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Mild drought in the vegetative stage induces phenotypic, gene expression and DNA methylation plasticity in Arabidopsis but no transgenerational effects

Tom JM Van Dooren^{1*+}, Amanda Bortolini Silveira²⁺, Elodie Gilbault³, José M. Jiménez-Gómez³, Antoine Martin², Liên Bach³, Sébastien Tisné³, Leandro Quadrana², Olivier Loudet^{3*} and Vincent Colot^{1*}

1. CNRS - UMR 7618 Institute of Ecology and Environmental Sciences (iEES) Paris, Sorbonne University, Case 237, 4, place Jussieu, 75005 Paris, France

2. Institut de Biologie de l'Ecole Normale Supérieure, (IBENS), Ecole Normale Supérieure, Centre National de la Recherche Scientifique (CNRS), Institut National de la Santé et de la Recherche Médicale (INSERM), PSL Université Paris, Paris, France

3. Institut Jean-Pierre Bourgin, INRAE, AgroParisTech, Université Paris-Saclay, 78000 Versailles, France

Email addresses, according to author order: Tom Van Dooren <vandoore@biologie.ens.fr>, Amanda Silveira <amandasilveira@gmail.com>, Elodie Gilbault <elodie.gilbault@inrae.fr>, Jose Jimenez-Gomez <jose.jimenez-gomez@inrae.fr>, Antoine Martin <antoine.martin@supagro.inrae.fr>, Liên Bach <lien.bach@supagro.fr>, Sébastien Tisné <sebastien.tisné@cirad.fr>, Leandro Quadrana <quadrana@biologie.ens.fr>, Olivier Loudet <olivier.loudet@inrae.fr>, Vincent Colot <colot@biologie.ens.fr>.

⁺Co-first authors

^{*}Corresponding authors:

vandoore@biologie.ens.fr (+31) 613186788

Olivier.Loudet@inrae.fr (+33) 130833217

colot@biologie.ens.fr (+33) 144323538

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Highlight

A comprehensive phenotypic analysis in five *Arabidopsis thaliana* accessions combined with methylome and transcriptome analyses in the Columbia reference accession find plasticity to mild drought but no transgenerational effects or inherited stress memory.

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Abstract

Whether environmentally-induced changes in phenotypes can be heritable is a topic with revived interest. In plants, heritable trait variation can occur without DNA sequence mutations through epigenetic mechanisms involving DNA methylation. However, it remains unknown if this other system of inheritance responds to environmental changes and if it can provide a rapid way for plants to generate adaptive heritable phenotypic variation. To obtain a comprehensive assessment of potential transgenerational effects induced by the environment, we subjected four natural accessions as well as the reference accession Col-0 of *A. thaliana* to mild drought in a multigenerational design. As expected, plastic responses to drought were observed in each accession, as well as a number of intergenerational effects of parental environments. However, after an intervening generation without stress, descendants of stressed and non-stressed plants were phenotypically indistinguishable, except for very few trait-based parental effects and irrespective of whether they were grown in control conditions or under water deficit. In addition, genome-wide analysis of DNA methylation and gene expression in the reference accession Col-0 demonstrated that, while mild drought induced changes in the DNA methylome of exposed plants, variants were not inherited. We conclude that mild drought stress does not induce transgenerational epigenetic effects.

Keywords: Arabidopsis, drought, epigenetics, maternal effects, methylation, plasticity, transgenerational effects.

Introduction

Being sessile organisms, plants are often exposed to environmental conditions they cannot escape from. Stress responses to these conditions are multivariate (Claeys and Inzé 2013), involving, for example, changed stomatal conductance or amounts of protective proteins. Fortunately, the presence of stress can be inferred in an integrative manner by comparing plant growth rates in different conditions (Claeys et al. 2014), such that the presence of a stress response cannot be missed.

Stressors can not only have a profound impact on the growth and development of exposed individuals but also on their offspring. These parental (typically maternal) environmental effects are well documented in general (Blödner *et al.*, 2007; Galloway and Etterson, 2007; Donohue, 2009; Herman and Sultan, 2011; Crisp *et al.*, 2016; Van Dooren *et al.*, 2016). However, when developing offspring (including the germ cells that produced the embryo) experience the same stressors as their parents, a transfer of information between generations is unnecessary for an effect in the offspring. This multigenerational exposure (Skinner 2008) has motivated the distinction between intergenerational and transgenerational effects (Heard and Martienssen 2014). We speak of intergenerational effects when the environments that provoke these effects are not only experienced by parents but also by germ cells or other developmental stages of the descendants in which the responses are assessed. Transgenerational effects occur in descendants which did not experience the environmental stressor in any phase of development (Heard and Martienssen 2014), which implies that information on the stressor was transmitted from previous generations. This distinction is not always respected or made explicit. Another way of characterizing parental effects is by speaking of transgenerational plasticity (Mousseau and Dingle, 1991; Herman and Sultan, 2011). This terminology assumes parental effects in offspring to be responses to environmental cues experienced by parents. Confusingly, such effects can be intergenerational. When so-called memory effects in stress responses (Lämke and Bäurle, 2017) span different generations, they could again involve intergenerational as well as transgenerational effects. Trait-based parental effects (Kirkpatrick and Lande, 1989) occur when phenotypes of parents determine phenotypes of offspring. These effects can be either intergenerational or transgenerational. They seem a natural framework to assess phenotypic stress responses, because they can investigate the determination of traits such as individual growth rates or other stress responses across generations. Trait-based parental effects can decay rapidly or persist longer due to evolutionary momentum, even without continual environmental change (Kirkpatrick and Lande, 1989).

Few experimental studies have been conducted to determine if a phenotypic memory of environmental stress can persist over multiple generations and whether such effects are truly transgenerational. In one early study, genetically identical *A. thaliana* lines grown under mild heat during the reproductive phase (from bolting onward) over two generations and then grown under normal conditions for an additional generation produced progeny with an ameliorated response to heat compared to control progeny from non-treated lines (Whittle *et al.*, 2009). However, as heat was applied during reproductive growth, the gametes and the developing seeds were also exposed to the environmental stressor (Whittle *et al.*, 2009). Therefore, intergenerational parental effects could be responsible for the ameliorated response to heat seen in the progeny (Blödner *et al.*, 2007; Pecinka and Mittelsten Scheid, 2012). Consistent with this possibility, another study found that when heat stress was applied during vegetative growth only, phenotypic effects did not persist for more than one generation (Suter and Widmer, 2013a,b). Similarly, when *A. thaliana* plants were infected with pathogens, increased resistance was reported in the immediate progeny and in the second generation, but only when infections were carried out until the reproductive phase (Boyko *et al.*, 2010; Luna *et al.*, 2012; Slaughter *et al.*, 2012). Salt stress memory across generations was also investigated and findings all point to an absence of *bona fide* transgenerational effects (Boyko *et al.*, 2010; Suter and Widmer, 2013a,b; Groot *et al.*, 2016; Wibowo *et al.*, 2016). Similarly, a transgenerational effect of drought on the speed of germination was reported (Ganguly *et al.* 2017), but in this case the effect diminished rapidly with each generation and is therefore unlikely responsible for a novel contribution to heritable variation. Furthermore, the experimental design did not allow for tests of transgenerational memory effects in plastic responses to drought and an analysis of maternal variation and maternal trait-based effects was lacking (Ganguly *et al.* 2017).

It is now well established that DNA mutations are not the only source of heritable phenotypic variation in plants. An additional system of inheritance often referred to as transgenerational epigenetics, typically involves stable differences of DNA methylation at or near transposable element (TE) sequences adjacent to genes (Quadrana and Colot, 2016). In the reference plant *A. thaliana*, most TE sequences are methylated at all cytosines, with methylation levels generally highest at CG sites (>80%), intermediate at CHG sites (40-60%) and lowest at CHH sites (<20%) (Cokus *et al.*, 2008; Lister *et al.*, 2008). TE sequences are methylated as a result of the combined activity of multiple DNA methyltransferases (Law and Jacobsen, 2010;

Stroud *et al.*, 2013; Stroud *et al.*, 2014) and can be actively demethylated by DNA glycosylases, which excise methylated cytosines from DNA (Law and Jacobsen, 2010). Demethylation is most pronounced in the central cell and leads to global hypomethylation of TE sequences in the endosperm, particularly on maternally-derived chromosomes (Satyaki and Gehring, 2017). In contrast, because methylation dynamics in TE sequences concerns almost exclusively CHG and CHH sites (Bouyer *et al.*, 2017; Kawakatsu *et al.*, 2017; Lin *et al.*, 2017), most TE sequences, thanks to the CG sites they contain, remain highly methylated in the female and male germlines as well as the embryo. Thus, the limited reprogramming of methylation patterns between generations during normal development implies a considerable potential for genome-wide transgenerational epiallelic variation following accidental loss of DNA methylation. However, because the *de novo* DNA methylation machinery targets distinct TE sequences with varying efficiency (Teixeira *et al.*, 2009; Zemach *et al.*, 2013), this potential is not uniformly distributed among TE-containing alleles. Specifically, while experimentally induced epiallelic variation can persist for at least eight generations and presumably many more at some TE-containing loci, it is fully erased within one or a few generations at others (Johannes *et al.*, 2009; Teixeira *et al.*, 2009; Colome-Tatche *et al.*, 2012). Consequently, *A. thaliana* accessions with different TE landscapes are expected to differ in their potential for transgenerational epigenetic variation. Finally, in the few cases where this was looked at, DNA methylation changes were observed in response to stressors and some of these changes were transmitted, but again transmission was limited to the immediate progeny (Secco *et al.*, 2015; Wibowo *et al.*, 2016; Ganguly *et al.*, 2017).

Here, we set out to determine if mild water deficit, a common stressor plants face in natural settings and to which plastic responses have been demonstrated at phenotypic and gene expression levels in *A. thaliana* (Tisné *et al.* 2013, Cubillos *et al.* 2014), could lead to new or altered intergenerational and transgenerational effects. We used a well-controlled multigenerational experimental design where the magnitude and timing of drought in the early part of the plant life cycle was replicated across generations for four natural accessions and the reference accession Col-0. We phenotyped two generations in detail and used individual relative growth rates as integrated measures of stress response. We carried out a validation of gene expression responses to drought using RNA-seq and an assessment of the likelihood of intergenerational epigenetics at the DNA methylation level in the reference accession Col-0. Our results show that mild drought induces phenotypic plasticity in each of the five accessions, but does not lead to any significant change in terms of heritable effects. In

addition, DNA methylome data indicate that mild drought induces intragenerational DNA methylation changes only, which are restricted to CHH sites and affect TE sequences predominantly. Taken together, our findings confirm that plants do not commonly generate transgenerational effects in response to changes in the environment, while intergenerational effects do occur.

Material and methods

Plant material and growth conditions

To investigate interactions between genotype and environment (G×E) in response to mild drought (Bouchabke *et al.*, 2008), we considered the accessions Col-0 (Col; Versailles stock # 186AV), Shahdara (Sha; 236AV), Bur-0 (Bur; 172AV), Tsu-0 (Tsu; 91AV) and Cvi-0 (Cvi; 166AV), which were obtained from the Versailles stock center (<http://publiclines.versailles.inra.fr/>). The four natural accessions were chosen because they show similar flowering time to Col-0 but extensive DNA methylation divergence among themselves and with Col-0 (Consortium, 2016; Kawakatsu *et al.*, 2016a), presumably in large part as a result of numerous differences in their TE landscapes (Stuart *et al.*, 2016; Quadrana *et al.*, 2016). Isogenic lines for each accession were grown under well-watered control and mild drought stress conditions for four generations (Figure 1), using the robotic platform Phenoscope (<https://phenoscope.versailles.inra.fr/>). This ensured uniform conditions during vegetative growth and enabled precise phenotype tracking (Tisné *et al.*, 2013). Control and mild drought stress conditions in each generation were chosen on the basis of phenotypic responses observed by Bouchabke *et al.* (2008) and Tisné *et al.* (2013) over a range of drought levels and on gene expression differences found by Clauw *et al.* (2015) and Cubillos *et al.* (2014) in the conditions used here. Growth conditions have been described in detail elsewhere (Tisné *et al.*, 2013). In the first generation (G1), 12 individuals (descending from the same mother plant) were grown per accession and per treatment. Half were used to establish 6 independent founder lines, which were maintained throughout the experiment by single seed descent propagation. In the following generations, six replicates per accession, treatment and treatment history were grown, with the exception of the third generation (G3) where only four replicates were grown due to space limitations on the robot (Fig. 1). Briefly, seeds were stratified for 4 days in the dark at 4°C and germinated for 8 days on peat moss plugs before these were transferred to the robot. Individual plants were then cultivated on the

Phenoscope for 21 days under short days (8 hours photoperiod) to minimize developmental differences between accessions and delay flowering transition (Mockler *et al.* 2003). The first week, during germination, soil was saturated with water. During the first week on the Phenoscope (days 9 to 15 after sowing), soil water content gradually decreased through controlled watering until it reached either 60% (control) or 30% (stress), which was then strictly maintained at these levels until day 29 after sowing. Then plants were moved to a standard growth chamber with optimal watering and long-day conditions to allow flowering and seed production. This strategy ensured that gametes or seeds were not themselves exposed to mild drought, minimizing intergenerational exposure effects. Seeds were collected from a single random individual per line \times treatment history.

For the subsequent generations, collected seeds were sieved to avoid sowing seeds that were in the top 10% or the bottom 10% of each line's seed size distribution. Seed size range varied between accessions (with Cvi-0 and Bur-0 having bigger seeds than Col-0 and Shahdara especially), but not between lines within accessions. Two phenotyping experiments were conducted (P3 and P4; Fig. 1) on lines from two treatment histories (P3: S1S2 versus C1C2 and P4: S1S2C3 versus C1C2C3).

Phenotyping

Zenithal rosette images of each individual plant were taken daily and segmented as described previously (Tisné *et al.*, 2013) to extract projected rosette area (PRA; a good proxy for rosette biomass at these developmental stages), rosette radius (the radius of the circle encompassing the rosette), compactness (the ratio between PRA and rosette circle area) as well as the red, green and blue components of the segmented rosette image. We report our phenotypic analysis of the last two generations of plants grown on the Phenoscope (P3 and P4).

P3&P4 Size and relative growth analysis

Data per accession and generation were separately analysed in detail. Sample sizes differ between P3 and P4. In simplified models covering both generations, this makes the P4 data weigh more on the results when parameters are shared between groups. We did analyse all data jointly for hypothesis testing on differences between generations and accessions.

Plant cohorts might contain groups with different properties and responses to treatments, while group membership might be unknown for each individual. To investigate such large initial heterogeneity among the plants selected for growth on the robot, initial PRA distributions on day 9 after sowing (essentially the summed cotyledon areas) were inspected

by finite mixture analysis (FlexMix library; Grün and Leisch, 2007). Gaussian models for initial log(PRA) with different numbers of component distributions (1 to 3) and different fixed treatment effects (stress/control in P3 or P4, stress/control in G1 and G2, i.e. G1/G2) for each component were compared using three information criteria (Akaike Information Criterion AIC, Bayesian Information Criterion BIC and Integrated Complete Likelihood ICL, Biernacki *et al.* 2000). Models with single Gaussian components consistently had the lowest values of the information criteria. There were no indications of hidden large heterogeneities among the plants installed on the robot.

Initial and final values of log(PRA) were further studied in detail using linear mixed models (Pinheiro and Bates, 2000). The maximal models fitted contained random line effects with different variances in G1/G2 control and drought groups. Models with more involved random effect specifications did not converge. Fixed effects were the exposure to drought in G1/G2 (intergenerational effect in the analysis of P3 and transgenerational effect in P4), treatment in P3 or P4 (plasticity), pot order effects on the robot and interactions of these variables. The maximal model contained heterogeneous error variances, different for each control/drought combination in G1/G2 and P3/P4. Model comparisons and simplifications were carried out using likelihood ratio tests LRT (using a REML fit for random effects, ML for fixed). Non-significant effects were removed one by one. Simplifications were first attempted in the random effects, then in the error variances and finally in the fixed effects, starting with the highest order interactions. Tests and estimates for random effects and error variances are reported for a REML model containing all fixed effects. P-values for fixed effects are reported with respect to the minimum adequate model (MAM) selected, i.e., the first model encountered that only contained significant effects.

We analyzed relative growth rates of PRA to understand the gradual response to environmental conditions. We first inspected the per-day relative increase of log(PRA) using generalized additive mixed models (gamms; Wood, 2017) and subsequently fitted mixed linear models (Pinheiro and Bates, 2000) to growth rates in age intervals where these appeared linear. Per treatment combination and accession, a gamm was fitted with smooths for day (age) and pot order effects (pot number). The gamm's assume random variation between individuals and exponentially decaying correlations between observations on the same individual. The mixed linear models fitted to restricted age intervals contained the same variables as the models for final size above, with the addition of random variation between individuals within lines, fixed age (day) effects and interactions of age with the other fixed effects.

When testing for differences of effects between generations and accessions, we fitted all data jointly, with the random effects and error variances as above but with different levels for generations and accessions added. In many cases this most elaborate model failed to converge and we reduced the amount of random effect and error variances fitted until it did. We then carried out model simplification and hypothesis testing on the fixed effects, which included interactions of the fixed effects above with generation and accession effects.

Phenotypic maternal trait-based effects

Our detailed analysis of PRA accounts for effects of ancestral environments in G1/G2, but does not include trait-based maternal effects (Kirkpatrick and Lande, 1989). These can lead to rapidly decaying or lasting transgenerational effects caused by individual parental variation. To investigate such effects and whether ancestral environments (i.e., memory) affect their strength and transmission, we analyzed all traits recovered from digital images in an equal manner. We restricted the analysis to the set of traits measured in both G2, P3 and P4, except for the image green component mean which had a correlation coefficient with the red component above 0.9. Per accession, we thus fitted linear mixed effects models to the log-transformed trait values after day 23. This part of the age trajectory of these traits was always approximately linear. We used individual maternal trait values on day 29 of G2 or G3 as explanatory variables to model the trait-based effects. For all traits, we tested whether trait-based maternal effects were present and differed between treatments in G1/G2 (ancestral environment \times maternal trait interaction) and treatments in P3 or P4 (plasticity \times maternal trait interaction). We removed data on a few plants with outlying patterns for the increase in log(PRA) before analysis: Observations with a Cook's distance value (in a simple regression on age) that was larger than one over the number of observations were removed. We fitted maximal mixed models to the data per accession and per trait with random effects of line and individual and heterogeneous error variances that could differ between environmental treatments experienced in G1/G2 and P3/P4. The model contained fixed effects of the G1/G2 and P3/P4 environmental treatments, pot order effects, age effects, the effects of the maternal trait values (difference from the overall mean) and interactions of these (except for age \times trait interactions and interactions between maternal traits). Model selection occurred as above. However, we observed that selected models often had confidence intervals for maternal effect slopes that still overlapped with zero or with each other and we simplified such effects out of the models. To have a simple graphical means to assess the validity of mixed model

predictions, we also fitted linear regressions to offspring trait – maternal trait combinations. All statistical analyses were conducted using R (R Core Team, 2005).

RNA-seq

To confirm effects of mild drought on gene expression, we performed RNA-seq on leaves isolated from Drought and Control Col-0 plants (3 replicates per treatment, 6 datasets). Leaf tissue was collected at 23 days after sowing from three Col-0 individuals grown on the Phenoscope under control and mild drought conditions and from the same seed batch as the founding Col-0 individuals used in the transgenerational design. Total RNA was extracted using the Qiagen RNAeasy extraction kit and sequenced at the Genome Center of the Max Planck Institute for Plant Breeding Research in Cologne, Germany. RNA-seq libraries were constructed using the standard Illumina Truseq protocol and sequenced in an Illumina HiSeq 2500 machine. Between 18.3 and 23.7 million reads were obtained per sample (average of 20.7) and aligned to the TAIR10 reference genome using TopHat2 with default parameters (Kim *et al.*, 2013). Reads aligning to multiple locations were removed using samtools' view with parameter -q 5 (Li *et al.*, 2009). After this filter, between 95.5 and 96.8 percent of the obtained reads were aligned to the reference genome. The number of reads per transcript was counted using the Bioconductor packages Rsamtools and ShortRead (Morgan *et al.*, 2009). Differential expression between samples in control and drought conditions was calculated with the DESeq2 package in R (Love *et al.*, 2014). Genes with *q*-values lower than 0.05 and log2FC above 0.5 were considered as differentially expressed. TE differential expression was analyzed using TETOOLS (Lerat *et al.*, 2016). We used the Panther classification system for Gene Ontology (GO) analysis (Mi *et al.* 2019) and used Fisher's exact tests with Benjamini–Hochberg false discovery rate (FDR) corrections to test for over-representation of functional classes corresponding to different biological processes. Only the results for genes with FDR < 0.05 are inspected.

Whole-genome bisulfite sequencing (WGBS)

To investigate plasticity of genomic DNA methylation patterns in response to mild drought, WGBS was performed on pooled DNA extracted at day 29 after sowing from mature leaves of 12 Col plants that were being subjected to control or water deficit treatments (1 pooled sample per treatment, two datasets). To assess intergenerational effects, we performed independent WGBS experiments on ten-day-old seedlings derived from 5 independent C₁C₂

and 5 independent S₁S₂ G2 lines grown under standard *in vitro* conditions (10 datasets). MethylC-seq library preparation and sequencing was performed by BGI (Shenzhen, China) using standard Illumina protocols. Adapter and low-quality sequences were trimmed using Trimming Galore v0.3.3. Mapping was performed on TAIR10 genome annotation using Bismark v0.14.2 (Krueger and Andrews, 2011) and the parameters: --bowtie2, -N 1, -p 3 (alignment); --ignore 5 --ignore_r2 5 --ignore_3prime_r2 1 (methylation extractor). Only uniquely mapping reads were retained. The methylKit package v0.9.4 (Akalin *et al.*, 2012) was used to calculate differential methylation in individual positions (DMPs) or in 100bp non-overlapping windows (DMRs). Significance of calculated differences was determined using Fisher's exact tests and Benjamin-Hochberg (BH) adjustment of *p*-values (FDR < 0.05) and methylation difference cutoffs of 40% for CG, 20% for CHG and 20% for CHH. Differentially methylated windows within 100bp of each other were merged to form larger DMRs. Cytosine positions covered by more than 100 reads were not considered. For DMP analysis only cytosines covered by a minimum of 6 (CG and CHG) and 10 (CHH) reads in all libraries were considered. Bisulfite conversion rates were estimated by the number of methylated cytosine calls in the chloroplast genome.

RNA-seq and MethylC-seq sequencing data have been deposited in the ENA short read archive (project number PRJEB27682).

Results

Mild drought induces stress: immediate phenotypic plasticity and gene expression changes

As expected (Tisné *et al.*, 2013), growth dynamics of the projected rosette area (PRA) showed clear phenotypic plasticity in response to mild drought for each accession analyzed and in each generation where we imposed it. PRA decreased significantly when accessions were grown under mild drought (in generation G1, Figure 2a). Depending on the accession, it reached values at an age of 29 days after sowing that were on average 27% to 40% lower than in control conditions (G1, Fig. 2b). All accessions therefore have reduced growth in drought conditions, which is evidence of stress (Claeys *et al.* 2014). Consistent with results of previous studies of the transcriptional response to mild drought (Cubillos *et al.*, 2014; Clauw *et al.*, 2015), differential analysis of the two RNA-seq datasets identified significant changes

in steady state mRNA levels for 468 genes (FDR < 0.05, log2FC > 0.5, 205 and 263 genes with lower and higher expression under mild drought, respectively; Supporting Information Table S9), but not for any of the annotated TE sequences. A possibility is that mild drought is too weak as a stressor to overcome the typically strong epigenetic silencing of TE sequences. Gene Ontology analysis (Supporting Information Table S10) revealed over-representation of several stress-related categories, including cell wall thickening and responses to water deprivation.

Intergenerational and transgenerational environmental effects

We then compared, in as much detail as possible, phenotypic traits of plants whose parents experienced drought treatments for two consecutive generations and of their progeny (Fig. 1). Phenotyping at P3 compares C₁C₂ vs. S₁S₂ and assesses intergenerational effects. Phenotyping P4 compares C₁C₂C₃ vs. S₁S₂C₃ treatment histories. The G3 generation without drought treatment allows only transgenerational effects to contribute to effects of treatment history in P4.

The effect of drought in G1/G2 on initial log(PRA) differs between accessions and generations ($\chi^2_4 = 11.9$, $p = 0.018$), with Bur-0 and Sha the most similar in their responses (Table 1). There are intergenerational effects but no transgenerational effects of drought on initial log(PRA), and the intergenerational effects differ between accessions. Regarding final log(PRA), we cannot demonstrate any significant interactions between factors and generation or accession effects when analysing all data together. We find significant overall plasticity ($\chi^2_1 = 92.8$, $p < 0.0001$) in agreement with the results on G1 (Fig. 2) and a trend for an overall effect of drought in G1/G2 ($\chi^2_1 = 3.50$, $p = 0.06$). When analysing accessions and P3 and P4 separately, we again detected intergenerational effects on initial log(PRA) and also on final log(PRA). Plants from a history with drought are smaller (Table 1). In this case, we did not find any transgenerational effect of exposure to drought on initial and final log(PRA) of P4. The trend for an overall effect of drought on final size in the analysis with P3 and P4 combined must therefore be ascribed to the intergenerational effects detected and not to any transgenerational effects. Unexpectedly, we sometimes found effects of drought on initial log(PRA) heterogeneity (residual variance). These effects must be spurious, as the stress treatment has not started yet at this stage. Therefore, we do model variance heterogeneity throughout but do not present nor interpret the results. For mean final size, we find that all

accessions except Sha show significant plasticity in each generation. Sizes are larger in the control treatment (Table 1).

Our gamm analysis indicated that relative growth rates (RGR) from days 13 to 16 and from days 25 to 28 could be considered linear per individual (Figure 3, representative results for accession Col-0 in P3 and P4). For early RGR we could not demonstrate differences between accessions in plasticity nor drought history effects. There are however significant plasticity and drought effect differences between P3 and P4 (day \times plasticity \times generation: $\chi^2_1 = 4.87$, $p = 0.03$; day \times drought in G1/G2 \times generation: $\chi^2_1 = 10.9$, $p = 0.001$). For late RGR, we found a weakly significant effect of drought history ($\chi^2_1 = 3.96$, $p = 0.05$). Plasticity in late RGR differs between accessions (accession \times plasticity $\chi^2_1 = 10.16$, $p = 0.04$) and between generations (day \times generation \times plasticity $\chi^2_1 = 12.2$, $p = 0.0004$). In the analyses per generation and accession separately, we did not detect transgenerational effects but did find significant intergenerational effects of drought on RGR from days 13 to 16 in three accessions and in a single accession from days 25 to 28 (Table 2, Fig. 3, Table 3). Plants from a history with drought grow relatively more. This could be seen as evidence of a stress memory. If we compare plastic responses for RGR from days 13 to 16, these are found to be identical in generations P3 and P4 (Table 2). Modeling RGR demonstrated a steeper decrease with age after the water supply reduction in all accessions (Table 2, Fig. 3). In control conditions and on day 13, plants grow equal or faster than in mild drought, and while the RGR of plants in drought goes down with age, it does much less so or not in control conditions. The plastic phenotypic responses in P3 show the same qualitative pattern for days 25 to 28 as for earlier RGR. However, for three out of five accessions in P4, plants subjected to mild drought acclimated and recovered RGR similar to that of control plants (days 25 to 28, Table 3, Fig. 3). For Cvi-0 we observe the same pattern as in P3. We found compensatory growth for Shahdara; RGR decreased less with age in the drought group and on day 28 the RGR of the drought group is faster than for the control. Strengths of RGR plasticity apparently can vary between generations, potentially in dependence on environmental history, but not at all ages. There were no transgenerational effects of exposure to drought in G1/G2 on average growth rate values in P4. Different magnitudes of between-individual variance across the drought histories did occur for Col-0, Cvi-0 and Bur-0. Individual variation in relative growth rates was larger from days 13 to 16 for the Col-0 individuals in P4 descending from parents in the G1/G2 drought group ($p < 0.001$); for Bur-0, it was larger in P3 ($p = 0.045$) and smaller in P4 ($p = 0.022$). For Col-0, the growth rate variance from days 25 to 28 was smaller among descendants of the G1/G2 drought group ($p < 0.001$). For Cvi, this variance was larger in P3

and P4 ($p = 0.027$, $p < 0.001$). This pattern points out that amounts of trait variation at the end of an experiment or at the moment where selection occurs could be determined by intricate time-dependent variances in growth processes. The gene expression responses in the plants thus can resolve stress, but this is not uniformly the case.

Limited presence and persistence of maternal trait-based effects

Models with maternal trait effects on individual phenotypes in P3 or P4 found in eleven out of 250 tests that the slope of the maternal trait regression depended on the environmental regime experienced by ancestors in G1/G2 (Supporting Information Tables S1 and S2). In two cases it depended on the environmental regime in P4. By inspecting the models per accession, we could make an assessment of whether these changes in maternal trait slopes would affect the persistence of these maternal effects. This could be the case when effects of traits on themselves have become larger in absolute value or when a causal chain of traits has obtained a stronger weight (Kirkpatrick and Lande, 1989). Only for compactness and mean blue value did we find a maternal trait-based effect where the trait has an effect on itself. There were no enchainned maternal trait effects that could lead to lagged responses. When we inspected the maternal trait dependency of compactness in Col-0 and Bur-0 (Fig. 4), we noted that the ranges of maternal trait values differed between descendants of stress and control treatments in G1/G2.

The results did show that maternal trait models have slopes that can depend on historical and current environments and that the scope for a change in the persistence of maternal effects due to mild drought was limited (Fig. 4). The slopes of the effects were not particularly strong nor general across accessions and they did not seem a valid candidate for persistent transmission of trait variation. The fraction of tests significant for effects of maternal traits on offspring and with interpretable confidence intervals, is very close to a 5% type I error rate (13/250). We therefore conclude that the number of heritable effects transmitted through maternal trait-based effects is negligible.

In 18 out of 50 trait×accession models, individual variation was enlarged in the drought environment in P3 or P4 (Supporting Information Tables S3 and S4). In 10 out of 50 models the variation between individuals was larger among descendants of individuals descending from drought in G1/G2. In five cases this variance was smaller.

Mild drought induces DNA methylation variation unrelated to known transcriptome changes associated with drought stress

To complement the phenotypic analysis, we investigated the impact of mild drought on genomic DNA methylation patterns using the reference accession Col-0, which showed the strongest phenotypic response (Fig. 2b) and for which a wealth of epigenomic data are available. To investigate within-generation plastic responses and capture as many cumulative changes as possible, WGBSeq was performed on DNA extracted at day 29 from leaves of pools of treated and control plants at P4 (C1C2C3 descendants, Fig 1; Supporting Information Tables S5 and S6). Overall, cytosine methylation levels are similar between control- and stress-treated leaves (Supporting Information Fig. S1a), albeit slightly higher than in previous reports (8.6% and 9% of methylated cytosines vs. 6.7%), presumably because of differences in mapping and methylation calling methods as well as in the organs examined (Cokus *et al.*, 2008; Lister *et al.*, 2008). Other global measures, such as the distribution of methylation between the three types of sites and annotations are also identical for control- and stress-treated leaves (Supporting Information Figs. S1b and c). We conclude that mild drought does not directly affect overall DNA methylation patterns in *A. thaliana*.

To identify local differences, methylation levels were compared at individual cytosine positions as well as in 100bp windows for each of the three types of sites (CG, CHG and CHH) separately (see Methods). Based on this approach, we could identify 286 differentially methylated positions (DMPs) and 1360 differentially methylated regions (DMRs), most of which are defined by single 100bp windows (Supporting Information Tables S7 and S8). All DMPs map to CG sites whereas most DMRs (95%) are CHH DMRs only (Fig. 5a). The vast majority of CG DMPs (93%) are within methylated gene bodies (Fig. 5a and b) and they reflect almost equally either increased or decreased methylation levels in treated plants compared to controls (Fig. S2), consistent with the notion that gene body methylation tends to vary stochastically across generations at individual CG sites (Becker *et al.*, 2011; Schmitz *et al.*, 2011; Jiang *et al.*, 2014). On the other hand, CHH-DMRs are mainly located over TE sequences and tend to reflect hypermethylation in plants experiencing drought (Fig. 5c and d, Supporting Information Fig. S2).

As different TE families may show different sensitivities to environmental cues (Pecinka *et al.*, 2010; Yu *et al.*, 2013; Grandbastien, 2015; Matsunaga *et al.*, 2015; Quadrana *et al.*, 2016), we assessed whether CHH-DMRs are preferentially localized over specific TE families. Out of the 326 TE and other repeat families annotated in the TAIR10 *A. thaliana* genome, 164 show at least one DMR and 18 families are enriched in DMRs compared to the random

expectation (Fig. 5e). These include the LTR-retrotransposon family *ATCOPIA78*, which is known to be sensitive to biotic and abiotic stress (Yu *et al.*, 2013; Quadrana *et al.*, 2016; Matsunaga *et al.*, 2015). On the other hand, only a small percentage of CHH DMRs caused by mild drought overlap with DMRs that arose spontaneously in mutation accumulation lines (9.3%; Hagmann *et al.*, 2015) or that were induced by hyperosmotic stress (1.9%; Wibowo *et al.*, 2016; Fig. 5f). Thus, we conclude that mild drought induces a limited number of robust DNA methylation changes over regions that are distinct from those subjected to stochastic or salt-induced DNA methylation variation.

To investigate further the CHH-DMRs induced by mild drought, we compared their CHH methylation level in different DNA methylation mutants (Stroud *et al.*, 2013) and found that most correspond to regions targeted by the RNA-directed DNA methylation (RdDM) pathway, which involves the DNA methyltransferase DRM2, rather than by the alternative CHH maintenance methylation pathway mediated by the DNA methyltransferase CMT2 (Fig. 5g). Consistent with these findings, TE sequences overlapping drought induced CHH-DMRs have a high abundance of matching 24nt small RNAs (Fig. 5h). Moreover, no association was detected between drought induced CHH-DMRs and regions subjected to active DNA demethylation (*rdd* mutant; Fig. 5g). In conclusion, mild drought directly affects mainly sequences targeted by RdDM.

None of the genes known to be involved in DNA (de)methylation appeared to be affected by mild drought, which leaves the question open as to which factors induce DNA methylation changes during mild drought.

Among the 468 genes detected as transcriptionally responsive to mild drought in our conditions (G1), only two are affected by CG DMPs, which are likely inconsequential given the lack of function associated with gene body methylation. Another two are located less than 500bp from a DMR (Supporting Information Fig. S3a). These two DMRs are of the CHH type but do not correspond to annotated TE sequences. One DMR maps to the promoter region of gene *AT5G35735*, which encodes an auxin responsive protein of unknown function. The other DMR is located within the first intron of gene *AT3G10340*, which encodes a putative phenylalanine ammonia-lyase that may be involved in plant defense against biotic and abiotic stresses (Raes *et al.*, 2003). Given the first intron's large size (1.3kb), it likely contains regulatory sequences (Morello and Breviario, 2008). Moreover, hypermethylation of the promoter DMR of *AT5G35735* and hypomethylation of the intronic DMR of *AT3G10340* in response to mild drought are associated with down and up regulation, respectively (Supporting Information Fig. S3b). Taken together, these findings suggest a causal link

between altered gene expression and altered DNA methylation for these two genes. However, the observation that most genes affected by mild drought are not proximal to drought-induced DMPs or DMRs indicates that plastic changes in DNA methylation have a marginal role in the phenotypic response of plants to mild drought. An alternative explanation for our results is that transcriptional and methylation patterns might be so heterogeneous and dynamic, that only analyses of both on the same individuals and very close in time can recover correlations. That would then immediately evidence the limited scope for propagation of effects.

No intergenerational effects on DNA methylation

Finally, we tested whether intergenerational DNA methylation changes occurred. Taking into consideration the possibility of cumulative effects over successive generations of growth under drought, G3 progenies of Col-0 C₁C₂ and S₁S₂ plants were chosen for further analysis (Fig. 1a). WGBseq was performed on DNA extracted from unstressed individuals of the progeny of five Col C₁C₂ and S₁S₂ founder lines (Methods and Supporting Information Tables S54 and S6). Differential DNA methylation was investigated as described above using the five C₁C₂ or S₁S₂ progenies as biological replicates. Following this approach, no single consistent DMR could be identified between the two types of progeny, hence no intergenerational effect could be identified. In addition, we carried out all possible inter-individual comparisons, which again yielded no single DMR. However, there was a marginal increase in the amount of stochastic variation in DNA methylation for the three sequence contexts among progenies derived from the five stressed parental lines (Supporting Information Fig. S4). In conclusion, our results suggest that there are no targeted and specific DNA methylation changes induced by mild drought that will persist into the next generation, although drought exposure may increase methylome heterogeneity among progeny of stressed plants and other intergenerational responses might still leave a trace in the methylome later.

Discussion

It has been proposed that exposure to environmental cues can trigger phenotypic changes that become inherited for more than one generation, and that this occurs through epigenetic mechanisms (Bossdorf *et al.*, 2008; Richards *et al.*, 2017). In this study, we showed that water deficit applied before the reproductive stage in two successive generations affects the vegetative growth of individuals negatively. As expected, we identified expression changes in genes involved in for example cell wall thickening and responses to water deprivation.

However, mild drought did not affect overall DNA methylation patterns and most genes with differential expression are not proximal to drought-induced DMRs, which suggests a marginal role of DNA methylation changes in the phenotypic response of plants to mild drought. We also detected intergenerational drought effects on the rosette sizes at the age where plants were placed on the robot and in relative growth rates. Transgenerational effects of mild drought after two successive generations of exposure are limited to changes in amounts of individual variation in phenotypic traits. The variance can increase or decrease depending on the accession and trait considered. Furthermore, the amount of stochastic DNA methylation variation increased marginally in lines with ancestral drought, suggesting that intergenerational effects on variability occur, which might persist into the next generation, but without any clear directional phenotypic effects. Finally, we found a limited number of maternal trait-based effects in any of the accessions. Their occurrence is overall close to the type I error rate. Therefore, our results add to the growing body of evidence against transgenerational epigenetic changes being a predictable and common response of plants to changes in the environment (Boyko *et al.*, 2010; Suter and Widmer, 2013a,b; Groot *et al.*, 2016; Wibowo *et al.*, 2016; Ganguly *et al.*, 2017).

Intergenerational plasticity is limited and does not lead to transgenerational effects

Stressors consistently affect the expression of a large number of genes and induce in a number of cases CHH hyper or hypomethylation of a variable number of TE and other repeat sequences (for example Downen *et al.*, 2012; Eichten and Springer, 2015; Secco *et al.*, 2015; Wibowo *et al.*, 2016; this study). However, gene expression is rarely associated with DNA methylation changes (Meng *et al.*, 2016) and for a given stressor, the extent of the latter as well as the mechanisms at play may differ radically between plant species (Secco *et al.*, 2015). While in *A. thaliana* many of the CHH-DMRs induced by salt stress are transmitted to the immediate progeny (Wibowo *et al.*, 2016), this is not the case for the CHH-DMRs induced by mild drought. In rice there was also no transmission of the CHH-DMRs induced by phosphate starvation (Secco *et al.*, 2015). Thus, evidence so far points to a clear effect of environmental factors in triggering DNA methylation changes, which however do not persist in offspring. Indeed, different mechanisms preventing transmission of environmentally induced epigenetic states across generations have been described (Baubec *et al.*, 2014; Crevillen *et al.*, 2014; Iwasaki and Paszkowski, 2014) and our design aimed to minimize intergenerational plasticity. Nonetheless, true transgenerational epigenetic variation exists in nature (Silveira *et al.* 2013; Quadrana and Colot, 2016) and what generates it remains

unresolved. Analyses of natural populations are now just beginning to investigate this question, with no clear answer so far, except that most DNA methylation variants seen in nature are likely caused by DNA sequence variation and therefore are by definition not truly epigenetic (Durand *et al.* 2012; Schmitz *et al.*, 2013; Li *et al.*, 2014; Dubin *et al.*, 2015; Kawakatsu *et al.*, 2016b; Niederhuth *et al.*, 2016; Quadrana *et al.*, 2016; Agorio *et al.* 2017).

At the morphological level, we detected different responses between accessions, with both plasticity, intergenerational effects and limited trait-based maternal effects playing a role. Maternal trait-based effects with potentially lasting effects occur at low frequency. Only for rosette compactness in a single accession (Bur-0) does an effect of a trait on itself occur in both P3 and P4. However, there is only an effect of ancestral drought on the slope in P3 and not P4. Mild drought changes trait variances but not consistently slopes of maternal trait-based effects. Therefore mild drought did not change this presumed mechanism of non-genetic heritability.

Plasticity is likely adaptive, a memory effect not

Modeling suggests a potential for DNA methylation-based transgenerational epigenetics to endow plants with a means to generate adaptive heritable phenotypic variation in response to changing environments (Bossdorf *et al.*, 2008; Geoghegan and Spencer, 2013a, b; Uller *et al.*, 2015; Kronholm and Collins, 2016). Models show that environmentally induced epiallelic variation can be favored over purely stochastic switching (Furrow and Feldman, 2014). We did not detect such phenotypic changes and did not even find specific intergenerational methylation changes. The general lack of such effects in our experiment might indicate that they are actually unnecessary in the range of imposed environments. In agreement with models of adaptation (Kuijper and Hoyle, 2015), we find that responses by means of phenotypic plasticity are stronger than by maternal effects. The rate at which intergenerational drought effects rapidly decrease with plant age in the P3 group, and the absence of transgenerational effects seems to indicate that mild drought stress does not induce strong physiological changes that persist for long and could affect transmission of information or resources. On the contrary, a number of days after drought set in, several accessions in P4 managed to return to the same relative growth rates in mild drought as in our control, demonstrating acclimation and overcompensation in one accession. This suggests that there is little remaining stress at that point. However, we did not find the same pattern in P3. Follow-

up experiments including additional accessions with incomplete recovery would allow testing whether lines with persistently incomplete acclimation have more transgenerational effects and whether indeed ancestral environmental history effects could explain this variability in strengths of plasticity. The adaptiveness of stress responses in certain conditions could be investigated further with Finlay-Wilkinson regressions (Finlay and Wilkinson 1963). This would require sampling seeds per individual in the experiment to have lifetime measures of fitness or yield and additional levels of (mild) drought to permit fitting the regressions.

The intergenerational responses observed could be consequences of environmental effects on seed investment. The changes in trait variance could indicate that mild drought stress affects the predictability of the near future, without much of a consistent trend in expectations. Our observed changes might then be more in support of a bet-hedging strategy (e.g., Crean and Marshall, 2009).

In conclusion, our study provides strong support to the notion that plants first respond to physiological stressors through well-defined and conserved transcriptional networks (Juenger, 2013; Ding *et al.*, 2014; Clauw *et al.*, 2015) or immediate parental influences on offspring phenotypes (Herman and Sultan, 2011; Wibowo *et al.*, 2016). Whether transgenerational epigenetic variation in nature is caused by more dramatic environmental conditions than those tested so far in the laboratory, or by combinations of several mild stressors, or by mutations in genes such as *A. thaliana* *DDMI* that are involved in the epigenetic control of TE remains to be determined (Quadrana and Colot, 2016).

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Author contributions

T.J.M.V.D., A.B.S., O.L., and V.C. planned and designed research; A.B.S., E.G., A.M., L.B and S.T. performed experiments and collected the data; T.J.M.V.D., A.B.S., L.Q., analyzed the data; J.J.G. contributed unpublished results; T.J.M.V.D., A.B.S., O.L. and V.C. wrote the manuscript with the help of all authors.

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Table 1. Effects of environmental states in G1/G2 and phenotypic plasticity on the initial and final average projected rosette area log(PRA) in generations P3 and P4. Confidence interval results from linear mixed models per accession, based on parameter estimates of minimum adequate models are shown. Symbol "*" indicates accessions where the variance between lines is retained in the model. LRT = Likelihood Ratio Test. All tail probabilities are from χ^2_1 tests.

Size on day 8 after germination								
Accession	Intergenerational effect of drought in G1/G2	LRT	Plasticity P3(Control - Drought)	LRT	Transgenerational effect of drought in G1/G2	LRT	Plasticity P4 (Control - Drought)	LRT
COL	[-0.143, -0.016]	p = 0.013	-	NS	-	NS	-	NS
BUR	[- 0.414, -0.129]	p = 0.001	-	NS	-	NS	-	NS
CVI*	[-0.128, -0.035]	p = 0.001	-	NS	-	NS	-	NS
TSU	NS	NS	-	NS	-	NS	-	NS
SHA	[-0.322, -0.167]	p < 0.001	-	NS	-	NS	-	NS
Size on day 29 after germination								
Accession	Intergenerational effect of drought in G1/G2	LRT	Plasticity P3(Control - Drought)	LRT	Transgenerational effect of drought in G1/G2	LRT	Plasticity P4(Control - Drought)	LRT
COL	-	NS	[0.175, 0.318]	p < 0.001	-	NS	[0.197 - 0.375]	p < 0.001
BUR	[-0.211, -0.092]	p < 0.001	[0.330, 0.448]	p < 0.001	-	NS	[0.317 - 0.546]	p < 0.001
CVI*	-	NS	[0.397, 0.509]	p < 0.001	-	NS	[0.394 - 0.557]	p < 0.001
TSU	-	NS	[0.305, 0.423]	p < 0.001	-	NS	[0.250 - 0.384]	p < 0.001
SHA	[-0.228, -0.026]	p = 0.013	[0.310, 0.449]	p < 0.001	-	NS		NS

Table 2. Effects of environmental states in generations G1/G2 and P4 on the mean relative growth rate (RGR) from days 13 to 16 in P3 and P4. Results from linear mixed model analysis and model after selection. Age in number of days is rescaled to value zero at day 13, to ease interpretability of estimates. In the table, accessions with random effects that differ in line/individual variance between the two G1/G2 treatment levels are indicated by symbol "**". Symbol "*" indicates accessions where the variance between lines is retained in the model. "#" indicates accessions for which the pot effects (linear effect of pot number on the robot) were retained.

Accession	Generation	Drought in G2 effect on intercept	Drought in G2 x Day slope	LRT test	Plasticity (Intercept difference between Control – Drought groups)	Plasticity Day slopes	LRT test
COL	P3 (inter-)	[-0.006, 0.047]	[-0.026, -0.000]	p = 0.042	[-0.032, 0.025]	Drought [-0.052, -0.026] Control – Drought [0.030, 0.059]	p < 0.001
COL**	P4 (trans-)			NS	[-0.003, 0.026]	Drought [-0.052, -0.041] Control – Drought [0.033, 0.046]	p < 0.001
BUR**	P3 (inter-)	[0.010, 0.029]	-	p < 0.001	[-0.011, 0.017]	Drought [-0.073, -0.060] Control – Drought [0.038, 0.053]	p < 0.001
BUR** #	P4 (trans-)			NS	[-0.020, 0.016]	Drought [-0.073, -0.060] Control- Drought [0.045, 0.060]	p < 0.001
CVI	P3 (inter-)			NS	[0.002, 0.045]	Drought [-0.077, -0.059]	p < 0.001

						Control – Drought [0.024, 0.046]	
CVI	P4 (trans-)			NS	[0.007, 0.061]	Drought [- 0.058 , - 0.037] Control – Drought [0.017, 0.046]	p < 0.001
TSU*	P3 (inter-)			NS	[-0.008, 0.034]	Drought [- 0.051, -0.036] Control- Drought [0.025, 0.044]	p < 0.001
TSU*	P4 (trans-)			NS	[-0.009, 0.024]	Drought [-0.062, - 0.049] Control – Drought [0.033, 0.049]	p < 0.001
SHA	P3 (inter-)	[0.007, 0.020]		p < 0.001	[-0.037, 0.015]	Drought [- 0.094, -0.067] Control – Drought [0.057, 0.085]	p < 0.001
SHA*	P4 (trans-)			NS	[-0.075, -0.000]	Drought [-0.107, - 0.080] Control – Drought [0.077, 0.106]	p < 0.001

Table 3. Effects of environmental states in G1/G2 and P4 on the mean relative growth rate from days 25 to 28 in P3 and P4. Results from linear mixed model analysis. Age in number of days is rescaled to value zero at day 28, to ease interpretability of estimates. In the table, accessions with random effects that differ in line/individual variance between G1/G2 groups are indicated by "***". Symbol "*" indicates accessions where the variance between lines is retained in the model. "#" indicates accessions for which the pot effects (linear effect of pot number on the robot) were retained.

Accession	Generation	Drought in G2 effect on intercept	Drought in G2 x Day slope	LRT test	Plasticity (Intercept difference between Control - Drought groups)	plasticity Day slopes	LRT test
COL	P3 (inter-)				[-0.001, 0.024]	Drought [-0.009, -0.003] Control - Drought [0.001, 0.010]	p = 0.011
COL***	P4 (trans-)			NS			NS
BUR	P3 (inter-)	[0.002, 0.012]	NA	p = 0.006	[0.020, 0.044]	[-0.025, -0.019] [0.006, 0.015]	p = 0.011
BUR*	P4 (trans-)			NS			NS
CVI***	P3 (inter-)			NS	[0.023, 0.048]	[-0.011, -0.006] [0.004, 0.012]	p < 0.001
CVI**	P4 (trans-)			NS	[0.019, 0.045]	Drought [-0.020, -0.015] Control - Drought [0.007, 0.015]	p < 0.001
TSU#	P3 (inter-)			NS	[0.028, 0.055]	[-0.030, -0.022] [0.009, 0.018]	p < 0.001

TSU*#	P4 (trans-)			NS			NS
SHA	P3 (inter-)			NS			NS
SHA	P4 (trans-)			NS	[-0.053, - 0.018]	Drought [-0.003, -0.002] Control - Drought [-0.014,- 0.001]	p = 0.021

Figure legends

Fig. 1. Schematic representation of the multigenerational experimental design. G, generation of growth; P, phenotyping experiment.

Fig. 2. Descriptive analysis of growth curves for projected rosette area (PRA) in the first generation (G1) for the five accessions grown under control or drought conditions. Individuals that remained below one cm² PRA by the end of the experiment or that died prematurely are not shown.

(a) Kinetics of shoot size estimated by daily measurements of PRA. Growth curves of plants in control conditions are in blue, those that experienced mild drought in red. Black lines represent the averages per group. (b) Box and whiskers plot showing PRA on day 29 for the plants in each accession × treatment combination. Inset photographs show representative plants per accession × treatment.

Fig. 3. The time-dependent pattern of relative growth rates in P3 and P4 as predicted for the first pot on the robot (the model accounts for pot order) and for accession Col-0, based on generalized additive mixed models (gamms). On the x-axis the age of the plants in days is given. Plants are nine days old after sowing when the recording starts. Four central panels for each phenotyping experiment show the raw data per treatment combination. Above and to the right of each central column and row, predicted trajectories are grouped for two panels each time so that pairwise comparisons between ancestral drought treatment levels and drought in the phenotyping generation (plasticity) can be made in both P3 and P4. The raw data values are shown in grey together with a predicted relative growth rate trajectory per combination of treatments in G1/G2 (Memory) and P3 or P4 (Plasticity). P3/P4 treatments are shown in blue (Control) or red (Drought). The graphs demonstrate clear growth plasticity in response to mild drought and that plants manage to compensate the initial drop in relative growth rate shortly after the mild drought has reached a stable level at day 20. Note that there are very few plants with outlying patterns and that these have very low growth rates for a restricted age window only.

Fig. 4. Effects of maternal traits in Col-0 and Bur-0, for P3 and P4. For each phenotyping experiment, the data points of both accessions are shown in grey in each panel. Dependencies

of offspring trait values on maternal trait values are shown for log-transformed rosette compactness. Linear regressions are estimated per ancestral drought environment. Data points are blue for individuals with ancestors under drought in G1/G2, grey for individuals with ancestors under control.

Fig. 5. Characterization of stress-induced local DNA methylation changes.

(a) Number of DMPs and DMRs for each sequence context (CG, CHG and CHH). (b) Annotation of DMPs and DMRs in relation to genes, TEs and intergenic regions. (c) Distribution of local gains and losses of DNA methylation across DMPs and DMRs. (d) Example of CHH DMRs on a TE. (e) Graphical representation of the 18 TE families that show more DMRs than expected by random (p -value < 0.01). (f) Overlap (including 500bp flanking windows) of DMRs induced by mild drought and DMRs found in mutation accumulation (MA) lines (Becker *et al.*, 2011; Schmitz *et al.*, 2011) or induced by hyperosmotic stress (Wibowo *et al.*, 2016) (g) Hierarchical clustering based on average CHH methylation levels in wild-type (wt) and mutants for the RdDM (*rdr2*, *ago4* and *drd1*), CMT2 (*ddm1* and *cmt2*) and DNA demethylation (*rdd*) pathways in regions overlapping hyper or hypermethylated CHH-DMRs. (h) Abundance of 24nt siRNAs in random or CHH-DMR containing TEs.

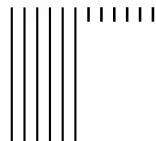
G1

C1 Control



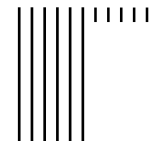
12 plants

lines 1-6



S1 Stress

lines 7-12

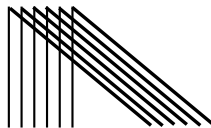


12 plants

G2

6 plants per line

C2 Control

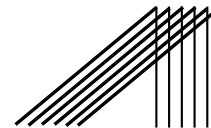


History C1C2

C3 Control

S3 Stress

S2 Stress



History S1S2

C3 Control

S3 Stress

G3

P3

4 plants per line



History C1C2C3

C4 Control

S4 Stress



History S1S2C3

C4 Control

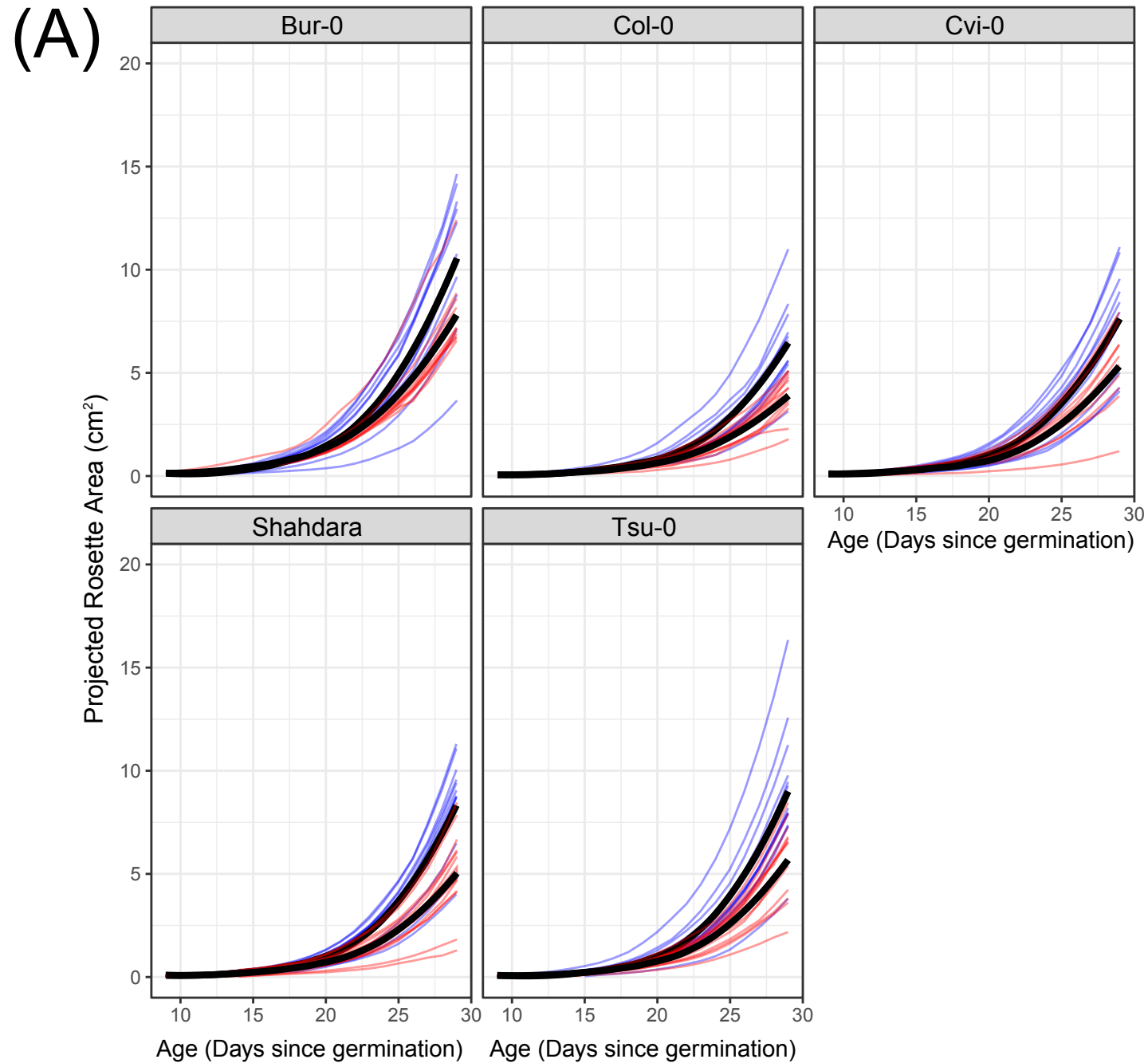
S4 Stress

G4

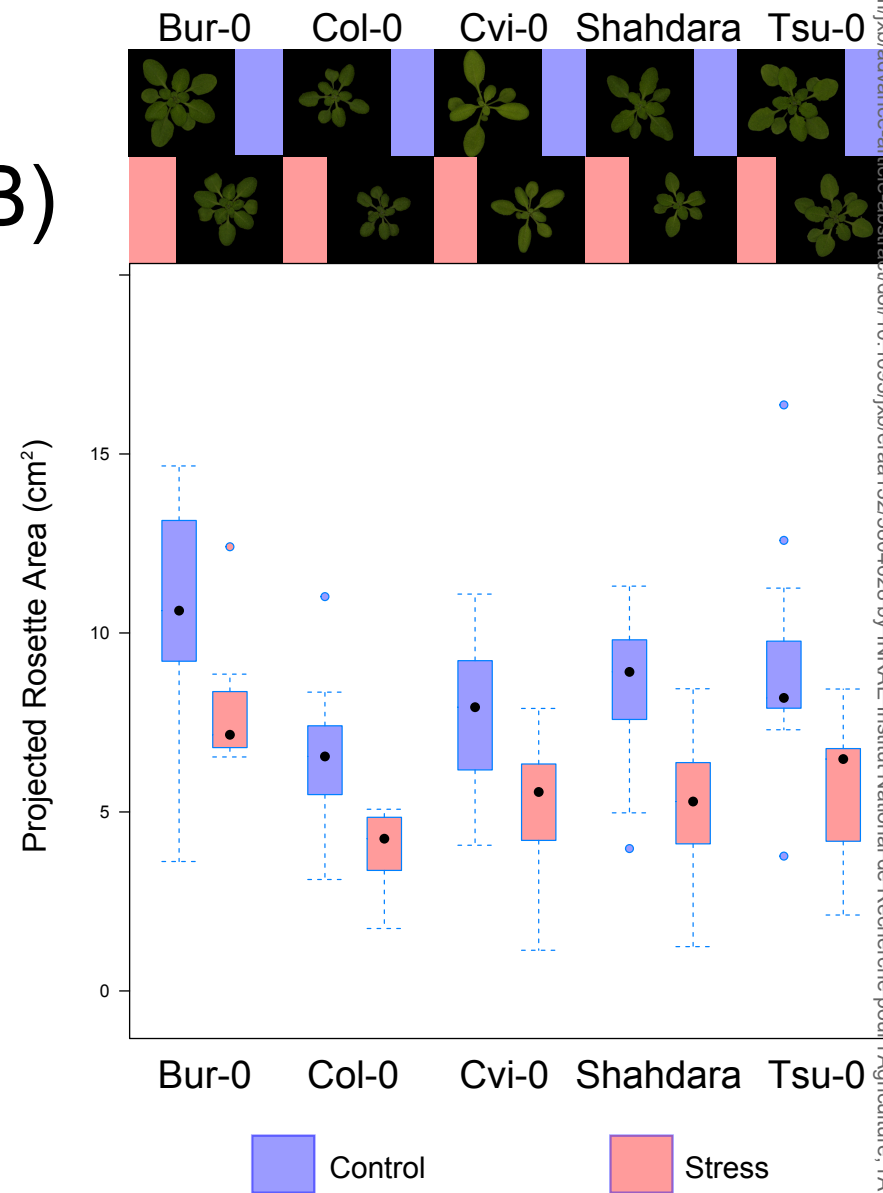
P4

6 plants per line

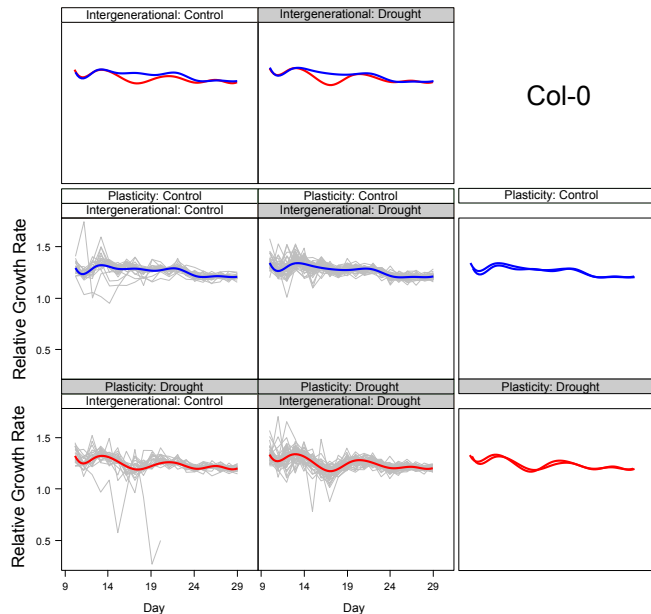




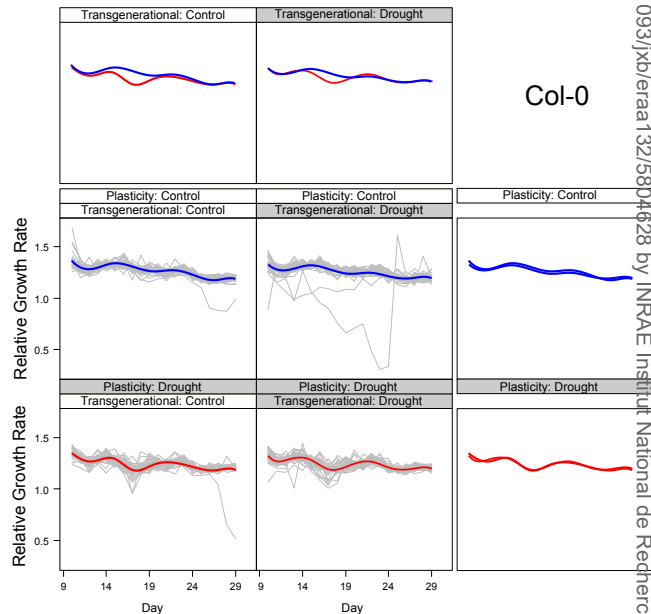
(B)



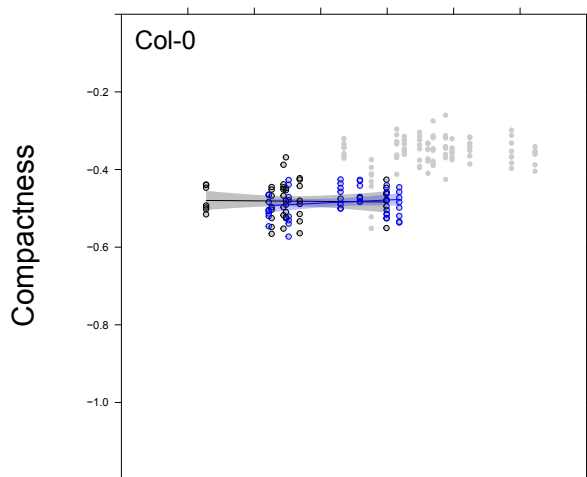
A. Growth rates phenotypes P3



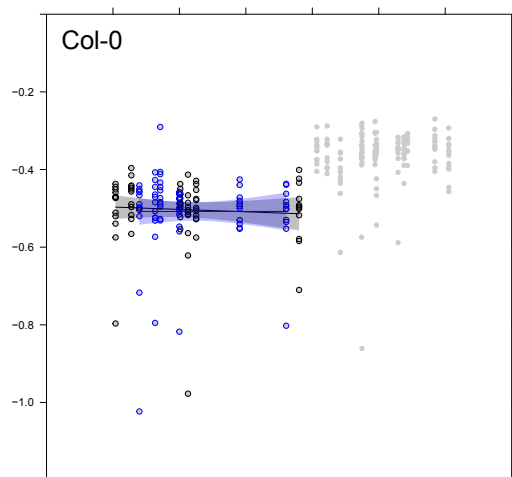
B. Growth rates phenotypes P4



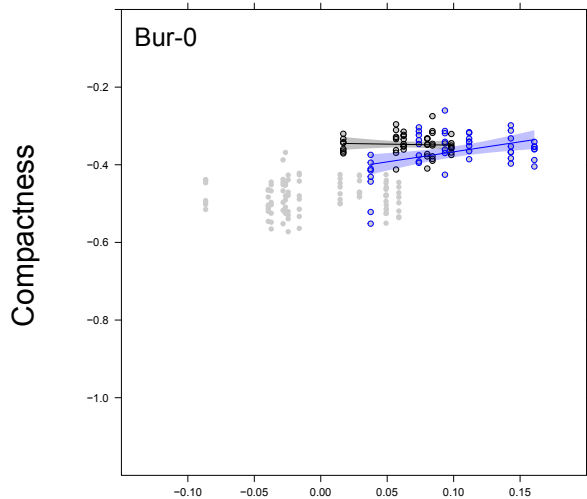
Parents G2 Phenotyping P3



Parents G3 Phenotyping P4



Bur-0



Bur-0

