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ORIGINAL PAPER

Experimental alteration of DNA methylation affects the phenotypic plasticity of ecologically relevant traits in *Arabidopsis thaliana*

Oliver Bossdorf · Davide Arcuri · Christina L. Richards · Massimo Pigliucci

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Abstract Heritable phenotypic variation in plants can be caused not only by underlying genetic differences, but also by variation in epigenetic modifications such as DNA methylation. However, we still know very little about how relevant such epigenetic variation is to the ecology and evolution of natural populations. We conducted a greenhouse experiment in which we treated a set of natural genotypes of *Arabidopsis thaliana* with the demethylating agent 5-azacytidine and examined the consequences of this treatment for plant traits and their phenotypic plasticity. Experimental demethylation strongly reduced the growth and fitness of plants and delayed their flowering, but the degree of this response varied significantly among genotypes. Differences in genotypes' responses to demethylation were only weakly related to their genetic relatedness, which is consistent with the idea that natural epigenetic variation is independent of genetic variation. Demethylation also altered patterns of phenotypic plasticity, as well as the amount of phenotypic variation observed among plant individuals and genotype means. We have demonstrated that epigenetic variation can have a dramatic impact on ecologically important plant traits and their variability, as well as on the fitness of plants and their ecological interactions. Epigenetic variation may thus be an overlooked factor in the evolutionary ecology of plant populations.

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O. Bossdorf (✉)
Institute of Plant Sciences, University of Bern, 3013 Bern, Switzerland
e-mail: bossdorf@ips.unibe.ch

D. Arcuri
Dipartimento di Biotecnologie (Bio.M.A.A.), Università Mediterranea di Reggio Calabria, 89061 Reggio Calabria, Italy

C. L. Richards
Department of Integrative Biology, University of South Florida, Tampa, FL 33620, USA

M. Pigliucci
Department of Philosophy, City University of New York-Lehman College, Bronx, NY 10468, USA

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Introduction

One of the established principles of biology is that evolution by natural selection requires heritable variation, and that the only source of such variation in natural populations is genetic. Therefore, the ability of plant populations to adapt to local habitat conditions or environmental change critically depends on the presence of genetic variation (Endler 1986; Falconer and Mackay 1996; Linhart and Grant 1996); without genetic variation, evolutionary change and adaptation are impossible. However, there is now increasing evidence that, even in the absence of genetic variation, phenotypic variation can be caused by variation in epigenetic modifications of the genome.

The term epigenetics generally refers to a set of molecular processes, including DNA methylation, histone modification, and RNA interference, that can alter gene function and, ultimately, the phenotype without changes in DNA sequence (Grant-Downton and Dickinson 2005; Berger 2007; Bird 2007). The best described of these mechanisms is DNA hypermethylation, where methylation of the 5′-carbon of the cytosine aromatic ring results in transcriptional silencing. Cytosine methylation is also strongly tied to the modification of histones and the condensation of chromatin, and is an important factor in the regulation of gene expression such as the silencing of duplicate genes after polyploid formation (Liu and Wendel 2003; Osborn et al. 2003; Chen 2007). Recent research has shown that there is natural variation in epigenetic modifications, very similar to that in DNA sequence, in many different plant species (e.g. Cervera et al. 2002; Riddle and Richards 2002; Keyte et al. 2006; Vaughn et al. 2007; Zhang et al. 2008), and that at least some of this variation is heritable and independent of genetic variation, and therefore potentially subject to evolution by natural selection (Kalisz and Purugganan 2004; Jablonka and Lamb 2005; Rapp and Wendel 2005; Grant-Downton and Dickinson 2006; Richards 2006; Johannes et al. 2008).

The study of epigenetic inheritance in natural populations is at an early stage. While it is possible that it will eventually significantly improve our understanding of the mechanisms underlying phenotypic variation and the responses of organisms to environmental change, currently there is a dearth of research that has addressed the causes and consequences of epigenetic variation in an evolutionary ecological context. In order to understand the relevance of heritable epigenetic variation, we need to: (1) investigate how much of it is present in natural populations; (2) verify whether epigenetic variation is at least partly independent of genetic variation; and in particular (3) study what sort of significant effects does epigenetic inheritance have on ecologically important traits, on fitness, and on ecological interactions. Here, we have attempted to contribute in particular to the third task.

A clear-cut demonstration of the ecological relevance of epigenetic variation is not a trivial undertaking because of its co-occurrence with genetic variation. To be able to unequivocally attribute phenotypic effects to underlying epigenetic variation, we must, by experimental or statistical means, discern genetic from epigenetic effects (Bossdorf et al. 2008; Johannes et al. 2008). However, the simultaneous analysis of the phenotypic effects of interrelated genetic and epigenetic variation, and their interactions, constitutes a significant methodological and statistical challenge (Johannes et al. 2008).

Previous studies that convincingly demonstrated phenotypic effects of epigenetic variation often utilized natural or artificial epimutations that allowed the comparison of different epigenotypes of the same genotype (e.g., Cubas et al. 1999). A related technique is to use the demethylating agent 5-azacytidine (5-azaC), a chemical that is incorporated into DNA during replication and thereby inhibits the enzyme methyltransferase and causes partial demethylation of the DNA (e.g., Jones 1985; Burn et al. 1993; Fieldes and Amyot 1999a; Tatra et al. 2000). The obvious advantage of such experimental epimutagenesis is that it allows researchers to create different epigenetic variants of the same genotypes. Because this can be done for many genotypes, it allows us to generalize across different genetic backgrounds, and it is much more amenable to controlled, multi-factorial experiments. The method is somewhat crude, but it can be a valuable first step in exploring the potential phenotypic consequences of epigenetic variation.

Here, we have used 5-azaC to manipulate DNA methylation in a set of natural genotypes of *Arabidopsis thaliana* and to examine the consequences of these modifications for plant traits and their phenotypic plasticity. Specifically, we have asked the following questions: (1) How does experimental alteration of DNA methylation affect the means and variances of ecologically important phenotypic traits in *A. thaliana*? (2) Does experimental demethylation alter the degree of phenotypic plasticity of plants in response to different nutrient levels? (3) Do these responses to 5-azaC vary among different *Arabidopsis* genotypes? (4) If yes, are genotypic differences in demethylation responses related to the genetic distances among genotypes?

Materials and methods

Plant material

Arabidopsis thaliana (L.) Heynh. (Brassicaceae) is a small annual weed in the mustard family (Brassicaceae). It is a predominantly selfing, ruderal species native to Eurasia but now widely naturalized in the USA and elsewhere. *A. thaliana* has long been a model species for plant genetics and molecular biology (Meyerowitz and Somerville 2002). However, because of the growing body of knowledge about its physiology, development and molecular biology, the logistic advantages it affords, and the availability of a broad array of molecular tools, the species is now also frequently used as a model for addressing basic questions at the interface of ecology and evolutionary biology (Pigliucci 1998; Mitchell-Olds 2001; Koornneef et al. 2004). In this study, we used a total of 22 genotypes of *A. thaliana*: 20 genotypes from natural populations, representing the species' widest geographical range (Table S1), and the two standard laboratory lines *Landsberg erecta* (Ler-2) and Columbia (Col). The seeds were obtained from the Arabidopsis Stock Center (TAIR; www.arabidopsis.org) where these genotypes, originally collected in the field, have been propagated and maintained under uniform conditions for several generations.

Experiment

We conducted an experiment in which the 22 *Arabidopsis* genotypes were subjected to a factorial combination of nutrient addition and experimental demethylation treatments. In January 2006, all seeds were cold-stratified in the dark at 4°C for 1 week. For each genotype, we used two petri dishes, one in which the filter paper was moistened with 1.4 ml water, and one in which it was moistened with 1.4 ml of a 50 μmol 5-azacytidine

(5-azaC; Sigma Scientific, St. Louis, MO) solution. After this stratification period, we transferred the petri dishes to an unheated greenhouse for another week under natural light conditions until most seeds had germinated. The seedlings were transplanted into 72-cell planting trays filled with a 50:50 mix of sand and vermiculite. The experiment followed a split plot design with trays as plots, 5-azaC and nutrient treatments as whole plot factors, and plant genotype as subplot factor. Low and high nutrient levels were created by adding either one or eight pellets of a slow-release fertilizer (15-9-12 Osmocote Plus 8–9 M, Scotts Miracle-Gro, Marysville, OH) to each cell in a planting tray. For each treatment combination, there were three planting trays, each with three replicates per genotype. Thus, there were 12 replicates of each genotype in each treatment combination, and a total of 1056 plants in the experiment. The trays were arranged randomly on two greenhouse benches and watered as needed throughout the experiment. After approximately 45 days, the first genotypes started flowering. Each plant was harvested at fruit maturity, 30 days after first flowering. For each plant, we recorded (1) time to flowering, as the number of days between the start of the greenhouse experiment and the first appearance of a white petal, (2) rosette diameter to the nearest millimeter as a measure of plant size at the time of flowering, (3) final plant height to the nearest millimeter, (4) the total number of basal and lateral branches, (5) the total number of siliques, and (6) the final aboveground biomass in milligrams after drying at 60°C for 24 h.

Statistical analyses

We used nested analysis of variance to test for the effects of demethylation, nutrient availability, genotype, and their interactions on each of the response variables. The effects of 5-azaC, nutrients and their interaction were tested at the plot level, using the tray mean squares as error term, whereas the genotype effects and their interactions were tested at the level of plant individuals, i.e. against the residual. To improve normality and homoscedasticity of the data, flowering time and plant height were square-root transformed, and all other variables were log-transformed prior to the analyses. In addition, we ran a generalized linear model with binomial error and logit link to test for the effects of treatments, genotypes and their interactions on plant mortality. We used the χ^2 deviances to calculate variance ratios that were approximately F-distributed (McCullagh and Nelder 1989). All analyses were done in JMP 6.0.3 (SAS Institute, Cary, NC).

To examine the effects of 5-azaC on phenotypic variation, we calculated coefficients of variation for each phenotypic trait in the control versus 5-azaC treatment, and we used the asymptotic test statistic by Miller (1991) to test for differences between these CVs. We ran these tests both for the raw phenotypic variation among plant individuals, and for the variation among genotype means, an estimate of quantitative genetic variation. As data transformations equalize variances, the CVs were calculated from untransformed data. The results of the genotype-level analyses did not qualitatively differ when CVs were calculated from raw genotypic means instead of least square means; we therefore present only the results for comparisons of CVs based on raw data.

Finally, we examined whether differences between genotypes in their phenotypic responses to 5-azaC were related to their genetic distance. We used data on 149 single-nucelotide polymorphisms (SNPs) provided by Justin Borevitz (available at http://naturalvariation.org/hapmap) and the dnadist program in PHYLIP (Felsenstein 2005) to construct a genetic distance matrix of the 22 genotypes. Next, we calculated seven Euclidean distance matrices based on the average percent phenotypic change of each genotype in response to 5-azaC treatment: one matrix for each of the six traits, and one in

which the changes in all traits were considered together. As the degree of change differed greatly among traits, the response data were standardized prior to distance calculations. We used Mantel tests to assess correlations between genetic and phenotypic distances. Distance calculations and Mantel tests were done using the ecodist package (Goslee and Urban 2007) in R (R Development Core Team 2007).

Results

Out of the 1056 individuals planted, 467 reached maturity. Plants treated with 5-azaC had more than 40% higher rate of mortality than plants that were not treated (Table 1). Analysis of deviance indicated that mortality was also significantly affected by plant genotype and the 5-azaC by genotype interaction (Table 2). However, the full statistical model explained only 15% of the total variation, so most of the mortality, which was mostly likely caused by temperature stress in the greenhouse, was unrelated to any of the experimental factors.

Table 1 Phenotypic differences between *Arabidopsis thaliana* plants germinated with or without 5-azacytidine

	Control	5-azaC	% Change
Mortality	45.83%	65.72%	+43.4
Flowering time (d)	50.19 (±0.78)	56.73 (±0.63)	+13.0
Size at flowering (cm)	1.91 (±0.08)	1.29 (±0.08)	−32.2
Plant height (cm)	15.77 (±0.50)	11.89 (±0.56)	−24.6
Branch number	5.39 (±0.25)	3.77 (±0.27)	−30.0
Biomass (mg)	49.96 (±3.90)	24.05 (±3.07)	−51.9
Fruit number	59.91 (±3.58)	37.71 (±4.58)	−37.1

Size at flowering = rosette diameter

The values are means ± standard errors from the raw data

Table 2 Summary of analysis of deviance of plant mortality

Source	DF	Mean deviance	Quasi-F
Plot level			
5-Azacytidine	1	46.78	30.36***
Nutrients	1	3.83	2.49
A × N	1	2.99	1.94
Tray	12	1.54	1.19
Plant level			
Genotype	21	3.09	2.38***
A × G	21	2.93	2.26**
N × G	21	0.54	0.41
A × N × G	21	0.53	0.41
Residual	956	1.30	

DF degrees of freedom. ** $P < 0.01$, *** $P < 0.001$

Table 3 Summary of analyses of variance of plant phenology, growth, and fitness

Source	DF	Flowering time ($r^2 = 0.557$)		Size at flowering ($r^2 = 0.482$)		Height ($r^2 = 0.422$)		Branch number ($r^2 = 0.387$)		Biomass ($r^2 = 0.479$)		Fruit number ($r^2 = 0.390$)	
		MS	*F*	MS	*F*	MS	*F*	MS	*F*	MS	*F*	MS	*F*
Plot level													
5-azaC	1	20.22	58.58***	16.50	91.03***	28.56	17.00**	14.05	45.78***	64.80	59.53***	24.42	13.65**
Nutrients	1	1.37	3.96(*)	11.69	64.48***	36.25	21.58***	18.06	58.84***	67.13	61.67***	43.35	24.23***
A × N	1	1.69	4.86*	0.96	5.31*	1.28	0.76	3.32	10.83**	4.11	3.78(*)	2.80	1.56
Tray	12	0.35	1.11	0.18	0.63	1.68	1.91*	0.31	0.61	1.09	0.95	1.79	1.80*
Plant level													
Genotype	21	2.33	7.42***	0.82	2.85***	2.41	2.74***	1.10	2.18**	3.25	2.83***	1.69	1.70*
A × G	21	1.08	3.45***	0.68	2.34***	1.24	1.41	0.45	0.88	2.08	1.81*	1.13	1.13
N × G	21	0.40	1.27	0.51	1.77*	0.70	0.79	0.44	0.87	1.35	1.17	0.88	0.88
A × N × G	21	0.74	2.36***	0.47	1.61*	1.02	1.15	0.60	1.18	1.35	1.17	1.26	1.27
Residual	367	0.31		0.29		0.88		0.51		1.15		1.00	

DF degrees of freedom. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, (*) $P < 0.1$

Experimental demethylation by 5-azaC strongly affected all of measured traits (Table 1). On average, plants treated with 5-azaC flowered over 7 days later, and were significantly smaller at the time of flowering than control plants (Table 1). Moreover, plants treated with 5-azaC suffered a significant reduction in plant height and biomass, as well as the numbers of branches and fruits produced (Table 1).

There was significant genetic variation in the degree to which 5-azaC affected flowering time, size at flowering, and plant biomass (Table 3; Fig. 1). While in most *Arabidopsis* genotypes, treatment with 5-azaC (averaged across both nutrient treatments) clearly delayed flowering, the opposite was true for at least four genotypes (Fig. 1). Similarly, size at flowering and total biomass were usually dramatically reduced by 5-azaC, but there were a few genotypes that maintained or even increased their size in response to demethylation.

Treatment with 5-azaC generally altered plant responses to nutrient addition (Table 3; Fig. 2). On average, demethylated plants that grew at high nutrient levels flowered 4.4 days later than demethylated plants at low nutrient levels, while in control (i.e., non-demethylated) plants there was a tendency for nutrients to have the opposite effect on flowering time (Fig. 2a). In addition, demethylated plants showed a significantly weaker response to nutrient addition with regard to plant size at flowering and average branch numbers (Fig. 2b/c). There was a significant 3-way interaction between the effects of 5-azacytidine, nutrients and genotype on flowering time, indicating that the degree to which 5-AzaC altered the plasticity of this trait to nutrient additions varied among *Arabidopsis* genotypes (Fig. 3). For instance, while control plants of the Bologna (Bl-1) and Coimbra (Co-1) accessions showed identical phenological responses to nutrient additions, treatment with 5-azaC induced a strong delay of flowering in response to nutrients in the Spanish population, but did not change the response of the Italian population (Fig. 3).

Experimental treatment with 5-azaC significantly increased the phenotypic variability among individuals in five out of six traits (Fig. 4a). The strongest effect was on reproduction, where treatment with 5-azaC increased the coefficient of variation by almost 60%. The picture was slightly different when we examined genotypic means (Fig. 4b). Overall coefficients were much lower, and while treatment with 5-azaC more than doubled the estimated genotypic variation in fruit production and increased genotypic variation in branch number, it did not significantly affect variability in other traits such as plant height and biomass, and it even decreased the estimated genotypic variation in flowering time.

When constructing genetic distance matrices, the genotypes Di-2 and Ler-2 turned out to be genetically identical, based on the SNP data used. The distance between these two genotypes constituted an extreme outlier in the matrix correlations, and we therefore omitted it from these analyses. When all phenotypic traits were considered together, there

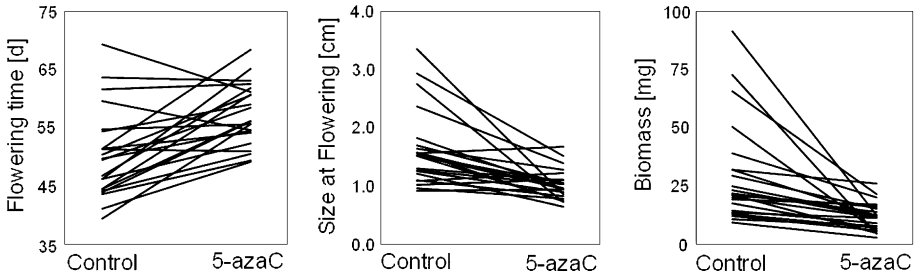

Fig. 1 The variation among 22 genotypes of *Arabidopsis thaliana* in their phenotypic responses to experimental demethlyation. *Each line* represents a different genotype

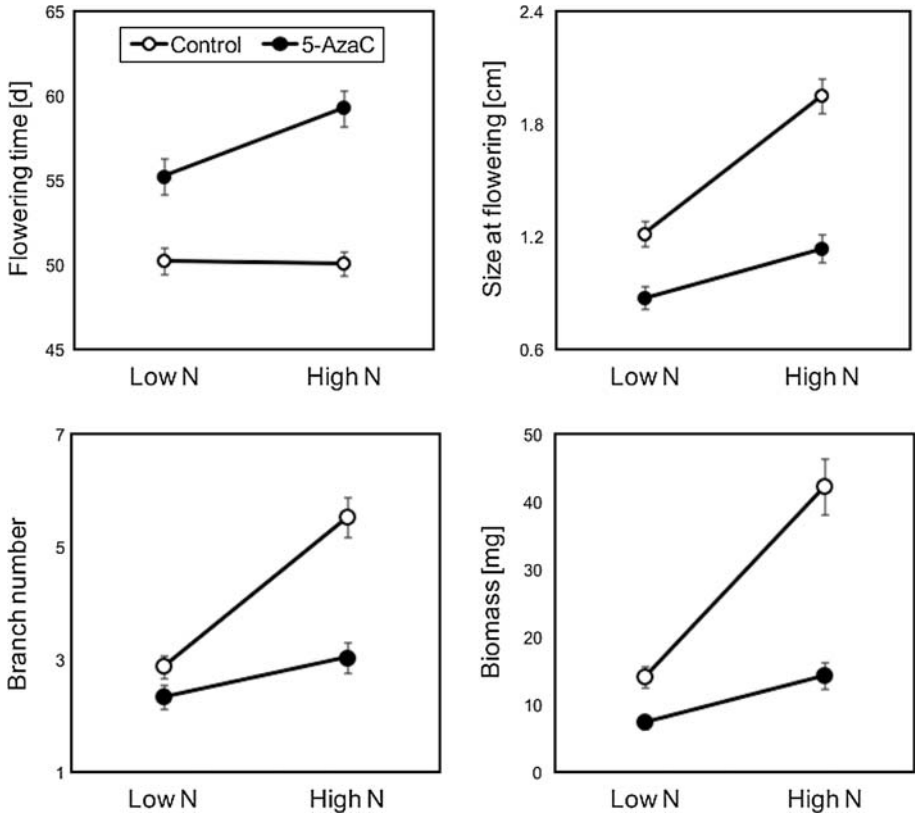

Fig. 2 The effect of experimental demethylation through 5-azacytidine on the average phenotypic plasticity of 22 genotypes of *Arabidopsis thaliana* in response to increased nutrient availability

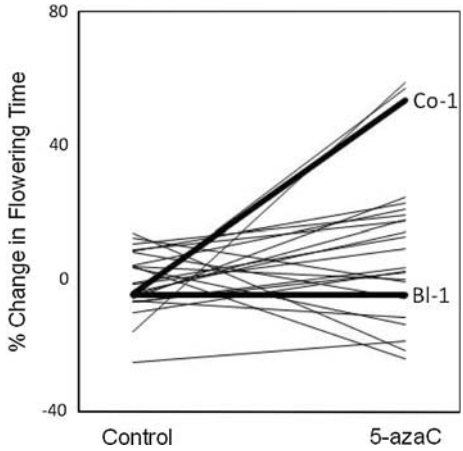

Fig. 3 Genotypic variation in the effects of experimental demethylation on plant phenotypic plasticity. The reaction norms illustrate how treatment with 5-azacytidine alters the phenological response to nutrient additions in 22 genotypes of *Arabidopsis thaliana*. Two genotypes with contrasting responses are highlighted in *bold*

was only a marginally significant correlation between the genetic distance of genotypes and the dissimilarity of their phenotypic responses to 5-azaC treatment (Table 4). When traits were considered separately, there was a significant correlation between genetic

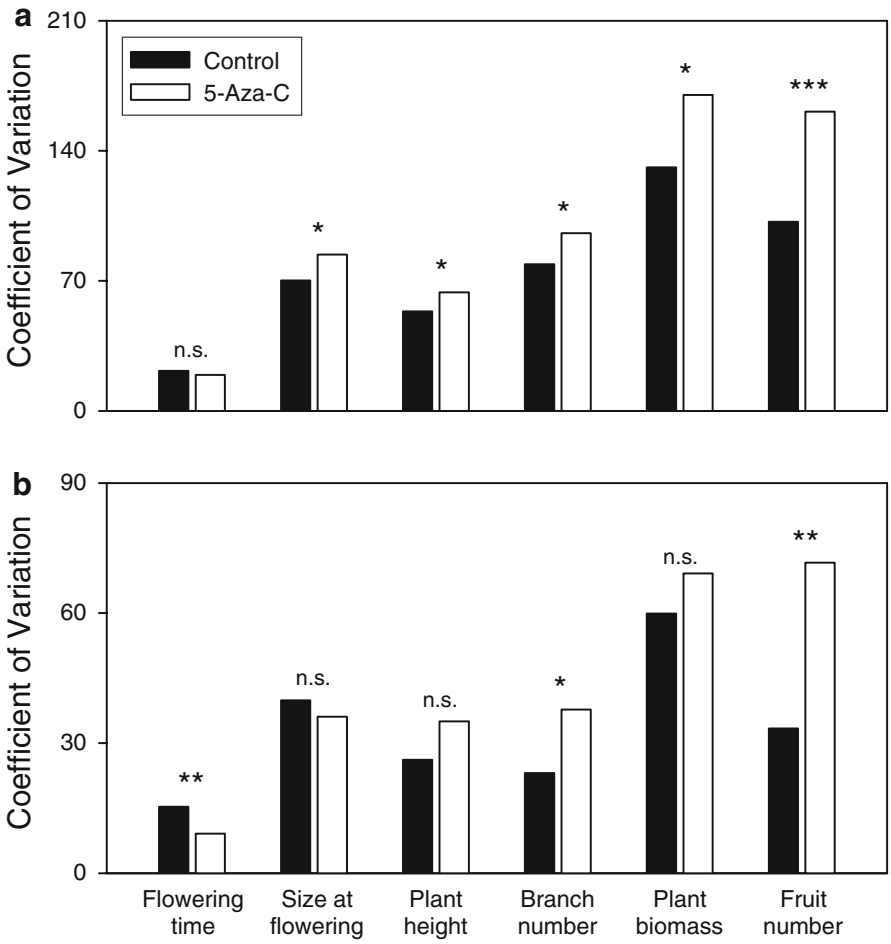

Fig. 4 The effects of experimental demethylation through 5-azacytidine on phenotypic variability among 22 genotypes of *Arabidopsis thaliana*. **a** Coefficient of variation among plant individuals. **b** Coefficient of variation of genotypic means. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, n.s. = not significant

Table 4 Mantel correlation tests between the genetic distances among 22 *Arabidopsis* genotypes and the dissimilarity of their phenotypic responses to 5-azacytidine treatment

Phenotypic response in terms of…	Correlation	Significance
ALL TRAITS	0.1421	0.0716
Flowering time	−0.0339	0.3364
Size at flowering	0.1417	0.0549
Height	0.1906	0.0158
Branch number	−0.0328	0.3603
Biomass	0.2030	0.0057
Fruit number	0.1147	0.1160

Significance levels are based on 100,000 permutations

distance and the height and biomass responses to 5-azaC, whereas correlations were non-significant for all other traits (Table 4). However, none of the significant correlations explained more than 4% of the variation in the data. These results were very consistent and did not change qualitatively when we re-ran the analyses using Jukes-Cantor genetic distances rather than simple distances based on the fraction of identical sites. We therefore present only the results for simple genetic distances in Table 4.

Discussion

Although there is increasing evidence of heritable epigenetic variation in plants (e.g. Cervera et al. 2002; Riddle and Richards 2002; Keyte et al. 2006; Vaughn et al. 2007; Zhang et al. 2008), few connections have been made so far between epigenetic variation and the variability and phenotypic plasticity of ecologically important plant traits (Bossdorf et al. 2008). To improve our understanding of the ecological relevance of epigenetic variation, we conducted a greenhouse experiment in which we used 5-azacytidine to manipulate DNA methylation in a set of natural genotypes of *Arabidopsis thaliana*.

In our study, we did not use molecular methods to verify demethylation at the molecular level. However, many previous studies have demonstrated this effect of 5-azaC (e.g., Burn et al. 1993; Tatra et al. 2000; Fieldes et al. 2005), and we think it is reasonable to assume that reduced DNA methylation was the main cause of the observed phenotypic changes in 5-azaC-treated plants. One alternative explanation we cannot rule out is that demethylation induced mobilization of transposable elements (Johannes et al. 2009), and that at least some of the observed phenotypic changes results from such induced DNA sequence changes. However, little is known about the frequency and phenotypic effects of such transpositions, and it is very difficult to evaluate the likelihood of this alternative.

We found that experimental alteration of DNA methylation strongly affected the growth, fitness and phenology of *A. thaliana*. There were large differences between demethylated and control plants of the same genotype. A few other studies have also demonstrated effects of demethylation through 5-azaC on ecologically important traits. Burn et al. (1993) showed that treatment of *A. thaliana* and *Thlaspi arvense* with 5-azaC significantly altered plant flowering times. In a series of experiments, Fieldes and colleagues (Fieldes 1994; Fieldes and Amyot 1999b; Fieldes et al. 2005) found that 5-azaC affected the growth, fitness and phenology of *Linum usitatissimum*. These studies, and the one presented here, show that epigenetic variation, independently of DNA variation, can cause major shifts in plant phenotypes.

In most genotypes, demethylation caused a decrease in overall plant performance. This general effect of demethylation or treatment with 5-azaC has been found by several previous studies (e.g., Burn et al. 1993; Fieldes 1994; Finnegan et al. 1996). Demethylation resulted in a significant delay of plant flowering: on average, 5-azaC-treated plants flowered 6.5 days later than control plants. This is in apparent contrast with several previous studies that found 5-azaC or demethylation to induce early flowering in *Arabidopsis* (e.g., Burn et al. 1993; Finnegan et al. 1996, 1998) and other plant species (e.g., Fieldes and Amyot 1999b; Kondo et al. 2007). However, recent research has shown that the transition to flowering in *Arabidopsis* is a complex developmental process that involves both methylation and demethylation of specific genes, and that, depending on the genetic background, hypomethylation may either cause early or late flowering, or have no effect on flowering time at all (Genger et al. 2003). Previous studies that found demethylation to induce early flowering in *Arabidopsis* had often been using the artificial Landsberg erecta

(Ler) line or Ler-based mutants. In our study, however, only two out of 22 *Arabidopsis* ecotypes (Abd-0, Oy-0) flowered earlier when treated with 5-azaC, whereas in 13 ecotypes flowering time was prolonged by over 5%. This suggests that the Ler ecotype may not be representative of natural variation in *Arabidopsis thaliana*.

There was significant variation among *Arabidopsis* ecotypes in their responses to experimental demethylation. However, these ecotypic differences in demethylation responses were only weakly related to the genetic distances between genotypes, which is consistent with the idea of natural epigenetic variation being independent of the corresponding genetic variation (Richards 2006; Bossdorf et al. 2008). Alternatively, ecotypic differences in the effects of 5-azaC could result from some kind of mechanical or physiological differences between ecotypes that affect the absorption, transport and/or incorporation of 5-azaC, but are at the same time uncorrelated to DNA sequence variation, or they could be—at least theoretically—due to ecotype-specific transposon activation. Both alternatives are rather speculative, and we think that epigenetic variation is indeed the most parsimonious explanation, particularly since its existence in *Arabidopsis thaliana* has already been shown several times (e.g., Cervera et al. 2002; Riddle and Richards 2002; Vaughn et al. 2007).

Treatment with 5-azaC increased phenotypic variability in most traits, which suggests that 5-azaC may not be a very precise demethylation agent and it may therefore not only reduce overall levels of methylation but also increase variation in methylation. Our result is consistent with a study of Fieldes and Amyot (1999b) who found such effects of 5-azaC were still present even in the fourth generation of untreated offspring. However, treatment with 5-azaC not only altered overall phenotypic variability but also the apparent variability of genotypic means of some traits. This has immediate implications for our understanding of evolutionary processes, as measures of genotypic variation are commonly used for estimating broad-sense heritability, one of the key parameters for predicting microevolution (e.g. Falconer and Mackay 1996). Thus, epigenetic variation can alter estimates of evolutionary relevant phenotypic variation and of evolutionary potential.

Altering DNA methylation in *Arabidopsis* not only changed the means and variability of ecologically important traits, but also their phenotypic plasticity. Demethylation decreased the overall plasticity of plants to nutrient addition with regard to several morphological parameters, and it substantially increased the plasticity of flowering time. We are only aware of one previous study that experimentally tested for the effects of variation in DNA methylation on plant phenotypic plasticity: Tatra et al. (2000) subjected two ecotypes of *Stellaria longipes* to a factorial combination of light and 5-azaC and found that the experimental demethylation significantly altered plants' response to light. In our study, as well as in the one by Tatra et al. (2000), there were significant differences between different genotypes in the way that demethylation affected phenotypic plasticity (significant three-way interaction between genotype, demethylation, and environmental treatment), which suggests that genotype and epigenotype may interact in complex ways to determine phenotypic plasticity.

In summary, epigenetic changes such as reduced DNA methylation can have a dramatic impact on the mean, variability and plasticity of ecologically important plant traits. Epigenetic variation may thus be an overlooked factor in the evolutionary ecology of plant populations.

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