Widespread long-range cis-regulatory elements in the maize genome

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Genetic mapping studies on crops suggest that agronomic traits can be controlled by gene–distal intergenic loci. Despite the biological importance and the potential agronomic utility of these loci, they remain virtually uncharacterized in all crop species to date. Here, we provide genetic, epigenomic and functional molecular evidence to support the widespread existence of gene–distal (hereafter, distal) loci that act as long-range transcriptional cis-regulatory elements (CREs) in the maize genome. Such loci are enriched for euchromatic features that suggest their regulatory functions. Chromatin loops link together putative CREs with genes and recapitulate genetic interactions. Putative CREs also display elevated transcriptional enhancer activities, as measured by self-transcribing active regulatory region sequencing. These results provide functional support for the widespread existence of CREs that act over large genomic distances to control gene expression.

The long-range transcriptional control of genes by distal cis-regulatory elements (CREs) is an important and well-studied feature of metazoan genomes. In contrast, many fundamental questions regarding distal CREs in plants—such as their prevalence, sequence and chromatin attributes, transcriptional regulatory behaviours and mechanisms of action—remain unanswered. In maize, agronomic quantitative trait loci (QTL) have been mapped to the intergenic space and a handful of domestication loci that were hypothesized to contain CREs have been fine-mapped to distal regions. Genetic evidence demonstrated that these fine-mapped loci controlled their target genes in cis. However, currently lacking are molecular characterizations of these loci and demonstrations of direct chromatin interactions between the hypothesized CREs and their target genes.

It has been widely observed that actively engaged CREs reside within accessible chromatin. This is partially due to the interactions between transcription factors (TFs) and DNA, which often disturb nucleosome stability and elevate chromatin accessibility. Nucleosomes surrounding accessible chromatin regions (ACRs) often exhibit histone modifications indicative of the transcriptional coregulators that have been recruited to the ACRs. Accordingly, flanking histone modifications provide insight into the regulatory mechanisms of the CREs contained within ACRs. Given that ACRs are enriched at intergenic QTL in the maize genome, we decided to take an ACR-centric approach to identify actively engaged CREs within the gene–distal intergenic space. Here, we combined assay for transposase-accessible chromatin sequencing (ATAC-seq) with multiple chromatin assays to demonstrate that distal CRE are abundant in the maize genome.

Results

Gene–dACRs are common in the maize genome. We first profiled chromatin accessibility in young Zea mays L. cultivar B73 leaves using ATAC-seq. We identified 32,111 ACRs (Supplementary Fig. 1a, and Supplementary Table 1), which ranged mostly from 300 to 1,000 base pairs (bp) in length (Fig. 1c) and occupied ~1% of the maize genome. Multiple chromatin accessibility datasets from comparable maize tissues were publicly available, allowing us to compare independent datasets that employed different enzymatic assays (Tn5, DNase14, and MNase15), allowing us to compare independent datasets that employed different enzymatic assays (Tn5, DNase14, and MNase15). Chromatin accessibility signals from the independent experiments were enriched at the ACRs identified in this manuscript (Supplementary Fig. 1a.b). These ACRs recapitulated 88% (18,789/21,384) of the accessible regions identified via DNase treatment14 (Supplementary Fig. 1c). These results indicated that systematic biases deriving from the Tn5 enzyme were negligible within our experimental context.

We split ACRs based on proximity to their nearest annotated genes (Fig. 1b). We found 12,495 (38.9%) of the ACRs overlapped genes (pACRs, defined as overlapping ≥1 bp with annotated genes) and 9,183 (28.6%) were within 2 kilobases (kb) of genes (pACRs, defined as overlapping ≥1 bp with the 2 kb regions flanking genes, but not overlapping the genes themselves). We also found 10,433...
ACRs (32.5%) occurred >2 kb from their nearest genes (distal ACRs (dACRs)) and 4,091 dACRs exceeded 20 kb from their nearest genes (Fig. 1d). Hypothesized long-range CREs that were previously identified by genetic mapping studies, such as those controlling tb1 (ref. 7), ZmRap2.7 (ref. 6), BX1 (ref. 8) and ZmCCT9 (ref. 5), were apparent in the ATAC-seq data (Fig. 1a and Supplementary Fig. 2a–c).
Gene–dACRs probably contain cis-regulatory elements. The elevated accessibility at dACRs could be caused by active mechanisms, such as the binding of nucleosome-displacing TFs or chromatin remodelers, or by inactive mechanisms, such as the presence of DNA sequences recalcitrant to nucleosome assembly. Our data suggested active mechanisms of dACR formation. The sequence content within dACRs was approximately 15% more GC-rich (better suited for nucleosome formation) than for negative control regions (control: randomly selected, uniquely mapping, non-ACR intergenic regions; Fig. 1e). Furthermore, dACRs were enriched for TF binding sites, which we identified empirically (using DAP-seq for 32 maize TFs) and computationally (using known TF binding motifs from Arabidopsis thaliana and de novo motif enrichment). pACRs and dACRs showed similar rates of DAP-seq peak overlap (Fig. 1f) and all 32 DAP-seq TFs were enriched at dACRs (Fig. 1g). Individual dACRs were predicted to contain multiple TF binding sites which corresponded to TFs from multiple families (Fig. 1h and Supplementary Fig. 2d–f).

Several lines of evidence suggested that many dACRs were functionally important and potentially enriched with CREs. First, DNA sequence diversity was markedly reduced at dACRs (Fig. 1i). Second, sequence variation within dACRs was more likely to be associated with phenotypic variation (Fig. 1j) and gene expression variation (Fig. 1k), as determined by genome-wide association data. Third, the nearest genes flanking dACRs were enriched for transcriptional regulatory functions and were tissue-specifically expressed (Supplementary Fig. 3a,b).

Gene–dACRs fall into chromatin classes suggestive of their regulatory functions. In mammalian genomes, transcriptional enhancers are associated with specific histone modifications (for example, H3K4me1, H3K27ac and H3K27me3). To determine if a typical chromatin signature existed for maize dACRs, we mapped DNA methylation (mCG, mCHG and mCHH, where ‘H’ indicates A, C or T, respectively) and histone covalent modifications (H3K4me1, H3K4me3, H3K27me3, H3K9ac, H3K27ac, H3K56ac and the histone variant H2A.Z) in maize leaves using MethylC-seq and ChiP–seq, respectively. The genic patterns of chromatin accessibility, histone modifications and DNA methylation were similar to those previously described in other plants. DNA cytosine methylation in all sequence contexts was markedly reduced at dACRs (Supplementary Fig. 3c–e). In contrast to H3K4me1, we found that mammalian enhancers, no histone covalent modifications in this study were common to the majority of maize dACRs, although nearly all dACRs were enriched for flanking nucleosomes containing the histone variant H2AZ.

K-means clustering of dACRs by their flanking histone modifications resolved four main groups (Fig. 2b–g and Supplementary Table 1). The majority of dACRs (51.2%) were depleted of flanking histone modifications (‘depleted group’; Fig. 2b and Supplementary Figs. 2c and 4). The histones flanking the depleted group dACRs were either lacking modifications or modified at low levels. We found 11.1% of dACRs contained primarily H3K27me3 at flanking histones (‘H3K27me3 group’; Figs. 1a, 2c and Supplementary Fig. 4). Similarly to the depleted group dACRs, other histone modifications were sometimes present at low levels, but H3K27me3 was the predominant modification. We also found 10.2% of dACRs were flanked by strong H3K9/K27/K56 acetylation and lacked other histone covalent modifications (‘H3Kac group’; Fig. 2d and Supplementary Fig. 4). Additionally, 27.5% of dACRs were flanked by multiple histone modifications typically found together at transcribed genes, including H3K4me1, H3K4me3, H3K63me3 and H3K9/K27/K56ac (‘transcribed group’, Fig. 2e,f and Supplementary Fig. 4). The assortment and strong directionalities of histone modifications at the transcribed group dACRs resembled the chromatin at transcribed genes (Fig. 2a). Furthermore, abundant transcripts colocalized with the histone modifications of the transcribed group dACRs (Fig. 2e,f).

The genes closest to the depleted, H3K27me3 and H3Kac group dACRs were enriched for developmental and transcriptional regulators that were expressed with high tissue specificity (Fig. 2h,i). The genes closest to H3K27me3 group dACRs were transcriptionally repressed, whereas the genes closest to the H3Kac and depleted group dACRs were expressed at low-to-moderate levels (Fig. 2j). In contrast, genes surrounding the transcribed group dACRs lacked significant functional enrichment or expression specificity. Due to the transcribed group’s resemblance to genes, we omitted the transcribed group dACRs from subsequent analyses. The omission of this group did not alter the functional enrichment results from Fig. 1 (Supplementary Fig. 5).

We sought to determine if tissue-specific changes in dACR accessibility correlated with changes in local histone modifications or the expression of nearby genes. We compared ATAC-seq, ChIP–seq and gene expression profiles between leaves and immature inflorescences. Evaluating ChiP–seq signals from both tissues at identical loci revealed that most dACRs (identified in leaf) retained accessibility and the same histone modifications in the second tissue (inflorescences) (Supplementary Fig. 3f,g). However, 15–21% of dACRs that were present in leaves were inaccessible in inflorescences (Fig. 2k and Supplementary Table 2). Tissue-specific dACRs that lost accessibility in one tissue also lost their flanking histone acetylation in the same tissue (Supplementary Fig. 3h,i). This association suggested that the factors responsible for acetylating the flanking histones could be causally linked to chromatin accessibility. In contrast, the relationship between accessibility and H3K27me3 was less clear and potentially decoupled. Tissue-specific dACRs also exhibited relationships with nearby genes. The closest genes to leaf-specific dACRs were more often differentially expressed between leaves and inflorescences (Fig. 2l). This did not hold true for the genes that were buffered from the dACRs by intervening genes. Furthermore, leaf-specific dACRs were more often located upstream, rather than downstream, of differentially expressed genes (Fig. 2m).

Chromatin loops connect gene–dACRs with genes. The locations of dACRs raised the question of how they might regulate target genes over large intergenic distances. To determine if dACRs interacted directly with their target genes through the formation of chromatin loops, we first performed Hi-C on young maize leaves. We focused on the characterization of chromatin loops involving dACRs and genes (Supplementary Tables 3 and 4). Due to technical constraints, we did not search for chromatin loops <20 kb in length; therefore, this was not an exhaustive characterization of all dACR–gene loops. However, 39.2% of dACRs—a sufficiently representative sample of the dACR population—were >20 kb from their nearest genes (Fig. 1d). Although dACRs comprised <0.2% of the intergenic space, more than half (614/1,177) of the identified intergenic–gene loops contained at least one dACR at their intergenic edges (Fig. 3b). Analysis of the Hi-C reads from self-ligated contact pairs demonstrated that the loop enrichment at dACRs was not an artefact arising from chromatin accessibility or mapping biases (Supplementary Fig. 6a,b). Therefore, dACR–gene loops spanning ≥20 kb were a common feature in the maize genome. These loops included interactions between the target genes tbo1, ZmRap2.7 and B1X1 and their genetically mapped controlling regions that have been hypothesized to contain long-range CREs (Fig. 3a and Supplementary Fig. 6c–e).

Although the Hi-C results provided evidence for dACR–gene interactions, relatively few chromatin loops were identified due to limited sequencing depth. Furthermore, because the Hi-C experiment was performed on whole leaves (which contained a diversity of cell types) it was not clear whether dACR–gene loops were formed in cells where the genes were expressed, silenced or both. To address these challenges, we performed Hi-C followed by ChiP (HiChIP) for another collection of dACRs.
using antibodies targeting histone modifications associated with transcriptional activation (H3K4me3) and silencing (H3K27me3), but largely absent from heterochromatin\(^25,27,29\) (Supplementary Table 3 and 4). Similar to the Hi-C loops, the intergenic edges of both H3K4me3- and H3K27me3-HiChIP loops were enriched for dACRs (Fig. 3b). Compared to immediately adjacent flanking regions, dACRs were strongly enriched for long-distance interactions (Fig. 3i and Supplementary Fig. 7a), indicating that the dACRs themselves (as opposed to nearby regions) were the focal points of the long-distance interactions. HiChIP detected more loops than

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**Fig. 2** | Chromatin attributes of dACRs and patterns among dACR-flanking genes.  

**a.** Meta-analysis of DNA methylation, ATAC-seq, ChIP-seq and RNA-seq signals at transcription start sites (TSS) and termination sites (TTS) of annotated genes, ranked by expression. Included are 2–kb upstream and downstream of TSS and TTS. Note that the bottom one-third of ranked genes probably correspond to pseudogenes. **b–g.** Chromatin attributes at dACRs, aligned at dACR summits and clustered into four groups: depleted group, H3K27me3 group, H3Kac group and transcribed group. Shown are \(\pm 2\)–kb from ATAC-seq peak summits. ChIP-seq and RNA-seq experiments for **a–g** were performed in duplicate and yielded identical results each time.  

**h.** Gene ontology (GO) term enrichment for the nearest genes flanking the dACRs on both sides.  

**i.** Expression Shannon entropy values (\(H\)) of the nearest genes on both sides of each dACR.  

**j.** Expression levels (transcripts per million) (TPM) of the nearest genes on both sides of each dACR.  

**k.** Percentage of first neighbour (primary) and second neighbour (secondary) genes that are differentially expressed among the genes flanking leaf-specific differential dACRs.  

**l.** Percentage of genes that are differentially expressed relative to neighbouring DEG.  

**m.** Percentage of differentially expressed genes for which the differential dACR occurs downstream or upstream of the 5' end of the gene. All figures use the same set of negative control regions. For **i, j, l, m**, percentages from genes flanking intergenic negative control regions were subtracted from the percentages of genes flanking dACRs. DEG, differentially expressed gene.
Fig. 3 | Hi-C and HiChIP identify dACR–gene interactions. a, Contact matrix heat maps showing the dACR–gene interactions at tb1 and ZmRap2.7. Red arrows indicate dACR–gene contacts. b, Percentage of intergenic–gene loop edges overlapping dACRs. The asterisks denote \( P < 2.2 \times 10^{-16} \) (Fisher’s exact test, two-sided). Leaf Hi-C, \( n = 1,177 \) total loops (within a single biological replicate); H3K4me3 HiChIP, \( n = 24,141 \); and H3K27me3 HiChIP, \( n = 18,106 \). c, Representative regions containing various HiChIP loops (top panel) and called loop numbers from Hi-C and HiChIP experiments (bottom panel). Grey curves indicate intergenic–intergenic interactions, pink curves indicate intergenic–gene interactions and green curves indicate gene–gene interactions. d, Regions demonstrating dACR interaction hubs (dACR anchors indicated by shaded pale blue regions in the upper panel). White squares in heat maps indicate loops. Yellow curves indicate dACR–gene loops and grey curves indicate intergenic–intergenic loops. e, Enrichment of TF on both ends of loop (–log10\( P\) value). f, g, h, i, j, k, l, m, n, o, p, q, r, s, t, u, v, w, x, y, z.
Hi-C and revealed webs of interactions among genes and dACRs (Fig. 3c). Thirty-four percent of all dACRs (excluding the transcribed group dACRs) looped to more than one gene. The dACR–gene loops that did not skip over genes occurred more often upstream than downstream of target genes (Fig. 3h and Supplementary Fig. 7b). In support of the biological relevance of these long-distance interactions, we found that the Hi-C and HiChIP loops could recapitulate links between intergenic eQTL and their target genes20. Compared to the background looping rates, dACRs that overlapped eQTL were more likely to loop with the target genes predicted by eQTL (Fig. 3j and Supplementary Fig. 7c). A subset of TFs also showed enrichment for binding (via DAP-seq) at both edges of the same loops (Fig. 3k), suggesting a potential mechanism for sequence-specific loop stabilization.

HiChIP allowed us to distinguish the chromatin-looping status of active (H3K4me3-enriched) and silenced (H3K27me3-enriched) genes within tissues containing mixed cell types. For example, strong chromatin interactions were detected between tb1 (silenced in leaves) and its distal CRE using H3K27me3-HiChIP, whereas the dACR–gene loop at ZmRap2.7 (expressed in leaves) was only detected by H3K4me3-HiChIP (Fig. 3a). To systematically explore such relationships, we catalogued dACR–gene loops that were

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**Fig. 4 | Loop strength identifies specific CRE–gene regulatory interactions.**

**a, b.** Genome browser shot of tb1 and its fine-mapped distal regulatory region (a) and a genetically mapped eQTL and its predicted target gene (b). Chromatin loops are represented as line segments with dots indicating $-\log_{10}[P]$. Black and red blocks represent loop edges for all loops interacting with the tb1 locus (indicated as anchor). The red boxes, connected by red line segments, represent the local loop with the highest contact significance that interacts with the anchor region; the black boxes, connected by black line segments, represent all of the other local loops that interact with the anchor region (that is, not the most significant loop). Panels a and b were not performed in replicate.

**c.** The statistical significance of all H3K4me3-HiChIP loops that link dACR-overlapping eQTL to their target genes versus all other dACR–gene H3K4me3-HiChIP loops.

**d.** The expression of target genes at one edge of the loop and dACR at the other end of the loop, split into the three chromatin groups, as classified in Fig. 2. Shown are loops at high and low $-\log_{10}[P]$. Boxplots in c and d include a median with quartiles and outliers above the top whiskers. All $P$ values were determined in the FitHiChIP program using a two-tailed binomial test.
enriched exclusively for H3K4me3-HiChIP loops, H3K27me3 loops, or no loop for an overlap of both (Supplementary Fig. 7d). In the H3K4me3-only loops, H3K4me3 was present at genes but absent from the flanking histones of the dACRs (Supplementary Fig. 7e). In contrast, many of the H3K27me3-only loops (219/632) contained H3K27me3 at both the genes and the interacting dACRs (Supplementary Fig. 7f). Additionally, genes in H3K4me3-only loops were expressed at higher levels than genes in H3K27me3-only loops (Fig. 3l). Although loop identification was not exhaustive, these results demonstrated that dACRs interacted with their target genes via chromatin loops during both transcriptional activation and repression.

Chromatin loop contact strength suggests loops involved in transcriptional regulation. The genes at the aforementioned agronomic loci formed multiple chromatin loops with local regions (Fig. 4a and Supplementary Fig. 8a,b). At each of these genes, the strongest chromatin loop (as measured by the loop statistical significance provided by FitHiChIP32) occurred between the genetically mapped control region and the target gene. For example, the chromatin loop connecting tb1 to its control region 65 kb upstream was stronger than the other loops that also interacted with tb1, even those spanning shorter genomic distances (Fig. 4a). Similarly, chromatin loops that connected eQTL to their predicted target genes were stronger than non-eQTL loops (Fig. 4b,c). Furthermore, strong H3K4me3-HiChIP loops preferentially connected highly expressed genes with the H3Kac group dACRs, a relationship that was not apparent for dACRs and genes connected by weaker loops (Fig. 4d). These results suggested that regulatory CRE–gene interactions could be predicted by the strength of the chromatin loops that connected them.

Nearly all the genetically mapped regulatory elements previously discussed resided upstream of their target genes with no intervening genes (Fig. 1a and Supplementary Fig. 2a–c) (the one exception was BX1, which had one intervening gene). Among the H3K4me3-HiChIP loops that connected eQTL to their target genes, ~75% of the dACRs were located upstream of the target genes and ~75% of the loops connected dACRs to adjacent genes (that is, no intervening genes). These spatial biases were consistent with the fact that strong loops preferentially contained dACRs located upstream of and adjacent to their interacting genes (Supplementary Fig. 8c,d). Collectively, these results suggested that long-range regulatory interactions were predictable based on loop strength, orientation and location relative to target genes.

Gene–dACRs display elevated transcriptional enhancer capacities. To obtain independent and empirical evidence for the transcriptional regulatory capacities of dACRs, we performed self-transcribing active regulatory region sequencing (STARR-seq)13 (a massively parallel enhancer reporter assay) in maize mesophyll protoplasts. We used the enrichment of transcriptional output (STARR-RNA) over DNA input (STARR-input) as a quantitative readout of transcriptional enhancer activity14 (Fig. 5a). We first performed STARR-seq on a ~150 kb bacterial artificial chromosome that contained the tb1 control region (encompassing the region shown in Fig. 1a). The tb1 control region had previously been demonstrated to function as an enhancer in maize protoplasts and could serve as a positive control for STARR-seq. A Hopschotch long-terminal repeat, previously identified as the enhancer-containing element within the tb1 control region, showed pronounced elevation of STARR-seq activity compared to the adjacent genomic regions (Fig. 5b). This demonstrated that the STARR-seq assay was sufficiently sensitive to detect a previously validated maize enhancer.

We then performed STARR-seq with a leaf ATAC-seq library as the input. This allowed us to quantify the enhancer activities of all ACRs in parallel (Fig. 5a). The enhancer activities of dACRs (excluding the transcribed group) were significantly greater than the activities of control regions (control regions were intergenic, non-ACRs containing sufficient STARR-input coverage, matched for length and GC content) (Mann–Whitney, P < 10−14) (Fig. 5c,d). dACRs and pACRs showed similar enhancer activities, with activities (regression coefficients) of dACRs and pACRs twice that of control regions (Fig. 5c). Further analyses suggested that many dACRs functioned as bona fide transcriptional enhancers. In 95% of cases, the enhancer activities of candidate DNA fragments were independent of their orientations relative to the minimal promoter within the STARR-seq vector (Fig. 5e,f). dACRs participating in long-distance chromatin loops were significantly more active than dACRs that were not within loop edges (Fig. 5g). The H3Kac group dACRs showed significantly greater enhancer activity than those of the depleted and H3K27me3 dACRs groups (Fig. 5h). Lastly, the binding of specific classes of TFs (via DAP-seq) was associated with increased enhancer activities and was enriched in highly active dACRs (Fig. 5i,j). Taken together, these results demonstrated that dACRs generally contained the capacity to act as transcriptional enhancers and that H3Kac group dACRs looping to genes showed the greatest enhancer capacities.

Discussion

Decades of studies on individual loci in the compact genome of A. thaliana suggested that CREs were predominantly located within or near genes41. However, emerging evidence in maize suggests that CREs can control genes located dozens of kilobases away. The most notable examples are several fine-mapped agronomic loci (including tb1 (ref. 1), ZmRap2.7 (ref. 8), BX1 (ref. 9) and ZmCCT9 (ref. 10)), which are hypothesized to contain CREs that act over large genomic regions.
Wallace et al. compiled thousands of agronomic QTL and found that approximately one-third of QTL were >5 kb from their nearest annotated genes. These QTL suggest a potentially substantial role for distal regulatory elements in controlling agronomic phenotypes. However, the QTL could also derive from unannotated genes or gene presence–absence variation. Rodgers-Melnick et al. and ourselves (Fig. 1j) demonstrated that dACRs are enriched for intergenic QTL, indicating that many of these QTL contain euchromatin, probably in the form of unannotated genes, non-coding transcription units or regulatory elements. We used histone modification data to identify 7,157 dACRs that did not resemble transcription units (Fig. 2b–d). These dACRs, which are unlikely to be annotation artefacts, are the most likely candidates for long-range CREs in the maize genome.

Multiple lines of evidence indicate that the non-transcription unit dACRs contain CREs: (1) the dACRs overlap fine-mapped hypothesized CREs; (2) dACRs display DNA sequence constraint, manifested as elevated GC content and depleted SNP frequency; (3) dACRs are enriched for TF binding sites and (4) eQTL; (5) dACRs loop to genes in cis, and these loops recapitulate genetically predicted interactions. The dACR–gene loops occur in a spatially non-random manner (dACRs containing putative CRE are
primarily adjacent to and upstream of target genes) and this resembles CREs in the proximal promoters of genes; (6) dACRs with acetylated flanking histones preferentially loop to highly expressed genes, thereby establishing a connection between the chromatin status and transcriptional status at distant loci and (7) dACRs contain sequence elements capable of acting as transcriptional enhancers. Collectively, these results indicate abundant CRE-containing dACRs in the maize genome.

dACRs display chromatin attributes that are useful for their discovery and classification. We found that all dACRs were depleted of DNA methylation (Fig. 2b–f). This finding was previously reported by Rodrigs-Melnick et al.14 and Oka et al.47 using MNase- and DNase-based assays, respectively. Because regions of depleted DNA methylation in plant genomes are developmentally stable, DNA methylation status can potentially be used to locate CREs within tissue-specific dACRs that are not detectable in bulk accessibility assays48. Flanking histone modifications allowed us to separate dACRs into transcribed, H3Kac, H3K27me3 and modification-depleted groups (Fig. 2). The non-transcribed group dACRs appear analogous to metazoan transcriptional enhancers, which are acetylated when active, enriched for H3K27me3 when inactive, and neither enriched for acetylation nor H3K27me3 when in a primed state. Our maize results, which demonstrate an association of H3Kac dACRs with highly expressed genes and an association of H3K27me3 dACRs with polycomb-silenced genes (Figs. 3 and 4), suggest that the chromatin marks at dACRs in maize are analogous to those in metazoans. However, the absence of H3K4me1 at maize dACRs (also found previously by Oka et al.14) contrasts with metazoan enhancers and suggests mechanistic differences in how TFs interact with chromatin pathways.

The prevalence of distal CREs raises the question of how long-range chromatin loops are established and maintained between CREs and their target genes. The loops may form as a consequence of compartmental segregation, in which euchromatic regions (primarily genes and ACRs) self-associate and exclude the intervening heterochromatin, thereby forming loops that span heterochromatin13–27. Alternatively, sequence-specific architectural proteins may play a role in loop formation or stabilization13–15. Both of these mechanisms appear to be common throughout eukaryotes49–57 and the dACR–gene loops described here can be explained by a combination of both. We speculate that the pervasive gene–gene and dACR–gene loops are a consequence of compartmental segregation. Loops with contact strengths elevated above local backgrounds may be brought together via compartmental segregation and then further stabilized by sequence-specific architectural proteins. The dACR–gene loops that are likely to contain specific CRE–gene interactions (such as the fine-mapped agronomic loci and the eQTL–gene interactions) display the greatest contact strengths (Fig. 4 and Supplementary Fig. 8). This leads us to speculate that specific CRE–gene interactions are stabilized by sequence-specific factors, such as TFs that form multimers. The contact strengths of loops can therefore be used to distinguish specific regulatory loops from non-specific compartmental loops. Furthermore, because the predicted regulatory loops preferentially reside upstream of and adjacent to their target genes, putative distal CREs can be assigned to target genes with reasonable confidence, even in lieu of Hi-C data.

A companion study in this issue (Lu et al., 2019 [DOI to come]) demonstrates that distal CREs exist across a wide range of evolutionarily diverse angiosperms and are particularly abundant in plants with large genomes. Even within the compactly sized genus A. thaliana, distal CREs are common in pericentromeric regions with low gene densities. A multispecies comparison of homologous ACRs (Lu et al., 2019 [DOI to come]) suggests that most distal CREs originate in gene–proximal regions (for example, the promoter) and become gene–distal as a result of transposable element proliferation. This is consistent with our observation that distal CREs in maize preferentially reside upstream of and adjacent to their target genes. Collectively, the results of both manuscripts indicate that long-range transcriptional regulation by CREs is a common phenomenon among angiosperms.

Methods

Experimental design. All experiments, except for Hi-C, HiChIP and STARR-seq, were replicated. We did not perform blind experiments or analyses. All assays, except for STARR-seq, were performed on the same tissues at the same developmental stages and grown in the same conditions. However, separate batches of plants were generated for separate experiments. Biological replicates were performed on separately grown batches of plants.

Plant material and growth conditions. Z. mays L. cultivar B73 was grown from seed collected from field-grown ears during summer 2017 in Athens, USA. ATAC-seq, RNA-seq, ChiP–seq, MethC–seq, Hi-C and HiChIP experiments were all performed on seedling tissue grown under the following conditions: kernels were sown in Sungro Horticululture professional growing mix (Sungro Horticulture Canada). Soil was saturated with tap water and placed under a 50/50 mixture of 4,100 K (Sylvania SupeSaver Cool White Deluxe F34CW/SS, 34 W) and 3,000 K (GE Ecolux with starcoat, F40x3MECO, 40 W) light. The photoperiod was 16 h of light and 8 h of dark. The temperature was approximately 25 °C during light hours. The relative humidity was approximately 54%. Seedlings were grown for approximately 6 d and harvested 4–6 h after Zeitgeber Time 0 (ZT0, lights on). Seedlings were harvested when the first leaf had emerged 2–3 cm above the apical meristem of the coleotile. The seedlings were cut 3 mm above the coleotile–mesocotyl boundary, excluding the shoot apical meristem, and the second leaf was removed from within the sheath of the first leaf. Only the inner second leaves, which contained the third and fourth leaves sheathed inside, were used for experiments.

For experiments on young inflorescences (which were ear primordia, hereafter inflorescence primordia), B73 maize was grown in the field or greenhouse. Plants were harvested approximately 1 month after sowing and inflorescence primordia were dissected from shoots. Inflorescence primordia were harvested from any node of the shoot if the length was 3–8 mm from the base to the apical tip of the inflorescence primordia.

ATAC-seq. ATAC-seq was performed as described previously58. For each replicate, approximately 200 mg of maize second leaves and several inflorescence primordia were harvested and immediately chopped with a razor blade and placed in 2 ml of pre-chilled lysis buffer (15 mM Tris–HCl pH 7.5, 20 mM sodium chloride, 80 mM potassium chloride, 0.5 mM spermine, 5 mM 2-mercaptoethanol and 0.2% TritonX-100). The chopped slurry was filtered twice through miracloth and once through a 40-μm cell strainer. The crude nuclei were stained with 4.6-diamidino-2-phenylindole and loaded into a flow cytometer (Beckman Coulter MoFlo XDP). Nuclei were purified by flow sorting, and washed in accordance with Lu et al.58.

The sorted nuclei (50,000 nuclei per reaction) were incubated with 2 μl of transposase in 40 μl of tagmentation buffer (10 mM TAPS–sodium hydroxide pH 8.0, 5 mM magnesium chloride) at 37 °C for 30 min without rotation. The integration products were purified using a Qiagen MinElute PCR Purification Kit and then amplified using Phusion DNA polymerase for 11 cycles. PCR cycle number was determined as described previously58. Amplified libraries were purified with AMPure beads to remove primers.

To make the ATAC-seq control, nuclei were sorted and genomic DNA was extracted from maize leaves using the Qiagen DNeasy Plant Mini Kit (cat. no. 69106). Then ~1 ng of gDNA was incubated with 2 μl of transposomes in 40 μl of tagmentation buffer (10 mM TAPS–sodium hydroxide pH 8.0, 5 mM magnesium chloride) at 37 °C for 30 min without rotation. All procedures after this were identical to the standard ATAC-seq library protocol described here.

RNA-seq. Second leaves and inflorescence primordia were flash-frozen with liquid nitrogen immediately after collection. Samples were ground to a powder with a mortar and pestle in liquid nitrogen. Total RNA was extracted and purified with TRIzol Reagent (Thermo Fisher Scientific) following the manufacturer’s instructions. For each tissue and replicate, 1.3 μg of total RNA was prepared for sequencing with the Illumina Truseq mRNA Stranded Library Kit (Illumina) following the manufacturer’s instructions.

ChiP–seq. ChiP was performed following the general protocol of Zhang et al.59. For a single chromatin extraction, which yielded sufficient chromatin for several ChIPs, approximately 300 mg of leaves and five inflorescence primordia were used for each replicate. After harvesting, the tissue was chopped into 0.5 cm × 0.5 cm sections and crosslinked in accordance with the referenced protocol. Samples were immediately flash-frozen in liquid nitrogen after crosslinking. Nuclei were extracted and lysed in 300 μl of lysis buffer. The lysed nuclei suspension was sonicated on a Diagenode Bioruptor on the high setting: 30 cycles of 30 s on, 30 s off. Tubes were centrifuged at 12,000 g for 5 min and supernatants transferred to new tubes. At this point, ChiP input aliquots were collected.

Dynabeads Protein A (Thermo Fisher Scientific, cat. no. 10002D) were washed with ChiP dilution buffer and then rotated with antibodies at a concentration of
1.5 μg of antibody (see Table 11 for antibodies used) per 100 μl of ChIP dilution buffer for 4 h at 4 °C. The antibody-coated beads were washed three times with ChIP dilution buffer.

Some of chromatin was diluted tenfold in ChIP dilution buffer to bring the SDS buffer concentration down to 0.1%. For all samples and replicates, 460 μl of diluted chromatin was incubated with 750 μg of Dynabeads Protein A coated with 1.5 μg of antibody. Samples were rotated at 4 °C overnight, then washed, reverse-crosslinked and treated with protease K in accordance with the referenced protocol. DNA was purified by a standard phenol–chloroform extraction followed by ethanol precipitation.

The DNA samples were end-removed using the End-H DNA End-Remove Kit (epicentre) following the manufacturer's protocol. DNA was cleaned up on AMPure beads (Beckman Coulter) with a size selection of 100 bp and larger. Samples were eluted into 43 μl of Tris–HCl and underwent a 50 μl A-tailing reaction in NEB buffer 1 at A-tailing conditions with Kleneow fragment (3′→5′ exo-) at 37 °C for 30 min. A-tailed fragments were ligated to Illumina Truseq adaptors and purified with AMPure beads. Fragments were amplified with Phusion polymerase in a 5 μl reaction following the manufacturer's instructions. The following PCR programme was used: 95 °C for 2 min, 98 °C for 15 cycles of 98 °C for 10 s, 60 °C for 30 s, 72 °C for 30 s and once at 72 °C for 10 min. PCR products were purified with AMPure beads to remove primers.

MethylC-seq. Several B73 second leaves were immediately flash-frozen after harvesting and ground to a powder in liquid nitrogen. DNA was extracted and purified with the DNeasy Plant Mini Kit (Qiagen) and 130 ng were used for MethylC-seq library preparation. MethylC-seq libraries were prepared as detailed in Urich et al.1; however, we used a final PCR amplification of eight cycles.

DAP-seq. DAP-seq experiments involving maize auxin response factor (ARF) samples were performed as detailed in Galli et al.2. All other TF were processed according to Bartlett et al.3, with the exception that 1 μg of pIX-HALO-TF plasmid DNA was used for protein expression, 1 μg of adaptor-ligated library prepared from B73 inflorescence genomic DNA was used for DNA binding and 1 μg of maize leaf genomic DNA was used for EREB71 and EREB17 binding.

Hi-C and HiChiP. We performed HiChiP as detailed in Mumbach et al.31, including the nuclear isolation, enzymatic reactions and ChIP steps. Hi-C was performed identically to HiChIP except after sonication, the chromatin was immediately reverse-crosslinked and the DNA purified. Fourteen B73 second leaves were harvested 4 h after ZT0 and were immediately crosslinked in 1% formaldehyde. Crosslinking was performed similarly to the ChIP protocol, except that the crosslinking times were extended: –84659.725 Pa for 20 min, followed by –84659.725 Pa for 10 min, then –84659.725 Pa for 10 min, then –84659.725 Pa for 10 min. PCR products were purified with AMPure beads to remove primers.

The B73 inflorescence genomic DNA was used for DNA binding and 1 μl of ATAC library, we followed the same method detailed in the ATAC-seq methods section; however, the protocol was scaled up to 1 million nuclei instead of 50,000. The fragmented tagmented product was size-selected on an 0.8% agarose gel to a range of 400–700 bp. The gel product was purified with the Monarch PCR and DNA Cleanup Kit (New England Biolabs). The eluate was split into a second PCR round of 12 μl which was used in a 50 μl PCR reaction following the manufacturer's instructions. The ATAC fragments and vector backbone were assembled with the NEBuilder HiFi DNA Assembly Mastermix (New England Biolabs) according to the manufacturer's instructions. The reaction product was precipitated using ethanol, washed twice with 20 μl of phenol and dried in an ultraviolet chamber. The precipitated DNA was resuspended in 80 μl of MegaX DH10B T1 Electrocompet Cells (Thermo Fisher Scientific) with 2 μl of HiFi Assembly product at 2,000 V and 25 μF. The cells were grown for 16 h in 11 of lysogeny broth with 100 μg/ml carbenicillin. Plasmids were isolated with the NucleoBond Xtra Midi EF Kit (Macherey-Nagel) following the manufacturer's instructions and the purified product was dissolved in ultrapure water to a concentration exceeding 1 μg/l.

For the generation and transfection of maize mesophyll protoplasts, we followed the maize-specific guidelines of the Jen Sheen lab (https://molbio.mgh.harvard.edu/sheenweb/protocols_reg.html); however, we used the polyethylene glycol transfection method detailed in Yao et al.4. Maize seedlings were sowed and grown under conditions detailed in the plant growth and materials section; however, once the coleoptiles emerged approximately 1 cm above the soil surface, trays of plants were transferred to total dark conditions and etiolated for approximately 1 week. Protoplasts were extracted, transfected and then incubated on petri dishes for 14 h at 22 °C at a concentration of 1 million cells ml⁻¹. An estimated 15 million protoplasts were transformed by STARR-seq plasmids.

Protoplasts were pelleted by centrifugation at 420 × g for 2 min and the cell pellets were immediately flash-frozen in liquid nitrogen.

Total RNA was extracted from protoplasts using the Monarch total RNA miniprep kit (New England Biolabs) using the cultured mammalian cell protocol in the manufacturer's instructions. On-column DNase treatment was performed. The RNA was eluted into RNase-free 6 μl HiPer pure water. To ensure that no total protoplast RNA was incubated with 4 μg of Dynabeads Oligo (dT)25 (ambion cat no. 61002) following the manufacturer's protocol. We then eluted 5.5 μg of polyA RNA into 160 μl of RNase-free water.

The polyA RNA was incubated in a 200 μl Turbo DNase reaction (Turbo DNA-free kit, Thermofisher Scientific) at 37 °C for 25 min. DNase was inactivated by the addition of 20 μl DNase inactivation reagent. The reaction was cleaned up and concentrated with the Monarch RNA cleanup kit (New England Biolabs) following the manufacturer's instructions.
The Supernscript IV reverse transcriptase kit (Thermo Fisher Scientific, cat. no. 18091050) was used for cDNA first-strand synthesis. We split 3.7 μg of polyA RNA into ten reactions, and 0.23 μg of polyA RNA was used for a no reverse-transcriptase negative control. The cDNA was primed with a plasmid-specific primer (5′-TTGGAGGTTCAACAAAAAGGAGG-3′). The samples were treated with RNaseH following cDNA synthesis. The cDNA was Monarch-purified and eluted into 4 μl of 10 mM Tris–HCl.

PCR was performed on the first-strand cDNA with Phusion polymerase. The cDNA library was split into 16 50-pb PCR with the following PCR parameters: 98°C for 1 min, ten cycles of 98°C for 15s, 63°C for 30s, 72°C for 1 min, then once at 72°C for 2 min. The reactions were pooled, Monarch-purified and selected for size (300–800bp, which encompassed the entire range of the library) on a 0.8% agarose gel to remove primers. The purpose of the size selection was to eliminate primers and small fragments that resided from RNA splicing. The DNA was purified from the gel with the Monarch DNA gel extraction kit. This product was split into eight more 50-pb PCR with the same parameters, except for a total of four cycles. This product was similarly size-selected on a 0.8% gel and DNA purified. To determine how much plasmid input to amplify, quantitative PCR was used to determine a computerized tomography of similar value to the cDNA. The plasmid input was subjected to the same PCR protocol and the samples were sequenced on an Illumina Nextseq500 platform with paired-end 35 bp reads.

**Sequencing Information.** Sequencing of ATAC-seq, RNA-seq, ChIP–seq, DAP-seq, Hi-C, HiChIP and STARR-seq was performed at the University of Georgia Genomics Facility and Illumina NextSeq500 instrument. MethC-seq was performed at the University of Minnesota, Twin Cities using an Illumina HiSeq 2500 instrument. ATAC-seq, MethC-seq, Hi-C, HiChIP and STARR-seq were sequenced in paired-end 35 bp, 125 bp, 75 bp, 75 bp and 35 bp, respectively. RNA-seq leaf and inflorescence replicates were sequenced in single-end 75 bp and 150 bp, respectively. ChIP–seq and DAP-seq were sequenced in single-end 75 bp. Information on read counts and alignment statistics can be found in Supplementary Tables 5–10.

**Data processing, quantification and statistical analyses.** Definition of intergenic negative control regions. To create the intergenic negative control regions, we first generated all possible simulated 75 bp fragments in the *Z. mays* v4 AGP4 reference genome\(^{46}\) by extending 75 bp downstream from every position in the genome. Then the uniquely mappable regions were identified by remapping all simulated fragments with the same parameters for ChIP–seq analysis. Genomic regions with mapped reads were considered as uniquely mappable. Annotated genes and their 2 kb flanking regions as well as gene–dACR were removed. Negative control regions with the same length distribution to dACR were then generated by the ‘shuffle’ command in BEDTools\(^{45}\), constrained to only the genomic space that was shared across species. The negative control regions were then filtered to only the genomic space that was shared across species.

Identifications of ACRs. MACS2 (ref. \(^{57}\)) was used to define ACRs with the ‘--keep-dup –bw 1000’ option by defining the STARR–RNA and –input libraries as treatment and control, respectively. The FDR was controlled to < 0.05 via the Benjamini–Hochberg method. Enhancer activity was defined as the ratio of reads in DNA input reads to input FPM. dACR enhancer activity was estimated as the maximum ratio of DNA to input within the dACR interval. This was done instead of calculating the activity of the entire dACR to account for the fact that only a small portion of a dACR may contain the cis-regulatory element of interest. Control regions (n = 6,808) were identified from random mappable regions, matched to dACR peak lengths and a similar composition of input FPM (median difference between dACR and control input FPM = 0.008). For calling dACR transcriptional directionality, forward to reverse ratios of fragments overlapping dACR were modelled as beta-binomial distributions independently for RNA and input fragments. Significant deviation of RNA forward to reverse fragment ratios from input ratios was estimated through empirical construction of P values by Markov Chain Monte Carlo sampling (n = 10,000) of the two beta-binomial distributions. H3K4me3 and H3K27me3–HiChIP loops were used to define dACR as upstream or downstream of target genes. Enhancer activities for DAP-seq–enriched dACR were estimated similarly as previous dACR activity analyses. We split dACR into three equal-sized groups based on activity (low, medium, high) for DAP-seq peak density analysis.

**Identification of ACRs.** MACS2 (ref. \(^{57}\)) was used to define ACRs with the ‘--keep-dup all’ function and with ATAC-seq input samples (Tn5 transposition into naked gDNA) as a control. The ACRs identified by MACS2 were further filtered using the following steps: (1) peaks were split into 50 bp windows with 25 bp steps; (2) the accessibility of each window was quantitated and corrected for the Tn5 integration frequency in each window with the average integration frequency across the whole genome to generate an enrichment fold value; (3) windows with enrichment fold values peaking a window (25-fold) were merged together by allowing 150 bp gaps and 4 possible false positive regions were removed by filtering small regions with only one window for lengths >500bp. The sites within ACRs with the highest Tn5 integration frequencies were defined as ACR 'summits'.

**Identification of dACR.** To call differential ACRs, MACS2 (ref. \(^{57}\)) was first used with ‘--keep-dup all’ function and with ATAC-seq input samples (Tn5 transposition into naked gDNA) as a control. The ACRs identified by MACS2 were further filtered using the following steps: (1) peaks were split into 50 bp windows with 25 bp steps; (2) the accessibility of each window was quantitated and corrected for the Tn5 integration frequency in each window with the average integration frequency across the whole genome to generate an enrichment fold value; (3) windows with enrichment fold values peaking a window (25-fold) were merged together by allowing 150 bp gaps and 4 possible false positive regions were removed by filtering small regions with only one window for lengths >500bp. The sites within ACRs with the highest Tn5 integration frequencies were defined as ACR 'summits'.
transcription polyA sites based on the 
maizeGDB63. GO terms under the ‘molecular function’ category were used 
ACRs. First, we obtained maize eQTL from a recent study20. We used the union 
we quantified the enrichment of best eQTL hits (relative to all SNPs) within 
Widespread-Long-range-Cis-Regulatory-Elements-in-the-Maize-Genome/. The code used for analyses can be accessed at https://github.com/schmitzlab/

GO terms under the ‘molecular function’ category were used to 
construct metaplots. Identification of dACR groups by K-means clustering. For K-means clustering, we only used dACRs that had ≥70% mapping coverage (from the 75 bp simulated reads; see Definition of intergenic negative control regions) in the ± 2 kb region flanking the dACR summits. We used this filtering step to ensure that none of the dACRs analysed were directly adjacent to unmappable regions.

Normalized values of 200 10-bp bins from upstream and downstream of distal ACR summits from heatmap analysis were extracted for the histone modifications H3K27me3, H3K36me3, H3K4me3 and H3K96ac. The values were concatenated into a single matrix with 1,600 columns. Finally, using the matrix as the input, dACRs were separated into different groups by the K-means method in R (https://www.r-project.org/) with ten random sets and 30 maximum iteration cycles7. The number of clusters were determined by the total within-cluster sum of square and subsequently manual inspection of identified histone patterns.

Identification of gene expression tissue specificity. Gene expression tissue specificity was determined by a modified entropy formula as described previously23. RNA-seq raw data from 23 Z. mays tissues (first replicate from each tissue) were downloaded from accession number GSE50191 (ref. 19). Raw data were processed as described in the RNA-seq raw data processing section of this publication. Transcripts per million values were used as the input to calculate an entropy value for each annotated gene.

GO enrichment analysis. GO enrichment analysis was performed using BinGO v3.0.3 (ref. 24) with the Z. mays AGPv4 GO annotation from maizeGDB5. GO terms under the ‘molecular function’ category were used for the analyses.

eQTL analysis. To test for a significant relationship between dACRs and 
nucleotides identified as genetic regulators of gene expression (that is, eQTL), we quantified the enrichment of best eQTL hits (relative to all SNPs) within ACRs. First, we obtained maize eQTL from a recent study26. We used the union of eQTL with higher effect and lowest P value for each gene in the maize genome across leaf tissues50. The set of all SNP were obtained from the maize hapmap 3.2.1 (ref. 19) for all taxa in the RNA-seq using a minimum read count of five (the same filtering criteria applied to run the eQTL analysis). We plotted the posterior distribution of eQTL SNP frequency, relative to all SNP, using a beta-binomial distribution with a Beta(1.1) prior. To test if enrichment was present within the dACRs, we estimated the same distributions for a group of control regions that were both gene–distal and uniquely mappable (see Definition of intergenic negative control regions).

Data availability

The data generated from this study has been uploaded to the Gene Expression Omnibus database and can be retrieved through accession number GSE120304. Additionally, the data from this study can be viewed interactively on the publicly accessible epigenome browser http://epigenome.genetics.uga.edu/

The STARR-seq plasmid sequence and additional information can be found at Addgene, deposit number 117379 (https://www.addgene.org/117379/).

Code availability

The code used for analyses can be accessed at https://github.com/schmitzlab/

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Author contributions
W.A.R., Z.L., M.J.R., V.G.C., J.Z., M.J.S., E.S.B., N.M.S., R.J.S. and X.Z. conceived and designed the experiments. W.A.R., Z.L., C.L.E., N.G.M., J.M.N. and M.G. performed the experiments. C.L.E., N.G.M., M.G. and A.G. performed the DAP-seq experiments. W.A.R., Z.L., L.J., A.P.M., M.K.M.-G., M.C.-T., E.J. and X.Z. performed the computational analyses. W.A.R., Z.L., L.J., A.P.M., M.K.M.-G. created the figures. W.A.R., A.P.M., R.J.S. and X.Z. wrote the manuscript. W.A.R., R.J.S. and X.Z. revised the manuscript. All authors read and approved the final manuscript.

Competing interests
R.J.S. and X.Z. are cofounders of REQuest Genomics, LLC, a company that provides epigenomics services.

Additional information
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Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- **n/a**
- **CONFIRMED**

☐ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement

☐ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

☐ The statistical test(s) used AND whether they are one- or two-sided

☐ *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*

☐ A description of all covariates tested

☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons

☐ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)

☐ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted

☐ Give P values as exact values whenever suitable.

☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

☐ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

**Data collection** Flow cytometry of nuclei was performed with a MoFlo XDP in conjunction with Summit (v6.3.1) software.

**Data analysis**

- Trimmomatic (v0.36); HISAT2 (v2.0.5); StringTie (v1.3.1b); DESeq2; Bowtie (v1.2.2); Bowtie2 (v2.1.1); Picard (v2.16.0); MACS2 (v2.1.2); BEDTools (v2.25.0); BEDTools (v2.26.0); SAMtools (v1.3.1); R (v3.4.3); RStudio (v1.1.383); Methylpy (v1.3); Cutadapt (v1.9.dev1); HiC-pro pipeline (v2.8.0); Homer (v4.10.0); Juicer (v0.7.0); FitzChIP (v5.3); GEM (v2.5)

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The raw data generated for this study has been uploaded to the Gene Expression Omnibus (GEO) database and can be retrieved through accession number GSE120304. Additionally, the data from this study can be viewed interactively on the publicly accessible genome browser http://epigenome.genetics.uga.edu/PlantEpigenome/. Processed data—including ACR coordinates, differential dACRs, called chromatin loops, and dACR-gene loop intervals—are available in Supplementary Data Tables S1–S4. The STARR-seq plasmid sequence and additional information can be found at Addgene, deposit number 117379 (https://www.addgene.org/117379/), where it is available for purchase. We have also used publicly available data for some analyses. Independently produced ATAC-seq datasets (Dong et al. 2017) (used in supplementary fig. 1) are available at the NCBI SRA project accession PRJNA391553. DNase-seq datasets (Oka et al. 2017) (used in supplementary fig. 1) are available at the NCBI SRA project accession PRJNA369253. M. scabrinodes datasets (Rodgers-Melnick et al. 2016) (used in supplementary fig. 1) are available at the NCBI SRA project accession PRJNA297204. Data for the maize SNP analysis (fig. 1i and supplementary fig. 5b) were from the maize
HapMap 3.1.1 (Bukowski et al. 2018), accessible at NCBI BioProject PRJNA399729. Coordinates of the GWAS QTLs used in fig. 1j and supplementary fig. 5c are available as supplementary data from Wallace et al. (2014). dACR-eQTL overlap analysis (fig 1k, 4c, and supplementary fig. 5d) used eQTLs generated from raw data (Kremling et al. 2018) available at NCBI BioProject PRJNA383416. Gene expression entropy analyses (fig. 2i and supplementary fig. 3b) used data from the gene expression atlas (Walley et al. 2016), available at NCBI BioProject PRJNA217053.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | The effective sample size is the number of loci studied, which are described throughout the manuscript. Generally, analyses are performed on thousands of loci. |
|-------------|------------------------------------------------------------------------------------------------|
| Data exclusions | From the STARR-seq analysis, we noticed some regions of the genome that were apparently contaminated from a separate experiment that was performed in parallel. We provide the coordinates of these genomic regions, totaling ~ 1 Mb of the genome, in supplemental table S12. These genomic regions were excluded from all analyses, with the exception of fig. 5b. |
| Replication | All experiments, except for Hi-C, HiChIP and STARR-seq, were performed in duplicate. All assays, except for STARR-seq, were performed on the same tissues at the same developmental stages and grown in the same conditions. However, separate batches of plants were grown for separate experiments. Biological replicates were performed on separately grown batches of plants, grown on different days. All attempts at replication were successful. |
| Randomization | Batches of plants were grown in approximately two-fold excess and plants were randomly sampled from different locations on the growth flat. Randomly selected plants were pooled together for each replicate. |
| Blinding | No blinding was used since measurements were not vulnerable to observer bias. Whenever separate groups were compared (i.e. dACR chromatin groups), the same analysis pipelines were performed in parallel. |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a Involved in the study | n/a Involved in the study |
| Antibodies | ChIP-seq |
| Eukaryotic cell lines | Flow cytometry |
| Palaeontology | MRI-based neuroimaging |
| Animals and other organisms | | |
| Human research participants | | |
| Clinical data | | |

Antibodies

For all antibodies, 1.5 μg antibody was incubated with 750 μg Dynabeads® Protein A.

H3: Abcam, cat # ab1791, lot # GR7182-1
H3K4me1: Abcam, cat # ab8895, lot # 889421
H3K4me3: Millipore, cat # 07-473, lot # 2839113
H3K9ac: Active Motif, cat # 61251, lot # 4812001
H3K27ac: Abcam, cat # ab7429, lot UNKNOW
H3K27me3: Millipore, cat # 07-449, lot # DAM1703508
H3K36me3: Abcam, cat # ab99050, lot # 826243
H3K56ac: Millipore, cat # 07-677-1, lot # 2514206

Validation

All antibodies used here have been validated by manufacturers. Furthermore, these antibodies have a long historical use in the ENCODE project and in plant genomics. H3K56ac, H3K27me3, and H3K27ac antibody have been independently validated with peptide array. We have also validated antibodies with computational analysis. The histone marks H3K56ac, H3K27me3,
H3K36me3, and H3K4me3 were used for cluster analysis. Gene metaplot analysis demonstrates that these marks have distinct enrichment profiles, indicating that cross-reactivity was not problematic in our experiments.

**ChIP-seq**

**Data deposition**

- Confirm that both raw and final processed data have been deposited in a public database such as GEO.

- Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

**Data access links**

May remain private before publication.

http://epigenome.genetics.uga.edu/PlantEpigenome/ for the interactive genome browser.

https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE120304 (token:kbstyyswzxupdg)

**Files in database submission**

- chip_B73_ear_H3K27me3_rep1.fastq.bz2
- chip_B73_ear_H3K27me3_rep2.fastq.bz2
- chip_B73_ear_H3K36me3_rep1.fastq.bz2
- chip_B73_ear_H3K36me3_rep2.fastq.bz2
- chip_B73_ear_H3K4me3_rep1.fastq.bz2
- chip_B73_ear_H3K4me3_rep2.fastq.bz2
- chip_B73_ear_H3K56ac_rep1.fastq.bz2
- chip_B73_ear_H3K56ac_rep2.fastq.bz2
- chip_B73_ear_H3_rep1.fastq.bz2
- chip_B73_ear_H3_rep2.fastq.bz2
- chip_B73_ear_input_rep1.fastq.bz2
- chip_B73_ear_input_rep2.fastq.bz2
- chip_B73_leaf_H2AZ_rep1.fastq.bz2
- chip_B73_leaf_H2AZ_rep2.fastq.bz2
- chip_B73_leaf_H3K27ac_rep1.fastq.bz2
- chip_B73_leaf_H3K27ac_rep2.fastq.bz2
- chip_B73_leaf_H3K36me3_rep1.fastq.bz2
- chip_B73_leaf_H3K36me3_rep2.fastq.bz2
- chip_B73_leaf_H3K4me1_rep1.fastq.bz2
- chip_B73_leaf_H3K4me1_rep2.fastq.bz2
- chip_B73_leaf_H3K4me3_rep1.fastq.bz2
- chip_B73_leaf_H3K4me3_rep2.fastq.bz2
- chip_B73_leaf_H3K56ac_rep1.fastq.bz2
- chip_B73_leaf_H3K56ac_rep2.fastq.bz2
- chip_B73_leaf_H3K9ac_rep1.fastq.bz2
- chip_B73_leaf_H3K9ac_rep2.fastq.bz2
- chip_B73_leaf_H3_rep1.fastq.bz2
- chip_B73_leaf_H3_rep2.fastq.bz2
- chip_B73_leaf_input_rep1.fastq.bz2
- chip_B73_leaf_input_rep2.fastq.bz2
- mapped_chip_B73_ear_H3K27me3_rep1.bed.bz2
- mapped_chip_B73_ear_H3K27me3_rep2.bed.bz2
- mapped_chip_B73_ear_H3K36me3_rep1.bed.bz2
- mapped_chip_B73_ear_H3K36me3_rep2.bed.bz2
- mapped_chip_B73_ear_H3K4me3_rep1.bed.bz2
- mapped_chip_B73_ear_H3K4me3_rep2.bed.bz2
- mapped_chip_B73_ear_H3K56ac_rep1.bed.bz2
- mapped_chip_B73_ear_H3K56ac_rep2.bed.bz2
- mapped_chip_B73_ear_H3_rep1.bed.bz2
- mapped_chip_B73_ear_H3_rep2.bed.bz2
- mapped_chip_B73_ear_input_rep1.bed.bz2
- mapped_chip_B73_ear_input_rep2.bed.bz2
- mapped_chip_B73_leaf_H2AZ_rep1.bed.bz2
- mapped_chip_B73_leaf_H2AZ_rep2.bed.bz2
- mapped_chip_B73_leaf_H3K27ac_rep1.bed.bz2
- mapped_chip_B73_leaf_H3K27ac_rep2.bed.bz2
- mapped_chip_B73_leaf_H3K36me3_rep1.bed.bz2
- mapped_chip_B73_leaf_H3K36me3_rep2.bed.bz2
- mapped_chip_B73_leaf_H3K4me1_rep1.bed.bz2
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- mapped_chip_B73_leaf_H3K56ac_rep2.bed.bz2
- mapped_chip_B73_leaf_H3K9ac_rep1.bed.bz2
- mapped_chip_B73_leaf_H3K9ac_rep2.bed.bz2
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- mapped_chip_B73_leaf_H3_rep2.bed.bz2
- mapped_chip_B73_leaf_input_rep1.bed.bz2
- mapped_chip_B73_leaf_input_rep2.bed.bz2
Genome browser session
(e.g. UCSC)
http://epigenome.genetics.uga.edu/PlantEpigenome/

Methodology

Replicates

Replicates were biological replicates. Two replicates were performed for each histone modification and tissue (listed below).

Sequencing depth

All ChIP-seq reads were single-end 75 bp. Single all profile histone marks are euchromatic, and only a small fraction of the maize genome (a few percent) are euchromatic, the effective genome coverage is much greater than the numbers indicated here (which correspond to the global genome coverage).

| Sample Type | Replicate | Read length (nt) | Total Reads | Aligned Reads | % Unique Aligned | Coverage |
|-------------|-----------|-----------------|-------------|---------------|-----------------|----------|
| B73 leaf H2AZ | rep1 | 75 | 27,732,267 | 26,486,801 | 95.5% | 0.902959125 |
| B73 leaf H2AZ | rep2 | 75 | 19,252,409 | 18,399,538 | 95.57% | 0.627256977 |
| B73 leaf H3K4me1 | rep1 | 75 | 48,198,327 | 46,208,231 | 95.87% | 1.575280602 |
| B73 leaf H3K4me1 | rep2 | 75 | 48,459,950 | 46,294,226 | 95.53% | 1.57821225 |
| B73 leaf H3K4me3 | rep1 | 75 | 14,706,000 | 13,658,703 | 92.88% | 0.465637602 |
| B73 leaf H3K4me3 | rep2 | 75 | 14,206,461 | 11,497,814 | 80.93% | 0.391970932 |
| B73 leaf H3K9ac | rep1 | 75 | 23,017,373 | 22,278,839 | 97.69% | 0.795905875 |
| B73 leaf H3K9ac | rep2 | 75 | 42,990,103 | 41,542,119 | 96.63% | 1.416208602 |
| B73 leaf H3K27ac | rep1 | 75 | 50,627,599 | 49,003,001 | 96.79% | 1.670556852 |
| B73 leaf H3K27ac | rep2 | 75 | 51,044,191 | 49,430,997 | 96.84% | 1.685147625 |
| B73 leaf H3K27me3 | rep1 | 75 | 24,095,651 | 23,474,756 | 97.42% | 0.800275773 |
| B73 leaf H3K27me3 | rep2 | 75 | 24,046,522 | 23,478,832 | 97.64% | 0.800414727 |
| B73 leaf H3K36me3 | rep1 | 75 | 49,208,879 | 47,424,742 | 96.01% | 1.610718477 |
| B73 leaf H3K36me3 | rep2 | 75 | 75,396,238 | 72,412,977 | 96.04% | 2.468624216 |
| B73 leaf H3K56ac | rep1 | 75 | 46,542,831 | 43,533,860 | 95.23% | 0.46154625 |
| B73 leaf H3K56ac | rep2 | 75 | 30,604,762 | 29,571,854 | 96.63% | 1.008131386 |
| B73 leaf H3 | rep1 | 75 | 14,631,000 | 13,604,127 | 92.98% | 0.463777057 |
| B73 leaf H3 | rep2 | 75 | 67,137,000 | 14,418,690 | 95.25% | 0.49154625 |
| B73 leaf input | rep1 | 75 | 22,716,315 | 21,335,884 | 93.92% | 0.727359682 |
| B73 leaf input | rep2 | 75 | 20,824,166 | 19,559,465 | 93.93% | 0.666799943 |
| B73 inflorescence H3K4me3 | rep1 | 75 | 42,571,199 | 41,517,523 | 97.52% | 1.415370102 |
| B73 inflorescence H3K4me3 | rep2 | 75 | 44,288,396 | 42,644,295 | 96.29% | 1.453782784 |
| B73 inflorescence H3K27me3 | rep1 | 75 | 78,963,021 | 75,414,392 | 95.51% | 2.570945182 |
| B73 inflorescence H3K27me3 | rep2 | 75 | 76,395,238 | 72,412,977 | 96.04% | 2.468624216 |
| B73 inflorescence H3K56ac | rep1 | 75 | 13,604,127 | 13,604,127 | 92.98% | 0.463777057 |
| B73 inflorescence H3K56ac | rep2 | 75 | 30,604,762 | 29,571,854 | 96.63% | 1.008131386 |
| B73 inflorescence H3 | rep1 | 75 | 14,631,000 | 13,604,127 | 92.98% | 0.463777057 |
| B73 inflorescence H3 | rep2 | 75 | 67,137,000 | 14,418,690 | 95.25% | 0.49154625 |

Antibodies

H3: Abcam, cat # ab1791, lot # GR71822-1
H3K4me1: Abcam, cat # ab8895, lot # 889421
H3K4me3: Millipore, cat # 07-473, lot # 2839113
H3K9ac: Active Motif, cat # A-M0101, lot # 4812001
H3K27ac: Abcam, cat # ab4729, lot # UNKNOWN
H3K27me3: Millipore, cat # 07-449, lot # DAM1703508
H3K36me3: Abcam, cat # ab9050, lot # 826243
H3K56ac: Millipore, cat # 07-677-1, lot # 2514206

Peak calling parameters

Parameter: MACS2, H2AZ/H3K27me3/H3K36me3/H3K4me1, "--nomodel --extsize 147 --broad --broad-cutoff 0.1" and FDR<0.05;
H3K9ac, H3K27ac, H3K56ac, H3K4me3, "--nomodel --extsize 147" and FDR < 0.05.

Data quality

ChIP peaks were not used for the study. The ChIP signal was only quantitatively assessed. However, we provide ChIP peaks here as a quality-control measurement. All ChIP experiments show high signal to background noise. Refer to the genome browser link to assess the ChIP quality. Called peak numbers are listed below:

- H2AZ: 98929
- H3K27me3: 116168
- H3K36me3: 48163
- H3K4me1: 42861
- H3K4me3: 71551
- H3K56ac: 51228
- H3K9ac: 67525
- H3K9me3: 68435
- H3K27ac: 69347

Software

Trimmomatic (v0.36); Bowtie (v1.1.1); Bowtie2 (v2.1.1); Picard (v2.16.0); BEDTools (v2.26.0); samtools (v1.3.1); MACS2 (v1.2.1).
Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Nuclei were sorted in order to purify them from other organelles prior to ATAC-seq. Approximately 200 mg freshly collected tissue was immediately chopped with a razor blade in ~1ml of pre-chilled lysis buffer (15 mM Tris-HCl pH 7.5, 20 mM NaCl, 80 mM KCl, 0.5 mM spermine, 5 mM 2-Mercaptoethanol, 0.2% TritonX-100). The chopped slurry was filtered twice through miracloth and once through a 40 μm filter. The crude nuclei were stained with DAPI and loaded into the flow cytometer.

Instrument

Beckman Coulter MoFlo XDP

Software

Summit version 6.3.1

Cell population abundance

For each library preparation, 50,000 nuclei were used.

Gating strategy

There are multiple DAPI signal peaks with high quality nuclei, reflecting the copy number of plant genomes. The nuclei with DAPI signal >= that of 2×n genomes were collected for the ATAC-seq.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.