Epigenetic features drastically impact CRISPR–Cas9 efficacy in plants

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Abstract
CRISPR–Cas9-mediated genome editing has been widely adopted for basic and applied biological research in eukaryotic systems. While many studies consider DNA sequences of CRISPR target sites as the primary determinant for CRISPR mutagenesis efficiency and mutation profiles, increasing evidence reveals the substantial role of chromatin context. Nonetheless, most prior studies are limited by the lack of sufficient epigenetic resources and/or by only transiently expressing CRISPR–Cas9 in a short time window. In this study, we leveraged the wealth of high-resolution epigenomic resources in Arabidopsis (Arabidopsis thaliana) to address the impact of chromatin features on CRISPR–Cas9 mutagenesis using stable transgenic plants. Our results indicated that DNA methylation and chromatin features could lead to substantial variations in mutagenesis efficiency by up to 250-fold. Low mutagenesis efficiencies were mostly associated with repressive heterochromatic features. This repressive effect appeared to persist through cell divisions but could be alleviated through substantial reduction of DNA methylation at CRISPR target sites. Moreover, specific chromatin features, such as H3K4me1, H3.3, and H3.1, appear to be associated with significant variation in CRISPR–Cas9 mutation profiles mediated by the non-homologous end joining repair pathway. Our findings provide strong evidence that specific chromatin features could have substantial and lasting impacts on both CRISPR–Cas9 mutagenesis efficiency and DNA double-strand break repair outcomes.

Introduction
CRISPR–Cas-based genome editing technologies have greatly advanced both basic and applied biological research. Among them, CRISPR–Cas9, the effector nuclease from a class 2 bacterial CRISPR system, has been the most widely adopted in eukaryotes (Jinek et al., 2012). The key steps in CRISPR–Cas9-mediated genome editing involve searching, binding and then cleaving a 20-nucleotide target site directed by a guide RNA (gRNA). The resulting cleavage product with double-strand breaks (DSBs) can then be repaired by either...
error-prone DNA repair pathways, such as classical nonhomologous end-joining (NHEJ) or micro-homology end-joining (MMEJ), or by a template DNA-dependent pathway, that is homology-directed repair (Chen et al., 2019). Thus, specific mutations, including insertions, deletions, or point mutations, can be introduced by employing distinct DNA repair machineries (Chen et al., 2019).

Previous studies indicated that the CRISPR target sequence is the primary determinant for mutagenesis efficiency and mutation profile (Allen et al., 2018; Lazzarotto et al., 2020). Several tools have been developed to predict the efficiency and mutation outcomes solely based on CRISPR targeted sequences (Allen et al., 2018; Concordet and Haeussler, 2018; Xiang et al., 2021). However, the predictability of these tools, primarily based on data from human cells, often varies and appears to translate poorly to other species, such as plants (Naim et al., 2020). This observation suggested that nonsequence features could influence CRISPR–Cas9 mutagenesis.

Increasing evidence has revealed negative associations between CRISPR–Cas9 mutagenesis rates and heterochromatic signatures or low chromatin accessibility in multiple systems, such as yeast (Saccharomyces cerevisiae), zebrafish (Danio rerio), mouse (Mus musculus), human (Homo sapiens), and rice (Oryza sativa) (Wu et al., 2014; Daer et al., 2017; Usui-Makelä et al., 2018; Yarrington et al., 2018; Liu et al., 2019). However, most of these studies were conducted at various genomic locations, making it difficult to separate the effect of chromatin context from those of DNA sequences. Recently, two studies investigated the impact of chromatin features by randomly inserting reporter constructs at multiple different genomic locations and then targeting an identical site within the reporter for cleavage (Gisler et al., 2019; Schep et al., 2021). Their finding confirmed previous observations that heterochromatin has a negative impact on CRISPR–Cas9 mutagenesis efficacy. Notably, specific chromatin features were also identified to impact both efficiency and mutation outcomes (Gisler et al., 2019; Schep et al., 2021). Nevertheless, these studies were limited by two factors: (1) bias in the genomic location of reporter constructs and (2) the ambiguity from whether the integrated sequences can faithfully adopt the local chromatin context. Furthermore, most of these previous studies were conducted in cell lines within a short time window (usually less than 72 h) using transiently expressed reporter constructs at multiple different genomic locations and then targeting an identical site within the reporter for cleavage (Gisler et al., 2019; Schep et al., 2021).

Results
 Identification of identical CRISPR–Cas9 sites in diverse chromatin contexts
 To identify identical CRISPR target sites in various chromatin contexts, the Arabidopsis Col-0 reference genome was scanned for 20-bp CRISPR–Cas9 recognition sequences with 3-bp NGG (the PAM sequence) at the 3′-end. Out of 7,376,476 distinct target sites, 19,161 were identified as repeating 7–25 times across the genome (Figure 1A). A series of filters were then applied to remove the sites with one of the following features: simple repeats, GC content outside the range of 40%–60%, matching sequences in mitochondria or chloroplast genomes, or containing no overlapping restriction enzyme site for subsequent mutation genotyping. The remaining 7,971 sequences, representing 92,117 total genomic sites, were assessed for three key chromatin features using 100-bp windows: chromatin accessibility indicated by ATAC-seq scores (Lu et al., 2016), DNA methylation patterns categorized as a DNA methylation domain (RdDM, heterochromatin, CG-only, unmethylated, and intermediate) (Crisp et al., 2017; Springer and Schmitz, 2017), and nine chromatin states (Supplemental Data Set S1; Sequeira-Mendes et al., 2014). Seven multicopy CRISPR sites (MCsites) were identified with individual sequences in each family having highly diverse chromatin contexts, including both open and closed chromatin, at least three different DNA methylation domains, and at least two distinct chromatin states (Figure 1, B and C and Supplemental Data Set S2).

Differential CRISPR–Cas9 efficiencies were associated with distinct chromatin features
 Next, we evaluated CRISPR–Cas9 efficacy for each of the seven MCSite families. T-DNA constructs, containing a CRISPR–Cas9 expression cassette, a firefly luciferase reporter and the bialaphos resistance (BAR) selection marker gene, were made to target each MCSite (Supplemental Figure S1A). Each construct contained two gRNA expression cassettes, one targeting the MCSite and the other as the CRISPR mutagenesis control targeting a single-copy endogenous gene located in unmethylated and accessible chromatin, the Chelatase I2 (CHL12) gene, as reported previously (Figure 2A; Mao et al., 2013). It is worth noting that we intentionally chose the CaMV 35S promoter to drive expression of the Cas9 and gRNA sequences because this promoter has much lower activity in Arabidopsis embryos than leaves (Wang et al., 2015; Yan et al., 2015). By reducing the mutagenesis potential in early embryo development
stages, we were able to capture more independent mutation events in somatic cells during leaf development.

After transformation of each T-DNA construct, the resulting CRISPR–Cas9 transgenic plants (T1) were first assessed for mutagenesis efficiency at the CHLl2 control site using the Cleaved Amplified Polymorphic Sequences (CAPS) method. Individual plants with detectable mutagenesis at the control site were then analyzed at each of the seven MCsites using a CAPS or NGS assay. We were able to identify two MCsites, MCsite4 and MCsite5, that produced notable mutagenesis for at least one of the CRISPR target sites (Supplemental Figure S1B). MCsite4 and 5, totaling 15 individual sites, were found spanning all five chromosomes (Supplemental Figure S1C). Among the 15 sites, four seemed to overlap with Arabidopsis genes, and only one site, MCsite5.8, was detected in transcribed RNA sequences (Supplemental Data Set S2).

When CRISPR–Cas9 mutagenesis efficiency was assessed at the individual sequences of MCsite4 and 5 using NGS, substantial variations were observed across individual target sites, consistent with the CAPS data. We were able to identify two MCsites, MCsite4 and MCsite5, that produced notable mutagenesis for at least one of the CRISPR target sites (Supplemental Figure S1B). MCsite4 and 5, totaling 15 individual sites, were found spanning all five chromosomes (Supplemental Figure S1C). Among the 15 sites, four seemed to overlap with Arabidopsis genes, and only one site, MCsite5.8, was detected in transcribed RNA sequences (Supplemental Data Set S2).

Phylogenetic analyses within each target site family found no evident associations between sequence similarity and the mutagenesis frequency groups. Thus, the local sequence context could not explain differential mutagenesis frequencies observed from individual target sites.

The initial association analysis of indel frequencies with DNA methylation domains and chromatin accessibility indicated unmethylated and accessible sites generally had higher mutagenesis levels than the methylated and inaccessible sites. Notably, negative associations between mutagenesis frequency and DNA methylation levels at both MCsite4 and 5 sites could also be observed when the cytosine methylation status was examined at the single nucleotide level of individual CRISPR target sites (Supplemental Figure S3). To systematically investigate the relationship between mutagenesis efficiency and chromatin

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**Figure 1** Identification of MCsites in various chromatin contexts. A, Bioinformatic pipeline used to identify MCsites. B, Characterization of the chromatin accessibility as closed or open at each MCsite using ATAC-Seq data. C, Characterization of DNA methylation domains at each MCsite. CG-only sites contain greater than 40% mCG; Heterochromatin sites contain greater than 40% mCG and mCHG methylation; RdDM sites contain mCG, mCHG, and mCHH, with at least 15% mCHH; unmethylated sites contain less than 10% mCG, mCHG, and mCHH; and intermediate sites are everything else with data that did not meet any of the above criteria.
features, we further characterized individual MCsites using all 23 chromatin features, including distinct histone modifications and histone variants (Supplemental Data Set S3; Liu et al., 2018). When hierarchical cluster analysis was performed, the lowly edited sites (group L) tended to cluster with the heterochromatic features, such as H2A.W, H3K9me1, H3K9me2, H3K27me1, and hyper DNA methylation, for the MCsite4 and 5 targets with exceptions observed for MCsite5.6 and MCsite5.8 (Figure 2, D and E). While they are in the lowly edited group, these two sites appeared to be associated with unmethylated and accessible chromatin features (Figure 2, C and E). On the contrary, the highly and moderately edited groups (groups H and M) appeared to be associated with accessible, active chromatin features such as histone acetylation, H3K36me3, and H3K4 methylation (Figure 2, D and E; Roudier et al., 2011). To examine the impact of individual features, we performed correlation analyses by plotting mutagenesis efficiency at all 15 target sites.
with each chromatin and DNA methylation feature (Supplemental Figure S4). Consistent with the hierarchical cluster analysis, strong positive correlations were observed between mutagenesis frequency and euchromatin-related features such as H3K56ac ($R = 0.85, P = 1.8e–13$), H3K9ac ($R = 0.82, P = 8.6e–12$), H3K36ac ($R = 0.75, P = 4.2e–9$), H3K27ac ($R = 0.71, P = 4.1e–8$), H3K36me3 ($R = 0.71, P = 3.5e–8$), H3K4me3 ($R = 0.65, P = 1.6e–6$), and accessibility ($R = 0.51, P = 0.00038$) measured with ATAC-Seq data (Supplemental Figure S4). Moreover, strong negative correlations were found between mutagenesis frequencies and the heterochromatin-related features H2A.W ($R = 0.57, P = 4.1e–5$), H3K9me1 ($R = 0.55, P = 0.00011$), H3K9me2 ($R = 0.55, P = 1e–4$), and cytosine DNA methylation ($R = 0.45, P = 0.0022$) (Supplemental Figure S4).

**Improving CRISPR–Cas9 mutagenesis efficiency through combined reduction of DNA methylation**

Because of the strong negative association observed between the lowly edited sites and heterochromatic features, we hypothesized that altering these chromatin states could improve mutagenesis efficiency at the refractory sites. In this study, we chose to perturb DNA methylation at the lowly edited sites because high levels of DNA methylation are often associated with heterochromatic features (Zemach and Grafi, 2003; Johnson et al., 2007; Crisp et al., 2020). We first sought to test the possible impact of CHG methylation on mutagenesis efficiencies because CHG methylation is often associated with H3K9me2 and heterochromatin (Springer and Schmitz, 2017). To this end, the *chromomethylase3* (*cmt3-11t*) mutant was chosen due to the well-documented genome-wide reduction in CHG methylation (Stroud et al., 2013). Analysis of the single-base resolution DNA methylation profiles indicated that a substantial reduction in CHG methylation alone is not sufficient to improve CRISPR–Cas9 mutagenesis at the refractory sites. Hence, we chose a chemical approach to reduce DNA methylation at the hypermethylated refractory sites.

Lastly, we tested the impact of combined reductions in DNA methylation using both the *cmt3-11t* mutant and 5-azacytidine treatments. The T2 siblings with the MCsite4-targeting T-DNA were obtained from a self-pollinated T1 plant in the *cmt3-11t* mutant background, grown for 2 weeks with or without 100-µM 5-azacytidine, and subjected to mutagenesis analyses using the NGS assay (Supplemental Figure S7B). No significant differences were observed for the normalized mutagenesis frequency at each MCsite4 site between the untreated and treated samples (Figure 3C). Thus, partial reductions in all DNA methylation contexts using 5-azacytidine did not reveal changes in mutagenesis frequencies.

**Differential CRISPR–Cas9 mutational profiles are associated with distinct chromatin features**

CRISPR–Cas9-induced mutations are typically composed of either small deletions or 1-bp insertions (Allen et al., 2018). Small deletions are mainly derived from the NHEJ or MMEJ pathway through exonuclease-mediated end processing and ligation, while the 1-bp insertions were mostly generated from blunt-end or 1-bp staggered cleavage by Cas9 followed by DNA polymerase-mediated end filling (Gisler et al., 2019). To investigate the potential impact of chromatin context on CRISPR–Cas9 mutation outcomes, we examined the insertion and deletion profiles for both MCsite4 and 5. As expected, most mutations contained 1-bp insertions or small deletions (<10 bp) for both MCsite4 and 5 (Figure 4A). MCsite4 was preferentially repaired as 1-bp insertions, while MCsite5 showed a strong bias towards...
**Figure 3** Comparisons of CRISPR–Cas9 mutagenesis frequencies using genetic and chemical approaches. A and B, Normalized mutation frequency was compared for MCsite 4 and 5 in wild-type and cmt3 mutant background. C, Normalized mutation frequencies for MCsite4 sites in wild-type plants were compared with or without 5-azacytidine treatment. D, Normalized mutagenesis frequencies were compared for the MCsite4 sites in the cmt3 mutant plants with or without 5-azacytidine treatment. The MCsite4 sites were categorized in High (H; site 8), Moderate (M; sites 7 and 4), and Low (L; sites 9, 3, 1, 5, and 2) mutagenesis groups. The standard error (SEM) was calculated for each target site with three replicates in (A)–(D). The fold changes in mutagenesis were calculated for the sites in group L in (D) (shown above bars). Mann–Whitney U test was performed in each panel, with asterisks indicating significant P-values (P < 0.05). If no asterisk is present, P-values are not significant.

**Figure 4** Analyses of CRISPR–Cas9 mutation profiles at MCsites. A, Distribution of mutation outcomes at MCsite4 and MCsite5. The mutations were categorized as 1-bp insertions (+1 bp, red), 1–5 bp deletions (Δ1–5 bp, dark blue), 6–10 bp deletions (Δ6–10 bp, blue), and deletions of more than 10 bp (Δ > 10 bp, light blue). B and C, The 1-bp insertion frequencies at individual MCsite4 and MCsite5 targets. The standard error (SEM) was calculated for each target site with replicates (n = 6 for MCsite4; n = 3 for MCsite5). Kruskal–Wallis analysis indicated the P-value for MCsite4 and MCsite5 are 0.17 and 0.016, respectively. D, Correlation plots between the insertion rate and three chromatin markers, H3K4me1, H3.3, and H3.1, with the blue dots for MCsite4 and the red dots for MCsite5. The trendline was plotted with gray indicating the standard error. The R- and P-values were calculated using Spearman’s rank correlation coefficient.
small deletion outcomes (Figure 4A). While the major mutation types at individual sites within each family seemed to be highly similar (Supplemental Figure S58, A–D), further analysis revealed substantial variations for the insertion rate between individual sites. The rate of insertion outcomes ranged from 56.25% to 81.73% and 7.57% to 30.27% in MCSite4 and MCSite5 sites, respectively (Figure 4B and C). We then conducted a correlation analysis using the 23 epigenetic features with the insertion rates at all 15 sites. Three histone H3-related features, H3K4me1 (R = –0.64, P = 0.01), H3.3 (R = –0.83, P = 3e–4), and H3.1 (R = –0.91, P = 2.8e–6), were identified with significant negative correlations with 1-bp insertional mutations (Figure 4D and Supplemental Figure S59). Thus, these findings strongly suggested that chromatin features could not only affect CRISPR–Cas9 mutagenesis efficiency but also influence mutation outcomes.

**Discussion**

In recent studies, chromatin contexts have been demonstrated to have significant impacts on CRISPR–Cas9-mediated genome editing (Wu et al., 2014; Daer et al., 2017; Yarrington et al., 2018; Liu et al., 2019). Most of these findings indicated that heterochromatic features at the CRISPR target regions could impede CRISPR–Cas9 mutagenesis efficiency in multiple systems, such as yeast, rice, mouse, and human cell lines (Wu et al., 2014; Daer et al., 2017; Yarrington et al., 2018; Liu et al., 2019). However, it remained unclear whether heterochromatic features could only temporally delay CRISPR–Cas9 mutagenesis (Kallimasioti-Pazi et al., 2018). In this study, we systematically characterized the impact of 23 distinct DNA methylation and chromatin features on CRISPR–Cas9 mutagenesis efficiency and mutation outcomes by investigating CRISPR–Cas9 transgenic Arabidopsis plants. Consistent with the previous studies, our results demonstrated that inaccessible and heterochromatic features were associated with low mutagenesis efficiency. Such repressive effects can be long-lasting through plant development leading up to a 250-fold difference between identical CRISPR target sites. However, it was worth noting that, although this observation broadly holds for both MCSite4 and 5, two lowly edited sites in MCSite5, MCSite6.6 and 5.8, appeared to be associated with open and active chromatin features. The weaker associations observed here could be due to the overall higher mutagenesis frequencies at the lowly edited MCSite5 targets than those at the MCSite4 targets. Furthermore, mutagenesis efficiency at the target sequences in accessible chromatin regions could also have substantial variations ranging from 1.53-fold to 10-fold at MCSite4 and MCSite5, respectively (Figure 2, B and C). These observations suggested that specific chromatin features other than just open or closed chromatin should be considered to account for CRISPR–Cas9 mutagenesis efficacy. Close examination of individual chromatin features identified several euchromatic marks, such as H3K9ac, H3K56ac, H3K36ac, H3K27ac, H3K4 methylation, and H3K36m3, that were positively correlated with mutagenesis efficiency. Further investigation with a larger data set will be needed to dissect their impacts on CRISPR–Cas9 mutagenesis in greater detail.

In this study, we observed strongly negative correlations between CRISPR–Cas9 mutagenesis efficiency and repressive chromatin features, such as DNA hypermethylation, low DNA accessibility, and H3K9 methylation. To test the hypothesis that modulating some of these features could improve mutagenesis frequency at the lowly edited sites, we sought to reduce DNA methylation by using both genetic and chemical approaches. Our results indicated that partial reduction of DNA methylation using either a mutant affecting CHG methylation or 5-azacytidine treatment alone was not sufficient to improve mutagenesis efficiency at any of the tested sites. When combining the CHG deficiency mutant with 5-azacytidine chemical treatment, 2.1- to 4.8-fold improvements in mutagenesis efficiency were found at the lowly edited sites but not at the highly and moderately edited sites. Combined reduction of DNA methylation in multiple contexts has been demonstrated to increase chromatin accessibility and even alter the higher order 3D chromatin organization in Arabidopsis (Zhong et al., 2021). Thus, mutagenesis efficiency improvement at lowly edited sites observed here could have resulted from increasing chromatin accessibility and/or changing 3D chromatin organization due to the substantial reduction of DNA methylation in multiple contexts; but this would need to be experimentally verified, for example using ATAC-Seq or DNase I hypersensitivity assays. Additionally, as discussed above, open/close chromatin structure alone could not explain all the variations in editing efficiencies. A systematic approach would be required to further dissect the relationship between epigenetic features and targeted mutagenesis efficiency. Furthermore, as reported previously, repressive chromatin features could act as the barriers to hinder CRISPR–Cas9 binding and cleavage (Wu et al., 2014). However, it is noted that the primary readout in this study, targeted mutation frequency, is an indirect measurement of CRISPR–Cas9 target binding and cleavage dependent on DSB repair. The mutation efficiency differences observed here could reflect differences in either CRISPR–Cas9 binding, cleaving, DNA repair, or a combination of these. Further investigation would be required to differentiate these possibilities.

In addition to the impacts on mutagenesis efficiency, chromatin features have been suggested to influence CRISPR–Cas9 mutation outcomes. For example, a recent study with more than 1,000 copies of identical insertion sites indicated the 1-bp insertions were found more prevalent in euchromatin than in heterochromatin, likely through recruiting different DNA repair machinery (Schep et al., 2021). However, conflicting results were also reported showing little impact of chromatin features on mutation outcomes (Kallimasioti-Pazi et al., 2018; Gisler et al., 2019). In this study, we observed significant variations for the 1-bp insertion rate in different chromatin contexts. Of the 23
chromatin features analyzed, three distinct histone H3 features, H3K4me1, H3.3, and H3.1, exhibited significantly strong negative correlations with the 1-bp insertion rate. Interestingly, H3K4me1 was also identified by Schep et al. (2021) as a marker to correlate with distinct mutation outcomes, while H3.1 and H3.3 were not included in their study. This suggested that the balance between 1-bp insertions and small deletions could be influenced by specific chromatin markers. Distinct chromatin features have been reported to recruit different DNA repair machinery and result in different repair outcomes in mammalian systems (Fnu et al., 2011; Jacquet et al., 2016; Luijsterburg et al., 2016). Further investigation is needed to address the potential roles of specific chromatin markers in determining DNA repair outcomes.

We propose a model to account for the impacts of chromatin features on CRISPR–Cas9 mutagenesis efficiency and mutation outcomes (Figure 5). In the first step, chromatin features are the key determinants for CRISPR–Cas9 recognition and binding efficiency (Figure 5). In general, heterochromatic features, such as H3K9 methylations, H3K27me1, H2A.W, and DNA hypermethylation, could substantially reduce chromatin accessibility and thus reduce the recognition and binding efficiency of CRISPR–Cas9. Such repressive effects could persist through cell division and development. After CRISPR–Cas9 locates and binds the genomic target site, it can introduce a DSB with either a 1-bp 5′ overhang (staggered cut), or blunt ends (Gisler et al., 2019). The cleaved product can be repaired to yield three outcomes: wild-type sequence by perfect ligation, 1-bp insertions, or small deletions (less than 10 bp) (Figure 5). It has been proposed that the staggered cut primarily leads to 1-bp insertion via template-dependent repair, facilitated by a DNA polymerase, while the blunt cut mainly results in small deletions through end resection (Gisler et al., 2019; Schmid-Burgk et al., 2020). The blunt ends could occasionally be repaired by DNA polymerase, likely the members from DNA polymerase family X, without DSB end resection, resulting in template-independent 1-bp insertions (Gisler et al., 2019). In this study, we observed both templated 1-bp insertion and template-independent insertions, as exemplified in MCSite5 and MCSite4, respectively (Supplemental Figure S8, A and B). Nevertheless, the balance between 1-bp insertion and small deletion products is primarily dependent on the repair choices between short-range DSB end resection and DNA polymerase end filling (Figure 5; Lemos et al., 2018; Schmid-Burgk et al., 2020). While sequence features are a key determinant in mutation profile, our data indicated that chromatin features, such as H3K4me1, H3.3, and H3.1, could also significantly impact the balance between 1-bp insertion and small deletion outcomes. These specific chromatin features may exert their influences on the balance between the staggered and blunt cut, or through modulating the balance between DNA polymerase end filling and short-range DSB end resection during NHEJ. In fact, previous studies have demonstrated that distinct chromatin features, such as the H3.3 variant, could impact DSB end resection (Luijsterburg et al., 2016).

Recently much effort has been made to develop computational tools to predict CRISPR–Cas9 editing efficiency and/or repair outcomes. To our knowledge, these tools primarily relied on sequence features (Allen et al., 2018; Concordet and Haeussler, 2018; Xiang et al., 2021). Yet, our results indicated that nonsequence features should also be taken into consideration for the prediction of both CRISPR–Cas9 efficiency and mutation outcomes. One implication from this study is that heterochromatin-related features, such as low accessibility, H3K9me1, H3K9me2, H3K27me1, H2A.W, and DNA hypermethylation, should be avoided in order to design gRNAs with high efficiency. When it comes to predicting CRISPR–Cas9 mutation profiles, chromatin features such as H3K4me1, H3.3, and H3.1 should also be considered. Future studies using genetic mutants that result in altered states of histone modifications will be interesting to further dissect the impact of distinct chromatin features on CRISPR–Cas9 mutagenesis. A better understanding of the interplay between chromatin dynamics and CRISPR–Cas9 will enable the development of more precise and efficient genome engineering technologies.

Materials and methods

Identification of multicopy CRISPR sites

To identify gRNAs that matched to multiple places in the genome, the Arabidopsis (A. thaliana) Col-0 reference genome (TAIR10) was parsed to identify every NGG PAM site using seqkit locate (Shen et al., 2016) with the search motif NNNNNNNNNNNNNNNGG. The number of occurrences of each nonredundant sequence in the genome was summarized using csvtk. The resulting distinct target sites were then filtered in R (v4.1.2) to retain gRNAs that had between 7 and 25 distinct matches. Sites were then eliminated if they had simple sequence motifs consisting of 5 As, Ts, Gs, or Cs in a row; CG content outside 40%–60% or if the sequence was found in the chloroplast or mitochondria genome; to give a final list of 9,902 candidate gRNAs. The gRNA identification script is available at https://github.com/pedrocrisp/Weiss_et_al_gRNA_chromatin. The potential gRNAs were also screened to identify those with restriction enzyme recognition motifs (from a list commercially available enzymes from NEB) that overlapped position 17–18 of the gRNA (between position 3 and 4 bp from the PAM) such that an indel mutation in this position would disrupt restriction enzyme recognition and cleave for efficient screening of edited transgenic lines. A final list of 7,971 gRNAs was then annotated with chromatin state information by overlapping the coordinates of the gRNA target sites with chromatin annotation files using bedtools (Quinlan and Hall, 2010). Chromatin data included nine histone states (Sequeira-Mendes et al., 2014); chromatin accessibility (Lu et al., 2016); and DNA methylation (Crisp et al., 2017;
DNA methylation data were converted to methylation domains for each 100-bp non-overlapping window of the TAIR10 genome using the method detailed in Crisp et al. (2020). Chromatin accessibility at the CRISPR target site was called either open or closed based on the presence of an overlapping ATAC-Seq peak or lack thereof, respectively, using the accessibility profiles in (Lu et al., 2016). Gene and transposable element annotations were downloaded from Araport v11.

**T-DNA vector construction**

The CRISPR–Cas9 constructs were created using the Golden Gate assembly method as outlined previously (Čermák et al., 2017). The gRNA sequences were first assembled into the
pMOD_B2301 vector containing the gRNA targeting the multiplex CRISPR site (MCsite) and the CHL2 positive control using the oligos listed in Supplemental Table S3. T-DNA constructs were assembled by combining pMOD_A0101 (the Cas9 component), pMOD_B2301 containing the gRNA array, pMZ105 (the luciferase reporter), and pTRANS230d (the T-DNA backbone) with the Golden Gate method as described previously (Cermák et al., 2017). The modular components used to build the T-DNA plasmids can be found at https://www.addgene.org/browse/article/28189956/ (Cermák et al., 2017). The T-DNA constructs described in this study are available through Addgene.

Plant materials and growth conditions
The A. thaliana Columbia ecotype (Col-0) was used in these experiments. The cmt3-11t (stock CS16392) genotype was acquired from the Arabidopsis Biological Resource Center. Floral dip transformation was performed according to the protocol as previously outlined (Zhang et al., 2006). Transgenic T1 seeds were sown on soil and exposed to BASTA selection to recover transgenic plants. Plants were grown in a growth chamber with the following conditions: 16-/8-h light/dark cycle, 22°C, and 55% humidity.

Characterization of chromatin features
Previously analyzed datasets (BigWig files) for the 23 chromatin features were downloaded from Plant Chromatin State Database (Liu et al., 2018). The data sets were wild-type Arabidopsis with and without 100-μM 5-azacytidine treatment were downloaded from Griffin et al. (2016). For each data set, the values for the 1-kb window (500-bp upstream and 500-bp downstream from the center of gRNA) were calculated using the Deeptools2 computeMatrix in the reference-point mode. Similarly, the values for each nucleotide of target sites were calculated using the scale-region mode at single base resolution. Each data set was then normalized to allow for comparison on a scale of 0–1 with 1 indicating the highest level of that feature.

5-Azacytidine treatment and luciferase screening
T2 seedlings from self-pollinated T1 Arabidopsis plants were grown on 1% (w/v) agar plates containing 0.5 Murashige and Skoog (PhytoTech Labs) and 100-μM 5-azacytidine (Griffin et al., 2016). After 2 weeks, seedlings were screened for the presence of the transgene using a luciferase reporter. The luciferase assay procedure was performed using the BioGlo Luciferase Assay System (Promega Corp., Madison, Wisconsin, USA) in accordance with the manufacturer’s instructions.

Mutation genotyping and characterization of mutation profiles
Genotyping was performed using two methods: genomic PCR followed by restriction enzyme digestion (CAPS) and the NGS assay using Illumina paired-end read amplicon sequencing. All tissues for genotyping were collected at 2 weeks post germination for the CTAB-base genomic DNA extraction. PCR was performed using GoTaq Green Mastermix (Promega Corp., Madison, Wisconsin, USA) according to the manufacturer’s instructions, with an annealing temperature of 55°C (CHL2 and MCsite4) or 60°C (MCsite5) with an extension time of 1 min. Primers to amplify CHL2, MCsite4, and MCsite5 target sites can be found in Supplemental Table S1. Amplicons were then subjected to restriction enzyme digestion using BsmAI (CHL2), AluI (MCsite4), or DrdI (MCsite5) according to the manufacturer’s instructions. PCR amplicons generated with the corresponding primers were subjected to Illumina paired-end read sequencing (Genewiz Inc., South Plainfield, New Jersey, USA). The raw NGS reads were analyzed using CRISPResso2 to estimate indel mutation rates (Clement et al., 2019). To analyze the mutation profiles for each sample, the NGS reads with indel mutations were extracted from the CRISPResso2 output files with a 2% threshold. The resulting output files were then loaded into R studio (version 4.1.0) for data visualization using ggplot (41). The total read counts for CRISPR–Cas9 editing frequency and repair profiles can be found in Supplemental Table S2. Normalized indel frequencies were calculated by dividing the indel frequency of each MCsite by the CHL2 positive control indel frequency within each replicate.

Data availability
All sequencing data analyzed in this article will be available at the National Center for Biotechnology Information under BioProject Accession PRJNA795172 (Supplemental Data Set S4).

Accession numbers
Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number PRJNA795172.

Supplemental data
The following materials are available in the online version of this article.

**Supplemental Figure S1.** Characterization of multiplex CRISPR sites (MCsites) for CRISPR–Cas9 mutagenesis.

**Supplemental Figure S2.** Non-normalized mutagenesis efficiency and sequence comparison for individual target sites in MCsite4 and MCsite5.

**Supplemental Figure S3.** Single nucleotide heatmap of DNA methylation levels at multiplex CRISPR site (MCsite) 4 (blue) and 5 (red) protospacer and PAM (bold) sequences from 0 (unmethylated) to 100 (fully methylated).

**Supplemental Figure S4.** Correlation analysis for CRISPR–Cas9 mutagenesis frequencies and chromatin features.

**Supplemental Figure S5.** Characterization of the single-based DNA methylation status at multiplex CRISPR site (MCsite) 4 and 5 in the wild-type and cmt3 mutant plants.

**Supplemental Figure S6.** Unnormalized mutagenesis frequencies for multiplex CRISPR site (MCsite) 4 (blue and gray) and 5 (red and gray) in the cmt3 mutant plants.
Supplemental Figure S7. 5-Azacytidine treatment of the wild-type and cmt3 T2 seedlings.

Supplemental Figure S8. Characterization of mutation outcomes for multiplicity CRISPR site (MCsite) 4 (blue) and 5 (red).

Supplemental Figure S9. Correlation analysis for 1-bp insertion rate and chromatin features.

Supplemental Table S1. Primer sequences to amplify each CRISPR target site analyzed in these experiments.

Supplemental Table S2. Summary of NGS reads count for each tested target site.

Supplemental Table S3. Oligos for cloning MCsite and CHL2 gRNAs.

Supplemental Data Set S1. Characterization of the sequences, DNA methylation, chromatin accessibility, and chromatin states for the 7,971 candidate CRISPR target sites identified.

Supplemental Data Set S2. Annotations of MCsite 4 and 5.

Supplemental Data Set S3. Plant Chromatin State Database (PCSD) files for MCsite 4 and 5.

Supplemental Data Set S4. PRJNA795172 accession key.

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