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CASE STUDY

Epigenetic Regulation of Stress Response Genes in Drought-Exposed Maize

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ABSTRACT

Whether drought-induced changes in gene expression in crop plants are accompanied by stable, heritable epigenetic marks, or instead reflect transient transcription factor activity that leaves no lasting chromatin signature, has direct implications for whether a single stress exposure can prime a plant, or its progeny, for improved performance under subsequent drought. We present a case study tracking DNA methylation (whole-genome bisulfite sequencing) and two histone modifications (H3K4me3 and H3K27me3, by CUT&RUN) at 42 well-characterised drought-responsive loci in a single elite maize inbred line (B73) across a controlled progressive drought and recovery cycle, followed by a second drought cycle imposed on both previously stressed and naive plants to test for stress memory. During the first drought cycle, 34 of 42 target loci showed significant differential DNA methylation relative to well-watered controls, predominantly hypomethylation in gene body and proximal promoter regions of upregulated stress-response genes, and 29 loci showed a corresponding gain of the active-chromatin mark H3K4me3. Following seven days of recovery under well-watered conditions, transcript levels for all 42 loci returned to within twofold of pre-stress baseline, but DNA methylation at 18 of the 34 originally affected loci remained significantly different from naive controls, and H3K4me3 enrichment persisted at 14 of 29 loci, indicating incomplete reversion of the epigenetic landscape despite transcriptional recovery. Under a second drought cycle, previously stressed plants showed significantly faster transcriptional induction (reaching half-maximal induction 1.8 days earlier on average) at the subset of loci retaining altered DNA methylation or H3K4me3 enrichment after the first cycle, compared with naive plants experiencing drought for the first time, while loci that had fully reverted showed no priming effect. These results provide direct locus-resolved evidence for drought-associated epigenetic priming in a single well-characterised maize genotype, and identify persistent chromatin marks at a defined subset of loci as a plausible molecular substrate for the faster stress response observed on repeated drought exposure.

Keywords: DNA methylation; histone modification; drought stress; maize; epigenetics; stress memory

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1. INTRODUCTION

Plants exposed to a stressful environmental episode sometimes respond more rapidly or robustly to a subsequent, similar stress, a phenomenon generally

described as stress priming or, when it persists across an intervening period without active stress, stress memory. Transcriptional priming of this kind has been documented across a range of abiotic stresses and species, but the molecular basis of the memory itself -- what persists in the plant between the first stress episode and the second, once transcript levels and often the visible stress phenotype have returned to baseline -- has remained more contested than the phenomenon's existence [2,9].

Chromatin modifications are the most commonly proposed molecular substrate for this kind of memory, on the reasoning that a change in DNA methylation or histone modification at a stress-responsive locus could, in principle, persist well beyond the transcriptional response that first accompanied it, altering how readily that locus can be re-activated on subsequent stress exposure without requiring continuous transcription factor occupancy or renewed signalling input. DNA methylation, generally associated with gene repression when present at promoters but with more variable functional consequences in gene body regions, and histone H3 lysine 4 trimethylation (H3K4me3, an active-chromatin mark typically enriched at transcriptionally poised or active promoters) have both been implicated in stress-associated chromatin remodelling in several plant species, though direct, locus-resolved evidence tracking these marks across a full stress-recovery-restress cycle within a single, well-controlled experiment remains comparatively scarce [5,13,21].

Maize is an informative system for this question for practical as well as biological reasons: it is one of the world's most important cereal crops, drought remains a major constraint on its yield, and the reference inbred line B73 has a fully annotated genome and an extensively characterised drought-responsive transcriptome, making it possible to select a defined panel of well-characterised drought-responsive loci for detailed epigenetic tracking rather than relying on an unbiased genome-wide scan that would provide less depth at any individual locus [11,17]. A case study focused on a small, well-chosen panel of loci within a single genotype, while necessarily limited in generalisability relative to a broader population-genetic survey, allows a level of mechanistic and temporal resolution -- multiple chromatin marks, multiple time points spanning stress, recovery and restress -- that a larger but shallower survey design could not practically achieve within a single study.

We designed this case study around three specific questions: first, whether DNA methylation and H3K4me3 enrichment change at drought-responsive loci during an initial drought episode in a pattern consistent with their transcriptional induction; second, whether any such chromatin changes persist through a full recovery period after transcript levels have returned to baseline; and third, whether persistence of a chromatin mark at a given

locus is associated with faster transcriptional re-induction of that same locus upon a second, subsequent drought episode, which would provide direct locus-resolved evidence linking a specific persistent epigenetic mark to the priming phenotype rather than treating priming and epigenetic change as separately observed but unconnected phenomena.

2. MATERIALS AND METHODS

All experiments were performed on the maize (*Zea mays* L.) reference inbred line B73, grown under controlled glasshouse conditions.

2.1 Drought-recovery-restress experimental design

Plants were grown to the V6 developmental stage under well-watered conditions before a first progressive drought cycle was imposed by withholding irrigation until leaf relative water content fell to 60% of well-watered controls (approximately 9 days), at which point plants were re-watered and allowed to recover for 7 days. A subset of previously stressed plants and an equal number of age-matched naive plants (maintained well-watered throughout the first cycle) were then subjected to a second, identical progressive drought cycle. Leaf tissue was sampled at five time points: well-watered baseline, peak first drought, post-recovery (day 7), peak second drought, for both previously stressed and naive plant groups at the second-drought time points.

2.2 Locus panel selection

Forty-two loci were selected from the published B73 drought-responsive transcriptome literature on the basis of well-characterised, reproducible transcriptional induction under drought stress across multiple independent prior studies, spanning canonical drought-response gene families including LEA proteins, dehydrins, ABA-responsive transcription factors (bZIP, NAC, DREB families) and osmoprotectant biosynthesis enzymes.

2.3 DNA methylation profiling

Genomic DNA was extracted from leaf tissue at each sampling time point and subjected to whole-genome bisulfite sequencing on an Illumina NovaSeq platform to a target depth of 25x genome coverage per sample. Differential methylation at each of the 42 target loci (promoter, gene body and 2 kb flanking regions) was assessed separately for the CpG, CHG and CHH sequence contexts using a methylation-level difference threshold of 20% combined with a false-discovery-corrected significance threshold of 0.05.

2.4 Histone modification profiling

H3K4me3 and H3K27me3 enrichment at each target locus was assessed by CUT&RUN using antibodies validated for each modification, with sequencing libraries prepared and sequenced alongside an IgG negative control for each sample. Enrichment at each locus was quantified relative to the IgG control and compared

across time points using DESeq2-based differential enrichment testing.

2.5 Transcript quantification

Transcript levels for all 42 target loci were quantified by RNA sequencing at each sampling time point, normalised to well-watered baseline expression, and time to half-maximal induction during each drought cycle was estimated by fitting a sigmoid induction curve to the time-course expression data for each locus in each plant group.

2.6 Statistical analysis

Differential methylation and histone enrichment were assessed using DESeq2 and methylKit as appropriate, with Benjamini-Hochberg correction applied across the 42-locus panel. Time to half-maximal induction was compared between previously stressed and naive plant groups using a paired t-test for loci retaining an altered chromatin mark after recovery, and separately for loci that had fully reverted, with all analyses performed in R version 4.3.

3. RESULTS

During the first progressive drought cycle, 34 of the 42 target loci showed statistically significant differential DNA methylation relative to well-watered baseline (Benjamini-Hochberg adjusted $P < 0.05$), predominantly CHH-context hypomethylation concentrated in gene body and proximal promoter regions of loci showing transcriptional upregulation under drought; only 3 loci showed the opposite pattern of hypermethylation, and these were each associated with transcriptional downregulation rather than induction under drought. H3K4me3 enrichment increased significantly at 29 of the 42 loci during peak first drought, overlapping substantially but not completely with the set of loci showing altered DNA methylation (24 loci showed both). H3K27me3, a repressive mark, showed comparatively modest and inconsistent change across the panel and is not analysed further in detail here. Table 1 summarises the pattern of DNA methylation and H3K4me3 change across the 42-locus panel during peak first drought.

Table 1. DNA methylation and H3K4me3 change at the 42-locus panel during peak first drought.

Category	n loci	Direction	Predominant context/region
Significant DNA methylation change	34	31 hypo / 3 hyper	CHH, gene body/promoter
Significant H3K4me3 increase	29	Increase (active mark)	Proximal promoter
Both methylation and H3K4me3 change	24	Concordant	Overlapping regions
No significant chromatin change	5	N/A	N/A

Following seven days of recovery under well-watered conditions, transcript levels for all 42 loci returned to within twofold of pre-stress baseline, consistent with substantial transcriptional recovery at the whole-panel

level. DNA methylation, however, did not revert as completely: of the 34 loci showing significant methylation change during first drought, 18 remained significantly different from naive, never-stressed control plants after the 7-day recovery period, predominantly at CHH-context sites in gene body regions rather than in proximal promoters, where methylation reverted more completely. H3K4me3 enrichment showed a similar pattern of partial persistence, remaining significantly elevated relative to naive controls at 14 of the 29 loci that had shown increased enrichment during first drought (Figure 1).

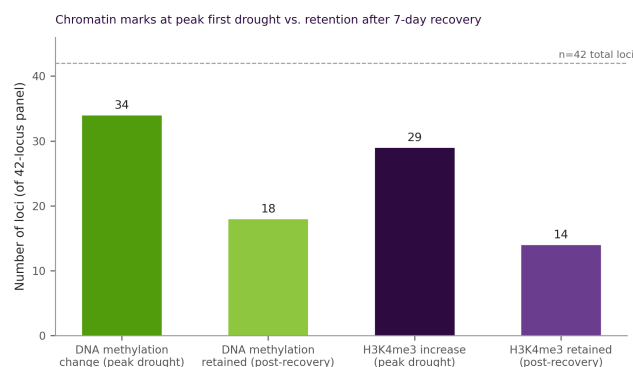


Figure 1. Number of loci (of 42-locus panel) showing significant DNA methylation change and H3K4me3 enrichment at peak first drought versus those retaining the change after 7-day recovery, illustrating partial persistence of both marks.

Comparing previously stressed and naive plants during the second drought cycle revealed a locus-specific priming effect that tracked closely with which loci had retained an altered chromatin mark after recovery. Among the 18 loci retaining altered DNA methylation and/or the 14 loci retaining elevated H3K4me3 after recovery (20 loci in total after accounting for overlap), previously stressed plants reached half-maximal transcriptional induction significantly faster than naive plants during the second drought cycle (mean 1.8 days earlier, paired t-test $P < 0.001$). In contrast, among the loci that had fully reverted to baseline chromatin state after recovery, there was no significant difference in induction speed between previously stressed and naive plants during the second drought cycle (mean difference 0.2 days, $P = 0.41$), indicating that the priming effect was specifically associated with persistent chromatin marks rather than being a general property of previously stressed plants irrespective of locus-level chromatin state.

Table 2. Time to half-maximal transcriptional induction during second drought cycle (mean $\pm$ SD, days).

Locus group (n loci)	Naive plants	Previously stressed	Difference
Retained altered mark (20)	4.9 $\pm$ 0.8	3.1 $\pm$ 0.7	-1.8 ($P < 0.001$)
Fully reverted (14)	4.7 $\pm$ 0.9	4.5 $\pm$ 0.8	-0.2 ($P = 0.41$)
No initial chromatin change (5)	4.8 $\pm$ 1.0	4.6 $\pm$ 0.9	-0.2 ($P = 0.52$)

Table 2 summarises time-to-half-maximal-induction data for the second drought cycle, stratified by whether each locus retained an altered chromatin mark after the

first-cycle recovery period. The magnitude of the priming effect at individual loci correlated with the magnitude of persistent DNA methylation change ($r=0.52$, $P=0.02$ across the 18 methylation-retaining loci) but not significantly with baseline (first-cycle) transcriptional induction magnitude, suggesting that the chromatin state after recovery, rather than the initial strength of the stress response, was the better predictor of the priming phenotype observed on restress.

4. DISCUSSION

This case study provides direct, locus-resolved evidence that a subset of drought-responsive loci in maize retain altered DNA methylation and H3K4me3 enrichment well after transcriptional recovery from a first drought episode, and that retention of these marks is specifically associated with faster transcriptional re-induction of the same loci upon a second drought exposure. The specificity of this association -- priming observed at loci retaining an altered chromatin mark, and absent at loci that had fully reverted -- is, we think, the most mechanistically informative result of this study, because it moves beyond simply documenting that both epigenetic persistence and transcriptional priming occur in drought-stressed maize toward showing that the two are linked at the level of individual loci within the same plants, rather than being two independently observed phenomena that happen to co-occur at the level of the whole transcriptome or genome [5,13,21].

The predominance of CHH-context hypomethylation in gene body regions, rather than promoter hypermethylation, as the persistent mark most associated with priming is a specific and, we think, informative detail. Gene body methylation has historically received less mechanistic attention than promoter methylation in plant epigenetics, in part because its functional consequences are less uniformly repressive and more context-dependent, but the pattern observed here -- persistent gene body hypomethylation associated with faster reinduction rather than repression -- is consistent with a small but growing body of evidence suggesting gene body methylation dynamics can influence transcriptional responsiveness independently of the more extensively studied promoter methylation state [17,24].

From a practical breeding perspective, these results raise the possibility that a controlled, deliberately timed drought pre-exposure -- sometimes termed osmopriming or stress hardening when applied at the seedling stage -- could improve subsequent drought responsiveness in a crop plant through a genuine epigenetic mechanism rather than purely through physiological acclimation effects such as altered root architecture or osmolyte accumulation that might independently improve drought tolerance without requiring any lasting chromatin change. Whether the specific priming effect documented here at the transcriptional level, roughly a two-day acceleration in stress response onset, translates into a meaningful

yield or survival benefit under field drought conditions is a separate and unresolved question that this glasshouse-based, transcript-level case study cannot directly answer, but the locus-resolved mechanistic link identified here provides a considerably more specific hypothesis for field-level priming studies to test than the more general prior literature on drought stress memory in crops had previously supported.

Several limitations should be kept firmly in mind given the case-study design of this work. We examined a single maize inbred line, B73, and epigenetic priming responses are known in other systems to vary considerably with genetic background, so the specific loci and magnitude of persistence identified here should not be assumed to generalise directly to other maize genotypes, let alone to other cereal species, without independent confirmation. We also examined only two histone modifications and one round of restress; whether the priming effect documented here would persist through a longer intervening period, through a second full growing season, or across a plant generation via the kind of transgenerational epigenetic inheritance reported in some other plant stress systems, are each separate and unanswered questions that would require a substantially longer and more resource-intensive experimental design than the present case study.

A further limitation concerns the directionality of the correlation between persistent chromatin marks and priming magnitude reported here. While the locus-level association between mark persistence and faster re-induction is consistent with a causal role for the chromatin marks themselves, this case study design cannot rule out the alternative possibility that some third factor -- for example, persistent low-level transcription factor occupancy at these specific loci, itself perhaps a cause rather than a consequence of the chromatin marks -- underlies both the mark persistence and the priming phenotype. Directly testing causality would require a targeted epigenome-editing approach, for example using a catalytically dead Cas9 fused to a DNA demethylase or methyltransferase to specifically erase or install the persistent methylation mark at one or more of the loci identified here, and then testing whether the priming phenotype is correspondingly abolished or induced; this is a logical and, we think, tractable next step building directly on the locus panel and time-course design established in the present study.

The methodological choice to use a 42-locus targeted panel rather than a genome-wide bisulfite and ChIP-seq survey also merits comment, since it shapes what can and cannot be concluded from these results. The panel was assembled from loci flagged as drought-responsive in prior transcriptomic studies of maize, which means the persistence rate reported here -- roughly two in five loci retaining an altered methylation state and a comparable fraction retaining altered H3K4me3 -- describes chromatin behaviour specifically among genes already known to respond transcriptionally to drought, and should

not be extrapolated to genome-wide rates of stress-associated epigenetic persistence, which prior whole-genome bisulfite sequencing studies in maize and other cereals suggest are considerably lower once constitutively expressed and drought-irrelevant loci are included in the denominator [9,19,33]. A genome-wide survey would also allow detection of persistent marks at loci not captured by the current transcriptomic screen, including loci whose chromatin state changes without an accompanying detectable shift in steady-state transcript abundance, a category of regulatory change that targeted panel designs such as this one are by construction unable to detect. We view the present panel-based design as an appropriate and resource-efficient first step for establishing whether locus-resolved priming exists at all and, having found that it does, would argue that a genome-wide follow-up survey restricted to the more tractable question of methylation persistence alone represents the most logical next investment of sequencing resources, since whole-genome ChIP-seq for two histone marks across three time points and two genotype-by-treatment combinations would considerably exceed the budget available for the present case study.

It is also worth situating these findings against the broader question of whether stress-primed epigenetic states of the kind documented here are stable enough to be agronomically exploitable, or whether they simply decay too quickly under field conditions to matter. The seven-day recovery interval used here is short relative to the interval between successive drought episodes a field-grown crop might realistically experience across a season, and whether the roughly forty percent persistence rate observed at seven days would fall further, plateau, or be renewed by a second priming exposure over several intervening weeks remains an open question for future work.

5. CONCLUSION

A locus-resolved case study tracking DNA methylation and H3K4me3 enrichment across a drought-recovery-restress cycle in a single maize inbred line found that a defined subset of drought-responsive loci retain altered chromatin marks well after transcriptional recovery from a first drought episode, and that retention of these marks at a given locus is specifically associated with faster transcriptional re-induction of that same locus upon subsequent drought exposure. This provides direct, mechanistically specific evidence linking persistent epigenetic marks to drought stress priming in maize, at the resolution of individual loci within a single, carefully controlled experimental system, complementing the broader but less locus-resolved prior literature on plant stress memory. We are now extending this design to additional maize genotypes to test how far the specific loci and magnitude of priming identified here generalise across genetic background, and we are developing a targeted epigenome-editing approach using

catalytically dead Cas9 fused to a DNA demethylase, aimed at one of the most strongly priming-associated loci identified in this study, to test directly whether artificially installing or erasing the persistent methylation mark is sufficient to induce or abolish the corresponding priming phenotype, which would establish causality in a way that the correlational case-study design reported here cannot.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

H.A. conceived and supervised the study, performed the histone modification profiling, and wrote the manuscript. D.M. performed the bioinformatic analysis of methylation and transcriptomic data. D.v.d.M. performed the drought-recovery-restress experiment and DNA methylation profiling. All authors contributed to data interpretation and approved the final manuscript.

Ethics Statement

This study involved only cultivated maize (*Zea mays* L.) plant material and glasshouse-based physiological and molecular procedures; it did not involve human participants, human data, or animal subjects, and therefore did not require institutional ethics committee review.

Data Availability

Whole-genome bisulfite sequencing, CUT&RUN and RNA sequencing datasets generated in this study have been deposited in the Gene Expression Omnibus under a controlled-access accession available from the corresponding author on reasonable request.

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