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Mutator transposon activation after UV-B involves chromatin remodeling

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Abbreviations: CA, cellulose acetate filter transmitting UV-B; PE, polyester plastic removing UV-B; qRT-PCR, quantitative real-time polymerase chain reaction; UV-B, ultraviolet-B radiation (280–315 nm); WT, wild-type

Spontaneous silencing of *MuDR/Mu* transposons occurs in ~10–100% of the progeny of an active plant, and once silenced reactivation is very rare. To date, only radiation treatments have reactivated silenced *Mu*; for example UV-B radiation reactivated Mutator activities. Here we have investigated possible mechanisms by which UV-B could reactivate *Mu* transposons by monitoring transcript abundance, epigenetic DNA marks, and chromatin factors associated with these elements. We demonstrate that both *mudrA* and *B* transcripts are expressed at higher levels after an 8 h-UV-B treatment, in both active Mutator and silencing plants, and that different chromatin remodeling events occur in the promoter regions of *MuDR* than in non-autonomous *Mu1* elements. Increased transcript abundance is accompanied by an increase in histone H3 acetylation and by decreased DNA and H3K9me2 methylation. No changes in siRNA levels were detected. In contrast, the decrease in H3K9me2 present at *Mu* elements after UV-B is significant in silencing plants, suggesting that early changes in H3 methylation in K9, chromatin remodeling, and transcription factor binding contribute directly to transposon reactivation by UV-B in maize.

Introduction

Epigenetic regulation permits changes in gene activity without changes in DNA sequence. Specific epigenetic changes are characteristic of developmental programs, and epigenetic states can be maintained through mitosis and meiosis, resulting in non-Mendelian inheritance.[1,2] Silencing of transposable elements (TE) has been proposed as a fundamental role of epigenetic silencing to suppress the deleterious impact of active TE on the genome.[3] Most TE contain tandem or inverted repeats, and when such repeated elements are present in transcripts they can form double-stranded RNA (dsRNA) regions that trigger silencing when processed into small interfering RNAs (siRNAs).[4] siRNAs are associated with post-transcriptional degradation of target mRNAs and with transcriptional silencing of target genes via DNA methylation and histone modification.[5]

In maize, transcriptional inactivation of transposase genes in *Ac*, *Spm* and *MuDR* correlated with decreased transposition and increased TE methylation in cytosine residues.[6] The causal relationship between methylation of maize TE and transposon activity is unknown; however, this epigenetic mark is a hallmark of TE silencing that accompanies loss of both excision and insertion in somatic and reproductive cells.

Epigenetic silencing plays a particularly important role in the multi-copy *MuDR/Mu* (*Mu*) system in maize.[7] The diverse *Mu* elements share similar ~215 bp terminal inverted repeats (TIRs), but each *Mu* TE family member has unique internal sequences. *MuDR* is the regulatory element, and a transcriptionally active *MuDR* is required for transposition of the non-autonomous elements.[8,9] *MuDR* encodes two genes, *mudrA* and *mudrB*.[10] *mudrA* encodes the transposase,[11] and *mudrB* encodes a gene with unknown function. Functional *MuDR* elements can be lost by genetic segregation[8,9] or through internal deletions and point mutations,[12] and in each case the non-autonomous *Mu* elements become methylated.[7] TE regulation involves both transposon and host-encoded gene products that participate in other aspects of gene regulation.[13] In maize, mutations in *mop1* (*Mediator of paramutation1*) can reverse *Mu* methylation.[13] *Mop1* encodes a putative RNA-dependent RNA polymerase similar to RDR2, whose activity is required for the production of small RNAs from the *MuDR* transcripts.[14] MOP1 is required to maintain *MuDR* methylation and silencing, but it is not required for the initiation of heritable silencing.[14]

Another feature of gene silencing is covalent modification of histones, such as methylation of lysine 9 of histone H3 (H3K9).[15] Methyltransferases that include a SET domain can catalyze this modification. In humans, H3K9 methylation is also mediated by Heterochromatin-associated Protein 1 (HP1). HP1 contains a chromodomain that specifically binds methylated H3K9.[16] In Arabidopsis, a reduction in H3K9 methylation is accompanied

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by a reduction in DNA methylation.[17,18] Recently, DNA and H3K9 methylation were shown to co-occur over large regions of the Arabidopsis genome, and these regions were enriched for siRNAs.[3]

Spontaneous *MuDR/Mu* silencing occurs in ~10–100% of the progeny of an active plant and once silenced reactivation is very rare.[19] To date, only radiation treatments have reactivated silenced *Mu*: UV-B radiation reactivated during plant growth and in pollen >100-fold above background spontaneous reactivation.[20-22] Left unanswered was the possibility that the TIRs, which contain the promoters for the *mudrA* and *mudrB* genes, contain an UV-B inducible promoter or whether host responses such as increased DNA repair and chromatin remodeling stimulated by UV-B indirectly contribute to TE reactivation.[23,24] To further explore the relationship between UV-B and *MuDR* reactivation, we have analyzed molecular events. We demonstrate that both *mudrA* and *B* transcripts are expressed at higher levels after an 8 h-UV-B treatment, in both active Mutator and silencing plants. This transcript increase is accompanied by an increase in histone H3 acetylation and by a decrease in DNA and histone H3 methylation. No changes in the siRNAs were detected. In contrast, the decrease in H3K9me2 present at *Mu* elements after UV-B is significant in silencing plants, suggesting that early changes in H3 methylation in K9 may be essential for *Mu* reactivation by UV-B in maize.

Results

Biological materials and experimental design. In active Mutator lines, *mudrA* and *mudrB* expression is high and proportional to *MuDR* copy number.[12] Transposase MURA-823 preferentially binds to hypomethylated TIRs and programs *Mu* excision late in plant development as evidenced by a high frequency of small purple dots when a mutable pigment allele is monitored.[12,25,26] Inactive fully silenced maize lines are stable at pigment reporter genes, and these TE are hypermethylated.[7] *MuDR/Mu* silencing is progressive during maize development.[19,27] Silencing plants in the process of losing activity exhibit a reduced frequency of somatic excision from reporter alleles while fully silenced individuals lack somatic excision; at the molecular level, loss of excision is correlated with the absence of detectable transposase transcripts.[12,26] Individual active and silenced plants within one progeny share 50% of the pre-existing parental insertion sites, but differ in *MuDR/Mu* copy number, as the inactive individuals have no new insertions. In this work, a Mutator active line and an epigenetically silencing sister line were used. The experimental strategy required initial characterization of independent maize stocks in which Mutator status was genetically verified. Subsequently, plant samples from selected lines were subjected to further analysis.

UV-B regulation of *mudrA* and *mudrB* transcripts in active and silencing Mutator plants. The *mudrA* gene is upregulated by sunlight UV-B in active Mutator plants as established by previous microarray analysis.[28] Moreover, after UV-B supplementation, the *mudrB* gene was also upregulated.[28] To validate these results, a qRT-PCR analysis was performed. Both active and F1 silencing plants were irradiated with UV-B radiation for 8 h, leaf samples were collected and RNA was extracted. Primers for a non-UV-B regulated gene, encoding a putative thioredoxin protein were used as a control.[29] This gene was previously chosen as a control from 22,000 ESTs printed in microarray slides because it showed no change in transcript levels in any of the UV-B treatments that we normally use in our experiments.[28,29] As shown in **Figure 1A**, after the treatment transcripts for both *mudrA* and *B* are increased 2.5 and 2.1-fold in active plants, respectively, while a slightly lower increase of 1.5-fold was observed for both genes in silencing plants. Both transcripts are higher in active than in silencing plants; this is true both under control conditions in the absence of UV-B and after an 8 h-UV-B-treatment (**Fig. 1B**).

mudrB primary transcript yields four alternatively spliced forms,[10] and these are differentially expressed in maize tissues (Fitzgerald M, Walbot V, unpublished data). To investigate if these alternatively spliced forms are differentially UV-B regulated, we did qRT-PCR using isoform-specific primers. *mudrB*-167, *mudrB*-207 and *mudrB*-231 showed increased levels after 8-h UV-B in both silencing (**Fig. 1C**) and active (**Fig. 1D**) plants; no expression of *mudrB*-191, the most infrequent alternatively spliced form[10] was detected in either control or UV-B treated plants (not shown). It is worth noting that the total increase in transcript levels for all *mudrB* forms shown in **Figure 1B** is lower than the increase measured for *mudrB*-167 and *mudrB*-207 (3.7 and 4.5-fold, respectively). For *mudrA*, three possible spliced forms have been described, *mudrA-736, mudrA*-823 and *mudrA*-854. We only detected expression of *mudrA*-823, previously described as the most abundant spliced product;[10] thus, the increase in *mudrA* transcript levels in **Figure 1A** corresponds entirely to an increase in this isoform.

DNA methylation in *Mu* transposons is decreased by UV-B. UV-B radiation can reactivate *Mu* during plant growth and in pollen.[20,21] With pollen, UV-B exposure for 3 min restored somatic excision behavior in 7 of 19 ears in progeny of recently silenced, inactive plants (F1 generation of inactivation). Reactivation was much lower in inactive individuals maintained for three generations (F3), but UV-B was still 14-fold more effective than crossing to an active Mutator plant.[21] Inactive *MuDR/Mu* transposons are highly methylated at CG and CNG residues based on restriction enzyme sensitivities[9,30] and bisulfite sequencing (Ono A, Walbot V, unpublished data). To determine if transposon DNA methylation was changed by UV-B, we analyzed active and F1 silencing plants after a UV-B treatment, with inactive plants as the control for fully methylated elements. In initial experiments, DNA blot hybridization was used to ask if there were methylation changes. To monitor the extent of cleavage, methylation sensitive restriction enzymes *Sac*I and *Hin*fI were used, these enzymes digest only once in the TIRs of *MuDR* and *Mu1*, respectively (**Suppl. Fig. 1**). The inactive state of Mutator was highly methylated as expected, and there was evidence of methylation loss after UV-B (**Suppl. Fig. 1**).

Thus, to quantify methylation changes more precisely, a sensitive PCR protocol was used to detect both methylated and unmethylated TIRs. Loss of methylation from *Mu* TIRs was quantified using qPCR with *Sac*I, *Hpa*II and *Hin*fI-digested DNA from control and UV-B treated plants (**Fig. 2A–C**). The

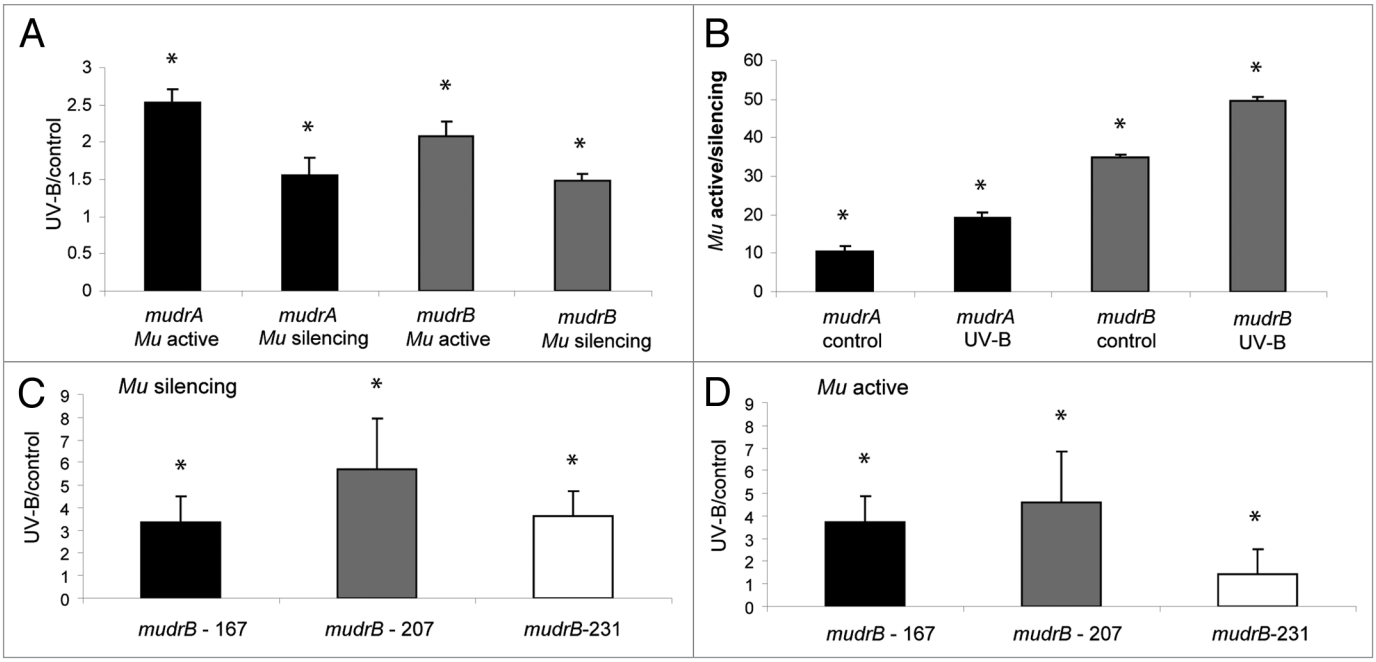

Figure 1. Transcript levels of *mudrA* and *B* in active and silencing Mutator plants after a UV-B treatment. (A) qRT-PCR analysis of *mudrA* and *B* transcript levels in control and UV-B conditions. This comparison was done using active and silencing Mutator plants. (B) qRT-PCR analysis comparison of *mudrA* and *B* transcript levels in active and silencing Mutator plants. This comparison was done using control and UV-B treated plants. (C and D) qRT-PCR analysis comparison of *mudrB* spliced transcripts in control and UV-B treated conditions in silencing (C) and active (D) Mutator plants. Three biological replicates were performed for each sample plus template-free samples and other negative controls (reaction without reverse transcriptase). Amplification of a thioredoxin-like transcript was used for data normalization. Error bars are standard errors. Statistically significant differences (t-*Student* test, significance level p = 0.05) are labeled with *.

percentage of methylation in the *MuDR* TIR methylation site recognized by *Sac*I was very low and not significantly changed in active *MuDR* TIRs, but decreased about 5% in silencing plants (**Fig. 2A**). Even if the percentage of methylation in the *MuDR* TIR methylation site recognized by *Hpa*II was higher in active *MuDR* TIRs (about 60%), it was not significantly changed by UV-B; but it was higher in silencing plants than in active plants, and decreased about 40% by UV-B (**Fig. 2B**). However, for *Mu1*, DNA methylation was decreased 7–8% for in both active and silencing plants. In inactive plants, DNA methylation in TIRs was high and not changed both for *MuDR* (**Fig. 2A and B**) and *Mu1* (not shown). Therefore, UV-B induces a decrease in DNA methylation of *Mu* elements in both active and silencing Mutator maize plants.

Changes in histone methylation in *Mu* transposons by UV-B. Using an 8 h UV-B treatment at levels that can reactivate transposition, we studied histone forms associated with the flanking regions of *MuDR/Mu* elements in active and silencing greenhouse-grown maize plants. Histone methylation, in particular in H3K9me2 and H3K27me2, are epigenetic marks that mediate gene silencing and are usually accompanied by DNA methylation. ChIP analyses were done using commercial antibodies against H3K9me2 and H3K27me2 using control and UV-B treated samples of active and silencing Mutator plants, and enrichment after immune precipitation was analyzed by qPCR with primers specific for *MuDR* and *Mu1* TIRs (**Fig. 3**). The coding region of a constitutive thioredoxin-like gene was used as an internal control: although amplification was very low, there was no significant change in the enriched fractions for the control gene with either antibody (**Fig. 3**). To evaluate nonspecific binding, the qRT-PCR reaction was done with samples incubated without an antibody; all ChIPed samples were also analyzed in parallel with total DNA from sonicated nuclei to evaluate the selective recovery of gene segments. The percentage of DNA recovered relative to the DNA input when experiments were done in the absence of antibodies was always lower than 5% of the DNA recovered when antibodies were used. After a UV-B treatment, TIR sequences from both *MuDR* and *Mu1* in silencing plants were decreased in the fractions immunoprecipitated with anti-H3K9me2; in contrast, similar levels of immunoprecipitate were found in active Mutator samples with or without a UV-B treatment (**Fig. 3A**). As a control, immunoprecipitation was done using antibodies against H3 histone (anti-H3). No enrichment was detected after the UV-B treatment in the controls (anti-H3 immunoprecipitate or control without antibody, **Fig. 3G**) for either active or silencing Mutator samples. Therefore, there is a specific decrease in H3K9me2 in the TIRs of *Mu* TE from silencing plants receiving UV-B. Despite this decrease, methylation in H3K9me2 around TIRs was considerably higher in silencing than in active Mutator plants (**Fig. 3B**).

For H3K27me2, there was decreased association with *MuDR* TIRs after UV-B, and this was true for both active and silencing plants (**Fig. 3C**). No changes in enrichment of H3K27me2 were found in *Mu1* TIRs after UV-B. Thus, it appears that decreased

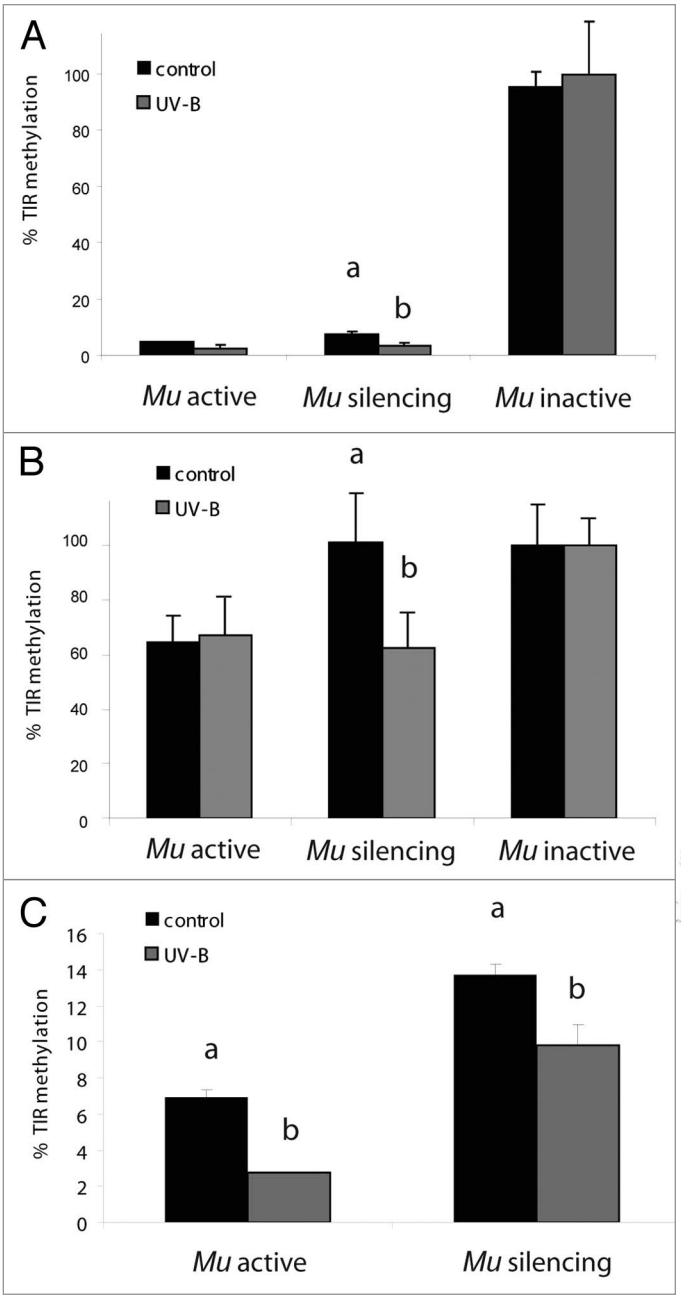

Figure 2. DNA methylation changes of *MuDR* (A and B) and *Mu1* (C) in active and silencing Mutator plants after UV-B. qPCR analysis of digested DNA from inactive, active and silencing Mutator plants under control conditions and after 8 h of UV-B irradiation with *Sac*I (A), *Hpa*II (B) and *Hin*fI (C) restriction enzymes. Primers were designed to amplify across the restriction sites; therefore, amplification is expected if DNA is methylated and not digested. Three biological replicates were performed for each sample, and three qPCR experiments were done with each sample. Amplification of a thioredoxin-like transcript was used for data normalization. Error bars are standard errors. If statistically significant differences exist (t-*Student* test, significance level p = 0.05), these are marked with letters a and b.

methylation on K27 by UV-B only occurs when TIRs act as promoters for transcription of both *mudrA* and *B* in the autonomous *Mu* elements, and this change does not occur in non-autonomous elements, whose TIRs lack detectable promoter activity.[31] Similarly, H3K27me2 association with *MuDR* and *Mu1* TIRs was significantly higher in silencing than in active plants; however, UV-B treatment decreased this association only for *MuDR* (**Fig. 3D**).

Because H3K9 methylation is mediated by the HP1 protein, and HP1 specifically binds H3K9me2,[16] we studied HP1 occupancy on TIRs after UV-B irradiation. Enrichment of HP1 on *MuDR* and *Mu1* TIRs paralleled H3K9me2: TIR sequences were decreased in the fractions from silencing Mutator samples after the UV-B treatment while similar levels of immunoprecipitate were found in active Mutator samples in the presence or absence of UV-B (**Fig. 3E**). From these results we hypothesize that HP1 may regulate the methylation of H3K9 during silencing of *Mu* TE.

Changes in histone acetylation and chromatin remodeling proteins occur in *Mu* transposons after UV-B treatment. Because increased *mudrA* and *B* transcript levels were measured after UV-B exposure (**Fig. 1A**), and increases in abundances often correlate with increased histone acetylation,[32,33] ChIP analysis was performed using antibodies specific for acetylated Lys residues in the N-terminal tails of histones H3 and H4 (anti-acetylated H3 and H4 antibodies). Antibodies against total H4 histone were used as one control, and the coding region of a constitutive thioredoxin-like gene was used as a gene control. Again, to evaluate nonspecific binding, the qRT-PCR reaction was also done with samples incubated without an antibody; and the percentage of DNA recovered relative to the DNA input when experiments were done in the absence of antibodies was always lower than 5% of the DNA recovered when antibodies were used. *MuDR* and *Mu1* TIRs were enriched in the fractions immunoprecipitated with anti-acetylated H3 antibodies after UV-B irradiation; this was true for both active and silencing samples. In contrast, both active and silencing lines yielded similar levels after immunoprecipitation with anti-acetylated H4 antibodies with or without UV-B exposure (**Fig. 4A and B**). No enrichment was detected in the controls (anti-H4 immunoprecipitate or control without antibody, **Fig. 4E**). Thus, the increase in acetylation of H3 during UV-B is correlated with an increase in transcript abundance from the genes monitored. It is surprising that no increase in H4 acetylation was measured, because other UV-B regulated genes in maize do show increased H4 acetylation after treatment.[24] It appears that changes in acetylation of H4 do not participate in the regulation *mudrA* and *B* transcription by UV-B.

To further understand the components of the altered chromatin landscape in *Mu* TE induced by UV-B, ChIP assays were employed to monitor two additional factors: SWI2/SNF2, a chromatin remodeling protein that facilitates binding of gene-specific transcription factors to their DNA targets, and CBP acetyltransferase, which can acetylate histones. Antibodies against these two factors were previously used in maize experiments with positive results (Casati et al. 2008).[24] DNA isolated from immunocomplexes obtained from control and UV-B treated nuclei from active

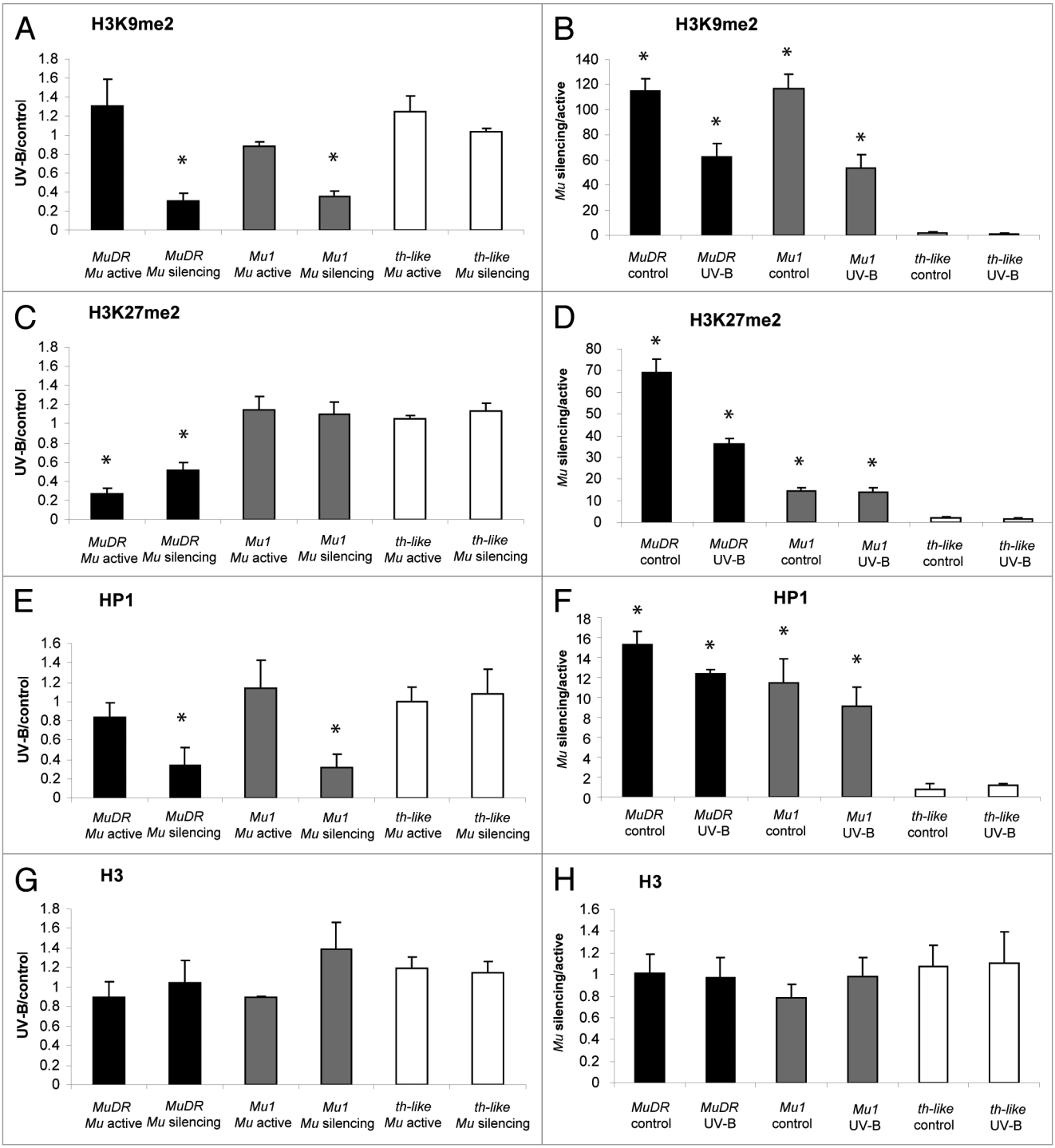

Figure 3. Methylation state of K9 and K27 residues when histone H3 is associated with *Mu* TIRs of active and silencing Mutator plants. ChIP assays utilized antibodies specific for H3K9me2 (A and B), H3K27me2 (C and D), HP1 (E and F) or total histone H3 (G and H) in nuclei prepared from active or silencing Mutator plants with UV-B treatment (UV-B) and control conditions (no UV-B). The immunoprecipitates were analyzed for the presence of the TIR sequences of *MuDR* and *Mu1* elements and a transcribed sequence of a control gene that is not UV-B-regulated (th-like) by qPCR. (A, C, E and G) Enriched fractions from UV-B treated vs. control plants were compared. (B, D, F and H) Enriched fractions from silencing vs. active plants were compared. ChIP data were normalized to input DNA before immunoprecipitation. The signal detected in samples incubated in the absence of any antibody as a control was less than 5% of the signal when antibodies were used. Error bars are standard errors. Statistically significant differences (t-*Student* test, significance level p = 0.05) are labeled with *. Three biological replicates of chromatin immunoprecipitation (ChIP) were performed from each genotype/treatment sample type, and three qPCR experiments were done with each sample.

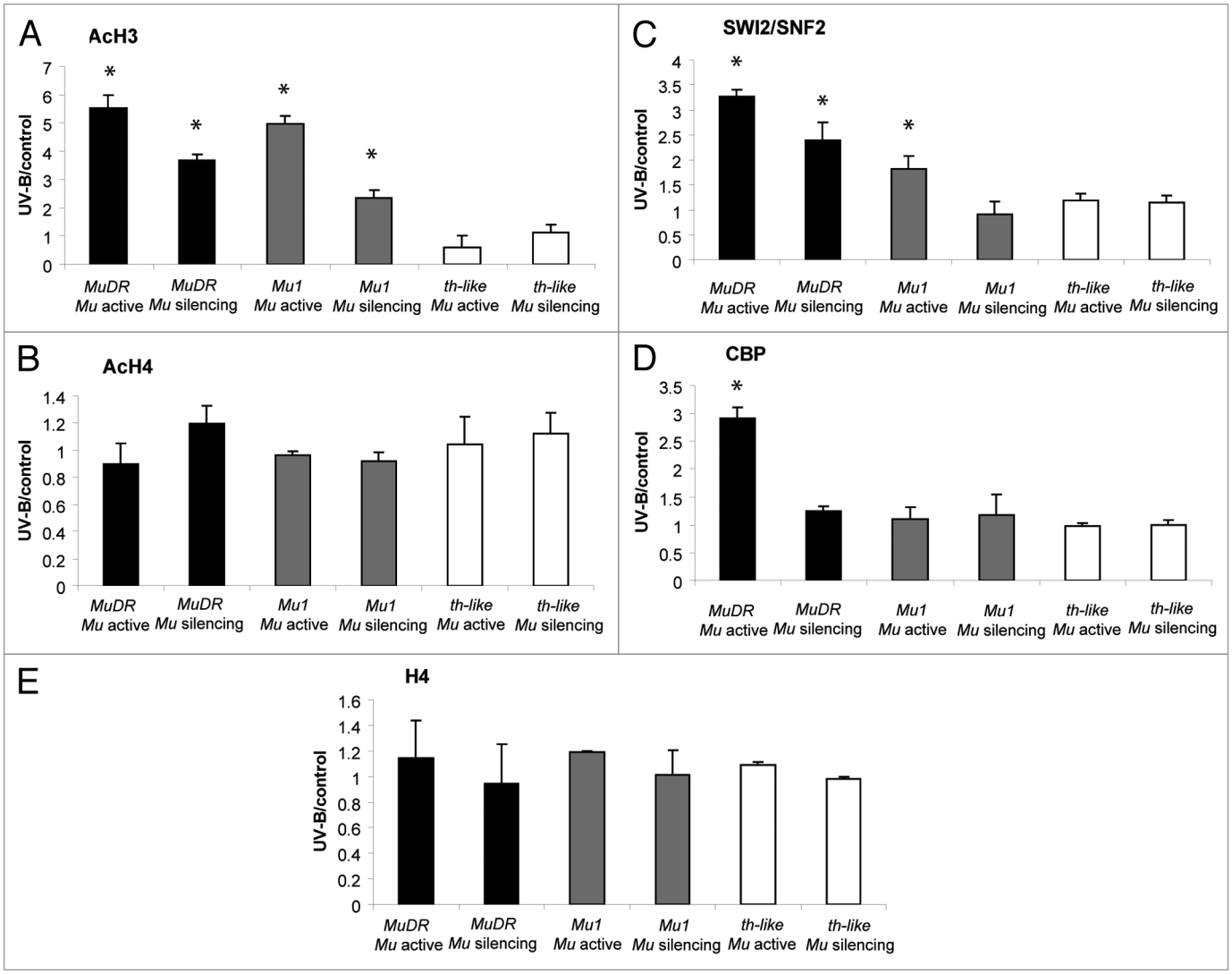

Figure 4. Acetylation state of histones H3 and H4 and other chromatin remodeling proteins associated with *Mu* TIRs of active and silencing Mutator plants. ChIP assays utilized antibodies specific for N-terminal acetylated histone H3 (A, AcH3), N-terminal acetylated H4 (B, AcH4), a chromatin remodeling factor (C, SWI2/SNF2), CBP acetyltransferase (D, CBP), or total histone H4 (E, H4) in nuclei prepared from active or silencing Mutator plants with UV-B treatment (UV-B) and control conditions (no UV-B). The immunoprecipitates were analyzed for the presence of TIR sequences of *MuDR* and *Mu1* elements and a transcribed sequence of a control gene that is not UV-B-regulated (th-like) by qPCR. Enriched fractions from UV-B treated vs. control plants were compared. ChIP data were normalized to input DNA before immunoprecipitation. The signal detected in samples incubated in the absence of any antibody as a control was less than 5% of the signal when antibodies were used. Error bars are standard errors. Statistically significant differences (t-*Student* test, significance level p = 0.05) are labeled with *. Three biological replicates of chromatin immunoprecipitation (ChIP) were performed from each genotype/treatment sample type, and three qPCR experiments were done with each sample.

and silencing Mutator plants was used for qPCR using primers specific for *MuDR* and *Mu1* TIRs and for the control thioredoxin-like gene. In ChIP experiments using anti-SWI2/SNF2, there was increased enrichment of *MuDR* TIR DNA sequences in all UV-B treated plants (3.2 and 2.4-fold enrichment for active and silencing plants, respectively); however, for *Mu1*, only active plants showed a significant enrichment (1.8-fold enrichment, **Fig. 4C**). As with the decrease in methylation on K27 by UV-B shown in **Figure 3C**, it seems that involvement of SWI2/SNF2 in chromatin remodeling in Mutator TE during UV-B treatment occurs when TIRs act as promoters. Using anti-CBP acetyltransferase antibodies, *MuDR* TIRs from active Mutator plants were enriched after irradiation, while no differences were measured between control and treated samples in silencing plants in TIRs from *MuDR* and *Mu1*, nor in *Mu1* TIRs in active plants (**Fig. 4D**). Because CBP is a histone acetyltransferase, the observed increased enrichment in active *MuDR* TIRs correlates with the higher transcript levels of *mudrA* and *B* after the 8-h UV-B treatment. The fact that there is no differential enrichment in the anti-CBP fractions assayed in other samples, suggests that changes in H3 acetylation as documented in **Figure 4A** may be mediated by other acetyltransferases.

Small RNA levels are not changed by UV-B. Increased DNA and histone methylation are proposed to be triggered by double-

A

Number in diagram	*Mu* siRNA	siRNA sequence
1	smRNA10831	GGGAACGGTAAACGGTGACAGGAC
2	smRNA11415	GAGAAGCCAACGCCAACGCCTC
3	smRNA16644	CCAACGCCAACGCCTCCATTTCGT
4	smRNA9697	CCCCTCTTCGTCTACAATGGCAAT

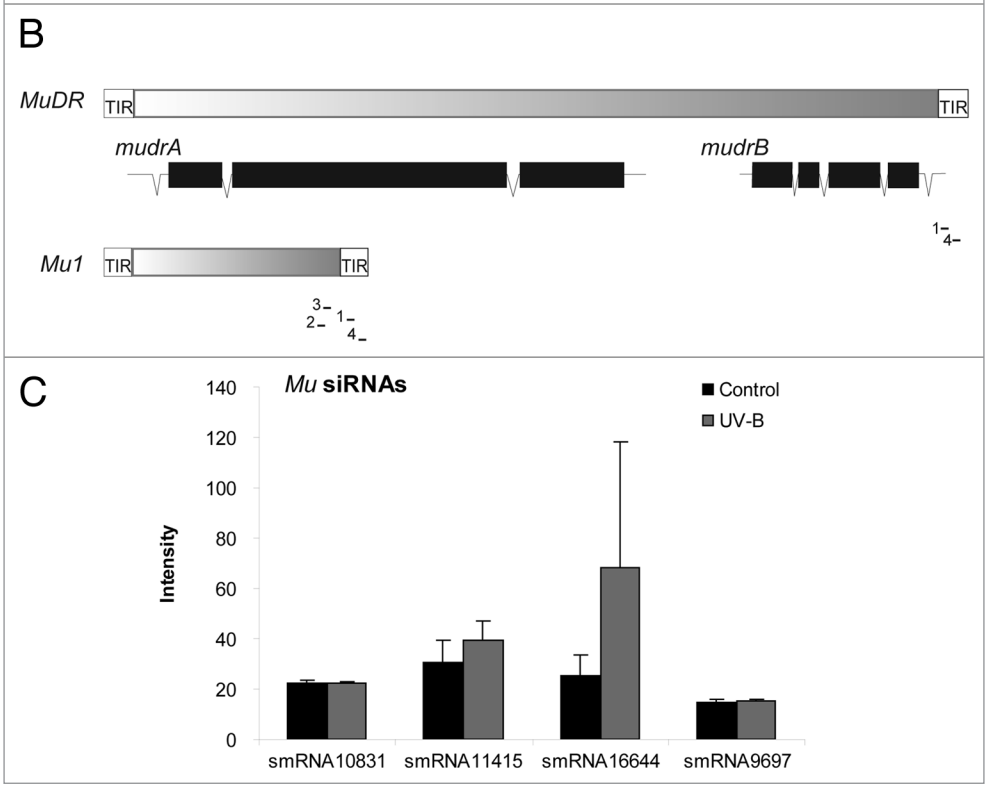

Figure 5. siRNA levels specific to *Mu* elements after UV-B. (A) siRNA sequences that were used for the microarray experiments. Sequences were retrieved from the Cereal small RNAs Database (http://sundarlab.ucdavis.edu/smrnas/) and probes were designed to hybridize to these targets. Microfluidic microarray assays were performed with probes complementary to the four siRNA sequences shown. (B) *MuDR* and *Mu1* representation, including the regions that are complementary to siRNA chosen. (C) Signal intensities of the siRNAs by microarray analysis using small RNAs from control and UV-B treated samples. Differences are not significant in any comparison. Experiments were run in duplicate using two biological replicates from each genotype/treatment sample.

stranded RNA, which is processed into 21–25 nucleotide RNA molecules.[34] To investigate whether changes in DNA methylation and chromatin in *MuDR* and *Mu1* are correlated with small RNA production, we measured levels of four siRNAs that correspond to different regions of *Mu1*/*MuDR* transposons. Maize siRNA sequences were retrieved from the Cereal small RNAs Database (http://sundarlab.ucdavis.edu/smrnas/). Using probes complementary to the four smRNA sequences (**Fig. 5A**), a microfluidic microarray-based assay was performed as shown in **Figure 5B**. After hybridizing with small RNAs extracted from silencing maize leaves from control and UV-B treated plants, the four mature siRNAs were scored positive for expression. No differences in intensity levels were measured for these siRNAs analyzed after the UV-B treatment (**Fig. 5C**). Thus, the expression levels of *Mu* element-derived siRNAs are not correlated with the changes in DNA and H3K9 methylation resulting from UV-B treatment (**Figs. 2 and 3**). The ubiquitous presence of small RNAs and the lack of correlation of siRNAs levels with active or silencing Mutator states was previously described by Rudenko et al.[35] and Woodhouse et al.[14]suggesting that either the silencing components are present but ineffective, or that small RNAs are involved in other aspects of regulation of this TE family.

Discussion

Silenced transposons populate eukaryotic genomes, yet we know little about the natural triggers that can reactivate them. We investigated the possible mechanisms that can participate in Mutator TE reactivation by UV-B radiation; in particular changes in transcript abundance and epigenetic marks associated with the *MuDR/Mu* elements. Previously, it was shown that field conditions simulating UV-B exposure at 33% ozone depletion can activate immobile *Mu* transposons.[20-22] These TE amplify the effects of UV-B exposure by producing an increased number of mutations.

Solar radiation is an important environmental factor, as it is not only a source of energy for photosynthesis and informational signals for growth and development but also contains potentially harmful UV-B radiation (UV-B, 280–315 nm) in sunlight.[36,37] Insight into the mechanisms plants use for sensing and responding to UV-B is scarce.[38] In *Arabidopsis thaliana*, the transcription factor long hypocotyl5 (HY5), which is required for normal growth in visible light, was identified as also participating in the long wavelength (300–315 nm) UV-B signal transduction pathway.[39] Increases in HY5 levels are mediated by UVR8, a UV-B specific factor, and COP1.[40,41] UVR8 and COP1 interact in the nucleus after UV-B exposure; this interaction is probably a very early step in UV-B signaling to ensure UV-B acclimation and protection.[42] At high intensities, UV-B radiation can cause cellular damage by generating DNA photoproducts and can damage proteins, lipids, and RNA molecules.[23,43,44] Plant responses to UV-B damage include mechanisms for repair and avoidance.[23,45-47]

Chromatin remodeling is clearly implicated in maize responses to UV-B by several lines of evidence. Transcriptome profiling of lines differing in UV-B sensitivity indicated that constitutively higher levels of genes predicted to encode chromatin remodeling factors are present in high-altitude, UV-B-tolerant landraces than in temperate zone maize. The landraces also showed greater UV-B mediated upregulation of these genes.[48] Transgenic plants with decreased expression of four UV-B regulated chromatin factors are hypersensitive to UV-B at doses that do not cause visible damage to normal maize.[48] Knockdown lines with decreased expression of a putative methyl-CpG-binding domain protein, MBD101, or a subunit of a putative chromatin remodeling complex, CHC101, were similarly hypersensitive to UV-B; however, they exhibited substantially different transcriptome changes compared to each other and to UV-B tolerant non-transgenic siblings both before and after 8 h of UV-B exposure.[49] By functional analysis, the largest category of genes with predicted functions affected by UV-B included DNA or chromatin binding proteins implicating differential activation of transcription factors as the likely explanation for the distinctive transcriptome profiles.[49] Moreover, UV-B-tolerant lines exhibited greater acetylation on N-terminal tails of histones H3 and H4 after irradiation;[24] H3 and H4 histone acetylation was enriched in the promoter and transcribed regions of UV-B-upregulated genes in UV-B tolerant lines. This enrichment was absent or much lower in the two knockdown lines.[24] However, H3K9me2 and H3K27me2 methylation on promoter and coding regions of a UV-B upregulated gene *nfc102* were not changed in different maize genotypes by this radiation.[24] Because UV-B tolerant and hypersensitive maize lines show specific chromatin states well-correlated with UV-B responsiveness, chromatin remodeling is a key process in UV-B acclimation.

Mu elements are defined by shared TIR ends and are regulated by autonomous *MuDR* elements. Both TIRs of *MuDR* contain promoters, resulting in convergent transcription of *mudrA* and *mudrB*.[10] When MURA transposase is present, *Mu* elements excise from existing locations and reinsert throughout the genome; when Mutator is inactive, *MuDR* transcripts are very low or undetectable and neither excision nor insertion occur.[34] In the active and sister silencing lines examined in this study, *mudrA* and *B* transcript levels in active plants were considerably higher than in the silencing plants, and after UV-B irradiation the differences increased further. These observations confirm and extend previous microarray results in which the *mudrA* gene was reported to be upregulated by sunlight UV-B in active Mutator plants, while the *mudrB* gene was upregulated only after UV-B supplementation.[28] We also found that three of the *mudrB* and one of the *mudrA* spliced forms increased after UV-B, and the encoded proteins are candidate participants in reactivation of Mutator activities. It is worth noting that the total increase in transcript levels for all *mudrB* forms shown in **Figure 1B** is lower than the increase measured for *mudrB*-167 and *mudrB*-207. It was previously described that alternative splicing is affected by several mechanisms, including chromatin remodeling.[50,51] UV radiation induces alternative splicing of the human MDM2 gene;[52] and in plants, splice variants of the respiratory burst oxidase homolog (*rboh*) gene accumulated markedly when maize seedlings were subjected to various abiotic stimuli including UV.[53] Thus, chromatin remodeling may be affecting alternative splicing of *mudrB* by UV-B.

The UV-B inducibility of *mudrA* and *mudrB* could be caused by transcription factors mediating UV-B responses that bind to TIRs. To explore this possibility, we analyzed candidate binding motifs in *MuDR* promoter regions. Previous work highlighted the presence of sequences similar to enhancers specific for pollen expression, as well as motifs important for the regulation of cyclin, kinesin and H3 and H4 histone genes in dividing cells of both monocots and dicots.[54] We identified a CGCG box in the 5' UTRs of both *mudrA* and *mudrB* (**Suppl. Fig. 2**). Previously, multiple CGCG cis-elements were found in promoters of genes involved in ABA and ethylene signaling, DNA repair after UV damage, signal perception and stress response.[55] In Arabidopsis, the (A/C/G)CGCG(G/T/C) elements are specific targets of CaM-regulated AtSR proteins. Each AtSR has a conserved structural feature with a DNA-binding region, the CGCG domain, in the N terminus and a CaM-binding domain in the C terminus.[55] A 6-base pair CCGCGC motif is located between two TIR sequences (sites I and II, **Suppl. Fig. 2**) previously identified as protein binding zones by gel-shift assays using maize extracts.[56] CGCG elements were also found in promoters of Arabidopsis UV-B regulated genes *Uvr3*, *Uvr7*, *Hyh* and *Chs.* A conserved sequence upstream of light-regulated genes in both monocots and dicots, the I-box (GATAA) core sequence, is also present in *MuDR* TIRs (**Suppl. Fig. 2**). The presence of these multiple motifs involved in rapid responses to radiation suggest that transcription factors induced by UV-B target *mudrA* and *mudrB* promoters, and that these factors in concert with chromatin remodeling could be responsible for increased transcription of *MuDR* in both active and silencing plants.

The higher transcript levels in active plants after UV-B treatment correlates with increased histone H3 acetylation in *MuDR* TIRs. Thus, increased *mudrA* and *B* transcript abundances can be mediated by an increased transcription rate after the recruitment of transcription activators that recognize acetylated H3 histone tails.[57] For example, increased association of a SWI2/

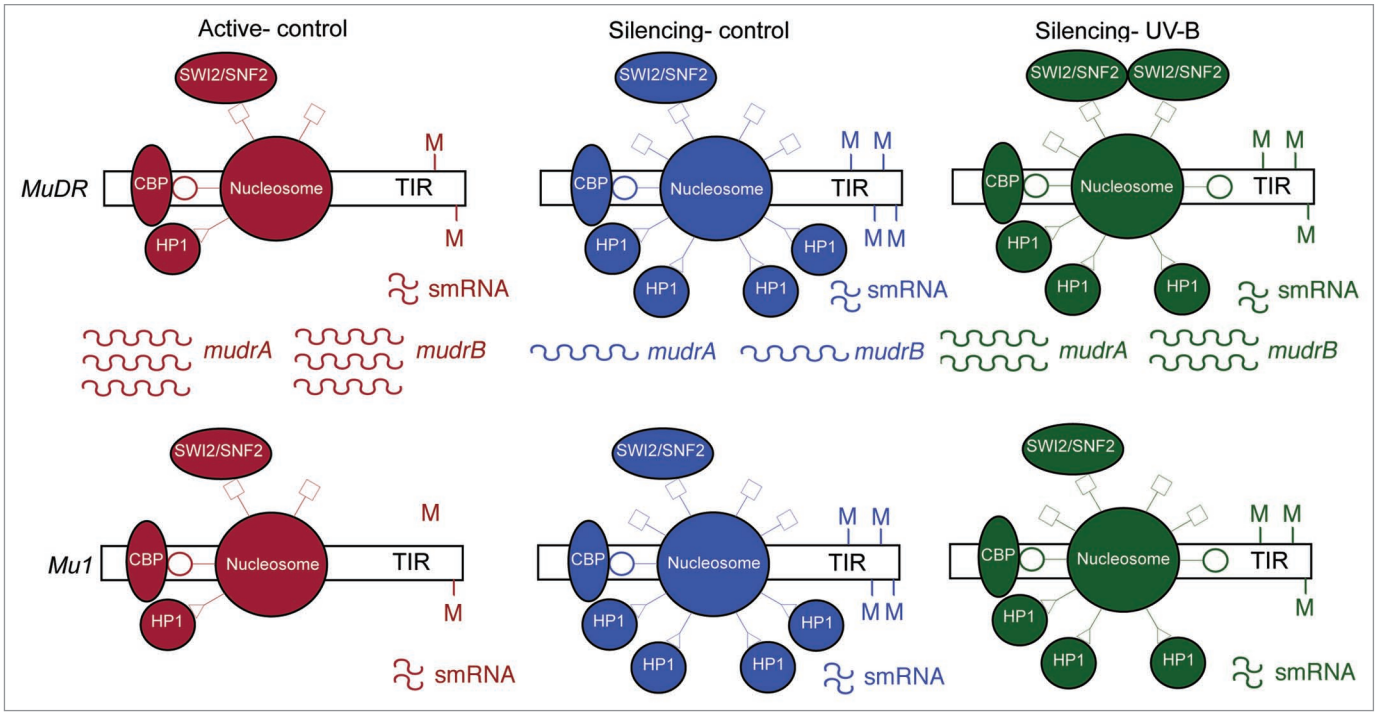

Figure 6. A model depicting epigenetic changes in *Mu* TIRs in silencing and active plants after UV-B. In purple, active plants under no UV-B; in blue, silencing plants under no UV-B; in green, silencing plants after 8 h of UV-B irradiation. Full circles represent nucleosomes, solid lines indicate the TIRs. Triangles represent H3K9me2, squares represent H3K27me2, and circles acetylated histone H3. M indicates DNA methylation. Other chromatin remodeling proteins are represented in full circles or ovals, and *mudr* transcripts and smRNAs are also shown.

SNF2 chromatin remodeling protein to *MuDR* TIRs was also detected after the UV-B treatment, and the enrichment was more extensive in active than in silencing plants. A similar result was observed in ChIP assays with anti-CBP acetyltransferase antibodies. The largest selective increase by UV-B was for precipitation with CBP acetyltransferase antibodies in the active *MuDR* TIRs, which is in accordance with the increase in H3 acetylation measured. Interestingly, the enrichment in *Mu1* or silencing plants was not significant with this antibody, suggesting that changes in histone acetylation in *Mu1* elements and in silencing plants may be mediated by a different acetylase(s). It is surprising that increased acetylation of H4 was not observed in our experiments. Previously, increases in both H3 and H4 acetylation in promoter and coding regions of four UV-B regulated genes were detected.[24] Apparently, changes in H4 acetylation states are not necessary for increased transcription of *MuDR* genes by UV-B, in contrast with what is observed for other maize genes.

We also detected minor but significant changes in DNA methylation states: unmethylated *MuDR* elements increase after UV-B in silencing plants; however, unmethylated *Mu1* elements increase in both active and silencing plants. Collectively, these results favor the slow imposition and removal of DNA methylation marks at *MuDR/Mu* TIRs, that is, the epigenetic switch from active to inactive status is more likely to be mediated by protein factors directly than by DNA methylation per se. This idea is further supported by ChIP experiments to detect H3K9me2. The association of this histone H3 isoform with *MuDR* and *Mu1* TIRs was significantly higher in silencing than in active plants, but only in silencing plants was this difference decreased by UV-B. Thus, there is a specific decrease in H3K9me2 in the TIRs of *Mu* TE from silencing plants receiving UV-B. In addition, HP1 enrichment at *MuDR* and *Mu1* TIRs was similar toH3K9me2. HP1 specifically binds methylated H3K9, and methylation of H3K9 is mediated by HP1.[15] We propose that HP1 may regulate the increase in H3K9me2 in silencing *MuDR/Mu* transposons. Previously, in screens for genes that relieve silencing of loci with inverted repeats in Arabidopsis, components of epigenetic processes were identified, including a H3K9 methyltransferase KYP, also called SUVH4.[58] When a mutation in the Arabidopsis ATP-dependent chromatin remodeling protein DDM1 was introduced, methylation of both DNA and H3K9 was sharply decreased at mobile elements, with concomitant increases in TE transcripts.[17,18] According to the results presented here, reactivation of silencing transposons could be initiated by changes in H3K9 methylation, and other epigenetic changes necessary to fully impose the silenced state, such as DNA methylation, can occur later.

We also investigated if changes in histone methylation in H3K27me2 occurred after UV-B irradiation. In contrast to what was observed for H3K9me2, there was a decreased association of H3K27me2 with *MuDR* TIRs after UV-B—this was true for both active and silencing plants. No changes in enrichment after UV-B was measured in any plant at the *Mu1* TIR. Thus, it appears that decreased methylation on K27 after UV-B only occurs when TIRs act as promoters, and this is another feature that distinguishes the TIRs of *MuDR* from those of *Mu1*.

Evidence is building that siRNAs are the initiating factors in TE silencing, because of their ability to confer sequence specificity for the silencing machinery.[59] In our work, we were unable to detect changes in siRNA abundances for four species derived from *Mu* elements after the UV-B treatment in silencing plants (**Fig. 5**). Previously, changes in siRNAs in different Mutator silencing stages were not detected—the siRNAs were constitutively present.[14,33] In the published maize genome[60] there are 155 families of *Mu* elements, nearly all of which are inactive; the ubiquitous siRNAs may be involved in surveillance against these elements. It is possible that another siRNA type will be involved in triggering *MuDR* transcriptional silencing. *Mu killer*, an internally deleted *MuDR* element with a central inverted duplication, can effectively silence *MuDR* transcription and *Mu* element movement, however, the nature of siRNAs derived from this element have not been elucidated.

Figure 6 summarizes the results shown in this paper. In active Mutator plants, *mudrA* and *B* transcripts are readily detected but levels are much lower in silencing plants; UV-B radiation upregulates the expression of both genes in active and silencing plants. This increase after UV-B correlates with increased association of H3 acetylation and other remodeling indicative of open chromatin within the TIRs. In addition, UV-B induces a decrease in DNA and histone H3 methylation, but no changes in the siRNA levels are detected. The decrease in H3K9me2 present at *Mu* elements after UV-B is significant in silencing plants, suggesting that early changes in H3 methylation in K9 may be essential for transposon reactivation by UV-B in maize.

Methods

Plant material, radiation treatments and measurements. Two independent Mutator-active and two silencing sister lines with *bz2-mu2* reporter alleles were used. Spotted (active) or non-spotted (inactive) kernels from appropriate ears were selected and grown in the greenhouse with supplemental visible light to 10% of summer noon radiation with approximately 15 h light/9 h dark without UV-B for 28 d. UV-B was provided once for 8 h, starting 3 h after the beginning of the light period, using fixtures mounted 30 cm above the plants (Phillips, F40UVB 40 W and TL 20 W/12) at a UV-B intensity of 2 W m^{-2}, UV-A: 0.65 W m^{-2}. The bulbs were covered with cellulose acetate to exclude wavelengths <280 nm. As a control, plants were exposed for 8 h under the same lamps covered with polyester film (no UV-B treatment, UV-B: 0.04 W m^{-2}, UV-A: 0.4 W m^{-2}). Lamp output was recorded using a UV-B/UV-A radiometer (UV203 A + B radiometer, Macam Photometrics, Ltd., Livingston, UK) to insure that both the bulbs and filters provided the designated UV dosage in all treatments. Leaf samples were collected immediately after irradiation.

Chromatin immunoprecipitation. Chromatin immunoprecipitation was done as described in Casati et al.[29] The following antibodies were used: 4 µL of anti-H3 (dimethylated K9) (ab1220), 4 µL of anti-H3 (dimethylated K27) (ab6002), 4.5 µL of anti-HP1 (ab14298); 4 µL of anti-H4 (ab7311);, 4 µL of anti-SWI2/SNF2 (ab5154); 4 µL of anti-CBP (ab18291) (all from Abcam, Cambridge, MA); two additional reagents were 4 µL of anti-N-terminal acetylated H4 or 4 µL of anti-N-terminal acetylated H3 (06-598 and 06-599, respectively, Upstate Biotechnology, Lake Placid, NY). The antibodies used were previously tested for crossreactivity against maize proteins.[24] Three biological replicates of chromatin immunoprecipitation (ChIP) were performed from each genotype/treatment sample type, and three qPCR experiments were done with each sample.

RNA isolation and reverse transcription reaction. RNA samples were isolated using Trizol (Invitrogen, Carlsbad, CA) as described by Casati and Walbot.[28] RNA was isolated from a pool of top leaves (which received the greatest UV-B exposure) from 6 plants; pooling minimizes organismal variation. Five µg of total RNA from each genotype/treatment combination was used for cDNA synthesis using Superscript II reverse transcriptase (Invitrogen).

Quantitative PCR and enzyme digestion. Primers for *MuDR* and *Mu1* TIRs, transcribed regions of *mudrA* and *B*, and the transcribed region of a thioredoxin-like gene were designed using Primer3 software.[61] The primer pairs were: *MuDR* TIRs: L: GAA GAG CGG AAG GGA TTC; R: TAA ACG GCG ACA GGA GAG; *Mu1* TIRs: L: TTG CCA TTA TGG ACG AAG A; R: GGT AAA CGG GGA CAG AAA A; *mudrA* total transcripts: L: AAA CAG GTG GCA GTG AGG, R: CGA ATC CCA GTC CAG TTG, *mudrB* total transcripts: L: GCT ATG GGA AAT GGT TTT TG, R: GGT CGT TTA TCT CTT CGA ACC, thioredoxin-like gene (AW927774); L: GGA CCA GAA GAT TGC AGA AG, R: CAG CAT AGA CAG GAG CAA TG. Primers used for amplification of alternative spliced transcripts were as follows: nBe4i3-L: GAG GAT GTT CAT CAT CTA CG; nBe2e3-R: CTC ATC GAA TGT GGA TCT TG; nBe3e4-L: TGT TCA TCA TCT ATC TTG CC; Be2i2-R: CCT CAT CGA ATG TGG TAT GG; A823-L: CTC ATT TTG GAT CCA TGG AC; A854-R: GTT GGC GTA CTC CTC TCC TC; *mudrA*-R: TCC AGT TGT GAT TCC CCA TC. To amplify the different spliced transcripts, the following primer combinations were used: nBe4i3-L and nBe2e3-R for *mudrB*-207; nBe4i3-L and Be2i2-R for *mudrB*-231; nBe3e4-L and nBe2e3-R for *mudrB*-167, nBe3e4-L and Be2i2-R for *mudrB*-191; A823-L and *mudrA*-R for *mudrA*-823 and A854-L and *mudrA*-R for *mudrA*-854.

Quantitative, real-time PCR (qRT-PCR) was carried out in a MiniOPTICON2 (Bio-Rad, Richmond CA) as described in Casati and Walbot.[29] For qRT-PCR, three biological replicates were performed for each sample plus template-free samples and other negative controls (reaction without reverse transcriptase). Amplification of a thioredoxin-like transcript was used for data normalization. To confirm the size of the PCR products and to check for correspondence to a unique and expected PCR product, the final PCR products were analyzed on a 2% agarose gel.

The isolation of DNA templates for DNA methylation analysis by qPCR, and calculation of the percentage of methylation was done as previously described.[62] 1 µg of DNA was used for each DNA digestion reaction. DNA was diluted to 50 ng μl^{-1} and digested overnight in a volume of 50 µl with 5 units of *Sac*I, *Hpa*II or *Hin*fI, or no enzyme as a control. After digestion, each PCR template was diluted 8-fold in water and incubated at 65°C

for 30 min to inactivate the enzyme prior to the reaction assay. Three biological replicates were performed for each sample, and three qPCR experiments were done with each sample.

DNA blot hybridization. Total genomic DNA was extracted from leaf tissue as previously described.[63] Eight µg of genomic DNA was mixed in 1x restriction buffer containing 5–8 units of each restriction enzyme per µg of DNA and incubated overnight at the optimal digestion temperature. The digested DNA was separated by electrophoresis in a 1% agarose gel and blotted onto Nylon membrane (Hybond N$^+$, GE Healthcare, Uppsala, Sweden). Probe labeling, hybridization and detection were done with AlkPhos labeling and detection (GE Healthcare) following the manufacturer's instructions using 1,010 bp *MuDR* and 426 bp *Mu1* probes generated by PCR amplification from appropriate *MuDR* and *Mu1* subclones. The *MuDR* probe was amplified with the following primer set: *MuDR*-3091L 5'-GAA AAT CAC CCC CAA GAA AAG A-3' and *MuDR*-4079R 5'-ACA AGG TGG CAA GAT AGG TAA G-3'. In samples from active Mutator lines, digestion with *Sac*I generated a 4.7 kb band indicative of unmethylated, intact *MuDR* element, plus a few other bands corresponding to non-functional *hMuD*R elements. The 426 bp *Mu1* probe was amplified from a plasmid containing *Mu1*,[64] with the following primer set: *Mu1*-444L 5'-TCC GCG TGC TCC TCA AGT TC-3' and *Mu1*-870R 5'-GCG GGG ACC TTC AGC TTG TT-3'. When elements are unmethylated, this probe generates a 1.4 kb band. Three biological replicates were performed for each sample, and DNA blot hybridization was done with each sample.

smRNA microarray. The miRNA microarray was custom synthesized by LC Sciences (Houston, Texas, USA) using their protocols.[65] Briefly, poly-A tails were added to the 3' ends of small RNAs using a poly(A) polymerase, and Cy3 and Cy5 was then ligated to the poly-A tails of samples from control and UV-B treated maize plants. The tagged RNAs were hybridized to the microfluidic hybridization chip. Hybridization images were scanned with a Gene-Pix 4000B microarray scanner (Molecular Devices, Sunnyvale, USA). Data extraction and image processing were performed using ArrayPro^{TM} image analysis software (Media Cybernetics, Silver Spring, USA). A smRNA was scored as detectable if the signal intensity was higher than 3 times the standard deviation of the background signal, and the spot CV <0.5. CV is calculated as standard deviation/signal intensity. Normalization was performed with a LOWESS method to remove system-related variations.

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/QuestaEPI5-4-Sup.pdf

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