The Plant Cell, Vol. 27: 337–348, February 2015, www.plantcell.org

Epigenetic Basis of Morphological Variation and Phenotypic Plasticity in *Arabidopsis thaliana*

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Epigenetics is receiving growing attention in the plant science community. Epigenetic modifications are thought to play a particularly important role in fluctuating environments. It is hypothesized that epigenetics contributes to plant phenotypic plasticity because epigenetic modifications, in contrast to DNA sequence variation, are more likely to be reversible. The population of *decrease in DNA methylation 1-2* (*ddm1-2*)-derived epigenetic recombinant inbred lines (epiRILs) in *Arabidopsis thaliana* is well suited for studying this hypothesis, as DNA methylation differences are maximized and DNA sequence variation is minimized. Here, we report on the extensive heritable epigenetic variation in plant growth and morphology in neutral and saline conditions detected among the epiRILs. Plant performance, in terms of branching and leaf area, was both reduced and enhanced by different quantitative trait loci (QTLs) in the *ddm1-2* inherited epigenotypes. The variation in plasticity associated significantly with certain genomic regions in which the *ddm1-2* inherited epigenotypes caused an increased sensitivity to environmental changes, probably due to impaired genetic regulation in the epiRILs. Many of the QTLs for morphology and plasticity overlapped, suggesting major pleiotropic effects. These findings indicate that epigenetics contributes substantially to variation in plant growth, morphology, and plasticity, especially under stress conditions.

INTRODUCTION

Epigenetics is thought to be one of the reasons why genome-wide association studies fail to explain a substantial part of the heritable variation within species (Johannes et al., 2008; Bergelson and Roux, 2010; Korte and Farlow, 2013). DNA methylation, together with other chromatin modifications, is most often associated with silencing of transposable elements (TEs), and when present in *cis*-regulatory regions, with reduced gene expression. Although DNA methylation and demethylation may occur spontaneously during development and in response to a changing environment, epigenetic patterns can be stably inherited through mitosis and meiosis and could thus play a significant role in evolutionary processes (Rapp and Wendel, 2005; Richards, 2006; Baubec et al., 2010; Eichten et al., 2013). When genetic resources are exhausted or genetic diversity within species is low, epigenetic variation could become an important resource for optimizing plant performance (Hauben et al., 2009; Mirouze and Paszkowski, 2011; Springer, 2013).

The genome-wide effects of epigenetic modifications on growth and development under stressful conditions have rarely been studied in detail. One of the main reasons is that the study of natural epigenetic variation is complicated due to the large contribution of DNA sequence variation to phenotypic variation within species. However, recently developed genome-wide bisulphite sequencing in natural and experimental populations of *Arabidopsis thaliana*, soybean (*Glycine max*), and maize (*Zea mays*) may open up new opportunities for studying epigenetic natural variation (Becker et al., 2011; Schmitz et al., 2011, 2013a, 2013b; Eichten et al., 2013). In addition, epigenetic recombinant inbred lines (epiRILs) provide an effective way to circumvent sequence variation. Two such epiRIL populations have been created in Arabidopsis by crossing wild-type Columbia-0 (Col-0) with the epigenetic DNA methylation mutants *decrease in DNA methylation1-2* (*ddm1-2*) or *DNA methyltransferase1* (*met1*) in the same Col-0 background (Johannes et al., 2009; Reinders et al., 2009). Loss of *DDM1* results in a substantial reduction in DNA methylation, an increase in TE transcription, and, although rare, transposition of TEs (Tsukahara et al., 2009). Loss of *MET1* results in almost complete loss of CG methylation and partial loss of non-CG methylation (Stroud et al., 2013). The epiRIL populations consist of nearly isogenic lines (the *ddm1-2* and *met1* mutations have been eliminated by backcrossing and segregation in the F2 progeny) with stretches of DNA being differentially methylated that can be tested in multiple experiments and environments.

The *ddm1-2*-derived epiRIL population has been analyzed for a number of growth-related morphological traits in both neutral and stressful conditions (Johannes et al., 2009; Reinders et al., 2009; Latzel et al., 2012; Zhang et al., 2013). The observed

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www.plantcell.org/cgi/doi/10.1105/tpc.114.133025

variation among the lines was found to be highly heritable, and recently, specific differentially methylated regions (DMRs) were shown to act as epigenetic quantitative trait loci accounting for most of the heritable variation in flowering time and root length (Cortijo et al., 2014). Besides phenotypic variation, phenotypic plasticity is an important property that can be induced or repressed through DNA methylation, as was recently demonstrated in epiRILs (Bossdorf et al., 2010; Mirouze and Paszkowski, 2011; Zhang et al., 2013). Phenotypic plasticity is defined as the ability of a genotype to express alternative phenotypes in different environments (Schlichting, 1986). Phenotypically plastic genotypes are able to display a variety of phenotypes, in both morphology and physiology, in response to changes in the environment and as such can have improved growth and reproduction (Lacaze et al., 2009). It has been proposed that this plasticity is hidden in wild-type plants through DNA methylation, and when unlocked, could be valuable for the improvement of plant performance in unfavorable conditions (Mirouze and Paszkowski, 2011). Indeed, phenotypic plasticity in response to drought and nutrient stress is significantly increased in epiRILs and this increase is heritable, indicating that it could be subjected to selection (Zhang et al., 2013).

In this study, a population of 99 *ddm1-2*-derived Arabidopsis epiRILs was grown under favorable and moderately saline conditions. The population was analyzed for a range of plant growth and morphology-related traits under both conditions. Ample variation between the epiRILs for all traits tested was observed, and this variation was found to be highly heritable. We show here that experimentally induced hypomethylation of chromosomes can render plants more sensitive to environmental variation and more plastic in their responses. DMR-based quantitative trait locus (QTL) mapping revealed many colocating QTLs regulating growth, morphology, and plasticity that were not affected by de novo TE insertions, suggesting pleiotropic regulation via epigenetic mechanisms.

RESULTS

Phenotypic Characterization: Morphological Traits

To assess the impact of DNA methylation on phenotypic variation in shoot growth and morphology, 99 epiRILs and their parents, Col-0 and *ddm1-2*, were analyzed under neutral and moderately saline (25 mM NaCl) conditions. Under saline conditions, the plants were smaller, flowered later, produced fewer branches, and had shorter internodes and inflorescence lengths (Figure 1A; Supplemental Figure 1A). The wild-type Col-0 parent was less affected by moderately saline conditions than its *ddm1-2* counterpart and the majority of epiRILs in almost all traits (Figure 1A; Supplemental Figure 1A), which indicates that DNA hypomethylation leads to higher sensitivity of plants to environmental perturbations. It should be noted that the *ddm1-2* phenotype is severely impaired, especially after numerous generations of inbreeding, and the comparisons between the two parents should therefore be analyzed with caution. We would like to emphasize here, however, that for almost all traits, Col-0 resembled the epiRIL population mean more than *ddm1-2*, and this provides evidence for a stable heritable basis in the epiRILs as it agrees with the expected segregation from a backcross scheme used for the population design (Johannes et al., 2009) (Supplemental Figure 1A). Substantial variation between epiRILs was observed for each of the analyzed traits, although the range of variation was similar under optimal and saline conditions (Table 1). Projected leaf area varied by a factor of five, whereas more than 2 weeks difference occurred between the earliest and latest flowering epiRIL. A 2-fold difference in total plant height was observed and some lines were heavily branched, while others had almost no lateral branches (Figure 1A).

A Spearman rank correlation matrix was constructed to compare the growth and morphology-related traits across the two conditions. Leaf area correlated very well between neutral and saline conditions (r_{LA20} = 0.84), implying that fast-growing epiRILs under control conditions also grow rapidly under saline conditions (Figure 2). All other traits also showed significant positive correlations between neutral and saline conditions. However, these correlations, ranging from 0.33 for total plant height to 0.74 for main stem branching, were much lower than for leaf area (r_{LA20} = 0.84), suggesting differential regulation of traits under control and saline conditions (Figure 2). Large plants produced more main stem branches and longer inflorescences, which suggests that these plants are also superior in terms of reproductive success (Clauss and Aarssen, 1994). However, large plants showed a much lower relative growth rate than small plants later in development, deduced from the highly negative correlation between leaf area and relative growth rate 20 d after germination (r_c = −0.46 and r_s = −0.48) (Figure 2).

Phenotypic Characterization: Plasticity

Phenotypic plasticity was measured for all epiRILs as the absolute difference in average trait values between control and saline conditions. For all traits except relative growth rate, *ddm1-2* showed higher plasticity levels than Col-0, with values predominantly matching the highest and lowest quartile of the population range distribution, respectively (Supplemental Figure 1B). These findings suggest that experimentally induced DNA hypomethylation augments phenotypic plasticity. Moreover, some epiRILs showed increased trait values under saline conditions, whereas others showed decreased trait values, further indicating that epiRIL variation can alter the response to saline conditions (Figure 1A).

The coefficient of variation (CV) was calculated for each epiRIL in both environments to quantify the within-line variation due to residual variation and developmental stability (Sangster et al., 2008). For most traits the average CV was higher under saline than under control conditions, indicating reduced stability in the saline environment (Figure 1B). CV values of the Col-0 parent again predominantly matched the lower quartiles of the population distribution, further supporting the suggestion that DNA methylation buffers phenotypic plasticity (Figure 1B; Supplemental Figure 1C).

QTL Analysis for Morphological Traits

To quantify to what extent the phenotypic variation among the epiRILs was heritable, broad-sense heritability (H^2) was estimated. In general, moderate to high heritability values were observed,

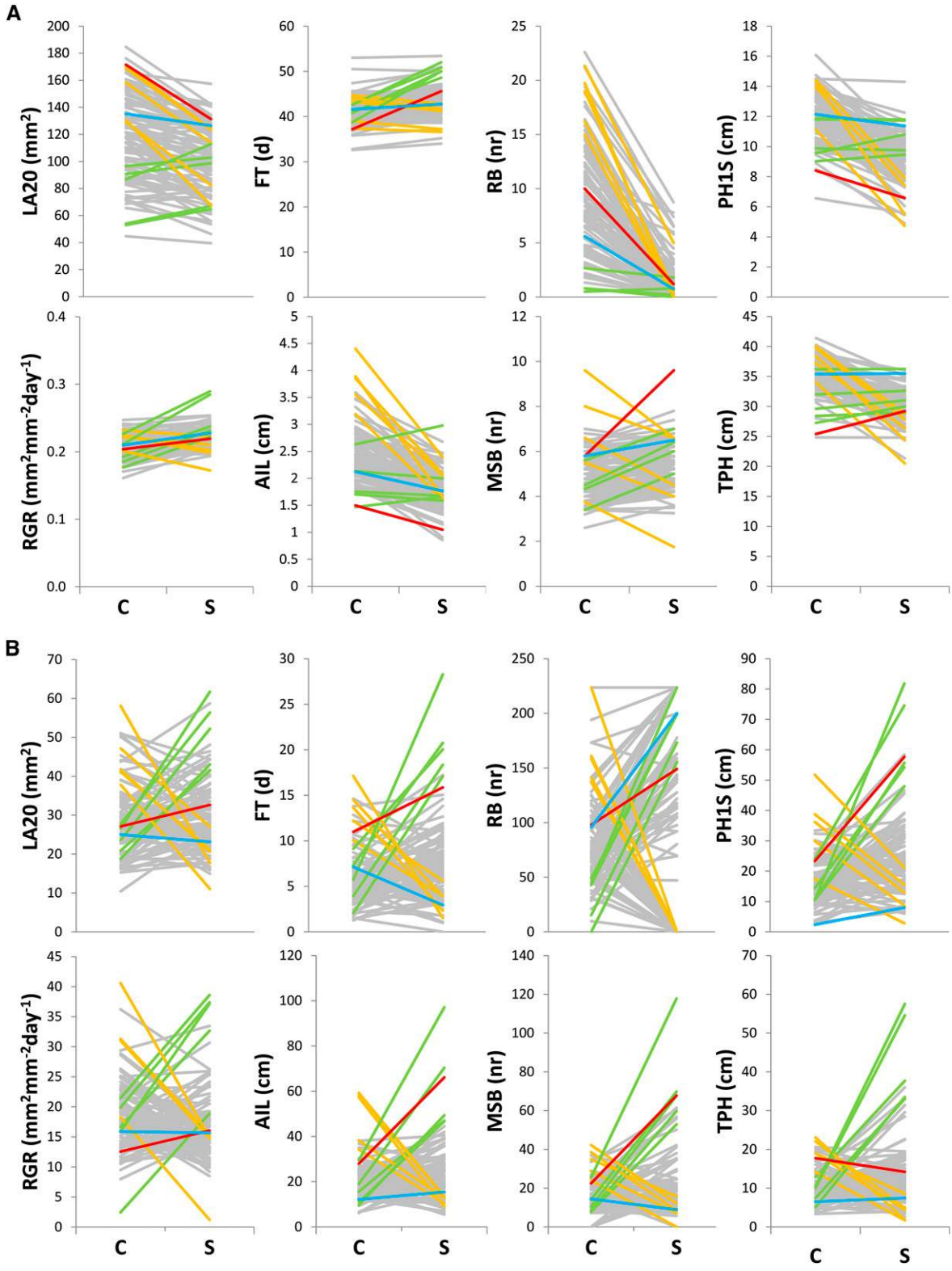

Figure 1. Reaction Norm Plots of epiRIL Morphological Variation and CV.

(A) Reaction norm plots for morphological traits.
(B) Reaction norm plots for CV for all morphological traits.

averaging at 0.37 and ranging from 0.14 to 0.58 for relative growth rate and main stem branching, respectively (Table 1). The H^2 for all traits, with the exception of relative growth rate, was higher under control than under saline conditions (across all traits, 0.41 and 0.33, respectively).

Previously, a genetic map was constructed for the epiRILs using DMRs as physical markers (Colomé-Tatché et al., 2012) (Supplemental Data Set 1). We employed this map to search for QTLs that could account for the heritable variation in the morphological traits. For most traits, at least one QTL was detected. In total, 15 QTL confidence intervals were detected for morphological traits under control and saline conditions (Figure 3, Table 2). The number and strength of QTLs varied between different traits and conditions with a maximum of five QTLs detected for leaf area under control conditions. Many QTLs were detected for multiple traits and under both conditions, indicating that these loci had pleiotropic effects independent of the growing conditions.

Six of the 15 QTL intervals were identified in both environments (QTL 2, 3, 4, 9, 10, and 15), while four QTL intervals were uniquely detected under control (QTL 6, 12, 17, and 18) and five under saline (QTL 7, 8, 11, 13, and 16) conditions (Figure 3, Table 2). The similarities in QTL profiles under neutral and saline conditions reflected the correlations between the two conditions. Traits with lower correlations showed higher numbers of unique QTLs (Figures 2 and 3). Significant LOD scores ranged from 2.6 for relative growth rate under saline conditions to 12.5 for average internode length under control conditions, explaining 12 and 46.4% of the variance, respectively. Large-effect QTLs, explaining more than 20% of the variance, were detected for leaf area, main stem branching, and average internode length in neutral and saline conditions, representing two pleiotropic loci on chromosome 1 and one pleiotropic locus on chromosome 4. Although most QTLs displayed positive effects, 9 out of 30 QTLs displayed negative effects, indicating that *ddm1-2*-inherited epigenotypes can both reduce and enhance plant morphological trait values. Positive-effect QTLs were detected for plant height, flowering time, and main stem branching, while solely negative-effect QTLs were detected for rosette branching and average internode length. Opposite-effect QTLs were detected for leaf area in control conditions and for relative growth rate in saline conditions, indicating that effects are locus dependent rather than trait specific. Although the effects were small, it suggests that the *ddm1-2*-inherited epigenotypes can increase relative growth rate (which can serve as a proxy for fitness) under saline conditions, providing a possible evolutionary benefit. It must be noted, however, that most of the QTLs for leaf area and relative growth rate are positive-effect QTLs; thus, in most cases, the *ddm1-2* inherited epigenotypes show impaired genetic regulation, which probably hinders their ability to adapt to the saline environment.

For a number of pleiotropic QTLs (QTL 3, 4, 9, and 15), opposite effects were observed for different traits, which was supported by the negative correlation between these traits (Figure 2, Table 2). The *ddm1-2*-inherited epigenotype in the QTL 3 interval, for instance, was associated with decreased main stem branching and increased rosette branching and internode length. Even though H^2 values were high, no significant QTLs were detected for total plant height under both control and saline conditions. Similarly, no QTLs were detected for flowering time and relative growth rate under control conditions and rosette branching under saline conditions. However, the QTLs for flowering time did resemble the highly significant QTL profiles from another epiRIL study (Cortijo et al., 2014) and might have gone undetected because of the smaller population size used here.

QTL Analysis for Phenotypic Plasticity

Large variation was observed in the CV and phenotypic plasticity (PP) values between epiRILs; thus, QTL mapping was subsequently performed on these traits. For PP, two QTLs were detected, one pleiotropic QTL on chromosome 1, explaining ~19% of the PP variation for internode length and rosette branching, and a second QTL on chromosome 5 explaining ~13% of the PP variation in rosette branching (Table 2; Supplemental Figure 2). Both QTLs colocated with the identified QTLs for morphological trait variation, implying that the regulation of PP is governed by the same loci (Figure 3, Table 2). Eight QTLs were detected explaining the variation observed in CV, of which two coincided with the chromosome 1 QTL for PP (Supplemental Figure 3). Two QTLs were pleiotropic and one QTL for relative growth rate was also detected for morphological trait variation (Table 2). Interestingly, a QTL was found for the CV of total plant height, for which no QTL was detected for morphological variation, most likely due to the large within-line variation. The majority of CV and PP QTLs showed negative effect signs, illustrating that the *ddm1-2*-inherited epigenotypes increase plant sensitivity to environmental variation.

An Epigenetic Basis for Pleiotropic QTLs

The QTL mapping results and the epigenetic makeup of the lines suggest that the variation for growth, morphology, and plasticity is due to DNA methylation differences in the epiRILs. However, DNA sequence variation due to transposable element transposition cannot be ruled out on the basis of these results. Therefore, resequence data for 53 epiRILs, obtained in a previous study (Cortijo et al., 2014), was analyzed for the presence of de novo TE insertions in the QTL confidence intervals. The analyses revealed 11 shared TE insertions within eight QTL confidence intervals (Supplemental Figure 4 and Supplemental Data Set 2). The TE insertions were never consistently inherited

Figure 1. (continued).

The blue line denotes Col-0, the red line denotes *ddm1-2*, the orange lines denote the highest negative-effect lines, and the green lines denote the highest positive-effect lines (in some cases least negative). LA20, leaf area after 20 d; RGR, relative growth rate; FT, flowering time; AIL, average internode length; RB, rosette branching; MSB, main stem branching; PH1S, plant height 1st silique; TPH, total plant height.

Table 1. Descriptive Statistics for the Morphological Traits Measured in the epiRIL Population

Trait	Environment	Count	AVG ± SE	$[V_G]$	$[V_E]$	$[H^2]$	$[CV_G]$
LA20	C	96	118.84 (3.20)	981.20209	1164.82954	0.46	26
	S	97	101.13 (2.57)	638.30060	1011.73169	0.39	25
RGR	C	96	0.21 (0.00)	0.00027	0.00158	0.14	8
	S	97	0.22 (0.00)	0.00030	0.00170	0.15	8
FT	C	97	40.67 (0.32)	9.96161	9.53814	0.51	8
	S	93	43.61 (0.37)	12.56121	16.48763	0.43	8
RB	C	97	9.70 (0.52)	26.53175	54.67491	0.33	53
	S	93	1.51 (0.21)	4.20662	9.76900	0.30	136
MSB	C	97	5.15 (0.12)	1.36351	0.98986	0.58	23
	S	93	5.35 (0.11)	1.15573	1.76559	0.40	20
AIL	C	97	2.48 (0.06)	0.30699	0.35506	0.46	22
	S	93	1.77 (0.04)	0.12766	0.24395	0.34	20
PH1S	C	97	12.11 (0.17)	2.92929	5.05219	0.37	14
	S	93	9.13 (0.17)	2.61269	5.71273	0.31	18
TPH	C	97	35.37 (0.33)	10.54974	13.81507	0.43	9
	S	93	30.19 (0.30)	8.32459	23.42437	0.26	10

AVG ± SE is population average ± SE of the population mean; V_G is among-genotype variance; V_E is residual variance; H^2 is broad-sense heritability calculated as $V_G/(V_G + V_E)$; CV_G is coefficient of genetic variation calculated as $\sqrt{V_G}/\bar{X}$*100%, where $\bar{X}$ is the population mean; LA20, leaf area after 20 d; RGR, relative growth rate; FT, flowering time; RB, rosette branching; MSB, main stem branching; AIL, average internode length; PH1S, plant height 1st silique; TPH, total plant height; C, control; S, saline.

from the *ddm1-2* parent, which suggests that they either arose in the F1 or that they already occurred in the *ddm1-2* parent and were lost by either segregation or excision in some epiRILs (Supplemental Figure 4).

Next, we assessed whether the TE insertions significantly affect the trait values and thus can regulate trait variation. In total, 11 TEs were tested for their contribution to eight QTLs, explaining variation in 15 different traits. In three of the 34 tested cases, a significant effect of the TE insertion on trait values could be detected, although the effect of the QTL DMR marker was always (much) stronger (Supplemental Data Set 3). However, the low contribution of TEs to the explained variance might also be due to sampling bias of only 53 of the 92 epiRILs used for the mapping. Therefore, we analyzed the presence or absence of four TE insertions in three QTL support intervals, including the most pleiotropic and significant QTLs, for all epiRILs used in this study (92 epiRILs) via PCR. The calling of TEs in the sequence data of the 53 lines could be confirmed with very few exceptions and the additionally analyzed lines provided more confidence for the epigenetic QTLs. In all but one case, the estimated effect of the TE insertion decreased with the higher number of observations, and for the QTLs in which the TE presence did show a stronger significant effect, the DMR effect was still larger (Supplemental Data Set 3).

Because the shared TE insertions that were found in this study were not consistently inherited from the *ddm1-2* parental line and were in most cases only weakly associated with the trait values, our results suggest that the QTLs are epigenetically regulated. It further gives strong supportive evidence for the pleiotropic regulation of morphology and plasticity by epigenetic mechanisms.

DMR-Based QTL Analysis of Epistasis

In the previous sections, it was outlined that morphological traits and phenotypic plasticity are to a large extent regulated by epigenetic loci. Because quantitative traits can be additively or epistatically regulated by different genetic factors (Kliebenstein et al., 2001), this might also hold for epigenetic regulation. To test for epistatic interactions, pairwise comparisons were made among all loci and the interaction effect (LOD_i) was estimated as the difference between the LOD score of an additive model (LOD_a, not including interactions) and a full model (LOD_f) (Table 3; Supplemental Data Set 4) (Broman and Sen, 2009; Manichaikul et al., 2009). Significant epistatic interactions were found between the loci on chromosomes 4 and 5 for leaf area under saline conditions and the loci on chromosomes 1 and 3 for the phenotypic plasticity parameter, CV, for total plant height under control conditions (Table 3; Supplemental Data Set 4).

DISCUSSION

DNA Methylation Affects Plant Growth and Productivity

In this study, we show that *ddm1-2*-induced DNA hypomethylation can give rise to a wide variety of highly heritable phenotypes. The high heritabilities were accompanied by strong epigenetic variation resulting in the detection of multiple QTLs, more or less similar to genetic variation, heritability, and number of QTLs found in conventional RIL populations (Ungerer et al., 2002; Bandaranayake et al., 2004; Keurentjes et al., 2007). For most traits, QTLs had positive additive effect signs, i.e., these loci increased trait values in the wild-type Col-0 background. However, negative-effect QTLs were also detected, e.g., for leaf area, relative growth rate, rosette branching, and average internode length, indicating that *ddm1-2*-induced hypomethylation can both reduce and enhance plant morphological trait values. It must be noted, however, that the impacts of negative-effect QTLs for leaf area and relative growth rate were

		LA20		MSB		PH1S		TPH		FT		RB		AIL		RGR	
		C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S
LA20	C	*	0.8	0.4	0.5	0.4	0.3	0.3	0.3	-0.1	0.0	0.0	0.0	-0.3	-0.3	-0.5	-0.4
	S	0.00	*	0.5	0.5	0.4	0.3	0.4	0.3	-0.1	-0.1	0.0	0.0	-0.2	-0.3	-0.5	-0.5
MSB	C	0.00	0.00	*	0.7	0.4	0.4	0.1	0.2	0.4	0.4	-0.4	-0.3	-0.8	-0.5	-0.3	-0.3
	S	0.00	0.00	0.00	*	0.2	0.5	0.1	0.3	0.3	0.3	-0.3	-0.3	-0.6	-0.6	-0.2	-0.2
PH1S	C	0.00	0.00	0.00	0.02	*	0.4	0.7	0.4	0.1	0.1	0.2	0.0	0.2	0.1	-0.2	-0.1
	S	0.00	0.00	0.00	0.00	0.00	*	0.2	0.6	0.2	0.2	0.0	0.0	-0.1	0.2	-0.3	-0.2
TPH	C	0.00	0.00	0.30	0.48	0.00	0.09	*	0.3	0.0	-0.1	0.4	0.1	0.3	0.1	0.1	-0.1
	S	0.00	0.00	0.07	0.00	0.00	0.00	0.00	*	0.1	0.0	0.0	0.2	0.0	0.2	-0.1	-0.1
FT	C	0.17	0.38	0.00	0.01	0.47	0.13	0.71	0.19	*	0.6	-0.5	-0.3	-0.4	-0.1	-0.1	0.0
	S	0.96	0.28	0.00	0.01	0.37	0.05	0.34	0.86	0.00	*	-0.4	-0.4	-0.4	-0.1	-0.1	-0.1
RB	C	0.95	0.88	0.00	0.01	0.04	0.69	0.00	0.69	0.00	0.00	*	0.5	0.6	0.2	0.2	0.0
	S	0.88	0.86	0.00	0.01	0.70	0.68	0.23	0.12	0.00	0.00	0.00	*	0.4	0.3	0.0	0.1
AIL	C	0.01	0.01	0.00	0.00	0.06	0.39	0.00	0.97	0.00	0.00	0.00	0.00	*	0.6	0.2	0.2
	S	0.01	0.00	0.00	0.00	0.24	0.05	0.33	0.14	0.17	0.30	0.02	0.00	0.00	*	0.1	0.1
RGR	C	0.00	0.00	0.01	0.02	0.12	0.01	0.56	0.15	0.35	0.22	0.03	0.80	0.02	0.49	*	0.4
	S	0.00	0.00	0.01	0.03	0.19	0.06	0.51	0.63	0.93	0.47	0.67	0.27	0.11	0.22	0.00	*

Figure 2. Spearman's Rho Correlations and Their Respective P Values among Morphological Traits.

The upper right panel shows the Spearman correlations; the lower left panel shows the significance values. LA20, leaf area after 20 d; RGR, relative growth rate; FT, flowering time; AIL, average internode length; RB, rosette branching; MSB, main stem branching; PH1S, plant height 1st silique; TPH, total plant height.

rather small. Because most of the QTL effects were positive, the *ddm1-2*-inherited epigenotypes are inferior in terms of leaf area, probably due to impaired genetic regulation. No QTLs were detected for flowering time and total plant height, most likely due to low levels of variation, which is in accordance with previous studies (Johannes et al., 2009; Zhang et al., 2013; Cortijo et al., 2014). However, strong epigenetic QTLs were detected for flowering time in a previous epigenetic QTL study on 123 epiRILs, and these QTLs resembled the QTL profiles in our study of 99 epiRILs (Figure 3; Supplemental Figure 5) (Cortijo et al., 2014). When linkage tests of the flowering time QTL markers on chromosomes 1, 4, and 5 from the previous study (Cortijo et al., 2014) were applied to our flowering time data, a highly significant association on chromosome 1 ($P < 0.01$) in neutral conditions was detected. In saline conditions, the QTLs on chromosomes 1 and 5 could be confirmed. These results indicate that the lack of genome-wide QTL detection for flowering time may simply be the result of reduced statistical power due to a lower number of epiRILs tested.

Because shared TE insertions were not consistently inherited from the *ddm1-2* parent and were only weakly associated with the trait variation, our data strongly suggest that the morphological traits are regulated by epigenetic mechanisms. However, we cannot exclude the possibility that the presence of a de novo TE in the F1 could have influenced the epigenetic state of the locus and that this state was maintained even after the TE got lost again in later generations (i.e., a paramutation of the locus), although we expect this to be a very rare and unlikely event. Moreover, it is unlikely that paramutation by TEs would affect large regions and even if this would be the case, the phenotypic variation can still be assigned to epigenetic variation of the locus. In addition, the frequency of observed TEs in QTL regions suggests that most TEs originated from the parental *ddm1-2* (Supplemental Data Set 3 and Supplemental Figure 4) and are inherited collectively with DMRs. This would indicate that the effects of DMRs and TEs are highly confounded and that loss of TEs in QTL support regions argues for a strong contribution of epigenetic mechanisms in the regulation of trait variation. Finally, a more detailed analysis by PCR and statistical assays for a number of TEs confirmed their low contribution to the total explained variance in most traits tested.

The colocation of multiple QTLs indicates pleiotropic epigenetic regulation of many different morphological traits. Furthermore, the major pleiotropic QTLs found in our study coincide with six QTLs detected in an epigenetic QTL mapping study on flowering time and root length (Supplemental Figure 5) (Cortijo et al., 2014). As to the nature of these pleiotropic loci, we can only speculate that these are transcription factor genes or genes involved in metabolism or circadian rhythms. It is well known that transcription factors, metabolism, and clock genes can be under epigenetic regulation (Manning et al., 2006; Alvarez-Venegas

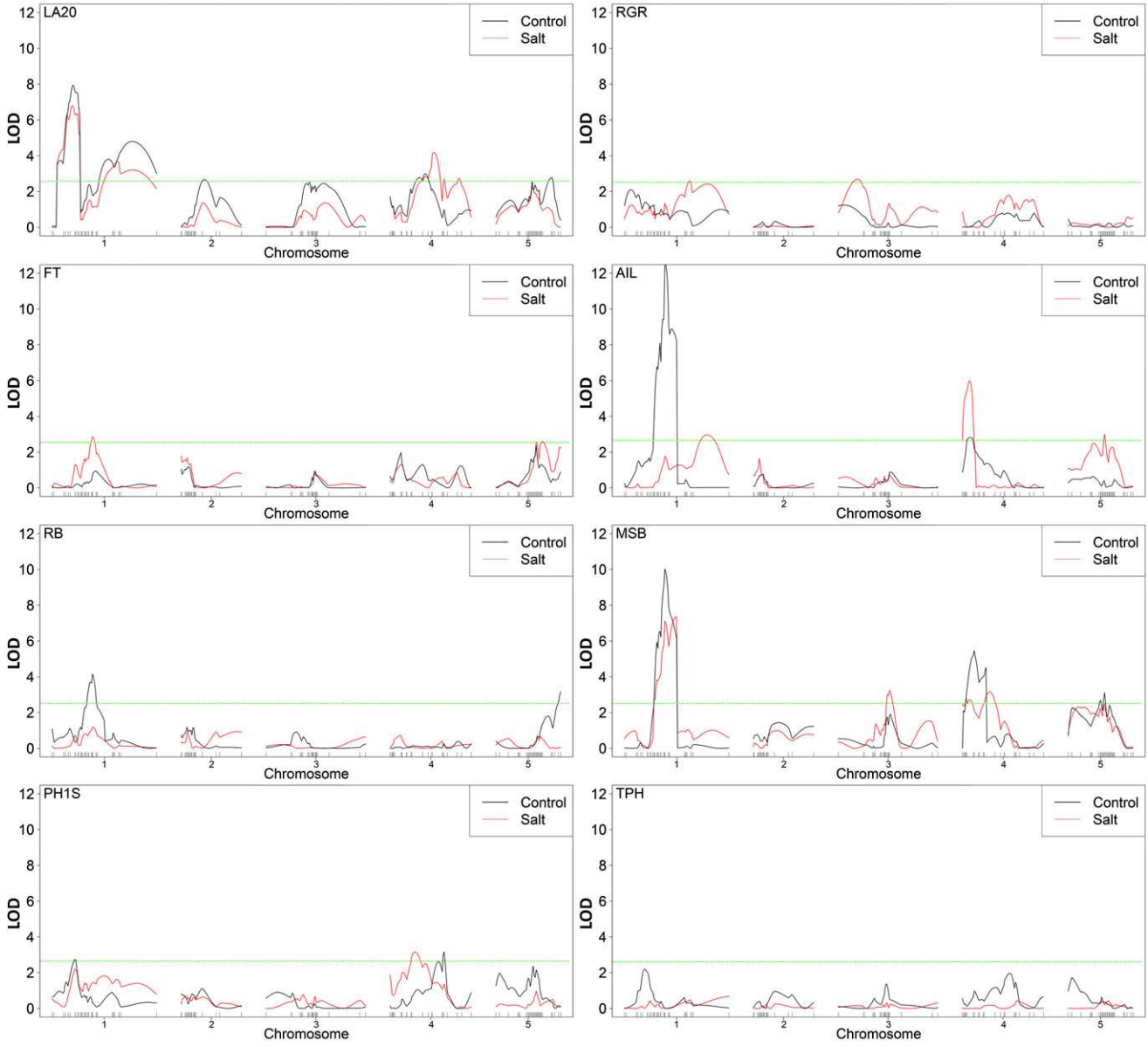

Figure 3. Epi-QTL Plots for Morphological Traits Tested in the epiRIL Population.

Traits were tested under control (black line) and saline (red line) conditions. LOD threshold was calculated using 1000 random permutations with α 0.05 as the genome-wide type I error level. The highest LOD threshold between the two conditions (horizontal green line) was used as LOD threshold in the figure and for determination of significance. LA20, leaf area after 20 d; RGR, relative growth rate; FT, flowering time; AIL, average internode length; RB, rosette branching; MSB, main stem branching; PH1S, plant height 1st silique; TPH, total plant height.

et al., 2007; Cazzonelli et al., 2009; Quadrana et al., 2014; Seo and Mas, 2014). Alternatively, *trans*-acting RNAs produced from differentially methylated TEs might affect multiple targets in the genome, causing pleiotropic effects. Most of the QTLs are found in pericentromeric regions because most of the DNA methylation variation is located here due to the stable loss of DNA methylation in *ddm1-2* in those regions. At pericentromeric boundaries, TEs are located in close proximity to genes more often than anywhere else in the genome, and loss of DNA methylation in these regions might therefore increase the likelihood of a stable epiallele.

DNA Methylation Can Alter Salinity Tolerance

Different lines of evidence illustrate that DNA hypomethylation decreases growth and reproductive success—in terms of branching and plant height (Clauss and Aarssen, 1994)—under saline conditions. For most traits, the wild-type Col-0 performed

Table 2. QTLs Detected in the epiRIL Population for Morphological Traits

nr	Trait	Morphology or Plasticity	Treatment	QTL	Chr	LOD	Peak Marker	Chr	Pos	QTL Support Interval	Explained Variance (%)	Effect (%)
1	LA20	CV	C	1	1	5.5	MM2	1	1.7	0–8	24.1	−13.8
2	LA20	M	C	2	1	7.9	MM11	1	23.2	15–28	32.8	13.4
3	LA20	M	S		1	6.8	MM10	1	18.2	15–28	28.7	12.3
4	PH1S	M	C		1	2.7	MM11	1	23.2	15–28	12.9	4.1
5	RB	CV	S	3	1	2.7	MM123	1	41.1	31–46	12.5	17.1
6	LA20	CV	C		1	2.7	MM91	1	37.0	31–46	12.8	−14.7
7	AIL	PP	–		1	5.9	MM123	1	41.1	31–46	25.6	−30.1
8	FT	M	S		1	2.9	MM123	1	41.1	31–46	13.4	3.1
9	RB	M	C		1	4.1	MM123	1	41.1	31–46	18.7	−22.7
10	RB	PP	–		1	4.2	MM123	1	41.1	31–46	18.9	−22.1
11	MSB	M	C		1	10.1	MM123	1	41.1	31–46	39.6	12.3
12	MSB	M	S		1	7.6	MM123	1	41.1	31–46	31.7	9.8
13	AIL	M	C		1	12.5	MM123	1	41.1	31–46	46.4	−13.3
14	LA20	M	C	4	1	4.8	MM160	1	70.1	50–101	21.3	13.1
15	LA20	M	S		1	3.7	MM158	1	67.8	50–101	16.8	12.0
16	RGR	M	S		1	2.6	MM158	1	67.8	50–101	12.0	−2.4
17	AIL	M	S		1	3.0	MM160	1	70.1	50–101	13.8	−7.4
18	TPH	CV	C	5	2	2.8	MM171	2	5.3	3–10	13.1	−19.2
19	LA20	CV	S		2	4.0	MM330	2	7.0	3–10	18.1	−13.9
20	LA20	M	C	6	2	2.7	MM382	2	21.5	14–50	12.5	−8.3
21	RGR	M	S	7	3	2.6	MM396	3	25.5	3–31	12.3	2.7
22	RGR	CV	C		3	2.8	MM396	3	25.5	3–31	13.0	−12.7
23	MSB	M	S	8	3	3.3	MM537	3	52.3	48–58	15.3	7.0
24	MSB	M	C	9	4	5.5	MM661	4	12.2	2–22	24.0	11.4
26	AIL	M	C		4	2.8	MM654	4	11.1	2–22	13.2	−10.6
27	AIL	M	S		4	6.0	MM587	4	4.5	2–22	25.9	−9.7
28	RB	CV	S		4	2.8	MM654	4	11.1	2–22	12.9	18.2
29	LA20	M	C	10	4	2.7	MM686	4	33.0	18–42	12.7	11.0
31	MSB	M	S		4	3.5	MM679	4	22.0	18–42	16.2	8.2
32	PH1S	M	S		4	3.2	MM679	4	22.0	18–42	14.6	7.0
30	LA20	M	S	11	4	4.2	MM693	4	43.0	33–52	18.8	12.4
33	PH1S	M	C	12	4	3.2	MM698	4	54.7	43–58	14.7	5.5
34	LA20	M	S	13	4	2.7	MM701	4	68.3	53–80	12.7	8.6
35	LA20	CV	C	14	5	4.5	MM707	5	3.6	1–16	20.0	−11.5
36	MSB	M	C	15	5	3.1	MM726	5	36.7	25–45	14.5	8.8
37	MSB	M	S		5	2.6	MM726	5	36.7	25–45	12.2	7.8
38	AIL	M	S		5	3.0	MM726	5	36.7	25–45	13.9	−6.7
40	FT	M	S	16	5	2.6	MM854	5	47.4	37–56	12.2	3.1
39	LA20	M	C	17	5	2.8	MM859	5	56.4	25–62	12.9	10.9
41	RB	M	C	18	5	3.2	MM867	5	65.9	58–66	14.7	−30.0
42	RB	PP	–		5	2.7	MM867	5	65.9	58–66	12.8	−27.2

The 1.5 LOD support interval is used. The explained variance is calculated according to the following formula: EV (%) = $(1 - 10^{(-2*LOD/n)})*100$, where LOD is LOD score for the particular trait and n is number of epiRILs (R/QTL FAQ). The effect (%) is calculated as effect size (a) divided by mean (x 100%). M, morphology; C, control; S, saline; LA20, leaf area after 20 d; RGR, relative growth rate; FT, flowering time; AIL, average internode length; RB, rosette branching, MSB, main stem branching; PH1S, plant height 1st silique; TPH, total plant height; Chr, chromosome; nr, QTL number; Pos, marker position in Mb.

better than the population mean of the hypomethylated epiRILs under saline conditions. Furthermore, the QTLs on chromosome 2, of which the hypomethylated allele increased leaf area under control conditions, was not observed under saline conditions. Also, all leaf area QTLs detected under saline conditions had positive additive effect signs, reflecting higher trait values for Col-0 alleles. The *ddm1-2*-inherited epigenotypes most likely have impaired genetic regulation, which impedes their ability to adapt to saline conditions.

Genetic induction of hypomethylation through the *ddm1-2* mutation also reduced salinity tolerance to some extent in Arabidopsis seedlings (Yao et al., 2012). In wheat (*Triticum aestivum*) seedlings, however, chemical induction of DNA hypomethylation enhanced biomass and the activity of antioxidant enzymes under salt stress conditions in two different cultivars (Zhong et al., 2010). DNA hypomethylation was higher in one of the cultivars, concomitant with increased activity of the antioxidant enzymes. Most likely, DNA hypomethylation can both increase and decrease salinity tolerance depending on the genotype and site of methylation in the genome.

An important observation in our study was the epistatic interaction between the two loci on chromosomes 4 and 5 for leaf

Table 3. Testing for Epistatic Interactions by Pairwise Comparisons between Loci in a Two-Dimensional, Two-QTL Model (Broman and Sen, 2009)

			Comparison between Loci on Chromosome		Position of Loci		LOD Score		
Phenotype	Morphology or Plasticity	Treatment	Chr 1	Chr 2	Pos_{1f}	Pos_{2f}	LOD_f	LOD_a	LOD_i
TPH	CV	C	1	3	23	101	**6.5**	2.1	**4.3**
LA20	M	S	4	5	41	38	**10.6**	**5.9**	**4.7**

The full model, LOD_f, in the two-QTL model includes the main effects of the two loci and their interaction; the additive model, LOD_a, only includes the main effects of the two loci; and the epistatic model, LOD_i, tests specifically for interaction effects between the two loci ($LOD_i = LOD_f - LOD_a$) (for full details of the models, see text, Methods, and Supplemental Data Set 4). LODs for the two-QTL model were found significant (in bold) above an arbitrary threshold based on Broman and Sen (2009): (LOD_f, LOD_a, and LOD_i) = (6.0, 5.0, and 4). M, morphology; Chr, chromosome.

area, detected solely under saline conditions. Although the detection of interactions among epigenetic features is quite unusual, our results clearly indicate that the regulation of complex traits may depend on the methylation status at multiple loci. DNA hypomethylation at one locus may, for instance, lead to the enhanced expression of a transcription factor whose functioning depends on the DNA methylation status of an unlinked target locus. The effect signs of both QTLs and their interaction are positive, indicating that methylation at the two interacting loci increased growth under saline conditions.

DNA Hypomethylation Amplifies Phenotypic Plasticity

In many cases, temporary adaptation to stressful conditions is beneficial for plants in fluctuating environments (Rando and Verstrepen, 2007). DNA mutations are irreversible and might thus be counterproductive in such environments, whereas epigenetic modifications could be rapidly induced and reversed. Phenotypic plasticity, or the ability of a species to display different phenotypes according to variation in the environment, is therefore hypothesized to be (partly) regulated via epigenetic means (Schlichting, 1986; Mirouze and Paszkowski, 2011). Our data strongly support this hypothesis. Col-0 was less affected by moderately saline conditions than the majority of epiRILs for most traits and matched (in most cases) the lower quartiles of the population distribution for both PP and CV. Furthermore, most PP and CV QTLs showed negative-effect signs, further supporting the observation that the *ddm1-2*-inherited epigenotypes have increased sensitivity to environmental variation. We would like to emphasize here that the variation in plasticity is most likely due to stable heritable variation and not due to plastic de novo variation in the epiRILs (Richards et al., 2010). Similar results were obtained in drought and nutrient stress experiments, in which phenotypic plasticity was much higher in epiRILs than in the Col-0 wild type and highly heritable (Zhang et al., 2013). However, PP is also observed in conventional RIL populations (Lacaze et al., 2009; Tétard-Jones et al., 2011; El-Soda et al., 2014). It is difficult to compare the epigenetic with the genetic contribution to phenotypic plasticity, as epigenetic variation might contribute to phenotypic variation in conventional RIL populations when the epigenetic variation associates with the genetic markers (Schmitz et al., 2013a). Recently, a RIL population in soybean was analyzed for genome, methylome, and transcriptome variation; indeed, the majority of the DMRs cosegregated with the genetic background, and for 90% of the DMRs, genetic QTLs explaining the methylation variation were identified (Schmitz et al., 2013a). Similar results were obtained for natural accessions of Arabidopsis (Schmitz et al., 2013b). Although this suggests that most epigenetic variants are dependent on genetic variation, rare examples of DMRs not linked to genetic variation were identified, and such DMRs could be epialleles (Schmitz et al., 2013a, 2013b). In another study, chemically induced hypomethylation in several Arabidopsis accessions differentially increased phenotypic plasticity, suggesting that genotypes and epigenotypes may interact to define plasticity (Bossdorf et al., 2010). These findings indicate that both genotype and epigenotype contribute to phenotypic plasticity.

In our study, large variation was detected in the plasticity response of the epiRILs to moderate salinity, and three QTLs were mapped related to PP. All PP QTLs coincided with QTLs explaining variation in the same morphological traits under control conditions. This indicates that the regulatory gene(s) underlying the QTLs are sensitive to variation in the environment and that modification of methylation profiles determines to some extent plasticity (Lacaze et al., 2009). In rice (*Oryza sativa*), a mutation in a gene leading to increased DNA methylation on repetitive sequences and decreased histone acetylation resulted in high expression variation in different environments, illustrating the regulation of PP through epigenetic processes (Zhang et al., 2012). PP is thus most likely regulated through a complex network of epigenetic and genetic factors, depending on environment and development.

In addition to environmental plasticity, within-line variation (Sangster et al., 2008) under both conditions was also surveyed for epigenetic regulation. The level of within-variation among epiRILs was significantly associated with certain genomic regions. Most of the trait variation QTLs did not overlap with the trait value QTLs, which indicates that different loci explain the variation within and between lines. For relative growth rate and total plant height, no QTLs were detected under control conditions, but QTLs were detected explaining differences in the level of variation within lines. This suggests that the biological variation or developmental stability within lines was higher than the epigenetic variation between lines but that part of the within-line variation is regulated through epigenetics.

In conclusion, the majority of plasticity and stability QTLs showed negative-effect signs, suggesting that DNA hypomethylation increases environmental sensitivity. In many genome-wide association studies and QTL analyses, high variation is often observed between replicates of isogenic lines, which could be due to subtle environmental differences. As outlined in this study, the differences in within-line variation detected in such genetic resources might be due to epigenetic components that modulate the level of susceptibility of plants to small changes in the environment.

METHODS

Plant Growing Conditions and Trait Descriptions

Seeds from 99 epiRILs and their parents, Col-0 and *ddm1-2* (both from the 5th generation), all in the *Arabidopsis thaliana* Col-0 genetic background, were sown on filter paper with demineralized water and stratified at 4°C in darkness for 5 d. Subsequently, seeds were transferred to a climate room (16 h light, 24°C) to induce seed germination for 42 h. Seventeen replicates of each epiRIL and parental line were completely randomized transplanted to wet Rockwool blocks of 4 × 4 cm under both control and saline conditions (different flooding tables in same chamber) in a climate chamber (16 h light, 125 μmol m^{-2} s^{-1}, 70% RH, 20/18°C day/night cycle). All plants were watered every morning for 5 min with 1/1000 Hyponex solution (Hyponex) supplemented with (salt) or without (control) 25 mM NaCl. Plants were photographed from above each hour for the entire growth period (until leaves started to overlap) to analyze leaf area after 20 d (LA20) and relative growth rate. Relative growth rate was calculated as RGR = ln(LA20)-ln(LA17)/d where LA20 is leaf area after 20 d, LA17 is leaf are after 17 d, and d is the number of days between the two time points. At 28 d after germination, the first plants started to flower and flowering time was recorded for five predefined replicates out of the 17. Two weeks after flowering, main stem branching, rosette branching, plant height at 1st silique, total plant height, and average internode length were measured for these five replicates.

Descriptive Statistics

Spearman's rho correlation coefficient was determined using SPSS 21 using a two-tailed significance test. Box plots were made using Excel 2010 based on the minimum (phenotypic value > first quartile − 1.5*IQR), first quartile, median, third quartile, and maximum (phenotypic value < third quartile + 1.5*IQR). The interquartile range (IQR) is the difference between the upper (third quartile) and lower quartiles (first quartile). Suspected outliers were classified as phenotypic values above the minimum and maximum. Phenotypic plasticity was calculated as the absolute difference in means between the two conditions. Coefficient of variation (CV_G) was calculated as $\sqrt{V_G}/\bar{X}$*100%; broad sense heritability (H^2) was calculated as $V_G/(V_G+V_E)$, where V_G is genetic variation, V_E is environmental variation, and $\bar{X}$ is the population average. Reaction norm plots were made using Excel 2010 based on the phenotypic values from neutral and saline conditions.

Multiple QTL Mapping

QTL mapping was performed with multiple QTL mapping implemented in the R/QTL software (Arends et al., 2010; Joosen et al., 2012). Cofactors were assigned to 42 out of the 126 markers based on the genetic map position and preliminary composite interval mapping on the data. Backward elimination was used to remove cofactors that did not contribute to the fit of the model. Multiple QTL mapping was performed on each trait and each treatment separately, and the results were compared with standard interval mapping, using Haley Knott regression (Haley and Knott, 1992). One thousand random permutations were generated for each phenotype to determine the LOD significance threshold with α 0.05 as the genome-wide type I error level. For Supplemental Figures 2 and 3, one hundred random permutations were used for the LOD significance threshold. For Table 2, Figure 3, and Supplemental Figure 3, the LOD threshold was determined for both neutral and saline conditions and the highest LOD threshold of both conditions was used for the significance determination. The explained variance per QTL was calculated as EV (%) = $(1 - 10^{(-2*LOD/n)})$*100 where LOD is the LOD score for the particular phenotype and n is the number of epiRILs (R/QTL FAQ).

Detection of de Novo TE Insertions

Illumina whole-genome resequence data (Cortijo et al., 2014) were used to determine whether de novo TE insertions were present in the QTL confidence intervals as described previously (Cortijo et al., 2014). Resequence data were available for 73 epiRILs, of which 53 were analyzed in our study. For four out of 11 shared TE insertions, PCR analysis was performed on 91 epiRILs according to Cortijo et al. (2014) to confirm the sequencing data and analyze the remaining epiRILs for TE insertions (see Supplemental Data Set 5 for primers).

Effect Analyses of de Novo TE Insertions

To assess whether the lines used for TE analysis were a representative sample of the epiRIL population and to determine the contribution of TE insertions to the explained variance, we compared various QTL models. First, we used the full QTL model on the available data sets for each trait:

$$y_i = \beta_0 + \beta_{1g}(MM_1)_i + \beta_{2g}(MM_2)_i + \ldots.\beta_{ng}(MM_n)_i + \varepsilon_{ij} \quad (1)$$

where y_i is the trait value and $_g(MM)_i$ is the epigenotype at the nth peak QTL marker for the ith individual, i = 1,…,91. The number of significant QTLs (n) for each trait determines the number of peak QTL markers (MM_n) taken up in the analysis of that trait. For instance, for relative growth rate under saline conditions, the full QTL model (marker; Supplemental Data Set 3) is described by:

$$y_i = \beta_0 + \beta_{1g}(MM158)_i + \beta_{2g}(MM396)_i + \varepsilon_i \quad (2)$$

where *MM*158 and *MM*396 are the peak QTL markers on chromosomes 1 and 3, respectively. To analyze the effect of the mth TE insertion in the QTL interval on chromosome 1, we tested the following model in the case of relative growth rate under saline conditions (transposon; Supplemental Data Set 3):

$$y_i = \beta_0 + \beta_{1g}(TE_m)_i + \beta_{2g}(MM396)_i + \varepsilon_i \quad (3)$$

where $_g(TE_m)_i$ is the TE insertion genotype of the ith individual. To analyze the effect of the mth TE insertion in the presence of the peak QTL marker, we analyzed the following model for relative growth rate under saline conditions (marker + transposon; Supplemental Data Set 3):

$$y_i = \beta_0 + \beta_{1g}(MM158) + \beta_{2g}(TE_m)_i + \beta_{3g}(MM396)_i + \varepsilon_i \quad (4)$$

We analyzed the significance of the β_j estimates, the F-value of the model, as well as the adjusted R^2 of the model for each trait that had a shared de novo TE insertion within the QTL confidence interval (Supplemental Data Set 3). If TE insertions are causal, the TE effects should be more significant than the peak QTL markers and the overall model fit should be the same or better compared with the model using only the peak QTL markers.

Two-Dimensional, Two-QTL Genome Scans

Two-QTL genome scans were performed using the *scantwo* function in the R/QTL software (Broman and Sen, 2009; Manichaikul et al., 2009; Arends et al., 2010). The output gives the results for five different QTL models: LOD_f, LOD_a, LOD_{fv1}, LOD_{av1}, and LOD_i. The QTL positions used for the models can differ between the interaction models (LOD_f, LOD_{fv1}, and LOD_i) and the additive models (LOD_a and LOD_{av1}). In the full model, LOD_f, main effects and interaction effects are included, whereas in LOD_a,

only the main effects are given. The interaction model, LOD_i, is the difference between LOD_f and LOD_a ($LOD_i = LOD_f - LOD_a$) testing for the significance of epistatic interactions. LOD_{fv1} compares the full model to the maximum single QTL model ($LOD_{fv1} = LOD_f - LOD_{QTLmax}$ in which LOD_{QTLmax} is the highest LOD of the two QTL [loci]) including interactions between the QTL, whereas LOD_{av1} compares the additive model to the largest single-QTL model ($LOD_{av1} = LOD_a - LOD_{QTLmax}$) excluding interactions. LODs are found significant above an arbitrary threshold based on Broman and Sen (2009): (LOD_f, LOD_{fv1}, LOD_a, LOD_{av1}, LOD_i) = (6.0, 5.0, 5.0, 2.5, 4.0).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL libraries under accession number At5g66750 (*DDM1*).

Supplemental Data

Supplemental Figure 1. Box Plots of epiRIL Variation and Phenotypic Plasticity.

Supplemental Figure 2. Epi-QTL Plots for Phenotypic Plasticity.

Supplemental Figure 3. Epi-QTL Plots for Coefficient of Variation.

Supplemental Figure 4. Locations of Insertions inside QTL Confidence Intervals.

Supplemental Figure 5. Overlap between QTLs Found in Our Study and Previously Validated QTLs for Flowering Time and Root Length.

Supplemental Data Set 1. Genetic Map Containing 126 DMR Markers.

Supplemental Data Set 2. Locations of TE Insertions on Chromosome and QTL Intervals.

Supplemental Data Set 3. Univariate Regression Tests for the Effect of DMR and TE Insertions on the Trait Values.

Supplemental Data Set 4. Comparison of Full Model to Additive Model and Interaction Model in a Two-Dimensional, Two-QTL Genome Analysis.

Supplemental Data Set 5. Primer Sequences Used for TE Insertion PCRs.

ACKNOWLEDGMENTS

This research was supported by the Centre for Biosystem Genomics, The Netherlands.

AUTHOR CONTRIBUTIONS

R.K. and J.J.B.K. designed the research. R.K. performed the experiments. R.K., R.W., and M.E. analyzed the data. R.K., J.J.B.K., and D.V. wrote the article with contributions from F.J. and V.C.

Received October 13, 2014; revised January 14, 2015; accepted January 30, 2015; published February 10, 2015.

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