2.3)</td>
<td>(6.77 ± 1.03)</td>
<td>(5.1 ± 2.1)</td>
<td>(5.3 ± 2.2)</td>
</tr>
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<td></td>
<td></td>
<td>(7.04 ± 1.02)</td>
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<td>(15.5 ± 1.8)</td>
<td>(16.15 ± 2.5)</td>
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<tr>
<td></td>
<td></td>
<td>Low</td>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(24.6 ± 2.5)</td>
<td></td>
<td>(5.3 ± 2.2)</td>
<td>(3.1 ± 0.8)</td>
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<tr>
<td></td>
<td></td>
<td>High</td>
<td></td>
<td>(4.0 ± 0.5)</td>
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</tr>
<tr>
<td><strong>Line</strong></td>
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<td>1.67</td>
<td>0.78</td>
<td>1.11</td>
</tr>
<tr>
<td><strong>Subline(line)</strong></td>
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<td>2.29***</td>
<td>0.88</td>
<td>1.20</td>
<td>1.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.91</td>
<td>0.84</td>
<td>1.01</td>
</tr>
<tr>
<td><strong>Final height (cm)</strong></td>
<td></td>
<td>0.85</td>
<td>1.90</td>
<td>1.10</td>
<td>1.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.95</td>
<td>1.80</td>
<td>1.01</td>
<td>0.71</td>
</tr>
<tr>
<td><strong>Dry seed weight (g)</strong></td>
<td></td>
<td>0.75</td>
<td>0.83</td>
<td>3.59***</td>
<td>7.13***</td>
</tr>
<tr>
<td><strong>Mean fruit size (cm)</strong></td>
<td></td>
<td>1.31</td>
<td>1.46*</td>
<td>1.14</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.83</td>
<td>1.14</td>
<td>0.60</td>
</tr>
</tbody>
</table>

*** $P < 0.005$, * $P < 0.05$, † $P < 0.1$.  

### Table 3. The contribution of mutation to the observed phenotypic variance, $V_M$, the relative magnitude of $V_M$ to the variance due to environment ($V_e$), and the mutational coefficient of variance (CV$M$) for the leaf number, final height, and mean fruit size.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Low nutrient</th>
<th>High nutrient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_M$</td>
<td>$h_M^2$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>($V_M/V_e$)</td>
</tr>
<tr>
<td><strong>Leaf number</strong></td>
<td>0.001</td>
<td>0.57</td>
</tr>
<tr>
<td><strong>Final height (cm)</strong></td>
<td>$2.86 \times 10^{-3}$</td>
<td>0.38</td>
</tr>
<tr>
<td><strong>Mean fruit size (cm)</strong></td>
<td>$1.70 \times 10^{-5}$</td>
<td>4.26</td>
</tr>
</tbody>
</table>
tional effects. Contrary to the suggestion of Kondrashov and Houle (1994), we found no greater decline in mean fitness in the harsh environment of reduced nutrients than in high-nutrient conditions (Fig. 1). We also did not find evidence for variation in mutational effects consistent with that suggested by Fry et al. (1996), in which mutations are deleterious in all environments, but to differing degrees. More generally, our results do not support the commonly held view (e.g., Lynch et al. 1999) that the overwhelming majority of spontaneous mutations reduces fitness. According to this view, fitness of MA lines is expected to decline through MA, due to the composite effects of their accumulated mutations. Consequently, fitness, averaged over late generation MA lines, is expected to be lower than that of the founder. Our results are not in accord with this expectation, even though we have demonstrated mutational increase in variation among lines (Shaw et al. 2000; this study).

In the clearest example from this study, mean fruit size varied significantly among lines in both environments (Table 2), yet there was no evidence from either nutrient treatment of a mutational decline in mean fruit size (Fig. 1d). Rather, mean fruit size increased slightly (< 1% in each environment) during the course of MA. For this trait, we can rule out a decline in the mean as great as 0.04% per generation in each environment, based on the lower bound of the 95% confidence interval (CI) for the difference between the means of generation 17 and generation 0. By this criterion, we can rule out rates of decline in total seed weight greater than 1.4% and 0.3% per generation in the low- and high-nutrient environments, respectively, in which the difference between the rates is fully explained by scaling to generation 0 means that differ by a factor of five between the two nutrient treatments. Our choice of representing generation 17 to a greater degree than generation 0 to focus on assessing variation among the MA lines in environmental responses reduces the precision for the estimates of mean differences between the generations. Thus, our lower bounds of the 95% CI for decline of mean seed weight include estimates for mean decline obtained in experiments with Drosophila (Mukai 1964; Fry 2001). For this reason, we cannot strictly rule out mean declines as great as in these experiments, but we note that, by the criterion of the 95% CIs, we also cannot rule out increases in mean mass of seeds produced of 1.76% and 0.31% per generation for the low- and high-nutrient environments, respectively. Thus, our estimates suggest that mutations that increase fruit size or total seed weight, as well as those that decrease these traits, contribute to the approximately symmetric divergence among the lines. This finding may apply widely, for this species at least. Even lines obtained by EMS mutagenesis of three distinct accessions of A. thaliana yielded no evidence of directionality in the effects of mutations (Camarina and Pigliucci 1999).

Kondrashov and Houle (1994) suggested that the effects of deleterious mutations may be more severely detrimental in harsh environments, defined operationally as conditions in which mean absolute fitness is low. They provided as evidence of this for D. melanogaster more extreme fitness decline over MA generations in more stressful conditions (see also Vassilieva et al. 2000). Similarly, Szafraniec et al. (2001), studying highly mutable strains of yeast, have dem-

### Table 4

<table>
<thead>
<tr>
<th>Variable</th>
<th>df</th>
<th>Location</th>
<th>Blend</th>
<th>Treatment</th>
<th>Nutrient</th>
<th>Seed size (mean)</th>
<th>Seed size (CI)</th>
<th>Seed size (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</table>

The denotator of fitness obtained by Satterthwaite's method were used for the analyses, but are not listed here. Degrees of freedom are the numerator of fitness for the significance tests.
TABLE 5. Multiple regression using the individual measurements (phenotypic selection) and least square means for each line (genetic selection) in high- and low-nutrient environments. The response variable is the dry seed weight. Values listed are partial regression coefficients for the predictor variables.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Low nutrient</th>
<th>High nutrient</th>
<th>Low nutrient</th>
<th>High nutrient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β</td>
<td>P</td>
<td>β</td>
<td>P</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.018</td>
<td>0.42</td>
<td>0.324</td>
<td>0.001</td>
</tr>
<tr>
<td>Bolting</td>
<td>-0.007</td>
<td>&lt;0.001</td>
<td>-0.017</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Bolting to flowering</td>
<td>-0.002</td>
<td>0.37</td>
<td>-0.002</td>
<td>0.82</td>
</tr>
<tr>
<td>Initial height</td>
<td>0.0005</td>
<td>0.68</td>
<td>-0.007</td>
<td>0.18</td>
</tr>
<tr>
<td>Leaf number</td>
<td>0.002</td>
<td>0.07</td>
<td>0.004</td>
<td>0.38</td>
</tr>
<tr>
<td>Leaf length</td>
<td>0.002</td>
<td>&lt;0.001</td>
<td>0.006</td>
<td>0.001</td>
</tr>
<tr>
<td>Final height</td>
<td>0.003</td>
<td>&lt;0.001</td>
<td>0.007</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

shown considerable mutational declines in mean fitness at elevated temperature, whereas differences in mean fitness were negligible at standard temperature. In our study, by contrast, there was compelling indication of directionality of mutational effects only for the phenological traits and only under the high nutrient conditions (Table 1), in which absolute fitness was higher (Table 2). For both traits in high nutrients, mean development time of plants in the generation 17 MA lines tended to be faster than for plants representing generation 0. Rapid development is common, although not necessarily (Zhang and Lechowicz 1994), associated with increased fitness in annual plants, and this interpretation is supported by both our phenotypic and genetic selection analyses, which indicate significant selection for earlier phenology in both environments (Table 5). Thus, where a difference between generation 0 and generation 17 means was detected, it was in the direction opposite from that expected based on the Kondrashov and Houle (1994) hypothesis.

Our study provided no clear evidence that newly arising mutations contribute strongly to previously hypothesized patterns of genetic variation in environmental response (norms of reaction) in Arabidopsis. For no trait was the line-by-treatment interaction detected as significant (Table 4). Accordingly, the estimates of genetic correlation across environments (although most were imprecise) tended to be very near one. We emphasize, however, that, as with other MA studies, we have here estimated the correlation between composite effects of accumulated mutations, rather than the correlation of effects of individual mutations. Keightley et al. (2000) have noted that estimates of the former may typically far exceed the latter when mutational effects are predominantly deleterious, because the among-line correlation can be heavily influenced by differences among the lines in the number of mutations they carry. Relatively few MA generations underlie the divergence of our lines, so the confounding of effects of individual mutations is expected to be less than in studies of extensive MA line advancement or those employing chemical mutagenesis to induce many mutations. Our estimates of genomewide mutation rate for number of fruits per plant (0.1) and number of seeds per fruit (0.2; Shaw et al. 2002), imply that our lines carry on average 1.7 and 3.4 new mutations, respectively, for each of these traits. By this reasoning, we suggest that our estimate of 0.88 as the among-line correlation of fruit size across nutrient environments can be interpreted as evidence of strong positive pleiotropy, indicating that effects of new mutations tend to be similar in the two nutrient environments we used.

Few previous studies have addressed the issue of mutational contribution to genetic variation in environmental responses. Studies of MA lines of D. melanogaster have yielded estimates of cross-environment fitness correlations that are significantly less than one (Fry et al. 1996; Fernández and López-Fanjul 1997), indicating that newly arising mutations generally contribute to differences in fitness ranking of genotypes among environments. Nevertheless, cross-environment correlations tended to be positive and, in the study by Fry et al. (1996) on MA lines advanced to 200 generations, large (>0.5). Those obtained by Fernández and López-Fanjul (1997) for MA lines advanced to 100 generations tended to be considerably smaller, and in one instance indicated a substantial trade-off in fitness across environments (standard vs. high saline conditions; Fernández and López-Fanjul 1997). Even if all mutations are deleterious, but to differing degrees in different environments, such patterns of mutationally induced correlations of fitness across environments could alone account for the evolution of ecological specialization, according to theoretical work of Kawecki et al. (1997). More recently, Fry and Heinsohn (2002) have obtained consistently high estimates of cross-environment genetic correlations (> 0.54) for MA lines advanced to generation 35. In this case, control lines maintained at larger effective population size and longer generation interval were available for comparison and consistently exceeded the MA lines in mean viability, evidencing a preponderance of deleterious mutations in these lines.

Apart from these studies of Drosophila, environment dependence of mutational effects has been assessed in two microorganisms. In a study of highly mutable strains of yeast, Korona (1999) found consistent mutational declines in growth rates and sizable positive cross-environment correlations. Remold and Lenski (2001) studied a set of 26 lines of Escherichia coli differing from their common progenitor by single insertion mutations. In assays of relative competitive fitness in four environments differing in temperature and resource (glucose or maltose), they detected extensive variation in responses to resource, but none in responses to temperature. Competitive fitness varied far more among lines in maltose, a resource novel to these lines, than in glucose. Among the 26 mutations, several (12%) significantly enhanced relative fitness in maltose.
We chose to vary soil nutrient content based on prior evidence of substantial variation among wild accessions of A. thaliana in their responses to nutrient availability. Zhang and Lechowicz (1994) documented considerable variation in the magnitude of response to nutrients among 13 wild accessions, but noted little variation in the pattern of plasticity. Pigliucci and Schlichting (1995), studying nutrient responses of nine traits in 26 wild accessions, found that five traits including time to bolting, number of basal leaves, final height, and numbers of basal and lateral branches exhibited highly significant interactions between population and nutrient level. Further evidence of variation among A. thaliana populations in their response to nutrient concentrations is given by Pigliucci and Schlichting (1995) and Pigliucci and Byrd (1998). Pigliucci and Schlichting (1998) have also demonstrated an influence of nutrient levels on correlations between traits.

Nutrient responses of A. thaliana lines obtained by chemical mutagenesis have also been assessed. In a study of five late-flowering lines, van Tienderen et al. (1996) found a significant interaction between lines and nutrient treatment for several morphological traits. Pigliucci and Byrd (1998), observing, as we did, a tendency toward greater line variance in high nutrient conditions, obtained suggestive evidence of such an interaction in a study of four lines with demonstrated reductions in nitrogen uptake.

We did not detect significant variation among lines in responses to the nutrient conditions. A larger experiment might well have revealed such variation for some of the traits but, given our present evidence, appears unlikely to have supported the model of Kawecki et al. (1997) in which mutations are generally deleterious but to differing degrees in different environments. In support of this model, we would expect to find in Figure 1b–d most generation 17 lines in the bottom left quadrant. In the case of fruit size (Fig. 1d), a small minority of the lines is included in this region; for seed weight (Fig. 1c), perhaps five of the 20 lines could be considered to exhibit expression of mutations that is deleterious strictly in the high-nutrient environment in which absolute fitness is high, contrary to the expectation of Kondrashov and Houle (1994). We also do not find evidence of a tendency toward trade-offs between fitness effects of new mutations in different nutrient regimes that could directly contribute to ecological specialization.

In conclusion, results of this study are consistent with our earlier finding (Shaw et al. 2000, 2002) that new mutations affecting fitness in these MA lines of A. thaliana are not strongly biased toward deleterious effects. Even in environmental conditions that severely curtailed absolute fitness, no directionality of mutational effects was evident. We also found no evidence for hypothesized patterns of mutational contribution to genotype-by-environment interaction. We cannot rule out the possibility that our study had insufficient power to detect mutational GEI. Nevertheless, our findings suggest that other evolutionary mechanisms, such as environment-specific selection, play an important role in establishing the GEI that have been documented in A. thaliana.

ACKNOWLEDGMENTS

We thank L. Rear, C. Kavanaugh, L. Nguyen, and L. Berkeley for their help in setting up the experiment and in caring for the plants; D. Adair for maintaining the greenhouse environment; and F. Shaw for providing invaluable help with data analyses. Comments from N. Armstrong, N. Gomez, K. Mercer, J. Hill, M. Lowe, C. López-Fanjul, and two anonymous reviewers improved the manuscript. Funding for this study was provided by National Science Foundation grant DEB-9981891 to RGS.

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Mackay, T. F. C., R. F. Lyman, M. S. Jackson, C. Terzian, and W.


Corresponding Editor: C. López-Fanjul