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Epigenetic variation creates potential for evolution of plant phenotypic plasticity

Yuan-Ye Zhang[1], Markus Fischer[1], Vincent Colot[2] and Oliver Bossdorf[1]

[1]Institute of Plant Sciences, University of Bern, Altenbergrain 21, CH-3013, Bern, Switzerland; [2]Institut de Biologie de l'Ecole Normale Supérieure, Centre National de la Recherche Scientifique Unité Mixte de Recherche 8197, Institut National de la Santé et de la Recherche Médicale Unité 1024, Paris F-75005, France

Author for correspondence:
Yuan-Ye Zhang
Tel: +41 31 631 4927
Email: zhang@ips.unibe.ch

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Summary

- Heritable variation in plant phenotypes, and thus potential for evolutionary change, can in principle not only be caused by variation in DNA sequence, but also by underlying epigenetic variation. However, the potential scope of such phenotypic effects and their evolutionary significance are largely unexplored.
- Here, we conducted a glasshouse experiment in which we tested the response of a large number of epigenetic recombinant inbred lines (epiRILs) of *Arabidopsis thaliana* – lines that are nearly isogenic but highly variable at the level of DNA methylation – to drought and increased nutrient conditions.
- We found significant heritable variation among epiRILs both in the means of several ecologically important plant traits and in their plasticities to drought and nutrients. Significant selection gradients, that is, fitness correlations, of several mean traits and plasticities suggest that selection could act on this epigenetically based phenotypic variation.
- Our study provides evidence that variation in DNA methylation can cause substantial heritable variation of ecologically important plant traits, including root allocation, drought tolerance and nutrient plasticity, and that rapid evolution based on epigenetic variation alone should thus be possible.

Introduction

A key question in ecology and evolution is to what degree variation in ecologically important traits is heritable, because heritability determines the potential for evolutionary change of traits (Fisher, 1930; Falconer & MacKay, 1996), and thus their ability to adapt to changing environments (Visser, 2008; Hoffmann & Sgro, 2011).

One particularly important class of ecologically important traits is phenotypic plasticity, the ability of a genotype to express different phenotypes in different environments (Pigliucci, 2005). This ability is considered particularly important for sessile organisms, such as plants, to adjust to spatial and temporal environmental heterogeneity (van Kleunen & Fischer, 2005; Pigliucci, 2005). Many kinds of plant phenotypic plasticity, such as induced resistance to herbivores or pathogens (Strauss & Agrawal, 1999; Weinig *et al.*, 2003), morphological and physiological responses to drought (Heschel *et al.*, 2004), or the response of root morphology to different nutrient conditions (Hodge, 2004), are considered to be adaptive and active reactions to the environment (Dorn *et al.*, 2000). Like most other traits, phenotypic plasticity is often highly variable in natural populations, (Pigliucci & Kolodynska, 2002; Bossdorf & Pigliucci, 2009), and it may evolve rapidly (Scheiner, 1993; Reboud & Bell, 1997; van Kleunen & Fischer, 2001, 2003).

Many traits of ecological importance, such as flowering time, yield, herbivore resistance and drought tolerance, are complex traits which are commonly thought to be determined by the joint action of, as well as interactions between, multiple genes (Lynch & Walsh, 1998). In addition, recent research indicates that heritable variation in ecologically important traits may also be caused by underlying epigenetic variation (Richards, 2011). Epigenetics is concerned with a suite of interacting molecular mechanisms that alter gene expression and function without changes in DNA sequence (Richards, 2006; Bird, 2007). In eukaryotes, these include chemical modification of DNA and histones, incorporation of histone variants and small or long noncoding RNAs (Grant-Downton & Dickinson, 2005; Rapp & Wendel, 2005; Berger, 2007). Out of these several mechanisms, DNA methylation is currently the best understood one. There is some limited evidence for naturally occurring single-locus DNA methylation variants (epialleles) that are transgenerationally stable and independent of DNA sequence variation (Cubas *et al.*, 1999; Morgan *et al.*, 1999; Rakyan *et al.*, 2003; Manning *et al.*, 2006), as well as for natural epigenetic variation among ecotypes of *Arabidopsis thaliana* (Cervera *et al.*, 2002; Vaughn *et al.*, 2007) and other plant species (Salmon *et al.*, 2005, 2008; Keyte *et al.*, 2006; Raj *et al.*, 2011). However, we still know little about the potential phenotypic and ecological consequences of epigenetic variation.

One complication in testing for phenotypic effects of epigenetic variation is that DNA sequence variation and epigenetic variation covary in most natural systems (Koornneef *et al.*, 2004), which makes it difficult to disentangle their effects on phenotypes (Johannes *et al.*, 2008; Richards, 2009; Richards *et al.*, 2010). However, there are several ways in which this problem may be avoided. First, one can study natural epialleles. Secondly, one can manipulate DNA methylation using chemical demethylation agents such as 5-azacytidine, and study the consequences (Bossdorf *et al.*, 2010). Thirdly, one can study systems that naturally lack DNA sequence variation, such as genetically uniform clonal plant species (e.g. Gao *et al.*, 2010; Raj *et al.*, 2011), or apomicts (e.g. Verhoeven *et al.*, 2010). A fourth possibility is the study of epigenetic recombinant inbred lines (epiRILs).

EpiRILs are lines that have been created through artificial crossings, and that are highly variable at the epigenetic level, but nearly identical at the DNA sequence level. Two sets of such epiRILs have been created in *A. thaliana* by crossing two near-isogenic parental lines, a methylation mutant and its wild type (Johannes *et al.*, 2009; Reinders *et al.*, 2009). The mutant is deficient in the DNA methylation machinery and, as a consequence, shows a genome-wide reduction of DNA methylation compared with the wild type. The resulting epiRILs are almost identical in DNA sequence, but inherit, through recombination, different DNA methylation patterns. Molecular analyses showed that these methylation patterns are surprisingly stable across many generations, and preliminary phenotypic analyses found the epiRILs to be significantly differentiated phenotypically (Johannes *et al.*, 2009; Reinders *et al.*, 2009). Thus, epiRILs are a powerful tool for proof-of-principle studies of the potential ecological and evolutionary consequences of plant epigenetic variation.

Here, we conducted a glasshouse experiment in which we subjected a large number of epiRILs of *A. thaliana* to different environmental conditions. In contrast to the few previous studies of epiRILs, we examined a broader range of ecologically important traits, including root allocation and different kinds of phenotypic plasticity, and we used quantitative genetics methods to estimate both heritabilities of traits and the direction and strength of selection acting on them. Specifically, we asked the following questions. How does epigenetic variation affect ecologically important phenotypic traits and their plasticities? Does epigenetic variation provide potential for microevolutionary change?

Materials and Methods

Plant material

Arabidopsis thaliana (L.) Heynh. (Brassicaceae) is a small and predominantly selfing annual weed typically growing in ruderal habitats such as fields, open spaces, and disturbed areas. *A. thaliana* has long been a model species for plant molecular and genetics studies (Pigliucci, 2002). Previous research has demonstrated a substantial amount of natural variation in ecologically important traits (Ungerer *et al.*, 2002; McKay *et al.*, 2003), including phenotypic plasticities (Dorn *et al.*, 2000; Ungerer *et al.*, 2003; Stinchcombe *et al.*, 2004; Bossdorf & Pigliucci, 2009).

In our study, we worked with epiRILs of *A. thaliana*. The construction of these lines has been described in detail in Johannes *et al.* (2009). Briefly, the epiRILs were created by crossing the methylation deficiency point mutant *Col-ddm1* with the *Col-wt*. These two parental lines were near-isogenic, but differed greatly in their overall methylation levels. The F1 generation was backcrossed with *Col-wt*, and F2 plants homozygous at DDM1 were then selected and and selfed for multiple generations to create epiRILs. They were found to have almost identical DNA sequences but to differ greatly in their heritable patterns of DNA methylation (Johannes *et al.*, 2009). Preliminary analysis of the epiRILs showed significant heritabilities for flowering time and plant height. To assess the potential influence of spontaneous mutations, there were also 24 control lines established along with the epiRILs, where the *Col-ddm1* parent was replaced by *Col-wt* and the offspring were subjected to the same multiple generations of inbreeding. These lines were initially nearly identical in both DNA sequence and DNA methylation. Variation among control lines must therefore result either from spontaneous mutations or from other hidden systematic influences that create line variation, such as biases caused by environmental heterogeneity and nonrandom spatial arrangement, or differently experienced transplanters. We thus used the control lines to estimate the effects of all other possible influences except for variation in DNA methylation.

For our experiment, we used a total of 135 epiRILs and 24 control lines of the F8 generation. The 135 epiRILs consisted of a sample of 75 lines that were randomly selected from all 505 epiRILs, plus another 60 lines that we included because their epigenomes were sequenced in another project and phenotypic information on these lines was thus very useful. The inclusion of the nonrandom set caused very little bias in our analyses. Except for the analyses of flowering time (see Results), results based on all 135 lines never differed from those based on the 75 randomly selected lines only. In the remainder of this paper, we therefore present the results of the analyses of all 135 epiRILs.

Experimental design

To test for differentiation in ecologically important traits among epiRILs and control lines, we carried out a glasshouse experiment in which we subjected these lines to three different environmental treatments: drought, increased nutrient availability, and control conditions. In May 2010, we sowed seeds on peat moss. After 4 d of stratification at 4°C, we transferred them to a long-day room growth chamber (16-h day; 20°C; 40% humidity) for germination. Ten days later, we moved the seedlings to an unheated glasshouse where we transplanted them individually into 0.25-l pots filled with a 3 : 1 mixture of sand and field soil. We initially planted six replicates per line and treatment, a total of 2862 plants. Plants that did not survive transplanting were replaced during the first week. One epiRIL and one control line with extremely low germination success were excluded from the experiment. After some mortality, the pots were reassigned to

treatments, with a preference for control and drought treatments, to ensure adequate replication for these two treatments. As a consequence, the nutrient treatment included plants from only 119 epiRILs. The plants were distributed across six glasshouse tables, with two glasshouse tables per treatment and two or three replicates per line per table. The pots were randomly arranged on the tables, with a minimum distance of 10 cm between pots.

Two weeks after transplanting, we started the experimental treatments. In the control treatment the tables were flooded every 2–3 d, depending on the weather conditions, whereas in the drought treatment the tables were only flooded when around half of the plants showed signs of water stress; that is, they started to wilt. The resulting flooding frequency in the drought treatment was about one-third of the control. In the nutrient treatment, watering was the same as in the control, but the water always contained NPK fertilizer (N-P-K: 18-12-18) at a concentration of 500 ppm.

On each plant, we recorded flowering time as the number of days from planting to first flower opening. The plants were then harvested sequentially, each plant *c.* 25 d after its first flowering. On each plant, we measured plant height and counted the number of siliques produced, which is closely correlated with seed production and thus plant fitness (Westerman & Lawrence, 1970; Mauricio, 1998). We cut the aboveground biomass and carefully washed the roots. Shoot and root biomasses were dried for 48 h at 70°C and weighed.

Statistical analyses

We analysed heritable variation in five phenotypic traits: flowering time; plant height; total biomass (shoot plus root biomass); root:shoot ratio (root divided by shoot biomass); and fruit number. For each trait, we first estimated its heritable variation within each of the three experimental treatments, and then the heritable variation of its plasticity in response to drought and nutrient environments. The same analyses were performed for epiRILs and control lines.

Within an environment, an individual phenotype (y_{ijn}) of replicate n of line i (1…134 for epiRILs; 1…23 for control lines) on table j is described as

$$y_{ijn} = \mu + b_i + t_j + e_{ijn} \quad \text{Eqn 1}$$

$$b_i \sim N(0, \sigma^2_L),\ t_j \sim N(0, \sigma^2_t),\ e_{ijn} \sim N(0, \sigma^2_e)$$

(μ, the population mean in that environment; b_i and t_j, the random effects of line and table, respectively; e_{ijn}, the residual.) If we fit this model using the *lmer* function in the *R* package *lme4*, we can obtain estimates of the variances of line ($\hat{\sigma}^2_L$) and table ($\hat{\sigma}^2_t$) effects. The broad-sense heritability (H^2_L) of each trait in an environment can then be calculated as $\hat{\sigma}^2_L$ divided by the total phenotypic variation $\hat{\sigma}^2_p$ in that environment.

Across two different environments, an individual phenotype (y_{ijkn}) of replicate n of line i on table j in response to environment k is described as

$$y_{ijkn} = \mu_1 + \beta_k + b_i + b_{ik} + t_j + e_{ijkn} \quad \text{Eqn 2}$$

$$b_i \sim N(0, \sigma^2_L),\ b_{ik} \sim N(0, \sigma^2_{L\times E}),\ t_j \sim N(0, \sigma^2_t),\ e_{ijn} \sim N(0, \sigma^2_e)$$

(μ_1, the population mean in the control environment; β_k, the main effect of the drought or nutrient environment; b_i and t_j, the random effects of line and table, respectively, with table nested within environment; b_{ik}, the line by environment interaction effect; e_{ijkn}, the residual.) If we fit this model using the *lmer* function in the *R*, we can obtain estimates of the treatment main effect $\hat{\beta}_k$ which describes the average (across lines) deviation of trait means in response to drought or nutrients from the control environment, and estimates of the variance of the line by environment interaction ($\hat{\sigma}^2_{L\times E}$). The broad-sense heritability of plasticity ($H^2_{L\times E}$) can then be calculated as $\hat{\sigma}^2_{L\times E}$ divided by the total phenotypic variance $\hat{\sigma}^2_p$ across environments. We carried out these analyse separately for trait plasticity in response to drought (i.e. using data from the control and drought treatments only) and plasticity in response to nutrients (using data from the control and nutrients treatments).

The significance of each fixed or random effect was tested through likelihood ratio tests that compared the full model to a model without the respective effect. Standard errors for H^2_L and $H^2_{L\times E}$ were calculated from 3000 parametric bootstrap samples (*simulate* and *refit* functions in *lme4*). Finally, whenever variance estimates for the control lines were > 0, we used *F*-tests to compare the magnitudes of $\hat{\sigma}^2_L$ and $\hat{\sigma}^2_{L\times E}$ estimates between epiRILs and control lines.

To obtain an idea of the consistency of our results with the initial study of Johannes *et al.* (2009), we calculated Pearson's correlation coefficients for the line means of flowering time and plant height in our control treatments vs the line means of the same traits in Johannes *et al.* (2009).

Next, we tested for phenotypic selection on traits within each environment by regressing the line means of our fitness proxy, fruit number, on the line means of the respective trait in an environment. For flowering time, plant height, total biomass, and root:shoot ratio, we fitted the following function:

$$W \sim S \cdot Z \quad \text{Eqn 3}$$

$$W \sim \beta_{s1} \cdot Z + \frac{1}{2} \cdot \beta_{s2} \cdot Z^2 \quad \text{Eqn 4}$$

(W, the relative line mean fitness in an environment; Z, the standardized line mean of the trait value in that environment.) The regression coefficient in Eqn 3 is equivalent to the selection differential S, which measures the covariance between fitness and the trait. If $S \neq 0$, this indicates either positive ($S > 0$) or negative ($S < 0$) directional selection. In the second regression, a quadratic term is included to test for possible nonlinearity of selection (partial regression coefficient $\beta_{s2} \neq 0$ in Eqn 4). To avoid underestimating the strength of stabilizing or disruptive selection, the quadratic term is divided by 2 (Stinchcombe *et al.*, 2008).

To test for selection on trait plasticities we used the following equation:

$$W \sim \beta_{pl1} \cdot Z + \beta_{pl2} \cdot plZ \qquad \text{Eqn 5}$$

(W, the average relative line fitness across the two environments (i.e. control and drought, or control and nutrients); Z, the standardized line mean of a trait averaged over the two environments; plZ, a standardized measure of plasticity (Scheiner & Berrigan, 1998).) For each trait, plZ is calculated as the line mean in the drought (or nutrients) treatment minus the line mean in the controls. Plasticity will be adaptive if it positively contributes to the cross-environmental fitness ($\beta_{pl2} \cdot plZ > 0$); that is, if a treatment increases the trait value ($plZ > 0$), $\beta_{pl2} > 0$ means that plasticity is adaptive, whereas if a treatment decreases the trait value, $\beta_{pl2} < 0$ indicates adaptive plasticity.

There were six epiRILs which consistently produced many small and apparently sterile fruits. As fruit number appeared to be an inadequate estimate of fitness in these lines, we excluded them from the selection analyses.

Results

We found significant line effects, indicating heritable variation among lines, for the epiRILs in all traits and environments except for root:shoot ratio in the drought environment (Table 1). Line effects were often nonzero also for the control lines, but the line effect variances of epiRILs were usually several times larger, and always significantly so, than those of the control lines (Table 1; see also Supporting Information Fig. S1, which shows the trait distributions). The broad-sense heritabilities of phenotypic traits (H_L^2) estimated for epiRILs within each environment ranged from 0.07 to 0.46, with averages of 0.31, 0.22, and 0.34 in the control, drought and nutrient environments, respectively (Fig. 1). For the control lines, these values were much lower and ranged from 0.00 to 0.17, with averages of 0.07, 0.08, and 0.09 in the three treatments, respectively. When we restricted our analyses to the randomly selected epiRILs only, the average heritability of flowering time was lower ($H_L^2 = 0.17$) than when estimates were based on all 135 epiRILs ($H_L^2 = 0.24$).

The drought treatment significantly affected all phenotypic traits except for flowering time. It decreased plant height, fruit number and biomass, but increased the root:shoot ratio (Table 2). These effects were very similar for epiRILs and control lines. The nutrient treatment, by contrast, had very little effect on the mean plant phenotypes (Table 2).

We observed significant heritable variation in phenotypic plasticity, estimated as the variance of the line by treatment interactions ($\sigma^2_{L\times E}$), for biomass, fruit number and root:shoot ratio of epiRILs in response to drought, and for flowering time and fruit number of epiRILs in response to nutrients (Table 2). The broad-sense heritabilities of phenotypic plasticity ($H^2_{L\times E}$) were lower than those of the trait means and ranged from 0.01 to 0.10 (Fig. 2). We observed no significant variation in phenotypic

Table 1 Summary of mixed model analyses of the phenotypic variation of 134 epigenetic recombinant inbred lines (epiRILs) and 23 control lines (Ctr lines) of *Arabidopsis thaliana* within each environment

		Means $\hat{\mu}$		Variances of line effects $\hat{\sigma}^2_L$			Variances of table effects $\hat{\sigma}^2_t$		Residual variances $\hat{\sigma}^2_e$	
	Trait	EpiRILs	Ctr lines	EpiRILs	Ctr lines	*F*-test	EpiRILs	Ctr lines	EpiRILs	Ctr lines
Control	Flowering time	29.6	30.4	0.94***	0.27^{NS}	3.54***	0.00^{NS}	0.00^{NS}	3.54	2.21
	Plant height	22.7	23.0	3.68***	0.64^{NS}	5.72***	0.46***	0.00^{NS}	6.71	3.88
	Fruit number	120	121	820***	0.00^{NS}	–	88.6***	19.4^{NS}	1286	859
	Total biomass	268	278	4345***	131^{NS}	33.27***	2355***	0.00^{NS}	6686	5764
	Root:shoot ratio ($\times 10^2$)	6.8	5.1	0.76***	0.28^{a}	2.70**	0.47***	0.00^{NS}	3.94	1.38
Drought	Flowering time	29.8	30.4	0.85***	0.13^{NS}	6.54***	0.22***	0.23^{NS}	3.15	2.95
	Plant height	15.4	15.3	2.11***	0.52^{NS}	4.06***	0.95***	1.58**	4.47	4.66
	Fruit number	42.0	39.6	85.2***	15.4^{NS}	5.52***	238***	186***	241	137
	Total biomass	110	107	613***	172^{NS}	3.57***	443***	886***	1346	1073
	Root:shoot ratio ($\times 10^2$)	9.2	7.1	1.58^{NS}	0.34^{NS}	4.62***	0.20^{NS}	0.03^{NS}	15.94	4.61
Nutrients	Flowering time	29.6	30.3	1.10***	0.34^{a}	3.23**	0.13**	0.18^{NS}	2.40	1.96
	Plant height	23.2	23.9	4.32***	0.39^{NS}	10.96***	0.21**	0.09^{NS}	4.95	4.81
	Fruit number	135	142	954***	81.5^{NS}	11.71***	14.5^{NS}	187^{a}	1505	1200
	Total biomass	304	326	4588***	833^{NS}	5.51***	1230***	0.00^{NS}	7278	6552
	Root:shoot ratio ($\times 10^2$)	7.1	5.6	0.52*	0.19^{NS}	2.79**	1.25***	1.03***	6.06	2.85

For each trait in each environment, the model estimates the trait means (intercept), as well as the random effects of lines and tables (see Eqn 1). Significance levels of variances are from likelihood-ratio tests. *F*-tests test whether the variances of the line effects differ between epiRILs and control lines.
Units in the means columns are days for flowering time, cm for plant height, and mg for total biomass.
Significance levels: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.

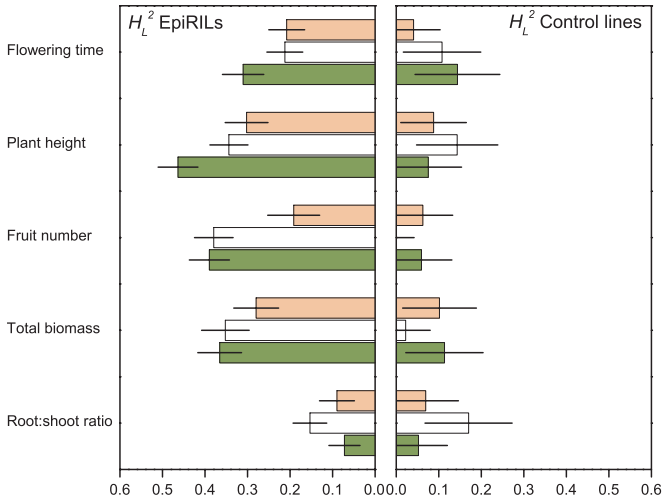

Fig. 1 Broad-sense heritabilities (H_L^2) ± SE of phenotypic traits in the control (white), drought (yellow) and nutrient addition (green) treatments, estimated for 134 epigenetic recombinant inbred lines (epiRILs) (left panel) and 23 control lines (right panel) of *Arabidopsis thaliana*.

plasticity in the control lines, with variances and heritability estimates close to zero for all traits (Table 2, Fig. 2).

The observed phenotypic variation among epiRILs appeared to be robust and repeatable across experiments. The line means of flowering time and plant height in the control treatment correlated with the data of Johannes *et al.*, 2009 ($r = 0.520$; $P < 0.001$ for flowering time; $r = 0.459$; $P < 0.001$ for plant height). Compared with the study of Johannes *et al.* (2009), the plants in our study generally flowered earlier, and there was less variation in flowering time among epiRILs. This is probably a consequence of the fact that from an *A. thaliana* point of view we conducted our experiment rather late in the season, and that the plants in our study experienced higher temperatures. This generally accelerated and homogenized phenologies.

We observed a significant positive selection coefficient (S) for plant height and total biomass in all environments, and a significant negative S for root:shoot ratio in the control treatment (Table 3), indicating that high root allocation is selected against under normal growth conditions. We also observed significant partial regression coefficients for the quadratic term, indicating a nonlinear shape of the selection function, for plant height in all environments, biomass in the nutrient treatment, and root:shoot ratio in the drought treatment (Table 3). Finally, we found significant selection on the plasticities of plant height and root:shoot ratio in response to drought, where stronger decreases of plant height and stronger increases of root:shoot ratio under drought are associated with higher fitness. There was no evidence of selection on plasticity to nutrients (Table 4).

Discussion

If epigenetic variation alone can cause heritable variation in ecologically important traits, then it could well be a significant, but hitherto overlooked, factor in the evolution and adaptation of populations, and their responses to environmental change. Here, we studied the responses of a set of epiRILs (lines with near-identical genomes but contrasted DNA methylation patterns) of *A. thaliana* to experimental drought and nutrient addition. We found substantial heritable variation among epiRILs in traits of ecological importance as well as their plasticities, and that selection could act on at least some of this variation, suggesting that DNA methylation variation alone can provide the potential for microevolution of plants.

Table 2 Summary of mixed model analyses of the phenotypic variation of 134 epigenetic recombinant inbred lines (epiRILs) and 23 control lines (Ctr lines) of *Arabidopsis thaliana* across two environments (control and drought, or control and nutrients)

		Environmental treatment effects $\hat{\beta}_k$		Variances of line effects $\hat{\sigma}_L^2$		Variances of plasticities $\hat{\sigma}_{L\times E}^2$			Variances of table effects $\hat{\sigma}_t^2$		Residual variances σ_e^2	
	Trait	EpiRILs	Ctr lines	EpiRILs	Ctr lines	EpiRILs	Ctr lines	*F*-test	EpiRILs	Ctr lines	EpiRILs	Ctr lines
Response to drought	Flowering time	0.2^{NS}	0.0^{NS}	0.67***	0.31*	0.22^{a}	0.00^{NS}	–	0.11***	0.11^{NS}	3.36	2.47
	Plant height	−7.3***	−7.6***	2.67***	0.60*	0.29^{a}	0.00^{NS}	–	0.70***	0.76**	5.61	4.25
	Fruit number	−78***	−82***	221***	17.9^{NS}	236***	5.75^{NS}	41.16***	162***	101***	775	491
	Total biomass	−155**	−171***	1187***	196^{NS}	1312***	0.00^{NS}	–	1405***	420*	4143	3455
	Root:shoot ratio (×10^2)	2.4**	2.0***	0.30^{NS}	0.36^{a}	0.89**	0.00^{NS}	–	0.34***	0.00^{NS}	9.67	2.91
Reponse to nutrients	Flowering time	-0.0^{NS}	-0.1^{NS}	0.83***	0.32*	0.21*	0.00^{NS}	–	0.07*	0.06^{NS}	3.00	2.07
	Plant height	0.5^{NS}	0.9*	3.85***	0.62*	0.10^{NS}	0.00^{NS}	–	0.35***	0.02^{NS}	5.89	4.24
	Fruit number	13^{a}	21*	730***	88.6^{a}	143**	0.00^{NS}	–	54.5***	99.8*	1390	977
	Total biomass	31.8^{NS}	49.4**	4131***	369^{NS}	286^{NS}	0.00^{NS}	–	1838***	0.00^{NS}	6961	6247
	Root:shoot ratio (×10^2)	0.4^{NS}	0.5^{NS}	0.74***	0.22^{NS}	0.00^{NS}	0.03^{NS}	–	0.88***	0.49***	4.83	2.10

For each trait, the model estimates the main effect of the treatment, the variances of the random effects of lines and tables, and the variance of the line by treatment interaction (= variance of plasticity; see Eqn 2). Significance levels of variances are from likelihood-ratio tests. *F*-tests test whether the variances differ between epiRILs and control lines.
Units in the treatment main effect are days for flowering time, cm for plant height, and mg for total biomass.
Significance levels: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.

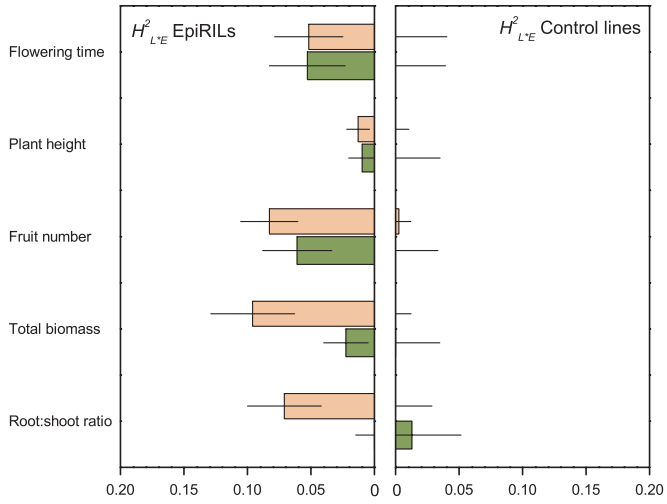

Fig. 2 Broad-sense heritabilities of trait plasticities ($H^2_{L\times E}$) ± SE in response to drought (yellow) and nutrients (green) estimated for 134 epigenetic recombinant inbred lines (epiRILs) (left panel) or 23 control lines (right panel) of *Arabidopsis thaliana*.

Heritability of traits and their plasticities

We found significant variance components as well as heritabilities in several phenotypic traits, including flowering time, plant height and total biomass, fruit number, and root:shoot ratio. This result agrees with those of several previous epiRIL studies which also found significant heritabilities of phenotypic traits (Johannes *et al.*, 2009; Roux *et al.*, 2011). Given the extent to which heritability estimates are influenced by the environments in which they are measured (Lynch & Walsh, 1998), heritabilities are surprisingly consistent across studies for those few traits that have now been measured repeatedly in several studies, for example, flowering time $H^2 = 0.24$ in our study and $H^2 = 0.26$ in Johannes *et al.* (2009). If we compare our heritability estimates with those obtained in studies of classical RILs or natural accessions of *A. thaliana*, then the heritabilities of flowering time and plant height seem to be lower in epiRILs, but for other traits, for example, fruit number, they are comparable or even higher than estimates from RILs or natural accessions (Ungerer *et al.*, 2002; Johannes *et al.*, 2009; Roux *et al.*, 2011).

We found that, in contrast to the initial study of Johannes *et al.* (2009), heritability estimates for the control lines were not zero (and variances of line effects even significant or marginally significant in a few cases). It is unlikely that this variation among control lines results from spontaneous mutations, but it probably also reflects other hidden systematic influences. For instance, during the setting up of the experiment, seedlings were planted by different transplanters, one line at a time. This may have caused some systematic differences among lines. We also know that there is environmental heterogeneity within the glasshouse that is not captured by the table effect, and we cannot rule out that this, too, influenced line variation. However, the differences among control lines do not compromise our study. The key question is whether line variation among epiRILs (effects of epigenetic variation plus spontaneous mutations plus other possible influences) is greater than that among the control lines (spontaneous mutations plus other possible influences), and this is consistently the case in our analyses.

One of the novel aspects of our study is that we also estimated variance components and heritabilities of trait plasticities in response to drought and nutrient addition, and for many traits we indeed found significant variation of plasticity among epiRILs. The magnitude of the variation of plasticity was generally larger for plasticity to drought than for nutrient plasticity, which could be explained by a low effect size of nutrient addition to an already nutrient-rich substrate. One general consistency with previous studies of genetic systems is that heritabilities of trait plasticities were usually lower than those of trait means (Scheiner, 1993; Agrawal *et al.*, 2002; Lacaze *et al.*, 2009). The exception was the root:shoot ratio, where variation in plasticity to drought was of a similar order of magnitude to the variation in the trait mean.

The phenotypic plasticity of a trait is a trait in itself which can vary and evolve independently of a trait mean (Scheiner, 1993;

Table 3 Phenotypic selection within each treatment in 128 (for nutrients: 113) epigenetic recombinant inbred lines (epiRILs) of *Arabidopsis thaliana*

Environment	Trait	*S*	*SE*	*P*	β_{s2}	*SE*	*P*
Control	Flowering time	−0.052	0.019	0.006	−0.031	0.025	0.223
	Plant height	0.083	0.018	**< 0.001**	−0.093	0.022	**0.000**
	Total biomass	0.152	0.014	**< 0.001**	−0.039	0.022	0.082
	Root:shoot ratio	−0.077	0.018	**< 0.001**	−0.052	0.030	0.080
Drought	Flowering time	0.020	0.024	0.405	−0.064	0.034	0.062
	Plant height	0.082	0.023	**< 0.001**	−0.062	0.025	**0.017**
	Total biomass	0.194	0.016	**< 0.001**	0.005	0.020	0.821
	Root:shoot ratio	−0.015	0.024	0.525	−0.080	0.027	**0.004**
Nutrient	Flowering time	0.010	0.017	0.564	−0.012	0.025	0.641
	Plant height	0.066	0.016	**< 0.001**	−0.082	0.018	**< 0.001**
	Total biomass	0.101	0.014	**< 0.001**	−0.049	0.017	**0.005**
	Root:shoot ratio	−0.006	0.017	0.735	−0.020	0.026	0.428

S is the linear selection coefficient from a regression of fitness against the mean trait value (Eqn 3); β_{s2} is the partial regression coefficient (Eqn 4) testing for nonlinear selection on a trait.
Significant *P*-values are in bold.

Table 4 Phenotypic selection on trait plasticities in response to drought and nutrient addition among 128 (for nutrients: 113) epigenetic recombinant inbred lines of *Arabidopsis thaliana* in a glasshouse experiment

	Plasticity to drought				Plasticity to nutrient			
Trait	β_{pl2}	*SE*	*P*	*plZ*	β_{pl2}	*SE*	*P*	*plZ*
Flowering time	−0.002	0.016	0.907	+	0.026	0.014	0.070	−
Plant height	−0.036	0.014	**0.013**	−	0.013	0.013	0.320	+
Total biomass	0.010	0.016	0.523	−	−0.005	0.011	0.663	+
Root:shoot ratio	0.068	0.019	**0.001**	+	0.022	0.014	0.114	+

β_{pl2}, selection gradient; *SE*, standard error; *plZ*, direction of plasticity (+/− = increase/decrease of trait in response to treatment). Note that plasticity is adaptive if *plZ* > 0 and $\beta_{pl2} > 0$, or if *plZ* < 0 *and* $\beta_{pl2} < 0$. Significant *P* values are in bold.

Pigliucci, 2005). Our study suggests that potential for evolution of plasticity can also be created by epigenetic variation. The logical next step will be to use molecular analyses and quantitative trait locus (QTL) mapping methods to search for the underlying DNA methylation loci associated with the observed variation in plasticity, similar to the search for 'plasticity genes' in genetic RILs or natural populations (Ungerer *et al.*, 2003; Juenger *et al.*, 2005). This will be particularly interesting for the plasticity of the root:shoot ratio in response to drought, as the root:shoot ratio is a key trait in crop breeding for drought tolerance (Manickavelu *et al.*, 2006; Reynolds *et al.*, 2007).

Phenotypic selection

Besides heritability, the other prerequisite for microevolution to be predicted in a phenotypic trait is that natural selection acts on it; that is, different heritable phenotypes must differ in their fitness (Endler, 1986; Conner & Hartl, 2004). We found significant covariances between phenotype and the fitness proxy, fruit number, for several of the measured traits in epiRILs: directional positive selection on plant biomass and height, and negative selection on root:shoot ratio in the control environment. The strength of these selection estimates tended to be somewhat lower than estimates typically found in natural populations (Scheiner & Callahan, 1999; Scheiner *et al.*, 2000). Nevertheless, if we combine the observed estimates for selection and heritability, then we should expect populations of multiple epiRILs to evolve towards increased biomass and plant height and, to a lesser extent (because of lower heritability estimates) and only under specific environmental conditions, towards decreased root:shoot ratio.

In addition to selection on trait under specific environmental conditions, we also found evidence that natural selection could act on the plasticity variation of epiRILs. While there was no selection on plasticity in response to nutrient addition, we found positive selection on the plasticities of plant height and root:shoot ratio in response to drought. Across lines, stronger decreases of plant height and increases of root:shoot ratio in response to drought were consistently associated with a higher fitness. This is consistent with previous work that found increased root allocation to be a common strategy of plants to cope with drought stress, because it improves access to the limiting water resource (e.g. Kamoshita *et al.*, 2002; Kozlowski & Pallardy, 2002; Dhanda *et al.*, 2004). Here, as there was also significant heritable variation among epiRILs in plasticities of the root:shoot ratio, we can again combine the evidence and predict that, in heterogeneous environments with (equally frequent) drought and nondrought periods, epiRIL populations should evolve towards increased plasticity of the root:shoot ratio.

Numerous previous studies of *A. thaliana* as well as other plant species have shown that the phenotypic plasticity of plant traits can be under selection, and that short-term evolution of plasticity can be observed in multi-generation experiments (Scheiner, 1993; Reboud & Bell, 1997; van Kleunen & Fischer, 2003; Callahan & Pigliucci, 2005). Our study indicates that evolution of phenotypic plasticity may also be possible on a purely epigenetic basis.

What is the underlying mechanism?

Having established that there is substantial heritable variation among epiRILs in several important phenotypic traits, a crucial question is which mechanism underlies this variation. Nonzero heritability estimates in the control lines suggest that part of the variation is probably attributable to spontaneous mutation or other hidden systematic influences, but it is only a minor part, as variance components and heritability estimates are much larger for epiRILs than for control lines. The only difference between control lines and epiRILs is that the former are derived from a cross between two epigenetically distinct (but near-isogenic) lines, while the latter are derived from a cross between two near-isogenic lines. Therefore, only two possible explanations for the higher variability of epiRILs remain: the phenotypic variation is created by underlying DNA methylation variation resulting from a segregation of the two distinct epigenomes, or it reflects DNA sequence variation resulting from increased transposon activity induced by the DNA methylation changes. We know that there is increased activity of transposable elements in the *ddm1* parent (Miura *et al.*, 2001; Singer *et al.*, 2001), and some of the transposable elements appear to maintain an increased activity in the epiRILs (Johannes *et al.*, 2009). However, preliminary observations indicate that DNA sequence differences caused by this are several orders of magnitude smaller than the variability of DNA methylation among epiRILs (V. Colot *et al.*, unpublished observations), and we therefore consider an epigenetic basis of the observed phenotypic variation to be the most likely and most parsimonious explanation. Detailed molecular analyses coupled with QTL mapping approaches should provide definitive answers about the respective

contributions of the two potential sources of phenotypic variation in the epiRIL population.

Conclusions

Our proof-of-principle study demonstrates that variation in DNA methylation can cause substantial heritability in ecologically important plant traits and their plasticities. Because there is selection acting on some of this variation, we predict rapid phenotypic evolution in this epigenetically based system. Our results suggest that phenotypic plasticity is not only – as commonly defined – the property of a genotype, but also that of an epigenotype. It is particularly interesting that we found strong variation in root:shoot ratio plasticity and drought responses among epiRILs, because these are key traits in crop breeding and plant adaptation to climate change. An important next step will be to identify the specific epigenomic regions underlying this variation. Another important question concerns the relative importance of DNA sequence vs epigenetic influences on plant phenotypes, at levels of natural variation, which can only be answered using natural accessions.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Histograms of line means of 135 epigenetic recombinant inbred lines (epiRILs) and 23 control lines for different traits measured in the control treatment.

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