The Arabidopsis DNA Methylome Is Stable under Transgenerational Drought Stress[1][OPEN]

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Improving the responsiveness, acclimation, and memory of plants to abiotic stress holds substantive potential for improving agriculture. An unresolved question is the involvement of chromatin marks in the memory of agriculturally relevant stresses. Such potential has spurred numerous investigations yielding both promising and conflicting results. Consequently, it remains unclear to what extent robust stress-induced DNA methylation variation can underpin stress memory. Using a slow-onset water deprivation treatment in Arabidopsis (*Arabidopsis thaliana*), we investigated the malleability of the DNA methylome to drought stress within a generation and under repeated drought stress over five successive generations. While drought-associated epi-alleles in the methylome were detected within a generation, they did not correlate with drought-responsive gene expression. Six traits were analyzed for transgenerational stress memory, and the descendants of drought-stressed lineages showed one case of memory in the form of increased seed dormancy, and that persisted one generation removed from stress. With respect to transgenerational drought stress, there were negligible conserved differentially methylated regions in drought-exposed lineages compared with unstressed lineages. Instead, the majority of observed variation was tied to stochastic or preexisting differences in the epigenome occurring at repetitive regions of the Arabidopsis genome. Furthermore, the experience of repeated drought stress was not observed to influence transgenerational epi-allele accumulation. Our findings demonstrate that, while transgenerational memory is observed in one of six traits examined, they are not associated with causative changes in the DNA methylome, which appears relatively impervious to drought stress.

Plants, being sessile organisms, must be phenotypically plastic in a dynamic environment. This ability is relayed by various intricate intercellular and intracellular mechanisms, including hormone signaling and organelle-nuclear retrograde pathways that allow plants to perceive and respond to biotic and abiotic stresses (Karpinski et al., 1999; Fernández and Strand, 2008; Cutler et al., 2010; Goodger and Schachtman, 2010; Chan et al., 2016; Martín et al., 2016). Additionally, repeated exposure to stress can lead to plant stress priming, whereby prior stress exposure conveys an enhanced ability to respond to future events (Conrath et al., 2006; Ding et al., 2012; Gordon et al., 2013; Sani et al., 2013; Wang et al., 2014; Virlouvet and Fromm, 2015; Hilker et al., 2016; Wibowo et al., 2016). This notion has been extended to numerous considerations of the formation of plant stress memory, in which a state of altered stress responsivity is mitotically or meiotically transmissible (Bruce et al., 2007; Hauser et al., 2011b; Probst and Mittelsten Scheid, 2015; Crisp et al., 2016; van Loon, 2016). There is much interest in plant stress memory, including the underlying molecular mechanism(s) and its potential to impact crop yields, particularly in harsh and variable environments (Springer, 2013; Ji et al., 2015; Mickelbart et al., 2015).

Stable propagation of DNA methylation states has been suggested as a possible mechanism for the formation of plant stress memory (Boyko and Kovalchuk, 2010; Probst and Mittelsten Scheid, 2015; Crisp et al., 2016). DNA methylation, occurring in three sequence contexts in plants (mCG, mCHG, and mCHH, where H is anything but G), is largely considered to function in transposable element (TE) silencing, maintaining genome stability, and possible regulation of gene expression (Reinders et al., 2009; Law and Jacobsen, 2010; Jones, 2012; Eichten et al., 2014; Niederhuth and Schmitz, 2017). This potential effect on gene expression

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has raised the proposition that DNA methylation could complement genetic variation, as a mode for transferring heritable information, to contribute to phenotypic variation (Molinier et al., 2006; Heard and Martienssen, 2014; Quadrana and Colot, 2016). Indeed, DNA methylation states can be maintained faithfully over both mitotic and meiotic cell divisions by a suite of pathways and enzymes (Probst et al., 2009; Law and Jacobsen, 2010; Stroud et al., 2013). It is unclear the extent to which genome-wide patterns of DNA methylation (DNA methylome) in plants are reset; rather, it appears that the parental DNA methylome is reestablished and propagated during gametogenesis and spermatogenesis (Slotkin et al., 2009; Calarco et al., 2012). Since these processes occur within postembryonic growth in plants (Boavida et al., 2005), any accumulated variations in the DNA methylome (epi-allele), either environmentally induced or spontaneous, have the potential to be carried over generations. DNA methylation state has shown stable heritability (Li et al., 2014; Dubin et al., 2015; Hagmann et al., 2015), with the documented appearance of epi-alleles (in the form of differentially methylated regions [DMRs]) to occur at a frequency comparable to genetic polymorphisms (Becker et al., 2011; Schmitz et al., 2011, 2013), which possibly arise at elevated frequencies under abiotic stress (Jiang et al., 2014).

This potential has led to many investigations of transgenerational stress memory mediated by environmentally induced epi-alleles, which could open exciting possibilities for crop (epi)genomics (Hauser et al., 2011b; Springer, 2013; Ji et al., 2015). However, bona fide examples of transgenerational methylation changes leading to substantially altered plant behavior remain a rare observation (Pecinka et al., 2009; Becker et al., 2011; Jiang et al., 2014; Seymour et al., 2014; Crisp et al., 2016), with the majority of DNA methylome variation attributable to underlying genetic differences rather than being truly epigenetic (Eichten et al., 2013, 2014; Schmitz et al., 2013; Li et al., 2014; Seymour et al., 2014; Dubin et al., 2015; Hagmann et al., 2015). Furthermore, there are a growing number of conflicting reports of short-term adaptation to abiotic stresses, including salt or drought stress, that occur independently of DNA methylation changes (Cayuela et al., 1996; Jakab et al., 2005; Rossel et al., 2007; Ding et al., 2012, 2013, 2014; Gordon et al., 2013; Sani et al., 2013). This is in contrast to the potential for DNA methylation-mediated transgenerational memory (Luo et al., 1996; Tricker et al., 2013; Herman and Sultan, 2016; Nosalewicz et al., 2016; Wibowo et al., 2016; Zheng et al., 2017). Thus, the nature of DNA methylation and its contribution to short-term and transgenerational stress adaptation remain enigmatic, which is confounded by the limited identification of causative changes in the DNA methylome correlating with enhanced stress tolerance (Secco et al., 2015; Meng et al., 2016).

Given the conflicting nature of past studies, we sought to systematically investigate the potential for environmentally induced changes in the DNA methylome in the model species Arabidopsis (*Arabidopsis thaliana*) that could convey transgenerational stress memory. First, the potential for drought-induced formation of heritable epi-alleles that correlate with drought-responsive gene expression was tested. Subsequently, with an experiment designed to minimize genetic variation and stochastic DNA methylome variation, the ability of plants to form transgenerational stress memory using a repeated drought stress over consecutive generations was investigated and coupled with in-depth DNA methylome profiling.

RESULTS

Stress-Associated Variation in DNA Methylation Observed under a Slow-Onset Mild Drought Stress within a Generation

A slow-onset water deprivation treatment (drought stress) was imposed on soil-grown plants by withholding watering for 9 d to assess the potential for drought stress to induce epi-alleles in the DNA methylome. This stress led to a drop in relative water content to around ~60% (measured in representative plants) and visible leaf wilting (Fig. 1A). Whole rosettes were harvested from unstressed plants ($n = 3$) and drought-treated plants ($n = 3$). The genome-wide DNA methylation patterns (DNA methylome) of these samples were assayed at single-base-pair resolution using whole-genome bisulfite sequencing (WGBS; Lister et al., 2008; Supplemental Table S1).

To explore variations in the methylome among our samples, pairwise comparisons of mean methylation levels, binned across 100-bp tiles, was performed to capture the full extent of variation between all samples (Eichten and Springer, 2015). This revealed 2,141 mCG, 1,039 mCHG, and 718 mCHH DMRs across all samples; however, hierarchical clustering of samples based on methylation levels at these regions did not cluster samples by treatment (Supplemental Fig. S1A). Instead, clustering revealed the existence of two to three putative preexisting DNA methylome states, herein referred to as epi-types. This suggests that the predominant source of variation in the DNA methylome between these samples arises from preexisting differences. As the seed stock for this experiment was derived from bulk seed harvestings, as opposed to single-seed descent, these differences are likely caused by distant relatedness between plants (Becker et al., 2011; Schmitz et al., 2011, 2013).

Notwithstanding the presence of epi-type DMRs, we hypothesized that, if the Arabidopsis DNA methylome is truly malleable to abiotic stress, then drought stress should induce conserved variations between control and treated plants among any epi-type variation. Despite the hierarchical clustering of 100-bp tile-based DMRs showing negligible evidence of conserved DMRs between treatments, we attempted to identify any evidence for statistically significant treatment-conserved changes through rank sum testing of DMRs (see "Materials and Methods"). However, upon correction for multiple testing, all of the observed changes were deemed to be insignificant. While tile-based DMRs are a powerful tool for exploring broad-scale methylome variation, it is limited in

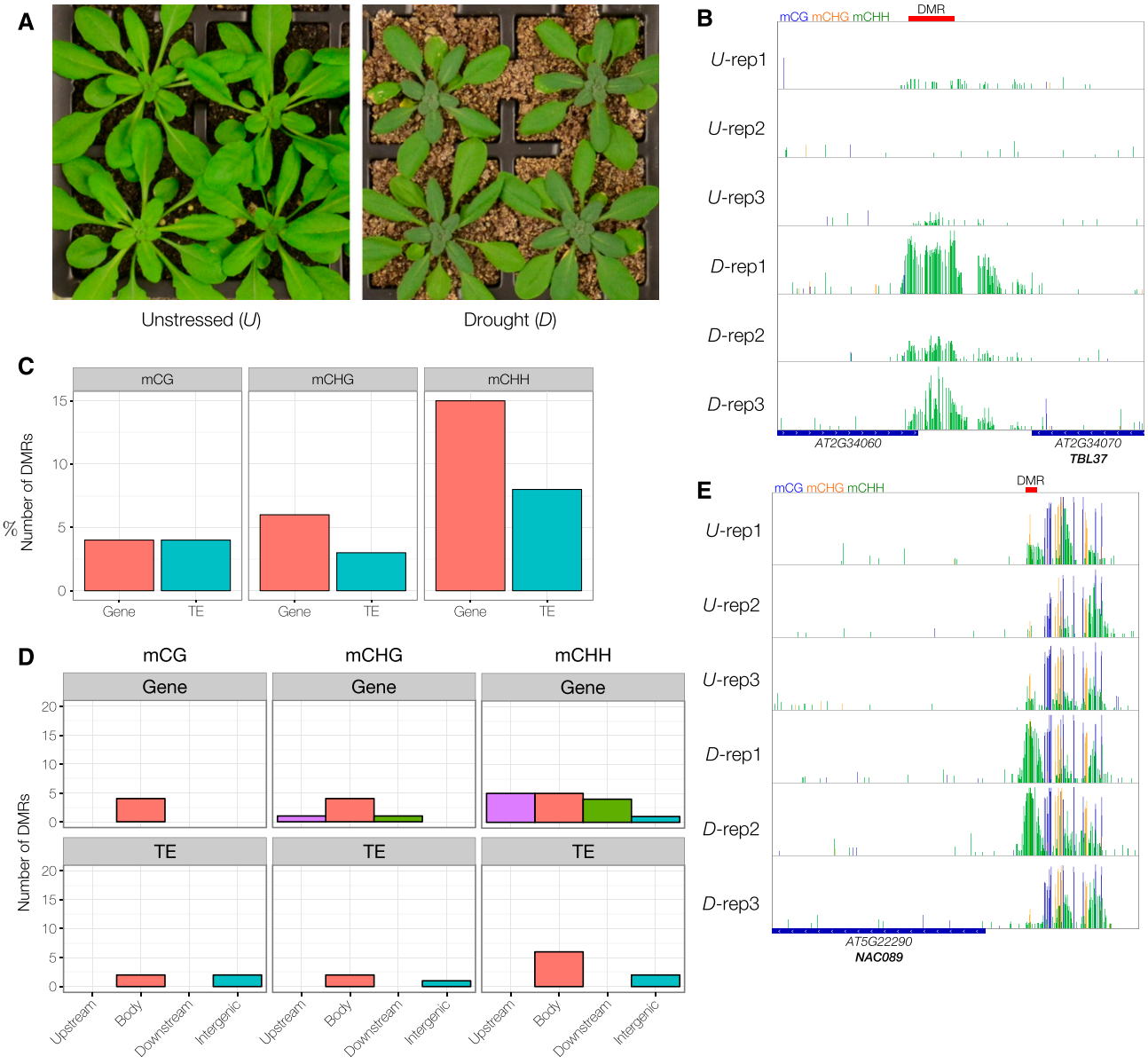

Figure 1. A mild drought stress is associated with variations in DNA methylation. A, Representative plants that were either unstressed (*U*) or underwent a drought stress (*D*) involving 9 d of withheld watering. B, Representative drought stress-associated DMR identified by DSS. Rows represent individual samples. DNA methylation is shown at single C resolution for mCG, mCHG, and mCHH as blue, orange, and green bars, respectively. Underlying genomic elements are presented at the bottom, and the exact region identified as a DMR is shown on top (red bar). C, Numbers of filtered stress-associated DMRs occurring near annotated protein-coding genes and TEs for each methylation context. D, Detailed mapping of filtered stress-associated DMRs within, or near, annotated protein-coding genes and TEs for each context of methylation. Body refers to DMRs occurring within the genomic feature, Upstream refers to DMRs within 1 kb near the 5′ end of the feature, Downstream refers to DMRs within 1 kb of the 3′ end of the feature, and Intergenic refers to DMRs that are farther than 1 kb away from the nearest genomic feature. E, Drought stress-associated DMR identified upstream of a gene encoding *NAC089*, identified previously as a locus exhibiting transcriptional memory to repeated dehydration stress. Rows represent individual samples. DNA methylation levels are shown at single C resolution for mCG, mCHG, and mCHH as blue, orange, and green bars, respectively. Underlying genomic elements are presented at the bottom, and the region identified as a DMR is shown on top (red bar).

its ability to appropriately attribute biological and technical information at single C residues, thus losing statistical power to identify differential methylation. Thus, an alternative approach to evaluate differential methylation was performed using DSS (Feng et al., 2014). This method employs Bayesian hierarchical modeling to incorporate the variation that exists both within and between biological replicates, at single C resolution, to identify bona fide treatment-associated DMRs with greater statistical rigor, including posthoc *P* value adjustments.

To identify stress-associated DMRs, we performed separate DMR calling between treatment groups accounting for within-group variation (Table I). First, to account for the contribution of preexisting methylome variation, DMRs were identified between epi-type groups (epi-type DMRs) using DSS (Table I; Supplemental Fig. S1B). The locations of epi-type DMRs were mapped relative to genomic features (Supplemental Table S2) based on the Araport11 genome reannotation (Cheng et al., 2017). Epi-type associated DMRs had comparable numbers mapping to annotated protein-coding genes and TEs; however, they were predominantly in the mCG context within gene and TE bodies (Supplemental Fig. S1, C and D).

Second, 49 stress-associated DMRs were identified using DSS, nine of which also were identified as preexisting epi-type DMRs and filtered from further analysis to produce a final list of 40 drought-associated DMRs (Table I; Fig. 1B; Supplemental Table S3). Positional mapping of drought- and epi-type-associated DMRs relative to protein-coding genes and annotated TEs was compared to explore whether they exhibited similar characteristics. Drought-associated DMRs were more likely to be found within 1 kb of genes (24 of 40; 60%) compared with epi-type-associated DMRs (91 of 218; 42%). Interestingly, there were proportionally fewer mCG stress-associated DMRs (eight of 40 DMRs; 20%) than mCG epi-type-associated DMRs (144 of 218; 66%), with the majority in the mCHH context (Fig. 1C). Beyond this, stress-associated DMRs located near genes were predominantly non-mCG (20 of 24; 83%; Fig. 1D; Table II). These results potentiate the involvement of the RNA-directed DNA methylation (RdDM) pathway as a source of stress-induced methylome variation near genes (Matzke et al., 2009; Schmitz et al., 2013). The exact mechanism underpinning mCG-DMRs remains elusive; however, they have been suggested to be more likely truly epigenetic (i.e. unlinked from underlying genetic variation; Schmitz et al., 2013).

mRNA Sequencing and Promoter Methylation Profiling of a Single Drought

Next, the 24 stress-associated DMRs mapping near protein-coding genes were further investigated for effects on the expression of neighboring genes. There were 4,369 differentially expressed genes under this drought treatment compared with unstressed controls (Crisp et al., 2017). Our comparison with this mRNA sequencing data set revealed only four significant differentially regulated genes correlating with drought-associated DMRs (Supplemental Table S4). Not only is there a negligible relationship of drought DMRs to drought-responsive genes (hypergeometric test; $P_{[X \geq 4]} = 0.54$), but three of the correlating genes (*ERD2*, *AT2G20920*, and *AT2G34060*) exhibited only weak gene expression changes. Interestingly, *NAC089*, which showed the strongest transcriptional response (approximately 7-fold up-regulated) under these conditions, has been reported to demonstrate transcriptional memory in response to repeated dehydration stress (Ding et al., 2013). While there is an observable increase in mCHH, the hypermethylated state is not conserved across drought-stressed samples, and similar levels of methylation remain in the adjacent downstream region from the identified DMR (Fig. 1E). Therefore, while this methylation difference may have biological significance, it is unclear whether this is truly associated with drought stress.

Our profiling of the DNA methylome suggests that it is relatively unresponsive to drought stress. Yet, this does not rule out an association; it is possible that the methylation profile of drought-responsive genes could distinguish them from other nonresponsive genes. For instance, given that most up-regulated genes do not display a change in DNA methylation, we would predict that their promoters are free from repressive DNA methylation to enable responsiveness. To investigate this possibility, the methylation state for all methylation contexts across the promoter region (considered as 1 kb upstream from gene annotation) of drought-responsive genes was profiled in unstressed and drought-treated plants. Methylation levels were averaged across genes clustered, using a k-means method, based on their $\log_2$ fold change in mRNA expression (Fig. 2A). There was no clear relationship between promoter methylation levels and the fold change in mRNA observed, although either strongly up- or down-regulated genes appeared to show lower levels of DNA methylation compared with other groups (Fig. 2B). There also appeared to be a slight, yet general, increase in promoter mCHG and mCHH levels in drought-treated samples. To test whether this increase reflected any characteristic of the promoters of drought-responsive loci, promoter methylation levels were averaged across 437 (mean group size from Fig. 2A) randomly selected loci that did not respond to drought stress (Fig. 2C). These loci, while generally having higher promoter methylation levels, also showed an increase in non-CG methylation, providing further evidence that this methylation difference was not reflective of gene expression changes. It was also apparent that some expression groups, with transcripts showing relatively small changes in expression, had higher levels of promoter methylation, possibly reflecting mRNA abundance under

Table I. *Numbers of DMRs between epi-type groups and treatments identified by DSS*

The numbers of drought stress DMRs exclude those that were also identified between epi-type groups. *U*, Unstressed; *D*, drought stressed.

DMR Class	Contrast	Sequence Context		
		mCG	mCHG	mCHH
Epi-type	*U*-1, *U*-3, *D*-2 versus *U*-2, *D*-1, *D*-3	144	41	33
Drought stress	*U*-1, *U*-2, *U*-3 versus *D*-1, *D*-2, *D*-3	8	9	23

Table II. *Numbers of drought stress DMRs mapping to protein-coding genes directly (gene body), within 1 kb (upstream/downstream) from the nearest gene, or greater than 1 kb from the nearest gene (intergenic)*

Location	Sequence Context		
	mCG	mCHG	mCHH
Gene body	4	4	5
Upstream region (less than 1 kb from nearest gene)	0	1	5
Downstream region (less than 1 kb from nearest gene)	0	1	4
Intergenic (more than 1 kb from nearest gene)	0	0	1

unstressed conditions. However, further inspection of transcripts in each expression group suggested that promoter methylation levels were not reflective of mRNA abundance under unstressed conditions (Supplemental Fig. S1E). We explored promoter non-CG methylation levels further to test whether there was a subset of drought-responsive genes driving this difference. However, these regions were found to be largely devoid of methylation, with the exception of a subset of loci (Fig. 2D). Despite the lack of association with drought-responsive mRNA expression, these findings implicate altered RdDM function under drought stress leading to elevated non-CG methylation upstream at a subset of genes (Matzke and Mosher, 2014).

Transgenerational Recurring Drought Stress

The experiment above highlights that DMRs do appear after the onset of a drought within a single generation. Although DMRs are present, the experimental design limits the ability to examine their biological relevance in a number of ways. First, the seed stock used contained existing epi-types that may interfere with stress-responsive changes to DNA methylation and/or prohibit detailed analyses by diluting any signal from stress-induced variation. Second, a single-generation experiment does not provide any insight into the heritable nature of methylation changes (Hauser et al., 2011b; Gutzat and Mittelsten Scheid, 2012). Third, biologically relevant DMRs may increase in number and persist over time if the stress is experienced repeatedly at different developmental stages both within a generation and across generations. Therefore, we performed a single-seed descent, recurring, and transgenerational drought stress experiment to directly address these experimental challenges.

Multiple independent lineages originating from a single inbred progenitor were propagated by single-seed descent, akin to previous mutant accumulation line experiments (Shaw et al., 2000; Becker et al., 2011; Schmitz et al., 2011). Plant lineages were subjected to either control growth conditions (unstressed lineages) or repeated drought stress composed of a 14-d drought, 5 d of recovery, followed by a second 12-d drought (Fig. 3A; Supplemental Fig. S2A). The first stress occurred during vegetative growth (D1) and the second during flowering (D2). This repeated drought treatment was performed through successive generations starting from founding plants (G_0) through to fifth generation plants (G_5; Fig. 3B). Direct progeny (P_1) and progeny one generation removed (P_2) of G_4 and G_5 plants, from independent lineages per treatment, were compared for altered growth and resilience. The DNA methylomes of six G_5 P_1 progeny per lineage condition, each from an independently propagated lineage, were assayed using WGBS. G_0 P_1 progeny also were assayed for a representation of initial DNA methylome patterns prior to generations of experimental treatment.

PSII performance was monitored using measures of chlorophyll fluorescence, allowing for nondestructive assaying of plant stress and vitality under drought, to maximize survival rate (Haitz and Lichtenthaler, 1988; Woo et al., 2008). Representative traces of various PSII parameters (see Table IV below) are shown for plants under control conditions, plants at the end of D1, and plants at the end of D2 (Supplemental Fig. S2, B–F). D1 and D2 plants demonstrated a corresponding reduction in both PSII quantum efficiency and photochemical quenching capacity, a reduction in the estimated fraction of open PSII centers, and some reduction in the maximal potential efficiency of PSII. For all these measures, D1 and D2 plants largely demonstrated similar trends, although the severity appeared greater after D2. For example, D2 plants showed a severely impaired nonphotochemical quenching profile, suggesting that plants after D2 were severely stressed to the point that they could not sufficiently activate photoprotective mechanisms. This suggests a greater impact of drought in mature plants undergoing the transition to reproduction.

Drought-Exposed Lineages Exhibit Enhanced Seed Dormancy

The progeny of G_4 and G_5 plants from unstressed and drought-treated lineages were compared to test whether sustained and repeated drought exposure over successive generations could lead to the formation of drought stress memory that might be evidenced as altered plant behavior or enhanced drought tolerance in drought-treated lineages.

The growth of 3-week-old descendants, from unstressed and drought-exposed lineages, was compared under control growth conditions for G_5 P_1 and P_2 progeny. There were no intragenerational differences in plant size, using either green pixel count or fresh biomass, between descendants of watered and drought-treated parents (Supplemental Fig. S3, A and B). The growth rate in G_5 P_1 progeny, using green pixel counts

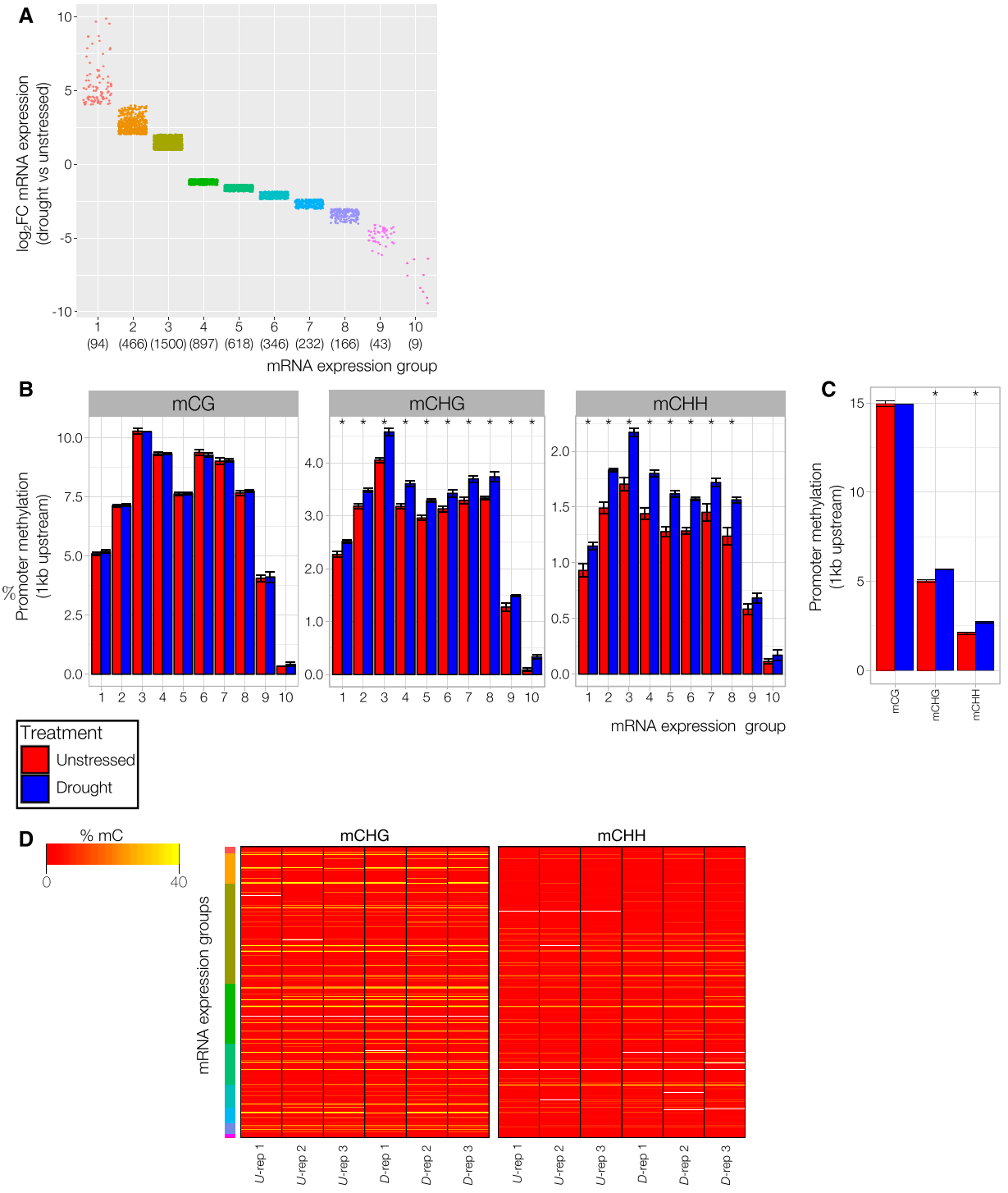

Figure 2. Promoter methylation levels at drought-responsive genes. A, Strip chart depicting $\log_2$ fold change in mRNA expression of all drought-responsive genes grouped based on k-means clustering. Dots represent individual drought-responsive loci. Numbers of genes in each cluster are presented in parentheses. B, Summarized methylation levels in the 1-kb region directly upstream of drought-responsive loci averaged across all genes in each expression group as defined in A. Bars denote mean, error bars denote standard error of the mean ($n = 3$), and asterisk denotes significant differences ($P < 0.05$) between treatments as determined by a Kruskal-Wallis rank sum test. C, Summarized methylation levels in the 1-kb region directly upstream of 437 randomly selected non-drought-responsive loci. Bars denote mean, error bars denote standard error of the mean ($n = 3$), and asterisk denotes significant differences ($P < 0.05$) between treatments as determined by a Kruskal-Wallis rank sum test. D, Heat maps of mCHG and mCHH levels summarized 1 kb directly upstream of drought-responsive loci for individual transcripts ordered by expression group (as defined in A) and subsequently by $\log_2$ fold change (top = highest; bottom = lowest).

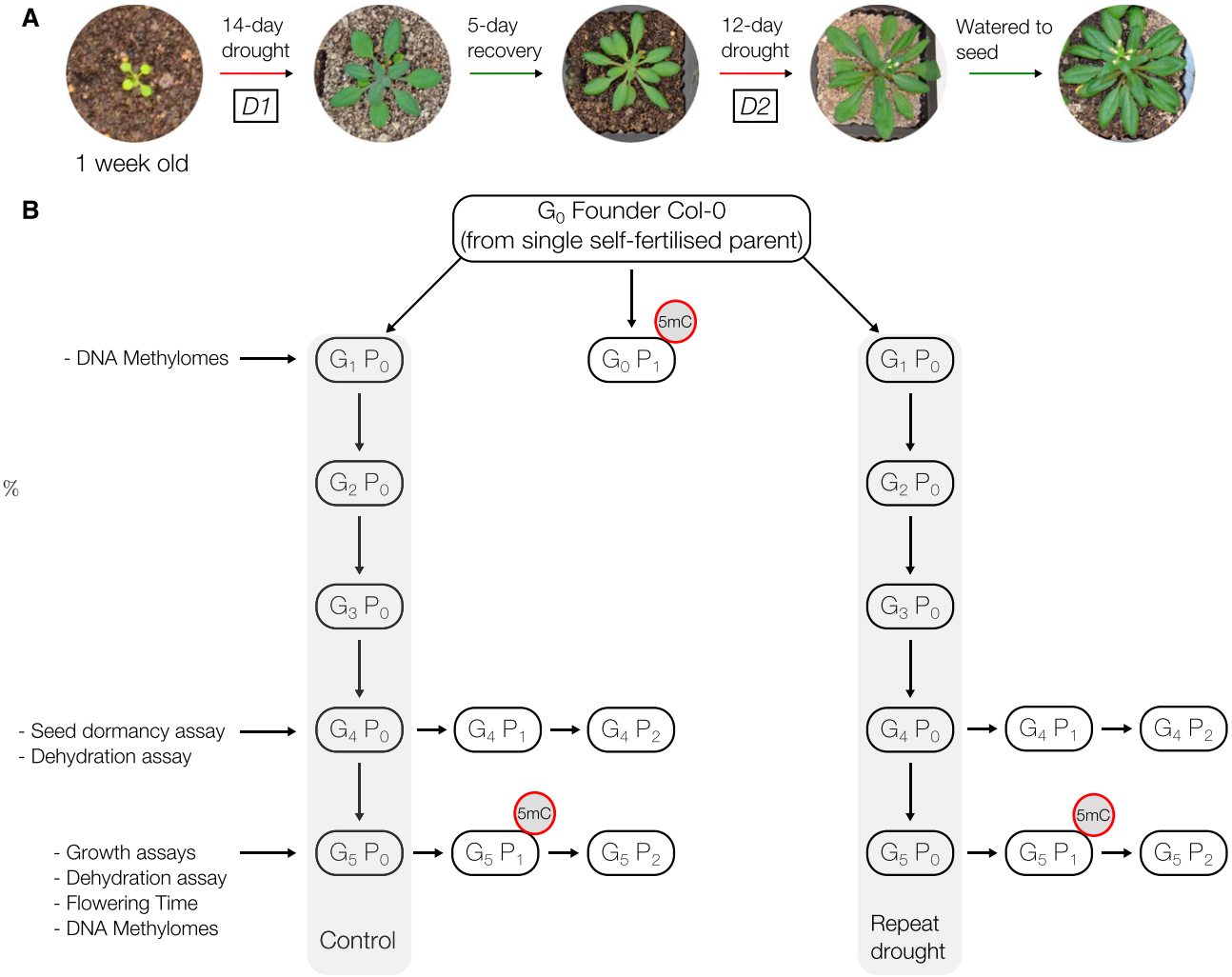

Figure 3. Transgenerational repeated drought stress experiment. A, Scheme of the repeated drought stress treatment performed every generation. Initial water deprivation (*D1*) began on 1-week-old seedlings and lasted for 14 d. After a recovery period of 5 d, a second treatment was performed (*D2*) that lasted for 12 d. B, Multiple independent lineages were propagated by single-seed descent for five generations, from a founding inbred progenitor, with half of the lines being exposed to the repeated drought stress treatment every generation. Testing for transgenerational stress memory was performed on the P_1 and P_2 progeny of G_4 and G_5 plants with plants from each independent lineage. WGBS was performed on P_1 progeny, each from an independent lineage, of G_0 and G_5 plants (red circles).

of plant area over 3 weeks, showed that progeny from unstressed and drought lineages had equivalent growth rates (Supplemental Fig. S3C) and flowering times (Supplemental Fig. S3D). Thus, gross plant growth and development appears unaltered after experiencing repeated drought stress over previous generations.

Seed provisioning is considered to be a significant mechanism for the transmission of adaptive transgenerational effects. For instance, seeds developed during periods of stress often have altered nutrient or hormone profiles, which holds important biological consequences such as propensity to germinate (Herman and Sultan, 2011). Indeed, previous transgenerational studies have reported phenotypes reliant on maternal exposure to stress (Murgia et al., 2015; Nosalewicz et al., 2016). Therefore, altered seed provisioning was tested by comparing dormancy in seed from P_0 and P_1 progeny from G_4 plants of both lineages (Fig. 4A). Seed dormancy was compared by constructing a Cox proportional hazards model producing a comparative HR (McNair et al., 2012). Seeds from G_5 P_0 drought were 72% less likely to germinate ($HR_D = 0.28$, $P < 0.001$) than seeds from unstressed lineages. It is possible that this was conveyed through maternal effects, such as increased abscisic acid (ABA) synthesis under drought stress, particularly since D2 occurred during early reproductive stages (Cutler et al., 2010). When seed dormancy was tested further in P_1 seed, one generation removed from stress, the size of this effect was reduced but still statistically significant ($HR_D = 0.69$, $P < 0.001$). While these observations are consistent with observations of maternal effects, in the form of altered seed provisioning, some dormancy is still retained in the

seed of P_1 progeny one generation removed from experimental drought.

It is possible that any form of transgenerational memory might only be observable in conditions of water limitation. One of the key responses to drought stress is stomatal closure (Verslues et al., 2006), and recent investigations have found that environmentally induced variation in stomatal development and index is regulated, at least in part, by DNA methylation, with some evidence for DNA methylation-mediated transgenerational transmission (Tricker et al., 2012, 2013). Greater stomatal control to prevent dehydration would be beneficial under water limitation; therefore, stomatal responsiveness was compared between lineages. A detached rosette dehydration experiment (see "Materials and Methods") was performed on G_4 and G_5 P_1 progeny. Independent experiments revealed that progeny from each lineage had very comparable rates of water loss, with lineage holding a very weak effect (α_2 = 1.3–1.71, $P > 0.05$; Fig. 4B). Ultimately, if any form of drought stress memory was conveyed to the progeny of drought-stressed plants, then these progeny would be expected to exhibit improved survivability under drought. However, G_5 P_1 progeny from both lineages demonstrated near identical survivability under a longer term drought measured using the fluorescence decline ratio (R_{fd}) as a vitality index (Fig. 4C; Haitz and Lichtenthaler, 1988). In total, phenotypic assessment of transgenerational drought lineages revealed enhanced seed dormancy to be the only form of drought stress

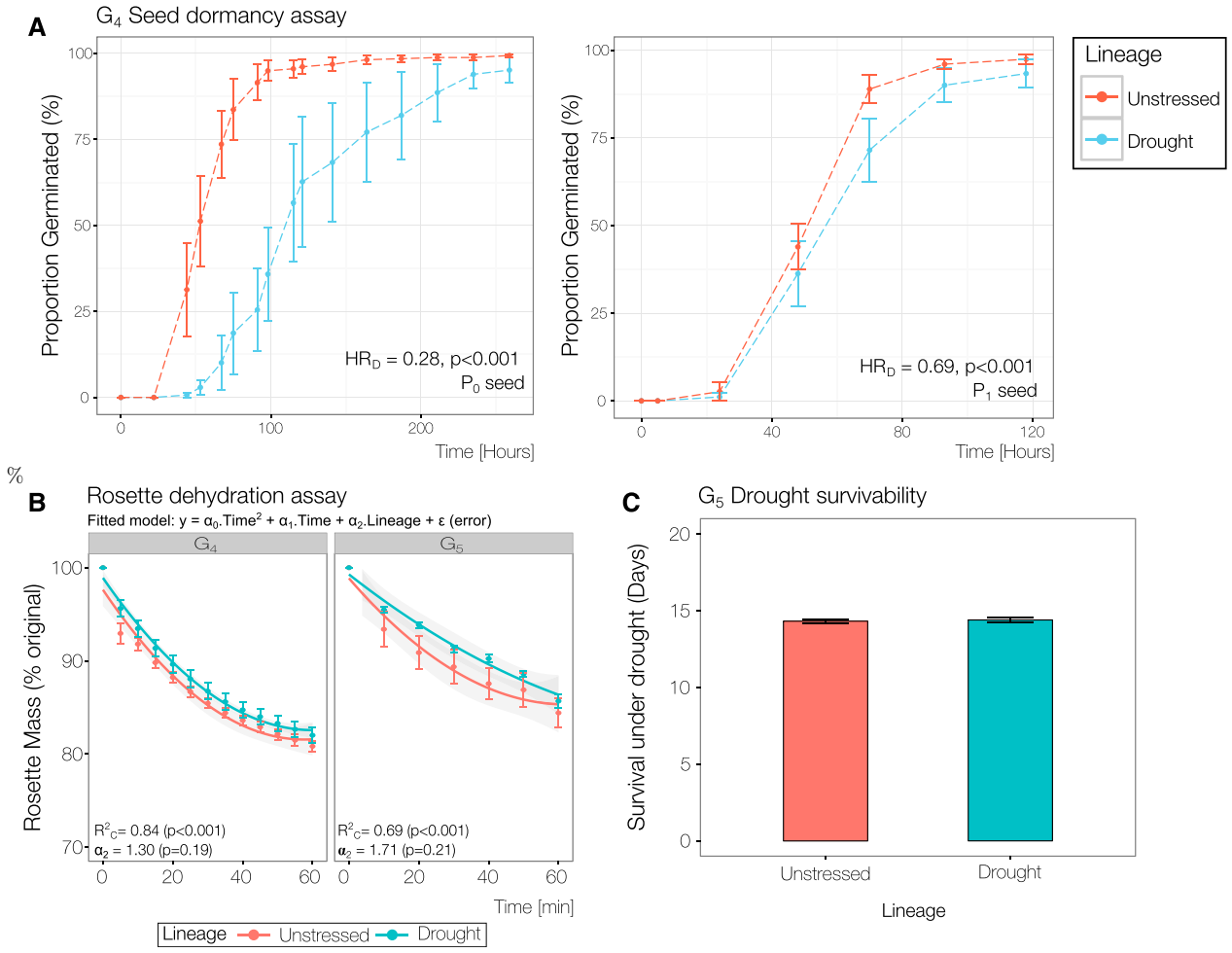

Figure 4. Progeny from drought-exposed lineages show enhanced seed dormancy. A, Independent dormancy assays performed on seeds from P_0 ($n = 6$; more than 25 seeds per plate) and P_1 ($n = 9$; more than 25 seeds per plate) progeny of G_4 plants (P_0 and P_1 seeds, respectively). Points denote mean proportions of seeds germinated; error bars denote SE. HR denotes the calculated hazard ratio from a fitted Cox proportional hazards model, representing the likelihood of germination between groups (HR_D = drought versus unstressed lineage). B, Dehydration assay performed on detached rosettes of P_1 progeny of G_4 ($n = 12$) and G_5 ($n = 11$) plants (independent experiments). A second-order polynomial regression, with a 95% confidence interval (shading), was performed to determine the coefficient for the lineage predictor term (α_2). R_2^C denotes the conditional R_2 calculated to assess model fit. C, Survival under the terminal drought experiment on transgenerational descendants (unstressed, $n = 44$; drought, $n = 51$). Bars denote means, and error bars denote SE across two independent experiments.

memory, which was partially retained in seeds one generation removed from stress.

Negligible DNA Methylome Epi-Alleles Associated with Transgenerational Drought Stress

Beyond phenotypic measures of memory, we were interested in the extent of DNA methylation variation between these lineages associated with the transgenerational repeated drought stress. DNA methylomes were produced from whole rosettes of approximately 3-week-old G_0 progeny (G_0 P_1) and G_5 progeny from six independent lineages per condition (G_5 P_1 unstressed and G_5 P_1 drought), grown under control growth conditions (Supplemental Table S1). Each progeny plant that was sequenced came from an independently propagated lineage. Hierarchical clustering of all G_5 P_1 samples by genome-wide methylation levels, binned into 100-bp regions, confirmed that broad methylome patterns were highly similar among all progeny, excluding the possibility of genetic contamination, such as seed contamination or outcrossing, which could affect the DNA methylome patterns observed (Fig. 5A). In contrast to the previous experiment (Supplemental Fig. S1A), no clear epi-types were detected in the profiled G_5 P_1 progeny, despite being derived from independent lineages, confirming the importance of comparing relatively closely related plants. To identify conserved drought-induced heritable changes in the DNA methylome, we utilized DSS to call DMRs between progeny of G_5 control and G_5 drought lineages. This yielded just four transgenerational drought stress-associated DMRs (Table III; Supplemental Table S5). None of these overlapped with the epi-type or stress-associated DMRs that were identified in the previous within-generation drought stress experiment. This lack of variation was unexpected, since 40 DMRs were observed within a generation from a single drought stress; however, this reinforces the notion that heritable stress-induced variations in the DNA methylome are rare. Despite this conservative approach, none of the identified DMRs demonstrated complete conservation within treatment groups, and three of the DMRs mapped to repetitive regions of the genome (Fig. 5B). The fourth DMR was in intergenic space, 800 bp upstream of *CEK3* (AT4G09760), and was present in only four of the six drought lineage progeny that were profiled.

Core ABA Signaling and Documented Memory Loci Remain Stable under Transgenerational Recurring Drought Stress

Given the negligible detection of transgenerational drought-associated DMRs using unbiased approaches, we undertook targeted analyses of subsets of the genome. The rationale for this strategy relates to the hypothesized biological relevance of methylation as a regulatory mechanism near, or within, annotated genes related to drought response and tolerance (Gutzat and Mittelsten Scheid, 2012). The directed approaches were used to examine DNA methylation levels at loci encoding the core signaling components in the ABA signaling pathway crucial for drought response and at previously characterized loci described to have stress-induced, transgenerational DNA methylome variation.

ABA induces a signaling cascade, involving both transcriptional and posttranscriptional changes, that activates drought-response mechanisms (Verslues et al., 2006; Cutler et al., 2010). Given the importance of this pathway and the observation of enhanced dormancy in progeny of drought-exposed lineages, it was postulated that loci encoding key components of the ABA signaling pathway (Hauser et al., 2011a) could be targets for memory formation. However, when differences in DNA methylation levels were assayed at these loci in G_5 P_1 progeny, between unstressed and treated lineages, they were found to be nearly identical, with the largest difference being a 4.45% decrease in mCG (Supplemental Table S6).

Low humidity-induced hypermethylation at the *FAMA* and *SPCH* loci was described to be transmitted to progeny in Landsberg *erecta* (Tricker et al., 2012, 2013). These loci were profiled across P_1 progeny of G_0 and G_5 plants to look for evidence of hypermethylation (Fig. 6, A and B). Interestingly, both loci were found to be largely devoid of DNA methylation across all experimental samples, comparable to the unmethylated nonstressed plants reported previously (Tricker et al., 2012). However, there was no evidence of any transmissible hypermethylation at these loci, neither lineage dependent nor drought dependent. Notably, there was a region of stochastic differences, in all three sequence contexts of DNA methylation, downstream from the protein-coding region of *FAMA*. This observation raises the following possibilities: (1) regulation of the DNA methylome can be stress type specific; and (2) different ecotypes within a species may have altered stress-induced regulation of the DNA methylome.

Transgenerational hyperosmotic stress (HS) was reported recently to induce enhanced salt tolerance in P_1 progeny of lineages exposed to salt stress for at least two generations (Wibowo et al., 2016). This enhanced tolerance was correlated with HS-DMRs, two of which occurred at TEs adjacent to *MYB20* and *CNI1*. In the case of *MYB20*, HS-induced hypermethylation across an upstream TE correlated with persistent down-regulated *MYB20* expression. In the case of *CNI1*, HS-induced hypomethylation across a downstream TE correlated with increased stress-responsive expression in the P_1. Thus, we investigated these loci in the context of transgenerational drought stress to see if any hypermethylation or hypomethylation was evident in G_5 P_1 progeny. The DNA methylation pattern across and upstream region of *MYB20*, in all samples assayed in this study, was similar to that of unstressed G_0 P_1 progeny (Fig. 6C). Interestingly, select drought lineages did appear to show hypomethylation in an upstream TE akin to the P_2 progeny, one generation removed from 75 mM salt stress, which did not exhibit enhanced salt tolerance (Wibowo et al., 2016). The *CNI1* locus also was largely devoid of DNA methylation; however, the

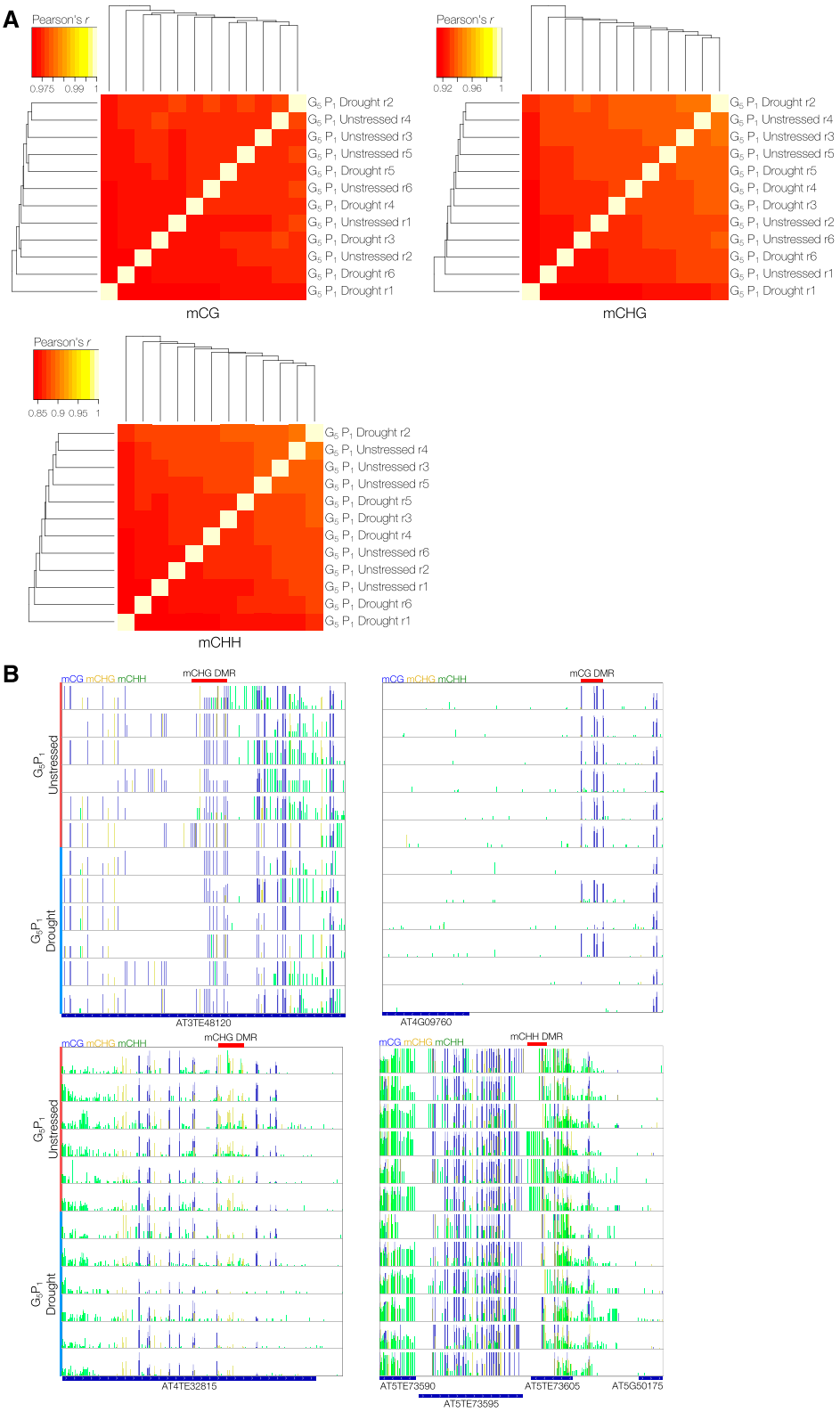

Figure 5. Limited methylome variation associated with transgenerational drought stress. A, Heat maps representing two-dimensional hierarchical clustering of correlations (Pearson's *r*) in genome-wide DNA methylation levels, in all sequence contexts, averaged across 100-bp bins confirms similar broad DNA methylome patterns between all G_5 descendants. B, Integrative Genomics Viewer (IGV) visualization of lineage-associated DMRs identified by DSS (red bars). Vertical blue, yellow, and green bars denote mean mCG, mCHG, and mCHH, respectively, at single C resolution.

downstream TE was partly methylated in all sequence contexts (Fig. 6D). However, there was no transgenerational drought-induced hypomethylation at this downstream TE, as was observed across P_0, P_1, and P_2 progeny of 75 mM salt-stressed parents. This supports the observed stochastic nature of DNA methylome variation in that methylome changes are not always universal and/or stable. Single studies may only

Table III. *Lineage-associated and spontaneous DSS-based DMRs identified in the transgenerational drought experiment*

DMR Class	Contrast	Methylation Context			
		mCG	mCHG	mCHH	Total
Lineage	G_5 P_1 unstressed versus G_5 P_1 drought	1	2	1	4
Spontaneous	G_0 P_1 versus G_5 P_1 unstressed	1	10	12	23
	G_0 P_1 versus G_5 P_1 drought	1	6	8	15

capture a portion of this and potentiate the possibility that different abiotic stresses induce changes in the DNA methylome to different efficacies.

Greater Stochastic Variation and the Appearance of Spontaneous DNA Methylome Epi-Alleles in Transgenerational Lineages

Having observed a limited number of transgenerational drought stress-associated DMRs, we explored the extent of stochastic variation in the DNA methylome. Using the aforementioned 100-bp tile-based analysis revealed extensive variation using pairwise comparisons of all G_5 P_1 progeny across lineages (2,871 mCG, 2,284 mCHG, and 1,292 mCHH DMRs; Supplemental Table S7). Almost all changes appeared to be unique to individual lineages, with negligible conservation within treatment groups (Fig. 7A). Rank sum testing was repeated on these 100-bp tile-based DMRs to test for association with treatment; however, none of the DMRs were significant after *P* value correction for multiple comparisons. Collectively, this suggests that the Arabidopsis DNA methylome is relatively impervious to transgenerational drought stress, with the predominant source of variation being due to stochastic differences between lineages.

The spontaneous nature of epi-allele appearance in the Arabidopsis DNA methylome is well characterized and also has been documented to increase in frequency under environmental stress (Becker et al., 2011; Schmitz et al., 2011; Jiang et al., 2014). The appearance of spontaneous DMRs in the lineages generated in this study was explored by comparing P_1 progeny from G_0 and G_5 plants using DSS (Table III; Supplemental Tables S8 and S9). Interestingly, more DSS-based DMRs were identified between G_0 and G_5 progeny regardless of lineage (Table III) at a magnitude comparable to previous observations of epi-allele accumulation (in the form of DMRs; Becker et al., 2011). Indeed, G_0 siblings were found to have more similar genome-wide DNA methylation patterns to each other than to G_5 descendants, particularly in the mCG context (Fig. 7B). Exposure to repeated drought stress for five successive generations did not lead to a greater number of DMRs; in fact, progeny from stressed lineages had fewer DMRs, than unstressed lineages, when compared with G_0 P_1 (Table III). Nine regions were in common between variations accumulated in unstressed and drought-exposed lineages, which may reflect truly labile DNA methylation sites from this data set. None of these nine regions were in common with previously identified labile regions. From the total 38 spontaneous DMRs identified here, only three were found to overlap with regions associated previously with spontaneous variation (Becker et al., 2011; Schmitz et al., 2011). Curiously, a handful of the overlapping sites occur across a hypothetical protein surrounded by TEs on chromosome 4 (AT4G19270), where there has been extensive non-mCG hypomethylation, yet unaffected mCG, in G_5 progeny (Supplemental Fig. S4A).

Labile regions of the Arabidopsis DNA methylome have been identified previously, whether spontaneous, stress induced, or driven by genetic divergence across diverse environments (Becker et al., 2011; Schmitz et al., 2011; Jiang et al., 2014; Hagmann et al., 2015; Wibowo et al., 2016). The four transgenerational drought stress-associated DMRs, identified in this study, were overlapped with regions identified across the aforementioned data sets to test whether any of the four regions were in common with previously reported stress-induced regions. One of these transgenerational drought-associated DMRs overlapped; however, this region was not associated with a stress-induced change (Supplemental Fig. S4B; Supplemental Table S10).

Stochastic DMRs, using the 100-bp tile-based method in this study, also were overlapped with previously published DMRs to look for conservation across methylation-labile regions. An overlap of the stochastic DMRs identified in this study showed that 617 of 6,447 (9.5%) occurred at regions identified previously. Overlaps with specific studies remained low, ranging from 0.2% to 4.4% of regions from this study being detected previously. Of particular interest was to compare the stochastic transgenerational DMRs identified here with previously identified transgenerational spontaneous DMRs across 30 generations of single-seed descent (Becker et al., 2011; Schmitz et al., 2011). Twenty-four of 72 regions characterized as a site hosting a spontaneous transgenerational epi-allele (Schmitz et al., 2011), unlinked from cis-genetic variation, were identified out of 6,447 stochastic transgenerational DMRs (Supplemental Table S10). These were predominantly changes in mCG, occurring largely at intragenic or repetitive regions, including TEs and pseudogenes.

DISCUSSION

Notions of transgenerational plant stress memory are often discussed alongside DNA and chromatin alterations, as a potential mechanism underpinning their

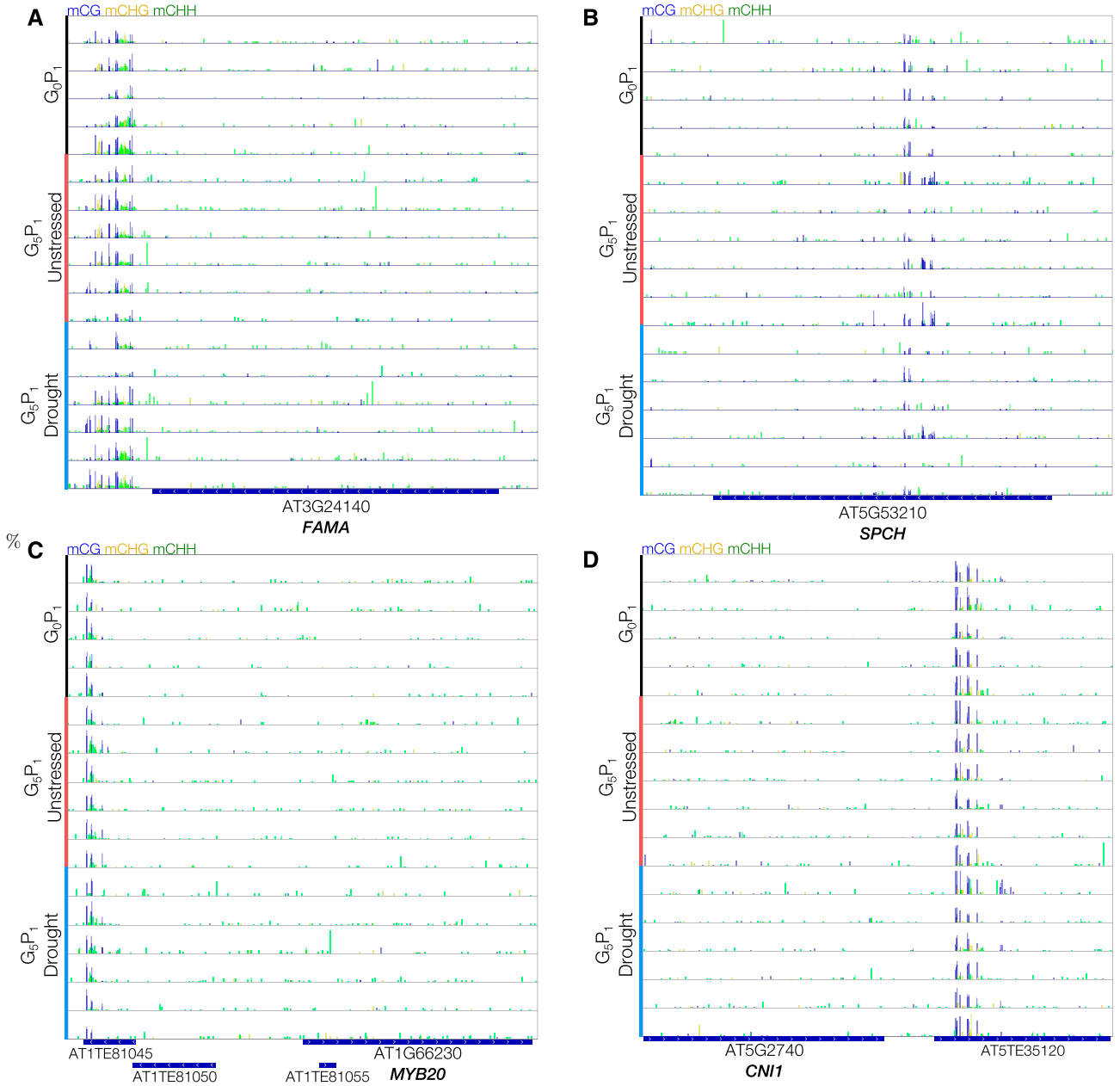

Figure 6. DNA methylation levels at loci reported to exhibit transgenerational stress-induced methylation variation. IGV visualization is shown for DNA methylation, in G_0 P_1 control plants and G_5 P_1 plants of both watered and drought-stressed lineages, across loci documented to exhibit transgenerational memory of stress-induced changes in DNA methylation: A, *FAMA* (low humidity-induced hypermethylation); B, *SPCH* (low humidity-induced hypermethylation); C, *MYB20* (upstream HS-induced hypermethylation); and D, *CNI1* (downstream HS-induced hypomethylation). Blue, orange, and green bars denote mean mCG, mCHG, and mCHH, respectively, at single C resolution.

storage and transmission (Herman and Sultan, 2011; Tricker, 2015; Crisp et al., 2016). In particular, DNA methylation is considered a key epigenetic mechanism for which there is now growing evidence (Luo et al., 1996; Boyko and Kovalchuk, 2010; Tricker et al., 2013; Herman and Sultan, 2016; Wibowo et al., 2016; Zheng et al., 2017). The specific contribution of DNA methylation at, or near, protein-coding genes toward basal plant growth and endurance remains unknown, albeit essential for proper development (Finnegan et al., 1996; Henderson and Jacobsen, 2008; Zemach et al., 2013; Yamamuro et al., 2014). Despite documentation of the stable inheritance of spontaneously occurring epialleles (Becker et al., 2011; Schmitz et al., 2011), there still remains uncertainty regarding the malleability of the DNA methylome to stress-induced variation

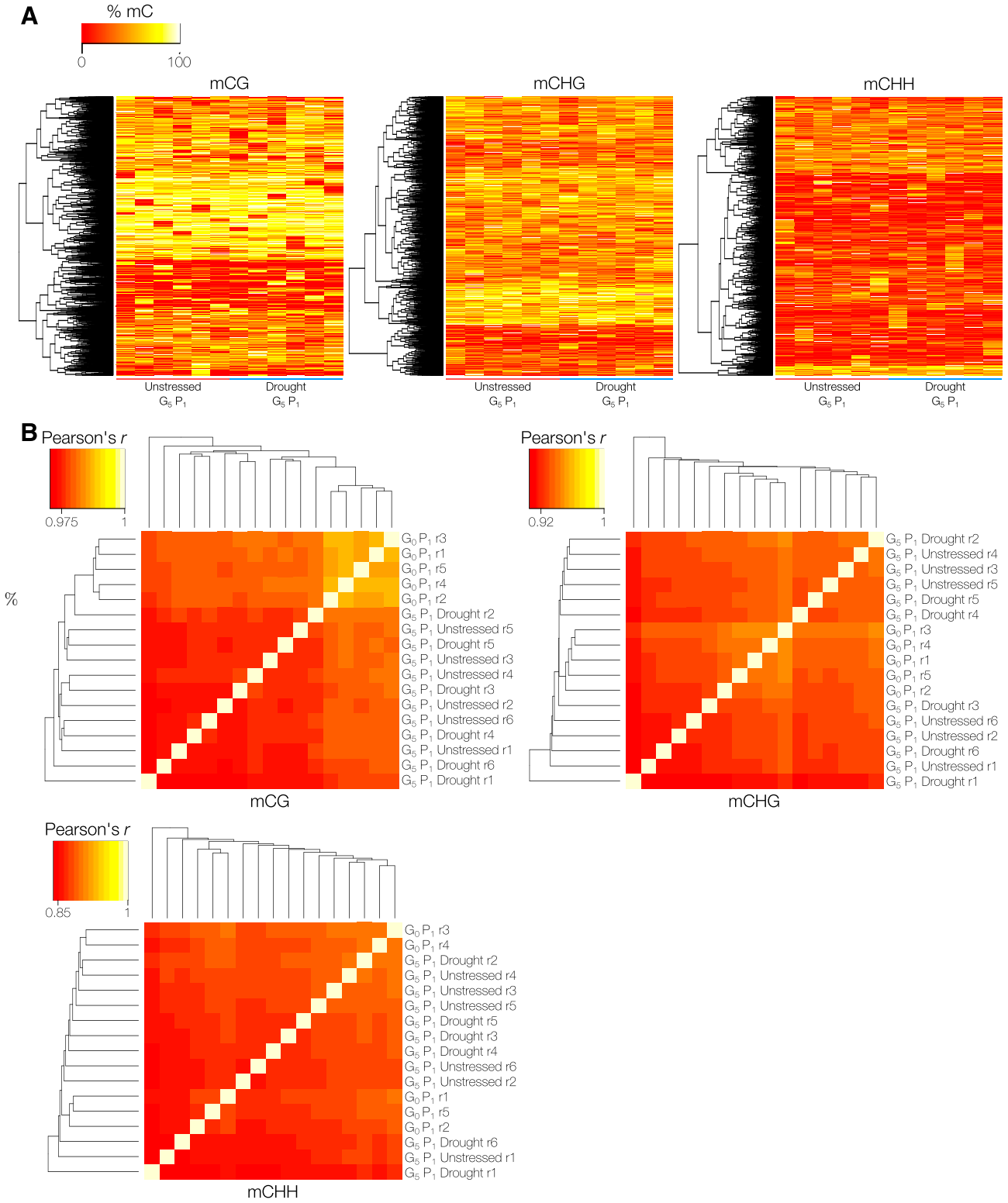

Figure 7. Exploring stochastic and spontaneous methylome variation across transgenerational lineages. A, Heat maps of average methylation across 100-bp tile-based DMRs identified from pairwise comparisons, with one-dimensional hierarchical clustering of rows, between all samples. B, Heat maps representing two-dimensional hierarchical clustering of correlations (Pearson's *r*) of genome-wide DNA methylation, in all sequence contexts, averaged across 100-bp tiles for all G_0 and G_5 progeny.

(Seymour et al., 2014; Eichten and Springer, 2015; Secco et al., 2015, 2017). Whether such DNA methylation changes are necessary for transgenerational stress memory is still unclear, with various memory traits not always aligning with changes in the DNA methylome (Ding et al., 2012; Sani et al., 2013; Murgia et al., 2015; Nosalewicz et al., 2016). In this investigation, we examined and compared the effect of drought stress on the Arabidopsis methylome both within and between generations.

Within-Generation Methylation Profiles in Response to Drought Stress

Within a generation, plants experiencing a mild drought stress that induced a substantial transcriptional response exhibited 40 stress-associated DNA methylation epi-alleles. However, these did not appear to correlate with drought-responsive gene expression changes. Further investigation of promoter methylation status at drought-responsive genes did not reveal any methylation features that distinguish drought-responsive genes. This did, however, reveal widespread non-CG hypermethylation in gene promoter regions in drought-treated plants, implicating altered RdDM performance under drought stress. Such observations are comparable to the non-CG hypermethylation, predominantly in the mCHH context, that occurred in the root tissue of rice (*Oryza sativa*) under phosphate starvation (Secco et al., 2015). The use of a *DCL3* knockdown line suggested that the phosphate-induced non-CG DMRs were largely RdDM independent, and a similar approach would be beneficial to address the putative involvement of RdDM here. In both cases, however, there was minimal evidence of such methylation changes affecting gene expression. It is worth noting that the effects of DNA methylation changes could be confounded by the complexity of interactions between all the chemical marks that contribute to chromatin state (Eichten et al., 2014; Crisp et al., 2016). It is also possible that preexisting epi-type differences could influence stress-inducible transcriptional changes. To systematically uncouple such effects would require a much larger scale sequencing effort, which may become a viable option in the future.

The identification of multiple epi-types within a seed stock derived from bulk harvesting further demonstrates the importance of appropriate experimental design when testing for DNA methylation-mediated stress memory. Furthermore, as the epi-type DMRs were predominantly mCG-DMRs, it is possible that these epi-type DMRs represent true epigenetic differences arising between distantly related plants rather than being reflective of genetic differences (Schmitz et al., 2013). Regardless, the relative lack of drought stress-associated DNA methylome epi-alleles observed within a generation aligns with other studies using phosphate, temperature, or UV radiation that all present a stoic DNA methylome unperturbed by abiotic stress (Seymour et al., 2014; Eichten and Springer, 2015; Secco et al., 2015; Meng et al., 2016).

Transgenerational Inheritance and Methylome Profiling

Between generations, descendants of lineages exposed to successive generations of recurring drought exhibited only four transgenerational drought stress-associated DMRs compared with unstressed lineages. Significantly, none of these were in common with the 40 drought-associated epi-alleles detected within a generation, reinforcing the notion that transgenerational adaptive DNA methylation is a rare occurrence, even under conditions of abiotic stress (Pecinka et al., 2009; Seymour et al., 2014; Eichten and Springer, 2015; Hagmann et al., 2015; Secco et al., 2015; Crisp et al., 2016). Three of the four mapped to repetitive and already heavily methylated regions of the genome. The fourth DMR also was in intergenic space, albeit 800 bp upstream from *CEK3*. *CEK3* encodes a protein, most highly transcribed in the hypocotyl, that is a part of the Choline/Ethanolamine Kinase family, for which *CEK4* has been implicated in phospholipid biosynthesis and embryo development; however, mutation of *CEK3* did not lead to the same phenotypes (Lin et al., 2015). It is unclear whether this DMR upstream of *CEK3* would be of biological significance; however, it does not appear to be required for transgenerational drought stress, as it was only evident in four of the six drought lineage progeny profiled. One possibility, since each progeny plant was derived from an independent lineage, is that this DMR is only weakly induced by drought stress; however, this would require further elucidation.

Targeted analyses of specific ABA-related loci were undertaken, as ABA-responsive genes are critical for drought responses. A recent study also reported that key ABA signaling kinases regulate the activity of a chromatin-remodeling ATPase (Peirats-Llobet et al., 2016). This regulation allowed for the fine-tuning of downstream components of the ABA pathway, in particular *ABI5*, further potentiating not only ABA-mediated chromatin variation but variation that feeds back onto the ABA signaling pathway itself. However, the targeted analysis of genes encoding ABA signaling components did not reveal treatment-specific methylation changes at any corresponding loci.

When compared with published data sets studying methylome variation, one transgenerational drought stress DMR overlapped with a previously identified spontaneous locus (Schmitz et al., 2011). Certainly, the nature of DNA methylome variation at all identified DMRs (stress associated and stochastic) is reminiscent of the spontaneous changes characterized previously comparing plants separated by approximately 30 generations (Becker et al., 2011; Schmitz et al., 2011). Whether those stochastic variations in the DNA methylome are tied to a particular lineage with biological consequence may warrant further investigation, despite not being tied to the experimental treatment. Furthermore, a vast majority of DMRs identified in this study mapped to TEs or unannotated genomic regions. This is unsurprising, given the expected relationship between DNA methylation and TEs. TE movement is

Table IV. *Photosynthetic parameters, and corresponding equations, to nondestructively assay the impact of the drought stress utilized in this study*

Parameter	Equation	Interpretation
F_v	$F_m - F_o$	Variable fluorescence, the ability of PSII to perform photochemistry
F_v'	$F_m' - F_o'$	
F_q'	$F_m' - F_t'$	Photochemical quenching of fluorescence by open PSII centers
F_v/F_m	$(F_m - F_o)/F_m$	Maximum quantum efficiency of PSII
F_v'/F_m'	$(F_m' - F_o')/F_m'$	Estimate of the maximum quantum efficiency of PSII under actinic light
Φ_{PSII}	F_q'/F_m'	PSII quantum efficiency: the proportion of light absorbed by chlorophyll used for PSII photochemistry
qP	F_q'/F_v'	Coefficient of photochemical quenching: relates PSII maximum efficiency to PSII operating efficiency
qL	$(F_q'/F_v')/(F_o'/F_t')$	Estimates the fraction of open PSII centers
NPQ	$(F_m/F_m') - 1$	Nonphotochemical quenching constant: estimates the rate constant for heat loss from PSII
R_{fd}	$(F_p/F_t') - 1$	Fluorescence decline ratio calculated using steady-state fluorescence: correlates with CO_2 fixation rate, with $R_{fd} > 3$ indicative of highly efficient PSII and $R_{fd} < 1$ reflecting negligible net CO_2 gain; this correlation allows this to be used as a vitality index; here, F_p is taken during the initial phase of the Kautsky effect, where the peak fluorescence is induced by actinic light

considered to be a driving force in the appearance of epi-alleles (facilitated or obligatory epi-alleles; Richards, 2006), and, indeed, documented environmentally induced epigenetic changes correlate with, although are not always necessary for, TE activity (Ito et al., 2011, 2016; Eichten et al., 2013; Ong-Abdullah et al., 2015; Stuart et al., 2016). Future studies should take into consideration the impact of TE regulation, under conditions of abiotic stress particularly in species with greater TE content, which possibly underpins at least a subset of the stochastic or spontaneous epi-alleles observed in this study.

There is evidence building for the possibility of transgenerational plant stress memory irrespective of chromatin variation (Agrawal, 2002; Rasmann et al., 2012; Murgia et al., 2015; Nosalewicz et al., 2016; Zheng et al., 2017). Indeed, a distinction has been made between transgenerational epigenetic effects, referring to nongenetically determined transgenerational phenotypes, and transgenerational epigenetic inheritance, referring to nongenetically determined transgenerational phenotypes attributable to heritable chromatin modifications (Youngson and Whitelaw, 2008). Thus, evidence for the formation of transgenerational drought stress memory was investigated in drought-exposed Arabidopsis lineages propagated by single-seed descent. Despite successive generations of repeated drought stress, during both vegetative and reproductive growth stages, no altered aboveground morphological growth phenotypes were observed. This also was surprising given the recent reports of transgenerational memory phenotypes observed in Arabidopsis for salinity and low humidity (Sani et al., 2013; Tricker et al., 2013; Wibowo et al., 2016). A caveat of this study was that root phenotypes were not investigated, as previously *Polygonum persicaria* and barley (*Hordeum vulgare*) roots demonstrated transgenerational memory phenotypes in response to drought (Herman and Sultan, 2016; Nosalewicz et al., 2016). However, root memory phenotypes are not a general occurrence, as demonstrated in studies of phosphate starvation in rice (Secco et al., 2015). Recently, propagation of rice under drought stress led to aboveground differences in generation 11 plants compared with the first generation (Zheng et al., 2017). However, in that study, critically, there were no unstressed lineages incorporated to enable analysis of the phenotypic changes to be considered alongside associated DMRs, as opposed to the stochastic methylome variability observed herein that can arise over such a long-term experiment.

Here, the only evidence of transgenerational memory was in the form of increased seed dormancy (72% enhanced dormancy), which persisted, to some extent, beyond a generation of drought stress exposure (31% enhanced dormancy). This seed-specific memory might be expected of a rapid-cycling annual species whose success is dependent on seed behavior (Grime et al., 1981; Thompson, 1994; Springthorpe and Penfield, 2015). Any effect of enhanced seed dormancy on other developmental phenotypes, in this study, would have been masked by the seed stratification treatment performed prior to experimentation. Although the potential adaptive advantage of increased seed dormancy was not tested directly in this study, it would not be inconsequential, as seed dormancy dictates the environment that progeny plants would germinate in, thus having a potentially critical impact on early growth (Finch-Savage and Leubner-Metzger, 2006; Shu et al., 2016). Such a trait also has been suggested to be an advantage for progeny whose parents were affected by herbivory (Agrawal, 2002; Rasmann et al., 2012).

Increased seed dormancy is a classic form of maternal imprinting, whereby environmental conditions experienced by the maternal plant can influence seed development, altering seed properties including propensity to germinate. For example, seeds that develop under conditions of stress induce maternal ABA production, which can increase seed ABA content, thus enhancing dormancy (Finch-Savage and Leubner-Metzger, 2006). This altered seed provisioning would be the simplest explanation for the enhanced seed dormancy observed, especially since the D2 treatment occurred during reproductive development. Indeed, independent transgenerational studies of Fe deficiency also have shown memory phenotypes to be carried through altered seed

provisioning that were lost in the absence of stress (Murgia et al., 2015). Here, however, the enhanced seed dormancy persisted in seeds developed in the absence of stress (P_1 seed), albeit to a weaker magnitude. This persistent memory is more consistent with the notion of transgenerational memory. The mechanism conveying this memory is not resolved; however, it appears to be DNA methylation independent. Histone modifications were not assayed in this study, but variations also may have been induced. Indeed, osmotic stress-induced variation in histone methylation has been reported previously to mediate stress priming to HS within a generation, lending support to this hypothesis (Sani et al., 2013).

CONCLUSION

Herein, we present a systematic and comprehensive investigation of the possibility for DNA methylation variants to act as heritable stress-induced epi-alleles to convey transgenerational drought stress memory for multiple physiological traits that could be associated with drought responsiveness. Overall, Arabidopsis showed one specific memory trait: elevated seed dormancy in both the direct seed of drought-stressed parents (72% enhanced dormancy) and in seed produced from P_1 progeny, from drought-exposed lineages, grown in the absence of stress, albeit to a lesser magnitude of dormancy (31% enhanced dormancy). Whether this conveys an adaptive advantage remains unclear, as seed stratification was done prior to experimentation for aboveground memory traits. Furthermore, there are likely to be cell type-specific responses that contribute to the complexity of plant stress memory, which will be important to consider in future investigations. Despite the appearance of 40 drought-associated DMRs within a generation, transgenerational drought stress-induced epi-alleles were rare and are unlikely to act as a mechanism to convey any form of transgenerational stress memory. Rather, the majority of DNA methylation states are highly stable, and the variation observed in this investigation, within and across generations, appears to occur stochastically. In conclusion, despite evidence of transgenerational drought stress memory for one of the six traits examined, the DNA methylome was relatively impervious to stress-induced changes.

MATERIALS AND METHODS

Plant Germplasm, Growth, and Drought Stress Treatments

All Arabidopsis (*Arabidopsis thaliana*) plants used in this study were in the Columbia background. Plants were cultivated on soil under a 12-h photoperiod (8 AM to 8 PM) of 100 to 150 μmol photons m^{-2} s^{-1}, 20°C ± 0.5°C, and 55% ± 5% relative humidity. The desired light intensity was achieved using 250-W metal halide lamps (Venture Lighting; MH 250W/U). Prior to light, seeds were sown onto moist soil and kept at 4°C for three nights to allow for seed stratification. A slow-onset water deprivation treatment (drought stress) was imposed, after saturating soil moisture, by withholding watering for 9 d for the within-generation drought treatment.

The growth conditions for propagation of lineages by single-seed descent were identical to those above, with the exception of a 16-h photoperiod (8 AM to 12 AM) to promote rapid cycling. All lineages were initiated from a common inbred G_0 progenitor to minimize genetic difference and stochastic DNA methylome variations. An extended version of the initial water deprivation treatment was applied twice every generation to lineages propagated under drought stress (Fig. 3A). The first treatment was applied at 1 week of age, which involved saturating soil moisture and subsequently withholding water for 2 weeks. Plants were then watered and allowed to recover for 5 d. The second treatment was repeated following recovery, although this time for only 12 d to minimize plant death. Plants were then watered until rosette leaf senescence and the appearance of dried, mature siliques for seed harvesting following the guidelines set by the Arabidopsis Biological Resource Centre.

High-Throughput Phenotyping Using PlantScreen

PlantScreen (Photon Systems Instruments), a platform for high-throughput phenotyping, was used to monitor plant growth and photosynthetic performance by measuring plant area and chlorophyll fluorescence (Humplík et al., 2015; Rungrat et al., 2016). An ANOVA with subsequent Tukey's honestly significant difference (HSD) method was utilized to test for size differences at single time points. A second-order mixed-effect polynomial model was constructed to test for differences in growth rate between lineages.

Monitoring PSII Performance Using Chlorophyll Fluorescence

To monitor PSII performance under drought stress, measures of chlorophyll fluorescence were obtained using a PSI FluorCam (Photon System Instruments). Images were analyzed using the accompanying FluorCam7 imaging software. Chlorophyll fluorescence was measured across the adaxial side of dark-adapted (30 min) plants for 7 min under actinic light (approximately 800 μmol photons m^{-2} s^{-1}) followed by 3 min in the dark, with regular measures of chlorophyll fluorescence induced by a saturating pulse (approximately 3,000 μmol photons m^{-2} s^{-1}) as well as minimal fluorescence in the presence of measuring light only (Humplík et al., 2015; Rungrat et al., 2016). Measurements on 30-min dark-adapted plants allowed measurements of base fluorescence (F_o), fluorescence immediately prior to a saturating pulse (F_t), maximal fluorescence after a saturating pulse (F_m), and variable fluorescence (F_v). Subsequently, the Kautsky effect was induced, with the initial signal giving peak fluorescence (F_p) as PSII activity engages. Regular saturating pulses occur under actinic light, allowing measurement of the light-adapted counterparts: F_o', F_m', F_v', and F_t'. The parameters shown in Table IV were calculated using these obtained values and the corresponding equations (Haitz and Lichtenthaler, 1988; Lichtenthaler and Miehé, 1997; Maxwell and Johnson, 2000; Lichtenthaler et al., 2005; Baker, 2008; Brestic and Zivcak, 2013; Murchie and Lawson, 2013).

Plant Biomass and Rosette Dehydration Assay

The rosette dehydration assay was performed as reported previously (Wilson et al., 2009). Briefly, rosettes of approximately 4-week-old plants, grown under control growth conditions as described above, were excised at the base and weighed on a five-digit fine balance (Mettler Toledo). This was used as the measurement of fresh biomass. An ANOVA with Tukey's HSD posthoc analysis was used to determine significant differences ($P < 0.05$) in rosette biomass. The mass of excised rosettes was then monitored at regular intervals for 1 h. A mixed-effect second-order polynomial model was constructed to test for significantly reduced rate of water loss between lineages ($P < 0.05$).

Flowering Time

Flowering time was measured on mature plants grown under the growth conditions described above. This was compared using two metrics upon emergence of the floral bud: (1) number of days since emergence; and (2) number of true leaves. Differences in flowering time were analyzed using ANOVA with Tukey's HSD posthoc for each metric.

Seed Dormancy Assay

Seed dormancy was tested on seeds collected from G_4 P_0 and G_4 P_1 plants using recommended methods (McNair et al., 2012). Dried and matured siliques were collected from senescing plants. Each silique was taken from an individual plant and considered as a single biological replicate, with six biological replicates per lineage. Seeds from an individual silique were released onto a 0.8%

agar plate and kept immediately in the growth cabinet under the growth conditions described above. For the first 5 d, photographs of the plates were taken twice daily; thereafter, they were taken only once daily. At each time point, all seeds per plate were scored as either germinated or ungerminated. To statistically compare seed dormancy, a Cox proportional hazards model was produced. From this model, calculation of the HR provides a comparative value between treatment groups. The HR_D was calculated relative to unstressed lineages.

Assaying Survival under Prolonged Drought

The length of survival under drought was tested by performing a terminal drought experiment. G_5 P_1 progeny from multiple independent lineages, per treatment, were grown under control growth conditions, as described above, to approximately 3 weeks of age. Subsequently, soil was watered to saturation and excess water was drained. Watering was withheld thereafter, and plant vitality was monitored nondestructively with chlorophyll fluorescence measurements. The parameter R_{fd} was utilized as a vitality index, where plants that demonstrated $R_{fd} < 1$ are considered to be dead (Table IV; Haitz and Lichtenthaler,1988).

WGBS Library Preparation

WGBS was performed from snap-frozen leaf tissue of harvested whole rosettes. Details for all samples are provided in Supplemental Table S1. Genomic DNA was extracted using the Qiagen DNeasy Plant Mini Kit, as per the manufacturer's instructions, and quantified using the ND-1000 Spectrophotometer (NanoDrop Technologies). One hundred to 200 ng of fragmented (Covaris) and purified genomic DNA was bisulfite converted using the Zymo DNA-Gold bisulfite conversion kit (Zymo Research). WGBS libraries were constructed using the Accel-NGS Methyl-Seq DNA Library Kit (Swift Biosciences) as per the manufacturer's instructions. All purification steps were performed using Seramag SpeedBeads (GE Healthcare). The concentration and size distribution of bead-purified libraries were quantified on the Perkin-Elmer GXII using a DNA High Sensitivity kit. Libraries were subsequently pooled equimolar, in six-sample pools, and sequenced across a HiSeq2500 (100-bp single end; Illumina), with a 5% to 10% spike in PhiX DNA, depending on sample complexity, at the ACRF Biomolecular Research Facility (Australian National University, Canberra).

WGBS Analysis

Raw sequencing reads were quality controlled and trimmed using Trim Galore! (version 0.3.7), Cutadapt (version 1.9), and FastQC (version 0.11.2). Reads were then aligned to the TAIR10 reference genome using Bismark (version 0.14.5; Krueger and Andrews, 2011) with the flags-bowtie1 -n 2, -l 20 (Bowtie1 version 1.1.2; Langmead et al., 2009). Methylated Cs were extracted from aligned reads using the Bismark methylation extractor with default parameters. Bisulfite conversion efficiency was calculated from the proportion of unconverted Cs in the mCHH context from the chloroplast genome and ranged from 99.3% to 99.7% per sample. The proportion of mCG, mCHG, and mCHH was determined as weighted methylation (Schultz et al., 2012) across reads at single C resolution and across 100-bp tiles for genome-wide comparisons. Pearson's correlation coefficient (r) of methylation levels, between samples, was performed on the 100-bp bin mean methylation levels in all sequence contexts. Methylation levels were assigned to annotated genes and TE features of the TAIR10 assembly using Bedtools (version 2.21.0; Quinlan and Hall, 2010) and the Araport11 genome reannotation (Cheng et al., 2017). Sequencing summary statistics are provided in Supplemental Table S1.

Identifying DMRs

This study employed two unbiased approaches to identify both stochastic and treatment-associated DMRs based on a previous combinatorial approach (Eichten et al., 2016).

First, to look at stochastic variation in the DNA methylome, regardless of treatment, DMRs were identified using pairwise comparisons employing a method based on average methylation binned to 100-bp tiles across the genome. In brief, pairwise comparisons were performed between corresponding 100-bp tiles in all samples. For each pairwise sample comparison, all 100-bp tiles were called differentially methylated if the absolute difference in methylation levels met a given threshold (mCG, 70%; mCHG, 50%; mCHH, 40%) alongside a minimum coverage and number of Cs (10× coverage, three Cs). Adjacent tiles identified as DMRs were collapsed into a single tile. All results were compared, and the largest region was kept for any overlapping DMRs between pairwise comparisons. A Kruskal-Wallis rank-sum test was performed to identify significant differences between treatment groups using 100-bp tile-based DMRs with a Bonferroni posthoc P value correction for multiple comparisons.

Second, a more conservative approach was used to identify statistically significant treatment-associated DMRs using a Bayesian hierarchical modeling approach, incorporating technical and biological variation at the individual C level, with the R package DSS (version 2.10.0; Feng et al., 2014). This was performed using the recommended default settings (with smoothing to allow for imputation of missing data) except for a reduced smoothing tile size (smoothing.span = 100). The threshold methylation difference for DMRs in each sequence context (delta) also was adjusted to 40% for mCG, 20% for mCHG, and 20% for mCHH. DSS calculates an adjusted P value (q value) based on the posterior probability that the difference specified (delta) is significant; DMRs were considered significant at $q < 0.05$.

Data Visualization and Statistical Analyses

Data visualization and statistical analyses, as described above and throughout the article, were conducted in R (version 3.3.2) using the appropriate packages (Wickham, 2007, 2009, 2011; Bache and Wickham, 2014; R Core Team, 2016; Warnes et al., 2016). DNA methylome patterns, including identified DMRs, were viewed using the IGV (Robinson et al., 2011). The R lme4 package (version 1.1; Bates et al., 2015) was used for generalized mixed-effects modeling with fixed (e.g. lineage treatment) and random (e.g. blocking design) effects. Model fit was assessed using the conditional R^2 value, calculated using piecewiseSEM (Lefcheck, 2016), and the Akaike information criterion to compare relative fit. Pearson's r was calculated using the R cor function (method = "pearson"). A hypergeometric test was performed using the R phyper function. Expression-based clustering of drought-responsive transcripts was achieved using the R kmeans function (centers = 10). Kruskal-Wallis rank-sum tests were performed using the R kruskal.test function. The R p.adjust function (method = "bonferroni") allowed for posthoc P value correction for multiple pairwise comparisons. Survival analyses to compare seed dormancy between lineages, using a Cox proportional hazards model, were performed using the R survival package (version 2.41; Therneau, 2015). All statistical analyses, including modeling, were produced on single data points. Biological replication, unless described otherwise, was considered to be on independent whole plants. The R DiGGer package (version 0.2.31; Coombes, 2011) was used to produce spatially optimized complete randomized experimental designs.

Accession Numbers

The next-generation sequencing data sets utilized for this article are available at the following National Center for Biotechnology Information Gene Expression Omnibus and Sequence Read Archive data repositories: GSE94075 (WGBS; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE94075) and PRJNA391262 (mRNA sequencing; https://www.ncbi.nlm.nih.gov/bioproject/PRJNA391262/). Bioinformatic pipelines are freely available on github (https://github.com/dtrain16/NGS-scripts). Other accession numbers are as follows: *ABI5* (AT2G36270), *ERD2* (AT1G29330), *MYB20* (AT1G66230), AT2G20920, AT2G34060, *FAMA* (AT3G24140), *CEK3* (AT4G09760), AT4G19270, *NAC089* (AT5G22290), *CNI1* (AT5G27420), and *SPCH* (AT5G53210).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Preexisting differences in the methylome define multiple epi-types.

Supplemental Figure S2. Representative plants and chlorophyll fluorescence profiles characterizing the impacts of D1 and D2.

Supplemental Figure S3. Characterizing the growth of G_4 and G_5 progeny from unstressed and drought-exposed lineages.

Supplemental Figure S4. DNA methylation-labile regions identified in the transgenerational drought stress experiment.

Supplemental Table S1. WGBS summary statistics.

Supplemental Table S2. Epi-type-associated DMRs.

Supplemental Table S3. Drought stress-associated DMRs.

Supplemental Table S4. Gene-mapping DMRs associated with drought-responsive genes.

Supplemental Table S5. Transgenerational drought stress-associated DMRs.

Supplemental Table S6. DNA methylation differences at core ABA signaling genes.

Supplemental Table S7. Stochastic DMRs between all G_5 progeny.

Supplemental Table S8. DSS-identified DMRs identified between G_0 P_1 progeny and G_5 P_1 progeny from lineages propagated under control conditions.

Supplemental Table S9. DSS-identified DMRs identified between G_0 P_1 progeny and G_5 P_1 progeny from lineages propagated with repeated drought stress.

Supplemental Table S10. Transgenerational drought-DMRs identified in public DMR data sets.

Supplemental Table S11. Overlap of stochastic DMRs with identifie epi-alleles.

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