RNA-DIRECTED DNA METHYLATION PREVENTS RAPID AND HERITABLE REVERSAL OF TRANSPOSON UNDER HEAT STRESS IN ZEA MAYS

by

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ABSTRACT

RNA-directed DNA methylation (RdDM) is a process by which epigenetic silencing is maintained at the boundary between genes and flanking transposable elements. In maize, RdDM is dependent on *Mediator of Paramutation 1 (Mop1*), a putative RNA dependent RNA polymerase. Here I show that although RdDM is essential for the maintenance of DNA methylation of a silenced *MuDR* transposon in maize, a loss of that methylation does not result in a restoration of activity of that element. Instead, heritable maintenance of silencing is maintained by histone modifications. At one terminal inverted repeat (TIR) of the element, heritable silencing is mediated via H3K9 and H3K27 dimethylation, even in the absence of DNA methylation. At the second TIR, heritable silencing is mediated by H3K27 trimethylation, a mark normally associated with somatically inherited gene silencing. I find that a brief exposure of high temperature in a *mop1* mutant rapidly reverses both of these modifications in conjunction with a loss of transcriptional silencing. These reversals are heritable, even in *mop1* wild type progeny in which methylation is restored at both TIRs. These observations suggest that DNA methylation is neither necessary to maintain silencing, nor is it sufficient to initiate silencing once it has been reversed. To leverage the specificity of our observations made at bench, I also performed a transcriptome analysis in *mop1* mutants under heat. I found that a substantial number of genes as well as a subset of TEs are reactivated in *mop1* mutants under heat, which is consistent with the effects I observed on *MuDR*. Interestingly, I found that *mop1*-specific reactivation of TEs is closely correlated with changes in expression of nearby genes, most of which are involved in metabolic transportation and sensing. This suggests that one function of *MOP1* is to prevent inappropriate expression of genes in this pathway when they are close to TEs. Taken together, my work will provide an opportunity to better understand the causes and consequences of TE silencing and reactivation, as well as the effects of TEs on gene regulation under stress conditions.

CHAPTER 1. INTRODUCTION

1.1 Epigenetics

Epigenetics is an area of study concerned with heritable changes that do not involve alterations in the DNA sequence (Pikaard & Mittelsten Scheid, 2014; T. Zhao, Zhan, & Jiang, 2019). It most often involves changes in gene activity, expression and phenotype, most of which can happen at cellular, physiological and phenotypic levels that may result from internal developmental factors or external environmental stimuli (Jaenisch & Bird, 2003). DNA methylation and histone modifications are two predominant mechanisms that can produce these heritable changes in gene activity and expression (Y. C. G. Lee & Karpen, 2017). These epigenetic changes may last temporarily, through somatic cell division, or for multiple generations (Pikaard & Mittelsten Scheid, 2014). Because epigenetics breaks the rules of Mendelian inheritance, it has long fascinated biologists.

Despite the obvious morphological differences between plants and mammals, there are many similarities in many aspects of genome and epigenome. Both plants and mammals employ a combination of DNA methylation and histone modifications to regulate gene expression (Rigal et al., 2016). Because of this conservation, epigenetic mechanisms discovered in either kingdom can be mutually informative for understanding in the other. However, plants have a haploid growth stage, a stage mammals do not have (Pikaard & Mittelsten Scheid, 2014). The male and female gametophytes consist of multiple cells that are produced mitotically from the initial haploid meiotic products. Unlike mammals, there is no evidence for a massive erasure of epigenetic marks during plant gametogenesis (Rigal et al., 2016). Indeed, repressive epigenetic marks in plant sperm and egg cells actually appear to be reinforced in these nuclei, which makes epigenetic changes transmissible through meiosis in plants (Rigal et al., 2016). Over the past decades, most major epigenetic mechanisms known to occur in eukaryotes have been studied in plants. In fact, plants have a particularly diverse set of mechanisms devoted to such regulation. Unlike animals, plants do not set aside a germline nor there is evidence that they undergo a global epigenetic resetting each generation, therefore epigenetic changes that occur in somatic cells are more likely to be transmitted to the subsequent generations (Quadrana & Colot, 2016). Because plants are unable to escape from an unfavorable environmental condition, they have to cope with these conditions.

Epigenetic mechanisms can fine-tune gene expression patterns and enable plants to survive and reproduce successfully under unfavorable conditions (Crisp., Ganguly., Eichten., Borevitz., & Pogson, 2016). The prominence of epigenetic regulation in plants mirrors their mode of development, stress response and evolutionary history, which in turn, makes it an excellent model for the study of epigenetics (Quadrana & Colot, 2016).

1.2 DNA methylation

DNA methylation is a conserved chemical modification at the 5' position in the deoxyribose ring of cytosine that is catalyzed by DNA methyltransferases (Eichten & Springer, 2015). In eukaryotic genomes, heterochromatin, which is enriched with transposable elements (TEs), is densely methylated (H. Zhang, Lang, & Zhu, 2018). In plants, DNA methylation occurs in all sequence contexts: CG, CHG and CHH, where H represents A, T, or C. Because both CG and CHG sequence context methylation are symmetrical, prior DNA methylation can be easily propagated by methylating newly synthesized DNA strands using the previous parent stand as a template (Pikaard & Mittelsten Scheid, 2014). As a result, DNA methylation can be heritably propagated, either following cell divisions during somatic development, or transgenerationally (Jaenisch & Bird, 2003). In combination with histone modifications and other protein complexes, DNA methylation defines the structure and accessibility of chromatin, which plays an essential role in gene expression, genome integrity, and inheritance (Quadrana & Colot, 2016). DNA methylation primarily occurs in the promoter regions of genes, and can repress gene transcription by directly inhibiting the binding of transcription activators or promoting repressive histone modifications (Erdmann & Picard, 2020). DNA methylation at gene promoters is often a result of the spreading of this modification from nearby TEs (D. Lisch, 2009). On the other hand, genes adjacent to TEs tend to be targeted by active DNA demethylation in order to protect them from silencing (OKamoto. & Hirochika, 2001). Likely due to these mechanisms, and due to purifying selection, in plants, very few genes are methylated in promoter regions(Gallego-Bartolome, 2020). As a result, DNA methylation may not regulate gene expression globally, and that may be the reason why most mutants in the DNA methylation pathways do not exhibit abnormal growth or development phenotypes (Sigman & Slotkin, 2016). In *Arabidopsis*, the gene bodies of over one-third of genes are methylated. In contrast to the DNA methylation pattern at TEs, which are usually densely methylated in all three contexts, DNA methylation at gene body show very little non-CG

methylation. Genome-wide analysis has revealed that genome-wide gene body methylation (gbM) requires CMT3, a chromatin methylase (C. Liu, Lu, Cui, & Cao, 2010). Although the biological significance of gbM is not clear and seems to be species specific, it has been observed that gbM may prevent transcription of aberrant transcripts in mice, and can increase pre-mRNA splicing efficiency in plants (Erdmann & Picard, 2020).

In addition to its role in gene regulation, DNA methylation also plays an essential role in TE silencing (Gent et al., 2013; Stroud et al., 2014). In plants, *de novo* DNA methylation is mediated through the RNA-directed DNA methylation (RdDM) pathway (Erdmann & Picard, 2020). The RdDM pathway maintains asymmetric CHH methylation at TEs, which is dependent on a chromatin remodeler, DDM1 and a methylase, CMT2 (Sasaki, Kawakatsu, Ecker, & Nordborg, 2019). Loss of CHH methylation can lead to transcription reactivation of some TEs near genes, which is also accompanied by CG and CHG hypomethylation, suggesting that RdDM is required to prevent silenced TEs from being activated by active nearby genes (Liang et al., 2020). Under heat conditions, *nrpd1* mutants, which are defective in RdDM, exhibited more frequent transposition of a retrotransposon, *ONSEN* (Masuta et al., 2017). However, reactivation of this TE has not been observed in mutants defective in CMT2 or RdDM mutants under normal conditions, suggesting that there are contributing factors other than CHH methylation that function in suppressing TE mobilization in the absence of heat (Bucher, Reinders, & Mirouze, 2012; Ito et al., 2011; Pooja Negi, Archana N Rai, & Penna Suprasanna, 2016).

1.3 Histone modifications

In eukaryotes, DNA is tightly compacted by histone proteins and forms a complex known as chromatin. Factors affecting chromatin structure can dramatically influence all DNA-templated processes (C. Liu et al., 2010). The basic unit of chromatin is the nucleosome, which consists of a length of ~146 base pairs of DNA wrapped around a histone octamer, which contains two copies of one of the four histone proteins H2A, H2B, H3, and H4 (C. Liu et al., 2010). The N-terminal of the tails of the core histones are subject to various chemical modifications, such as acetylation, methylation, phosphorylation, ubiquitination, ADP-ribosylation, glycosylation, and sumoylation (Deal & Henikoff, 2011). The histone code hypothesis proposes that these chemical modifications might provide information that guides the execution of gene expression and other biological processes (Deal & Henikoff, 2011). Although most histone modifications are conserved among

different species, the establishment and maintenance of these modifications in plants are not identical to those in fungi and animals (C. Liu et al., 2010). Overall, histone methylation plays an essential role in various biological processes (Tariq & Paszkowski, 2004). Histone methylation only occurs at lysine and arginine (Cedar & Bergman, 2009). In *Arabidopsis*, histone methylation occurring at lysine is mainly at Lys4 (K4), Lys9 (K9), Lys27 (K27), and Lys36 (K36) . Histone methylation at lysine is an important epigenetic mark that influences either transcriptionally silenced or active chromatin domains depending on which lysine residues are methylated and the degree of that modification (Du, Johnson, Jacobsen, & Patel, 2015). In general, histone H3K9 and H3K27 methylation are associated with silenced chromatin regions, whereas H3K4 and H3K36 methylation are mostly associated with active genes (Frapporti et al., 2019). In mammalian systems, histone H3K9 methylation occurs at H3K9 monomethylation, H3K9 dimethylation, and H3K9 trimethylation levels, each of which can result in different biological outcomes (Cheung & Lau, 2005). Consistent with various genetic studies of the function of H3K9me2, genome-wide ChIP sequencing has revealed that this modification is largely enriched around transposons and other repetitive sequences (Sigman & Slotkin, 2016; Weinhofer, Hehenberger, Roszak, Hennig, & Kohler, 2010). In plants, the first plant histone H3K9 methytransferase identified was KRYPTONITE (KYP) (Du et al., 2014). In *kyp* mutants, many loci that are silenced by DNA methylation become transcriptional reactivated, suggesting that H3K9 methylation plays a role in DNA methylation-mediated silencing (Jackson et al., 2004). Similar to H3K9me2, H3K27 methylation is also a repressive mark found in both animals and plants (Liang et al., 2020). Both H3K27me3 and H3K9me3 are primarily localized to euchromatin (Lindroth et al., 2004). H3K27me3 is particularly interesting as it has been implicated in regulation of development, by providing cellular memory in order to maintain a repressed transcriptional state after cell division (Borg et al., 2020; J. Liu et al., 2019). Importantly, H3K27 trimethylation at genes is reset every generation (Johnson, Cao, & Jacobsen, 2002). The best characterized model for H3K27me3-mediated regulation is the epigenetic setting of *FLC*, a negative regulator of flowering in *Arabidopsis* (Yang, Howard, & Dean, 2014). In a process known as vernalization, prolonged exposure to cold results in somatically inherited silencing of that gene, which in turn promotes flowering (C. Liu et al., 2010). Silencing of *FLC* is initially triggered by non-coding RNAs, which recruit the components of POLYCOMB complex 2 (PRC2) that catalyze H3K27 trimethylation (Angel et al., 2015). Recently, it has been observed that not only does H3K27me3 targets genes,

but can also it target transposons (Montgomery et al., 2020; Tian et al., 2019; T. Zhao et al., 2019).

1.4 RNA-directed DNA methylation (RdDM) in plants

In plants, the RdDM pathway, which is unique to plants, mediates *de novo* DNA methylation (Erdmann & Picard, 2020). Over the past two decades components in the RdDM pathway, including the subunits of Pol IV and PoI V, have been identified through genetic and biochemical approaches (Aufsatz, Mette, van der Winden, Matzke, & Matzke, 2002; M. B. Wang & Dennis, 2009). There are two essential parts in the RdDM pathway: PoI IV-dependent siRNA biogenesis, and PoI V-mediated *de novo* DNA methylation, which involves histone modifications, nucleosome positioning and changes of chromatin structure (Mahfouz, 2010). PoI IV defective mutants fail to produce the precursors of most 24-nucleotide siRNAs that eventually guide methylation and target transposons, suggesting that the siRNA mediated methylation is PoI IV-dependent (Q. Li et al., 2015). Although *in vivo*, PoI IV transcripts have not been observed, it is assumed that PoI IV transcribes single-stranded RNAs at the target loci, which are then copied by the RNA-dependent RNA polymerase RDR2 to form double-stranded RNAs (dsRNAs). Dicer-like protein 3 (DCL3) processes dsRNAs into 24-nucleotide siRNAs that are stabilized by HUA ENHANCER 1 (HEN1) (Matzke & Mosher, 2014). The siRNAs-protein complex is loaded onto a member of the AGO4 clade. A second member of this clade, *AGO6*, together with *AGO4*, can directly interact with PoI V, and this association can be enhanced by RNA-directed DNA methylation 3 (RDM3) (Yaari et al., 2019). Similar to PoI IV, the production of PoI V transcripts requires the protein complex DDR, which consists of the chromatin remodelers RDM3 and RDM1 in association with AGO4 and DRM2(Matzke, Kanno, & Matzke, 2015). Studies from various genome-wide chromatin immunoprecipitation sequencing have revealed that transposons and other repeats associated with 24-nucleotide siRNAs and DNA methylation are the primary targets of PoI V-mediated RdDM (Anderson et al., 2018; Matzke & Mosher, 2014). However, other methylated sites that are not occupied by these chromatin modifications are not targeted by PoI V, suggesting that PoI V alone is not sufficient for RdDM genome-wide (Yaari et al., 2019). In the RdDM pathway, H3K9me2 is a key player because around 70% of RdDM targets are targeted and modified it, which can also act in a feedback loop with DNA methylation to mutually reinforce transcription gene silencing (T. Zhao et al., 2019). In *Arabidopsis*, it has been found that the histone methytransferases SUVH4, SUVH5 and SUVH6 are all closely associated with non-CG

methylation, which is catalyzed by CMT3 (Yamaguchi et al., 2020). Indeed, the SRA domain of the SUVH protein binds to methylated DNA. In the addition, the chromodomain of CMT3 binds to H3K9 methylase, which creates a self-reinforcing loop that facilitates both epigenetic modifications (Johnson et al., 2002). In addition to a repressive histone marks that play a key role in RdDM, some active marks, such as acetylation, H3K4me3 and H2B ubiquitylation are important as well (Song, Zhang, Han, Zhou, & Liu, 2021). Some RdDM targets need histone-modifying enzymes to remove these active marks prior to silencing. For instance, HISTONE DEACETYLASE 6 (HDA6) acts in together with MET1 to maintain CG methylation, and deacetylate histones in order to promote H3K9 methylation (Q. Li et al., 2015).

1.5 Transposable elements (TEs)

Transposable elements (TEs) are DNA fragment that can change position in a genome, which may create or reverse mutations and alter the local epigenetic environment (OKamoto. & Hirochika, 2001; Z. Zhang et al., 2020). Genomes, particularly those of higher eukaryotes, have been proved to be considerably more complex that once anticipated because many of them are composed largely of transposable elements (Johns, Mottinger, & Freeling, 1985; Zilberman. & Henikoff, 2004). TEs are often considered to be “selfish” elements because their replication and activity are often correlated with a negative impact on host genomes (Fedoroff, 2000). Because of this, most organisms have evolved mechanisms to silence them through epigenetic mechanisms, and to keep them that way, primarily DNA methylation and histone modifications (Hirsch & Springer, 2017).

TE content varies in various species (S. N. Anderson et al., 2019; Sarah N Anderson et al., 2019). Unlike most model species, which have relatively small genomes, TEs account for 85% the maize genome (R. K. Slotkin & Martienssen, 2007). Although a majority of those TEs are located in pericentromeric regions and heterochromatic maize knobs, there are many TE insertions interspersed between maize genes (Makarevitch et al., 2015). Recent studies concerning the molecular mechanisms by which TEs are epigenetically silenced have enhanced our understanding of another aspect of their contribution to heterochromatin, which make people think of the possible regulatory role of TEs in gene expression, possibly through epigenetics mechanisms (Fedoroff, 2000; Y.-S. Lee et al., 2021). Indeed, McClintock referred to TEs are “controlling elements” because of their potential ability to influence the expression of nearby genes (Bourque et al., 2018).

A subset of TEs is known to respond to various of abiotic stress treatments and can influence nearby gene expression under stress conditions (Arnault & Dufournel, 1994; Makarevitch et al., 2015). There are several proposed potential mechanisms for this, including providing novel cis-regulatory sequences that can directly facilitating transcription factor binding, or influences by the TEs on the local chromatin state of gene promoter regions ((Warman et al., 2020). These, along with allelic variation in different maize inbred lines, make maize a powerful model system to study the relationship between TEs epigenetics, and gene regulation (Fedoroff, 2000).

1.6 Epigenetic regulation of TEs

Transposons are potentially dangerous genomic parasites (Feschotte, Jiang, & Wessler, 2002; Stitzer, Anderson, Springer, & Ross-Ibarra, 2019). Given the vast number of TEs in most eukaryotic genomes, particularly in the maize genome, global activation of TEs could be lethal (Fedoroff, 2000; J. Liu, Feng, Li, & He, 2015; Okamoto & Hirochika, 2001). In response to this threat, most host organism have evolved mechanisms to silence TEs, and most TEs in all species examined to date are silenced, both transcriptionally and transpositionally, through epigenetic mechanisms. These mechanisms involve two distinct stages, recognition of active TEs to be silenced, and maintenance of this silencing over long periods of time, even in the absence of initial trigger, which can be propagated through multiple rounds of mitotic cell division or meiotically, over multiple generations (Quadrana & Colot, 2016). Because TEs are the primary target of epigenetic silencing, they are an ideal model to study the means by which DNA sequences are targeted for silencing, and how such silencing can be maintained from one generation to the subsequent (Okamoto & Hirochika, 2001). In addition, TEs have proved to be sensitive to various abiotic and biotic stresses, which can affect their epigenetic status, which can in turn be associated which changes in growth, development and stress response. This gives us an opportunity to study the relationship between stress response, epigenetically encoded memory of stress and development (Bennetzen & Wang, 2014; H. Ito et al., 2016).

In plants, the primary targets of RdDM are small TEs and TEs immediately adjacent to genes, which are usually located in open and accessible euchromatic regions (Singer, Yordan, & Martienssen, 2001). In these regions, the open state associated with expressed genes can spread to nearby silenced TEs, which can potentially cause those TEs to become activated (Dubin, Mittelsten Scheid, & Becker, 2018; Makarevitch et al., 2015). Conversely, because of the self-reinforcement

loop between RdDM and DNA methylation, RdDM activity in turn can recruit other pathways that help establish and propagate the heterochromatic state to nearby genes (Makarevitch et al., 2015; R. K. Slotkin & Martienssen, 2007). Once silencing is established, maintenance of silencing is almost invariably associated with DNA methylation (Thiebaut, Hemerly, & Ferreira, 2019). This is particularly true because both CG and CHG sequence context methylation are symmetrical so that prior DNA methylation can be easily propagated by methylating newly synthesized DNA strands using the previous parent stand as a template (Kenchanmane Raju, Ritter, & Niederhuth, 2019). As a result, DNA methylation can be heritably propagated through cell divisions in the somatic tissues or transgenerationally. Maintenance methylation of CHH is much complicated and involves RdDM and CHROMETHYLASE (CMT2) that works in conjunction with H3K9me2 to methylate non-CG cytosines (Fu et al., 2013). Chromatin remodelers such as DDM1 are also required for maintenance of TE silencing (H. Zhang et al., 2018). In fact, effective silencing of TEs requires a coordination between DNA methylation and histone modifications (Wittmeyer et al., 2018). In plants, loss of DNA methylation is often associated with TE reactivation(Anderson et al., 2018). A number of TEs, including MULEs (*Mutator*-like elements) are reactivated in *ddm1* and *met1* mutants in Arabidopsis (H. Ito et al., 2016; Singer et al., 2001). In addition to chromatin-remodeling factors, genes required for DNA methylation, histone modification, and RNAi also play a role in TE silencing in both plants and animals (P. Negi, A. N. Rai, & P. Suprasanna, 2016). TE silencing actually requires a network of almost all epigenetic components (Le et al., 2014). Although most TEs are epigenetically silenced, under stress conditions TEs can become transcriptionally or transpositionally reactivated. Because genes in many plant species are interspersed with TEs, the resulting reactivated TEs can affect adjacent genes (Joly-Lopez et al., 2017). Heat stress, has drawn particular attention because of its global effect on both genes and TEs (Pooja Negi et al., 2016; T.-Y. Zhao, Meeley, & Downie, 2003). For instance, the ONSEN retrotransposon is more sensitive to heat in RdDM pathway mutants and new insertions can be transmitted to the next generations (O. V. Popova, H. Q. Dinh, W. Aufsatz, & C. Jonak, 2013). Indeed, many TEs become reactivated upon heat stress, and this activity is associated with a global change in 3D structure of genome (Sun et al., 2020).

1.7 The minimal *MuDR* system

To study transposon biology, *Arabidopsis* has some advantages because of its smaller

genome, short life cycle, a number of available mutants that affect TE activity. However, most autonomous elements, which can move on their own, have not been identified, and, because the genome is smaller, there are fewer TEs. In this respect, maize is a unique system, because 85% of its genome is composed of TEs, and many autonomous elements have been characterized (Damon Lisch, 2002). However, due to the different classes and copy numbers of those TE, it is difficult to perform genetic analysis, which requires an understanding of cause effect relationships between known autonomous elements and mutants and environmental conditions (Nuthikattu et al., 2013). For my dissertation work, I use the minimal *Mutator* line as a model to study TE silencing. *Mutator-like* Elements (MULEs) have been identified in plants, fungi, and bacteri, and are generally characterized by relatively long, 220-bp terminal inverted repeats (TIRs) and are regulated by autonomous elements encoding transposases (Q. Li et al., 2015; R. K. Slotkin, Freeling, & Lisch, 2005). The *Mutator* family in maize consists of the autonomous *MuDR* element as well as several classes of non-autonomous elements (Damon Lisch, 2002). The high copy numbers of active autonomous and non-autonomous elements in most *Mutator* lines makes it difficult to perform genetic analysis (H. Li, Freeling, & Lisch, 2010). Therefore, I use a minimal *Mutator* line, which contains a single functional autonomous element *MuDR* at position one on chromosome 2, and a single nonautonomous element, *Mu1*, in the promotor region of an allele of the *A1* color reporter gene (*a1-mum2*) (O'Reilly et al., 1985). *MuDR* elements carry two genes, *mudrA*, a transposase-encoding gene, and *mudrB*, a helper gene that is required for insertional activity (Burgess, Li, Zhao, Kim, & Lisch, 2020). The *MuDR* TIRs contain the promoters for both *mudrA* and *mudrB*, of which methylation is typically associated with silencing of *Mutator* elements (Woodhouse, Freeling, & Lisch, 2006). This system provides a monitoring system in which to easily track the excision and duplication events of a single active *Mutator* element (Burgess et al., 2020). Excision of *Mu1* from *a1-mum2* results in pigmented spots in the aleurone of the kernel, which is an indicator of transposase activity (R. K. Slotkin et al., 2005). Unlike high copy *Mutator* lines, the minimal line does not exhibit spontaneous silencing, which makes it a stable system to perform genetic analysis of factors influencing epigenetic regulator of the *Mutator* elements (H. Li et al., 2010; Woodhouse et al., 2006).

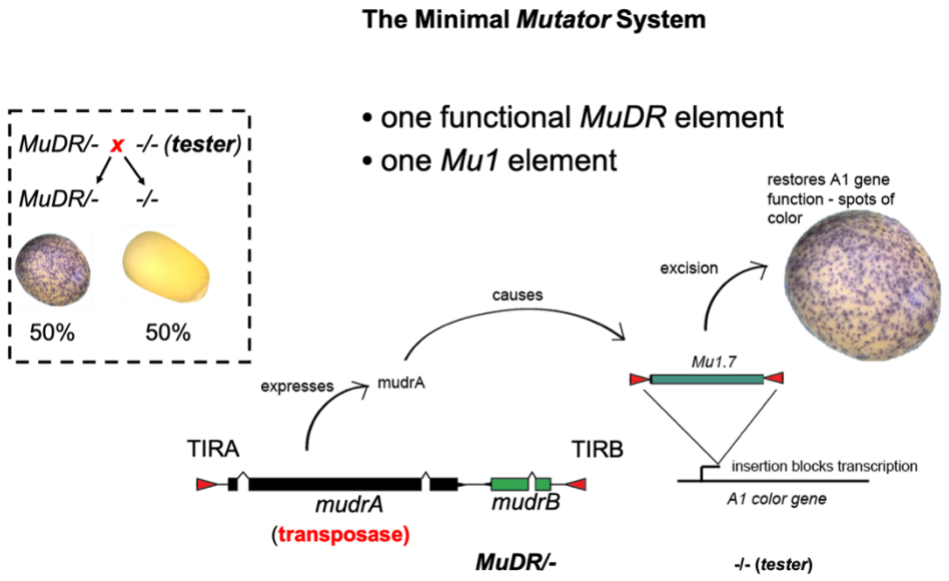

Figure 1.1 The minimal *Mutator* system

Mu killer (*Muk*) is a single dominant allele that can heritably and reliably silence the *Mutator* system. *Muk* is a variant of *MuDR* that carries an inverted and duplicated portion of the *mudrA* gene and its activity can result in silencing of *mudrA* in the first generation, and in the next generation, *mudrB* is also be silenced subsequently (Damon Lisch, 2002; R. K. Slotkin et al., 2005). In the presence of *Muk*, all *Mu* TIRs are methylated, which is associated with loss of *Mutator* activity. Because silencing of *MuDR* can be stably maintained in the absence of *Muk*, the *MuDR/Muk* system can be used as a model to study both initiation and maintenance of silencing (Woodhouse et al., 2006). Our previous work has demonstrated that DNA methylation in all three sequence contexts at TIRA is associated with silencing of *mudrA* element, which corresponds to *Muk*-derived 22 nt siRNAs that trigger silencing. Silencing of *mudrA* is also associated with H3K9me2, another hallmark of epigenetic silencing (H. Li et al., 2010). Interestingly, the second gene encoded by *MuDR*, *mudrB*, is silenced through distinct mechanisms (Woodhouse et al., 2006). Indeed, experiments using deletion derivatives of *MuDR* that only carry *mudrB* are not silenced by *Muk*, and siRNA profiling indicates that *Muk*-derived siRNAs are specifically targeteed to *mudrA*, which suggests that silencing of *mudrB* is likely triggered by *mudrA* silencing in *cis* and this involves spreading of silencing information between these two genes (Burgess et al., 2020; R Keith Slotkin, Freeling, & Lisch, 2003).

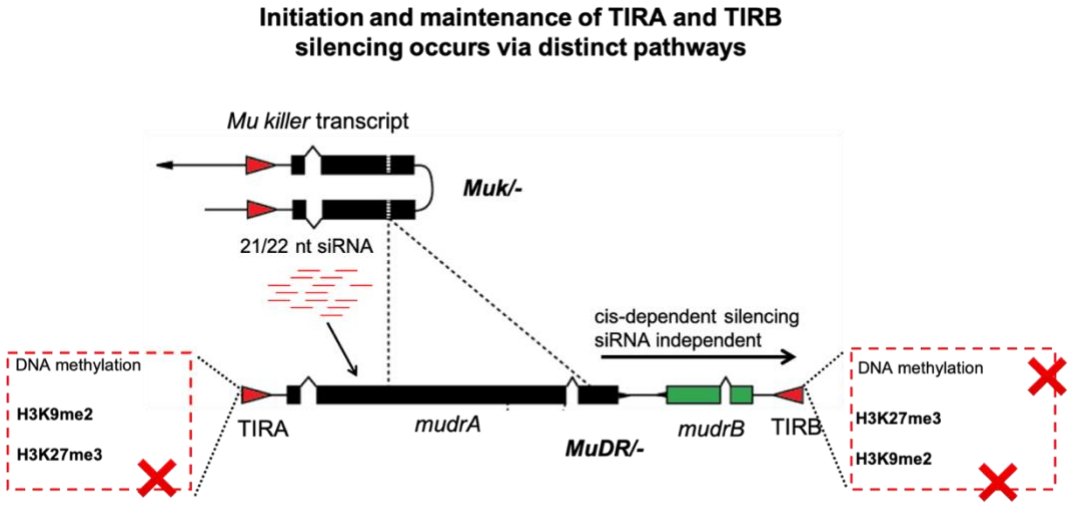

Figure 1.2 The minimal *Mutator-Muk* system

1.8 *Mediator of paramutation 1* (*Mop1*) and TE silencing

Paramutation is the phenomenon one allele can induce a heritable change in the other allele possibly via changes in histone modifications or DNA methylation via trans-acting small RNAs (Damon Lisch, Carey, Dorweiler, & Chandler, 2002; Pikaard & Mittelsten Scheid, 2014; Woodhouse et al., 2006). Mutations of *Mediator of paramutation 1* (*Mop1*), a homolog of *RNA-DEPENDENT RNA POLYMERASE2* (*RDR2*), can prevent establishment of paramutation of the paramutable alleles of *b1*, *r1*, and *pl1*, and increase the expression of the paramutagenic *b1* and *pl1* alleles (Damon Lisch et al., 2002). In the maize genome, RdDM activity is primarily associated with TEs near genes (Matzke & Mosher, 2014). Mutations in components of the RdDM pathway affect both paramutation and transposon silencing, likely because both paramutation and TE silencing are mechanistically linked. Mutations in *Mop1*, result in the loss of almost all 24-nucleotide small RNAs (Burgess et al., 2020; Madzima, Huang, & McGinnis, 2014; P.-H. Wang, Wittmeyer, Lee, Meyers, & Chopra, 2017; Woodhouse et al., 2006). Previous work has shown that this mutant can reverse *Mutator* silencing (Damon Lisch et al., 2002; Woodhouse et al., 2006). Although *mop1* affects both paramutation and *Mutator* TIR methylation, it does not affect global methylation except for CHH methylation nor does it influence the methylation pattern of TEs in deeply silenced heterochromatin. Silenced *MuDR* elements can be progressively reactivated in *mop1* mutants, but only after multiple generations (Woodhouse et al., 2006). However, although

mudrA becomes transcriptionally reactivated, *mudrB* is remains silenced, suggesting that maintenance of silencing of these two genes are via distinct mechanisms (Woodhouse et al., 2006). Despite high levels of somatic activity as it revealed by spotted kernels, we do not see any new insertions in progeny, which is consistent with the observation that *mudrB* is required for those insertions (H. Li et al., 2010; Woodhouse et al., 2006). The observation that a silenced TE gene in a *mop1* background is only progressively reactivated suggests that changes in the repressive epigenetic state can be gradually lost, which provides an opportunity for us to study the transmission and maintenance of a memory in general.

1.9 Heat stress response in plants

Abiotic stresses such as heat, salinity and drought can have a devastating impact on plant growth, development and reproduction. Among all these stress conditions, heat stress, likely to be an increasing problem as the planet warms, has profound and diverse effects on plants (Bita & Gerats, 2013). Because of their sessile nature, in order to cope with environmental conditions, plants have evolved sophisticated mechanisms to perceive and respond to those conditions (X. Wang et al., 2016). Great progress has been made on identification of genetic and epigenetic factors, pathways and network in heat stress signaling pathways (Bäurle, 2016). This has enhanced our understanding of gene regulation, epigenetic memory and has begun to make it possible to begin to breed more heat tolerant plant.

The mechanisms of plant heat stress response have been well studied (H. C. Liu et al., 2018). It is a network involving perception, transmission and response, which can globally affect flowering, modulation of circadian clock and immune responses (Scharf, Berberich, Ebersberger, & Nover, 2012). MADS-box genes are key regulators of the genetic regulatory network involved in flowering time regulation. *SHORT VEGETATIVE PHASE* (*SVP*) and *FLOWERING LOCUS M* (*FLM*) can modulate flowering time in response to a mild temperature change (J. Liu et al., 2015). These genes act as flowering repressors and can trigger early flowering time independent of the photoperiod pathway (J. Zhao, Lu, Wang, & Jin, 2021). Interestingly, it has been observed that *FLM* is subject to temperature-dependent alternative splicing, which is consistent with the observation that the RNA processing-related genes are induced upon heat stress (Horváth, Merenciano, & González, 2017). In order to coordinate with photocycles and thermocycles, the circadian clock, a cellular time-keeper mechanism, allows plants to fine-tune endogenous

biological rhythms via three interlocked transcriptional feedback loops (Bita & Gerats, 2013). Higher temperatures can increase the binding affinity of *circadian clock associated 1* (*CCA1*) to the promoters of the oscillator genes, which is antagonized by the protein kinase *CASEIN KINASE2* (*CK2*) and the transcription factor *FLOWERING BASIC HELIX-LOOP-HELIX 1* (*FBH1*) (J. Liu et al., 2015). In addition, the activities of *CCA1* and *LATE ELONGATED HYPOCOTYL* (*LHY*), a *MYB* transcription factor, are counter balanced in a temperature-dependent manner, which in turn, can promote signal transduction through the network (Sun et al., 2020). In natural settings, plants routinely experience a combination of two or more different abiotic and biotic stresses. Extreme environmental conditions, such as heat, salinity and pathogen attack can have a severe impact on plant growth, development and reproductivity due to the cumulative effects of multiple stressors (He et al., 2019). Recent studies have revealed that in response to a single heat stress treatment applied to a single leaf, plants can mount a global stress-specific systemic response. Heat stress can inhibit the effector triggered immunity (ETI) and enhance RNA-silencing mediated resistance (J. Liu et al., 2015). *SUPPRESSOR OF npr1-1, CONSTITUTIVE 1* (*SNC1*) can mediate inhibition of resistance under high temperature (Alcázar & Parker, 2011; S. Zhang & Wang, 2011; Zhu, Qian, & Hua, 2010). In addition, abscisic acid (ABA) positively mediates inhibition of disease resistance in response to heat stress (Sanchez & Paszkowski, 2014).

1.10 Genetic regulation of heat and other abiotic stress response

Many key regulators and pathways associated with heat stress have been identified, including *heat shock factors* (*HSFs*), *heat shock proteins* (*HSPs*), reactive oxygen species (ROS), phospholipids, calcium signaling pathways and the network of hormone pathways (Sanchez & Paszkowski, 2014). Thermotolerance consists of basal and acquired phases (Scharf et al., 2012). Basal thermotolerance is an immediate response after exposure to high temperatures, while acquired thermotolerance refers to the ability to cope with extreme temperatures after acclimatization to mild high temperatures (J. Liu et al., 2015). Although great progress has been made in elucidating the molecular mechanisms of thermotolerance, the means by which plants sense and transmit the signal is still poorly understood (J. Zhao et al., 2021). Several putative heat sensors have been proposed, including a plasma membrane cyclic nucleotide gated calcium channel (CNGC) and two protein sensors located in the endoplasmic reticulum (ER) and the cytosol (Bita & Gerats, 2013). In response to heat, two basic leucine-zipper domain-containing

transcription factors, bZIP17 and bZIP28, are translocated to the nucleus and activate ER chaperone genes and brassinosteroid signaling, which is required to promote subsequent heat adaption(J. Liu et al., 2015). Taken together, these observations indicate that the plant heat sensor may be primarily located in the plasma membrane, ER and cytosol and function in transmitting the signal to the downstream network. HSFs are a family of transcriptional activators that act upstream of hormone signaling pathways and can also form a protein complex to directly regulate *HSP* expression under heat stress, which in turn can stabilize proteins and facilitate protein refolding during the heat stress response (J. Liu et al., 2015). In addition, a variety of signaling molecules, such as, nitric oxide, calcium, H_2O_2, and phospholipids are also involved in plant thermotolerance as well, which can directly regulate the expression of HSFs and HSPs, protecting cells against heat stress-induced damage(J. Liu et al., 2015). Additionally, retrograde signaling in chloroplasts also appears to be involved in heat stress response. In response to heat, the *chloroplast ribosomal protein S1* (*RPS1*) is induced and plays a critical role in regulating the translation of thylakoid proteins in order to maintain the stability and integrity of thylakoid membranes(Scharf et al., 2012). Thus, the protein products translated in the chloroplast can send signals back to the nucleus and activate HsfA2-dependent genes, rendering plants heat tolerant(J. Liu et al., 2019).

1.11 Epigenetic regulation of heat stress response

There is a great deal of evidence that environmental stresses can alter the epigenetic state globally (J. Liu et al., 2015; Yamaguchi et al., 2020). In cotton anthers, *S-ADENOSYL-LHOMOCYSTEINE HYDROLASE1* (*SAHH1*) and DNA methy-transferases (*DRM1* and *DRM3*) are down-regulated in response to heat, which results in the global hypomethylation at the tetrad stage. However, in *Arabidopsis*, *DRM2* and *NUCLEAR RNA POLYMERASE D 1* (*NRPD1*) are up-regulated in response to heat stress, and this may contribute to an observed increased genome-wide methylation in this species (Ferrafiat et al., 2019). Previous genetic analysis has shown that *nrpd2* mutants are hypersensitive to heat stress, suggesting that the RdDM pathway plays a role in maintaining global DNA methylation and helping to ameliorate the effects of heat stress (Olga V Popova, Huy Q Dinh, Werner Aufsatz, & Claudia Jonak, 2013). In *Brassica napus*, it has been observed that global levels of DNA methylation are increased more in the heat-sensitive accessions compared with that in the heat-tolerant lines under heat stress (Gao et al., 2014; J. Li et al., 2016; G. Liu, Xia, Liu, Dai, & Hou, 2018). Although global DNA methylation is subject to change under

stress conditions, there is no consistent trend of this modification in various species(Bäurle, 2016). Methylation at some loci may be the key to trigger downstream signals under stress conditions.

Similar to DNA methylation, histone modifications can be affected by stress conditions (Gao et al., 2014; Sallam & Moussa, 2021; J. Zhao et al., 2021). In rice, for instance, histone modifications are involved in seed development at high temperature (Bej & Basak, 2017). For instance, the level of H3K9me2 at *OsFIE1*, a polycomb group gene, is sensitive to heat stress during seed development; heat stress reduces H3K9me2 at *OsFIE1*, which results in an increase in H3K27me3 (Folsom, Begcy, Hao, Wang, & Walia, 2014; Ye et al., 2015). In cotton anthers, it has been reported that one histone methyltransferase and two JUMONJI C (JMJC) domain-containing genes are down regulated in response to heat stress(Gan et al., 2014). In *Chlamydomonas reinhardtii*, the global level of H3/4 acetylation and H3K4me1 is changed under heat stress conditions (Song et al., 2021). In *Arabidopsis*, the transcription factor *HSF1* mediates a global histone3 H3/4 acetylation and recomposition of H3K4 methylation(Hu et al., 2015; J. Lin et al., 2020). Indeed, multiple studies suggest that heat-induced release of silencing seems to be associated with histone acetylation. However, in the histone deacetylase mutant, *hda6*, both the level of H3K9me2 and H3K4me3 is significantly reduced in response to heat (Hu et al., 2015; Hung et al., 2018; J. Lin et al., 2020). After long term heat stress, nucleosomes and all the histone marks associated with genes were removed and reloaded to the chromatin, which exhibit a return to that observed under ambient temperatures, suggesting that histone modifications may not play an important role in heat-induced release of epigenetic silencing. Very recently, it has been shown that JUMONJI (JMJ) proteins are involved in histone H3K27 trimethylation, which is necessary to maintain heat induced activity in *jmj* mutants, suggesting that the knowledge underlying the role of histone modification in heat stress response remain elusive (J. Liu et al., 2019).

Small RNAs can also play a role in sensing environment stimuli and regulating genes (Pan et al., 2018; Yan et al., 2012). micro-RNAs, for instance, appear to be involved in a broad range of responses to stress in addition to their well-documented effects on various aspects of development (He et al., 2019; J.-S. Lin et al., 2018). Although many miRNAs are known to be induced by stress conditions, few of them have been validated (J.-S. Lin et al., 2018) (Barciszewska-Pacak et al., 2015). A well characterized example of involvement of a microRNA in response to stress is miRNA156. miRNA156 is dramatically induced upon heat stress and targets *SQUAMOSA-PROMOTER BINDING-LIKE* (*SPL*) transcription factor genes, which are essential

for plant development under stress conditions (Chao et al., 2017). Natural antisense siRNAs (nat-siRNAs) are also known to be involved in stress response (Borsani, Zhu, Verslues, Sunkar, & Zhu, 2005; Xu et al., 2017). For instance, nat-siRNAs derived from a natural cis-antisense transcript pairs of genes, *SRO5* and *P5CDH* are induced by salt stress, and target the antisense transcript of *P5CDH* for degradation (Borsani et al., 2005). Under heat stress conditions, the pattern of diverse exogenous and endogenous siRNAs is changed dramatically. The abundance of many endogenous tasi-RNAs is significantly reduced under heat, which is correlated with an increased abundance of transcripts of tasiRNA-target genes, along with a global release of epigenetic silencing (Zhong et al., 2013). Recently, it has been reported that phased plant 22-nt siRNAs can mediate translational repression, development and stress response. Under heat stress, MALE-ASSOCIATED ARGONAUTE-1 and -2 are associated with heat-induced phased secondary siRNAs in pre-meiotic maize anthers, which is essential to control the activity of TEs (Casacuberta & González, 2013; Wheeler, 2013).

1.12 Stress memory and inheritance

Because plants are sessile they must cope with and even anticipate recurring stresses in the natural environment (Hidetaka Ito et al., 2016; Quadrana & Colot, 2016). Long-term agronomic practices have revealed that plants can be primed to become more tolerant to recurring stresses by a transient stress treatment, possibly by establishing an "alarm state" that render plants broadly resistant to subsequent stresses (H. C. Liu et al., 2018; X. Wang et al., 2016). Indeed, some modifications at certain responsive loci have been observed to be primed under recurring stress conditions, and these changes tend to become both stronger and more rapid. In this way, plants may have the capacity to remember how to cope with stresses and to transmit this memory to the new emerging tissues or even to subsequent generations (Heard & Martienssen, 2014; Migicovsky, Yao, & Kovalchuk, 2014; Pikaard & Mittelsten Scheid, 2014; Quadrana & Colot, 2016). Transmission of this memory may offer an adaptive advantage (Crisp. et al., 2016). In *Arabidopsis*, exposure to UV light can lead to a transgenerational increase of *HRF* gene expression (Boyko et al., 2010; Pikaard & Mittelsten Scheid, 2014; Rahavi & Kovalchuk, 2013). Heat-induced changes can also exhibit a transgenerational behavior. In the progeny of *dicer-like* mutants *dcl12* and *dcl3*, the heritable phenotypic and epigenetic changes are partially deficient, suggesting that these changes are dependent on DNA methylation and the function of Dicer-like proteins

(Duempelmann, Skribbe, & Bühler, 2020; Migicovsky et al., 2014; Rahavi & Kovalchuk, 2013). Under stress conditions, many genes and TEs can be induced by stress (Feng, Leem, & Levin, 2013; Horvath, Merenciano, & Gonzalez, 2017). Once reactivated, it will be interesting to see how many exhibit transgenerational inheritance of that activity, which will allow us to better understand how of epigenetic memory is maintained or lost (Dubin et al., 2018; Horvath et al., 2017; Lamke, Brzezinka, Altmann, & Baurle, 2016; Lang-Mladek et al., 2010; Rahavi & Kovalchuk, 2013). Indeed, transgenerational of heat-induced retrotransposition of *ONSEN* is defective in the RdDM pathway mutants, suggesting the RdDM pathway plays a key role in genome defense under stress conditions (Duempelmann et al., 2020; Ito et al., 2011; Migicovsky et al., 2014). In contrast to TEs, most genes that are silenced during development in plants are targeted by H3K27 trimethylation, which is largely mediated by the PRC2 and PRC1 complexes (Borg et al., 2020; Tian et al., 2019; Yang et al., 2014; T. Zhao et al., 2019). A well-studied model of H3K27me3 in plants is *FLC*, an *Arabidopsis* floral repressor protein (Angel et al., 2015; Quadrana, 2020). In the process of vernalization, prolonged exposure to cold can result in somatically heritable silencing of *FLC,* which in turn results in flowering (Angel et al., 2015; Tian et al., 2019). A forward genetic screen has identified two key components, *DDM1* and *MOM1*, chromatin remodelers that function redundantly in preventing the transmission of stress-induced changes to the progeny of stress-treated plants (Annacondia, Magerøy, & Martinez, 2018; Iwasaki, 2015; Iwasaki & Paszkowski, 2014; Singh & Roberts, 2015). Unlike animals, plants do not experience a global wave of DNA demethylation either in the germinal cells or in the early embryo, which means that DNA methylation and histone modification may be a possible mechanism for stably propagated silencing (Gallego-Bartolome, 2020; Heard & Martienssen, 2014). Indeed, there is evidence that mutants that trigger a global loss of DNA methylation can a heritable reactivate of a subset of previously silenced TEs (Cheung & Lau, 2005; Friedrich, Faivre, Baurle, & Schubert, 2019; Lamke et al., 2016; H. C. Liu et al., 2018; Mosammaparast & Shi, 2010). However, the role of DNA methylation and histone modification in transgenerational memory is inconclusive (Annacondia et al., 2018). Similar to what being discussed previously, the changes of a chromatin modification at certain sites may be responsible for maintaining or transmitting a stress memory.

1.13 Research significance and objectives

Because epigenetics breaks the rules of Mendelian inheritance, it has long fascinated geneticists (Dubin et al., 2018; Fedoroff, 2000). Although epigenetic phenomena have been observed for many decades, we are only beginning to understand the mechanism by which genes can be heritably silenced or reactivated (Jaenisch & Bird, 2003; Lamke & Baurle, 2017; Pikaard & Mittelsten Scheid, 2014). TEs are mobile genetic parasites that inhabit the genomes of all known life forms, from viruses to humans (OKamoto. & Hirochika, 2001; Zilberman. & Henikoff, 2004). When they increase their copy number, TEs can cause mutations and diseases (Dubin et al., 2018; P. Negi et al., 2016). Because of that, all organisms have evolved strategies to epigenetically silence these invasive parasites (Jaenisch & Bird, 2003). The presence of high numbers and multiple types of transposons in plant genomes make it difficult to perform genetic analysis of these elements (Damon Lisch, 2002; Woodhouse et al., 2006). Here, I am using a minimal *Mutator* line that only contains a single functional *MuDR* element to dissect the precise mechanism of the maintenance of transposon silencing, and the intersection of epigenetic regulation and environmental stress using the *MuDR* line as a model. To do that, I am applying a combination of genetics, biochemistry, and molecular biology approaches to characterize an example of transgenerational transmission of environmentally induced reactivation of a silenced TE that is not associated with changes in DNA methylation. This is intriguing because my dissertation results suggest that heat stress integrates two distinct epigenetic pathways to reactivate a silenced transposon. These observations give me an opportunity to examine global effects of stress on plants that are compromised with respect to DNA methylation. To leverage the specificity of my observations made at bench, I also performed a transcriptome analysis in *mop1* mutants under heat. I found that a higher numbers of TEs are reactivated in *mop1* mutants under heat, which is consistent with the effects I observed on *MuDR*. Taken together, my dissertation work will provide an opportunity to better understand the causes and consequences of TE silencing and reactivation, as well as the effects of TEs on gene regulation under stress conditions.

CHAPTER 2. MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF A SILENCED MUDR ELEMENT IN *MOP1* MUTANTS

2.1 Introduction

In plants, heritable epigenetic silencing of TEs is almost invariably associated with DNA methylation. The vast bulk of TEs in plant genomes are methylated and, with some notable exceptions, epigenetically silenced. DNA methylation has a number of features that makes it an appealing mechanism by which silencing can be heritably propagated, either following cell divisions during somatic development, or transgenerationally, from one generation to the next. Because methylation in both the CG and CHG sequence contexts (where H = A, T or G) are symmetrical, information concerning prior DNA methylation can be easily propagated by methylating newly synthesized DNA strands using the parent strand as a template (Pikaard & Mittelsten Scheid, 2014). For CG methylation, this is achieved by reading the methylated cytosine using VARIANT IN METHYLATION 1-3 (VIM1-3) and writing new DNA methylation using the methyl transferase MET1. For CHG, methylation is read indirectly by recognition of H3K9 dimethylation (H3K9me2) by CMT3, which catalyzes methylation of newly synthesized DNA, which in turn triggers methylation of H3K9 (C. Liu et al., 2010).

Maintenance methylation of most CHH involves RNA-directed DNA methylation (RdDM) (Erdmann & Picard, 2020; Matzke et al., 2015). The primary signal for *de novo* methylation of newly synthesized DNA from previously methylated DNA sequences is thought to be transcription by DNA POLYMERASE IV (POLIV) of short transcripts from previously methylated templates(Q. Li et al., 2015). This results in the production of small RNAs that are tethered to the target DNA by DNA POLYMERASE IV (POLV), which is targeted by SU(VAR)3-9 homologs SUVH2 and SUVH9, which bind to methylated DNA (Aufsatz et al., 2002). This in turn triggers *de novo* methylation of newly synthesized DNA strands using the methyl transferases DRMT1/2. In addition to the RdDM pathway, CHH methylation can also be maintained due to the activity of CHROMOMETHYLASE (CMT2), which, similar to CMT3, works in conjunction with H3K9me2 to methylate non-CG cytosines, particularly in deeply heterochromatic regions of the genome (Deal & Henikoff, 2011). Finally, because both histones and DNA must be accessible in order to be modified, chromatin remodelers such as DDM1 are also often required for successful

maintenance of TE silencing. In plants, effective silencing of TEs requires coordination between DNA methylation and histone modifications (Quadrana, 2020). Together, these pathways can in large part explain heritable propagation of both DNA methylation and histone modification of TEs (Mahfouz, 2010).

In large genomes such as that of maize, much of RdDM activity is focused not on deeply silenced heterochromatin, which is often concentrated in pericentromeric regions, but on regions immediately adjacent to genes, referred to as "CHH islands" because genes in maize are often immediately adjacent to silenced TEs (Erdmann & Picard, 2020; Q. Li et al., 2015). In maize, mutations in components of the RdDM pathway affect both paramutation and transposon silencing. Mutations in *Mutation of Paramutation 1* (*Mop1*), a homolog of RNA DEPENDENT RNA POLYMERASE2 (RDR2), result in the loss of nearly all 24 nucleotide small RNAs, as well as the CHH methylation that is associated with them (Burgess et al., 2020; Erdmann & Picard, 2020; Woodhouse et al., 2006). Despite this, *Mop1* has only minimal effects on gene expression in any tissue except the meristem, and the plants are largely phenotypically normal (Q. Li et al., 2015). This, along with similar observations in *Arabidopsis*, has led to the suggestion that the primary role of RdDM is to reinforce boundaries between genes and adjacent TEs, rather than to regulate gene expression (Burgess et al., 2020; Erdmann & Picard, 2020; Woodhouse et al., 2006). However, it should be noted that the *mop1* mutation can in some cases have effects on plant phenotype. Further, *mop1* mutants can enhance the effects of exogenously applied ABA and mutants of Required to maintain repression6 (*Rmr6*), a homolog of the PolIV subunit NRPD1, are altered in their response to drought, suggesting that the RdDM pathway may play a role in buffering stress responses in maize (Damon Lisch, Guo, & Wang, 2021). Further, even in wild type backgrounds, there is evidence that the process of heritable paramutation of an allele of *R1*, which is known to be dependent on RdDM, is sensitive to changes in temperature and light during specific stages of development (Erdmann & Picard, 2020; Mahfouz, 2010; Matzke et al., 2015; Woodhouse et al., 2006).

Our model for epigenetic silencing is the *Mutator* system of transposons in maize. When *mudrA* is silenced, DNA methylation in all three sequence contexts accumulates within the 5' end of the TIR immediately adjacent to *mudrA* (TIRA). Methylation at the 5' and 3' portions of this TIR have distinctive causes and consequences. The 5' end of this TIR is readily methylated in the absence of the transposase, but this methylation does not induce transcriptional silencing of *mudrA*.

Methylation in this end of TIRA is readily eliminated in the presence of functional transposase (Damon Lisch, 2002). However, the loss of methylation in a silenced element in this part of the TIRA does not result in heritable reactivation of a silenced element. In contrast, CG and CHG methylation the 3' portion of TIRA, which corresponds to the *mudrA* transcript as well as to *Muk*-derived 22 nt small RNAs that trigger silencing, is not eliminated in the presence of active transposase and is specifically associated with heritable transcriptional silencing of *mudra* (Burgess et al., 2020; Wheeler, 2013; Woodhouse et al., 2006). The second gene encoded by *MuDR* elements, *mudrB*, is also silenced by *Muk*, but the trajectory of silencing of this gene is entirely distinct, despite the fact that the *Muk* hairpin has near sequence identity to the TIR adjacent to *mudrB* (TIRB) (R. K. Slotkin et al., 2005). By the immature ear stage of growth in F1 plants that carry both *MuDR* and *Muk*, *mudrA* is transcriptionally silenced and densely methylated (Damon Lisch et al., 2021; Woodhouse et al., 2006). In contrast, *mudrB* in intact elements remains transcriptionally active in this tissue, but its transcript is not polyadenylated. It is only in the next generation that steady state levels of transcript become undetectable (Damon Lisch et al., 2021; R Keith Slotkin et al., 2003; Woodhouse et al., 2006). Further, experiments using deletion derivatives of *MuDR* that carry only *mudrB* are not silenced by *Muk* when they are on their own, or when they are in trans to an intact *MuDR* element that is being silenced by *Muk* (Damon Lisch, 2002; Woodhouse et al., 2006). This suggests that heritable silencing of *mudrB* is triggered by the small RNAs that target *mudrA*, but the means by which this occurs is indirect and involves spreading of silencing information from *mudrA* to *mudrB* (Burgess et al., 2020; Wheeler, 2013; Woodhouse et al., 2006).

Silencing of *mudrA* can be destabilized by the *mop1* mutant, a homolog of *RNA-DEPENDENT RNA POLYMERASE2* (*RDR2*) that is required for the production of the vast bulk of 24 nt small RNAs in maize, including those targeting *Mu* TIRs (Burgess et al., 2020; Q. Li et al., 2015; Singer et al., 2001; R. K. Slotkin et al., 2005; Woodhouse et al., 2006). However silencing of *MuDR* by *Muk* is unimpeded in a *mop1* mutant background, likely because *Muk*-derived small RNAs are not dependent on *MOP1* (Burgess et al., 2020; Wheeler, 2013; Woodhouse et al., 2006). Further, although reversal of silencing of *MuDR* in a *mop1* mutant background does occur, it only occurs gradually, over multiple generations, and only affects *mudra* (Burgess et al., 2020; Woodhouse et al., 2006). In contrast, *mudrB* is not reactivated in this mutant background and, because *mudrB* is required for insertional activity, although these reactivated

elements can excise during somatic development, they cannot insert into new positions (Q. Li et al., 2015; Woodhouse et al., 2006).

Previous observations showed that *MuDR* elements are only reactivated after multiple generations in a *mop1* mutant background, and that silencing of *mudrA* can be maintained in the absence of DNA methylation in this mutants (H. Li et al., 2010; Woodhouse et al., 2006). Because transposon silencing is often associated with H3K27me2, and DNA methylation that is usually linked with H3K9 dimethylation via a self-reinforcing loop (Q. Li et al., 2015; D. Lisch, 2009; Mahfouz, 2010), I hypothesize that additional repressive histone modifications may be responsible for maintaining the silencing state at TIRA in *mop1* mutants. To test this hypothesis, I decided to examine both DNA methylation and histone modifications, H3K9me2 and H3K27me2 at TIRA by performing genomic bisulfite sequencing and chromatin immunoprecipitation assays. Unlike *mudrA*, *mudrB* is never reactivated in a *mop1* mutant background under normal conditions (Woodhouse et al., 2006), suggesting that maintenance of silencing of these two genes is through distinct epigenetic mechanisms. Because our lab previously found that silencing of *mudrB* is not associated with DNA methylation. Instead, it's associated with H3K27 trimethylation, I will examined H3K27me3 at TIRB to understand how silencing of *mudrB* is maintained in *mop1* mutants as well.

2.2 Materials and Methods

2.2.1 Plant materials and growth conditions

Maize seedlings and adult plants were grown in MetroMix under standard long-day greenhouse conditions at 26°C unless otherwise noted. The minimal Mutator line consists of one full-length functional *MuDR* element and one nonautonomous Mutator element, *Mu1*. *Mu* killer (*Muk*), a derivative version of the *MuDR* transposon, can heritably trigger epigenetic silencing of that transposon. Mutator activity is monitored in seeds via excisions of a *Mu1* element inserted into the *a1-mum2* allele of the *A1* gene, resulting in small sectors of revertant tissue, or spots, in the kernels when activity is present. When *MuDR* activity is absent, the kernels are pale. All plants described in these experiments are homozygous for *a1-mum2*. Although *MuDR* can be present in multiple copies, all of the experiments described here have a single copy of *MuDR* at position 1 on chromosome 2L. All of the crosses used to generate the materials examined in this paper are

depicted in Fig 2.1. Active *MuDR/-;mop1/mop1* plants were crossed to *Muk/-;mop1/+* plants. The resulting progeny plants were genotyped to screen for plants that carried *MuDR*, *Muk* and that were homozygous for *mop1*, which were designated F1 plants. F1 plants were then crossed to *mop1* heterozygotes. Progeny plants lacking *Muk* but carrying silenced MuDR elements, designated *MuDR**, were designated F2 *MuDR** progeny. F2 *MuDR** progeny that were homozygous for *mop1* were crossed to *mop1* heterozygotes.

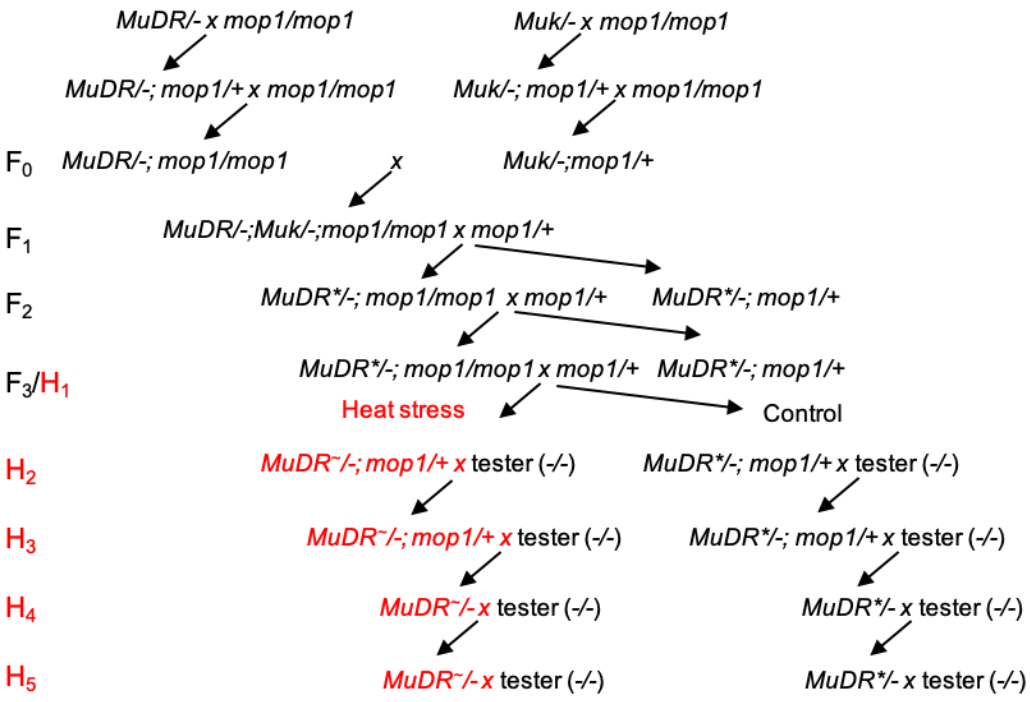

Figure 2.1 Diagram of the crosses and generations used in this study. F1 refers to the first generation during which *MuDR* was exposed to *Muk*. H1, which corresponds to F3, is the generation in which a brief heat treatment was applied. *MuDR* indicates an active *MuDR* element. *MuDR** indicates an inactive *MuDR* element. *MuDR*~ indicates a reactivated *MuDR* element.

2.2.2 RNA isolation and qRT-PCR analysis

Total RNA was extracted using TRIzol reagent (Invitrogen) and purified by Zymo Direct-zolTM RNA Miniprep Plus kit. 2 µl of total RNA was first loaded on a 1% agarose gel to check for good quality. Then, RNA was quantified by a NanoDropTM spectrophotometer (Thermo Fisher Scientific) and reverse transcribed using an oligo-dT primer and GoScriptTM Reverse

Transcriptase (Promega). Quantitative RT-PCR was performed by using SYBR Premix Ex TaqTM (TaKaRa Bio) on a ABI StepOnePlusTM Real-Time PCR thermocycler (Thermo Fisher Scientific) according to the manufacturer's instructions. Expression of *ZmHsp90* (Zm00001d024903) shown in Fig S3 was measured using primers HSP90-qPCR_F and HSP90-qPCR_F. Relative expression values for all experiments were calculated based on the expression of the reference gene, *ZmTub2* (Zm00001d050716) using primers TUB2-qPCR_F and TUB2-qPCR_R and determined by using the comparative CT method. Sequences for all primers used for RT-PCR are available in Table S1

2.2.3 Genomic Bisulfite Sequencing

These experiments were performed as previously described. In brief, genomic DNA was isolated and digested with RNase A (Thermo Fisher Scientific). 2 µl of this DNA was loaded on a 1% agarose gel to check for good quality and then quantified using a Qubit fluorometer (Thermo Fisher Scientific). 0.5-1 µg of genomic DNA from each genotype and treatment were used for bisulfite conversion. The EZ DNA Methylation-Gold kit (Zymo Research) was used to perform this conversion. Fragments from TIRA and TIRB were PCR-amplified using EpiMark Hot Start Taq DNA Polymerase (New England BioLabs). For TIRA, the first amplification was for 20 cycles using p1bis2f and TIRAbis2R with an annealing temperature of 48 °C, followed by re-amplification for 17 cycles using TIRAbis2R and TIRAmF6 with an annealing temperature of 50 °C. Amplicons from TIRB were amplified for 30 cycles using methy_TIRBF and methy_TIRBR with an annealing temperature of 55 °C. The resulting fragments were purified and cloned into pGEM®-T Easy Vector (Promega). Ligations and transformations were performed as directed by the manufacturer's instructions. The resulting colonies were screened for the presence of insertions by performing a colony-based PCR using primers of pGEMF and pGEMTR with an annealing temperature of 52 °C. The sequences of all primers are provided in Table S1. Plasmid was extracted from positive colonies using the Zyppy Plasmid Kit (Zymo Research). Plasmid from at least 10 independent clones were sequenced at Purdue Genomics Core Facility. The sequences were analyzed using kismeth (http://katahdin.mssm.edu/kismeth/revpage.pl).

2.2.4 Chromatin Immunoprecipitation (ChIP)-qPCR assay

The ChIP assay was performed as described previously with some modifications (H. Li et al., 2010). Briefly, leaf samples were treated with 1% methanol-free formaldehyde for 15 minutes under vacuum. Glycine was added to a final concentration of 125 mM, and incubation was continued for 5 additional minutes. Plant tissues were then washed with distilled water and homogenized in liquid nitrogen. Nuclei were isolated and resuspended in 1 mL nuclei lysis buffer (50 Mm tris-HCl pH8, 10 mM EDTA, 0.25% SDS, protease inhibitor). 50 µl of nuclei lysis was harvested for a quality check. DNA was sheared by sonication (BioruptorTM UCD-200 sonicator) sufficiently to produce 300 to 500 bp fragments. After centrifugation, the supernatants were diluted to a volume of 3 mL in dilution buffer (1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl pH8, 167mM NaCl). Each sample of supernatant was sufficient to make 6 immunoprecipitation (IP) reactions. Every 500 µl sample was precleared with 25 µl protein A/G magnetic beads (Thermo Fisher Scientific) for 1 hour at 4 °C. After the beads were removed using a magnet, the supernatant was removed to a new pre-chilled tube. 50 µl from each sample was used to check for sonication efficiency and set aside to serve as the 10% input control. Antibodies used were anti-H3K9me2 (Millipore), H3K27me2 (Millipore), H3K27me3 (Active Motif), H3K4me3 (Millipore) and H3KAc (Millipore). After incubation overnight with rotation at 4°C, 30 µl of protein A/G magnetic beads was added and incubation continued for 1.5 hours. The beads were then sequentially washed with 0.5 mL of the following: low salt wash buffer (20 mM Tris (pH 8), 150 mM NaCl, 0.1% (wt/vol) SDS, 1% (vol/vol) Triton X-100, 2 mM EDTA), high salt wash buffer (20 mM Tris (pH 8), 500 mM NaCl, 0.1% (wt/vol) SDS, 1% (vol/vol) Triton X-100, 2 mM EDTA), LiCl wash buffer (10 mM Tris (pH 8), 250 mM LiCl, 1% (wt/vol) sodium deoxycholate, 1% (vol/vol) NP-40 substitute, 1 mM EDTA), TE wash buffer (10 mM Tris (pH 8), 1 mM EDTA). After the final wash, the beads were collected using a magnet and resuspended with 200 µl X-ChIP elution buffer (100 mM NaHCO3, 1% (wt/vol) SDS). A total of 20 µl 5M NaCl was then added to each tube including those samples used for quality checks. Cross-links were reversed by incubation at 65 °C for 6 hours. Residual protein was digested by incubating with 20 µg protease K (Thermo Fisher Scientific) at 55 °C for 1 hour, followed by phenol/chloroform/isoamyl alcohol extraction and DNA precipitation. Final precipitated DNA was dissolved in 50 µl TE. Quantitative RT-PCR was performed by using SYBR Premix Ex TaqTM (TaKaRa Bio) on an ABI StepOnePlusTM Real-Time PCR thermocycler (Thermo Fisher Scientific) according to the manufacturer's instructions.

The primers used in this study are listed in Table S2. The primers used to detect H3K9 and H3K27 dimethylation of Copia retrotransposons and H3K4 trimethylation of actin that were used as internal controls in this study have been validated previously. Primers used for TIRA (TIRAR and TIRAUTRR) and TIRB (Ex1 and RLTIR2) were those used previously to detect changes in chromatin at these TIRs. Expression values were normalized to the input sample that had been collected earlier using the comparative CT method.

2.3 Results

2.3.1 DNA methylation is not required to maintain silencing of *MuDR* elements in *mop1* mutants

Given that *MuDR* elements are only activated after multiple generations in a *mop1* mutant background (Woodhouse et al., 2006), I wanted to understand how silencing of *MuDR* is maintained in *mop1* mutants prior to reactivation. To do this, I examined the transcriptional expression level of *mudrA* and DNA methylation at TIRA by performing bisulfite sequencing of TIRA of individuals in families that were segregating for a single silenced *MuDR* element, designated *MuDR**, and that were homozygous or heterozygous for *mop1*.

In control plants carrying an active *MuDR* element, all cytosines in TIRA were unmethylated, which was consistent with our previous results and which indicated that bisulfite conversion was efficient (Fig 2.2B). Also consistent with previous results, F_2 *MuDR*/-; mop1/+* plants, whose F_1 parent carried both *MuDR* and *Muk,* exhibited dense methylation at TIRA. In contrast, DNA methylation in the CG, CHH and CHG contexts at TIRA was absent in *mop1* mutant siblings. Interestingly, *mop1* had effects on TIRB that are more consistent with the known effects of this mutant specifically on CHH methylation. While F_2 *MuDR*/-; mop1/+* plants exhibited dense methylation at TIRB in all sequence contexts, *mop1* homozygous siblings exhibited a loss of methylation only in the CHH context. Despite the effects of *mop1* on *MuDR* methylation at both TIRA and TIRB, qRT-PCR results demonstrated that these *mop1* mutant plants did not exhibit reactivation of *mudrA* or *mudrB* (Fig 2.2A).

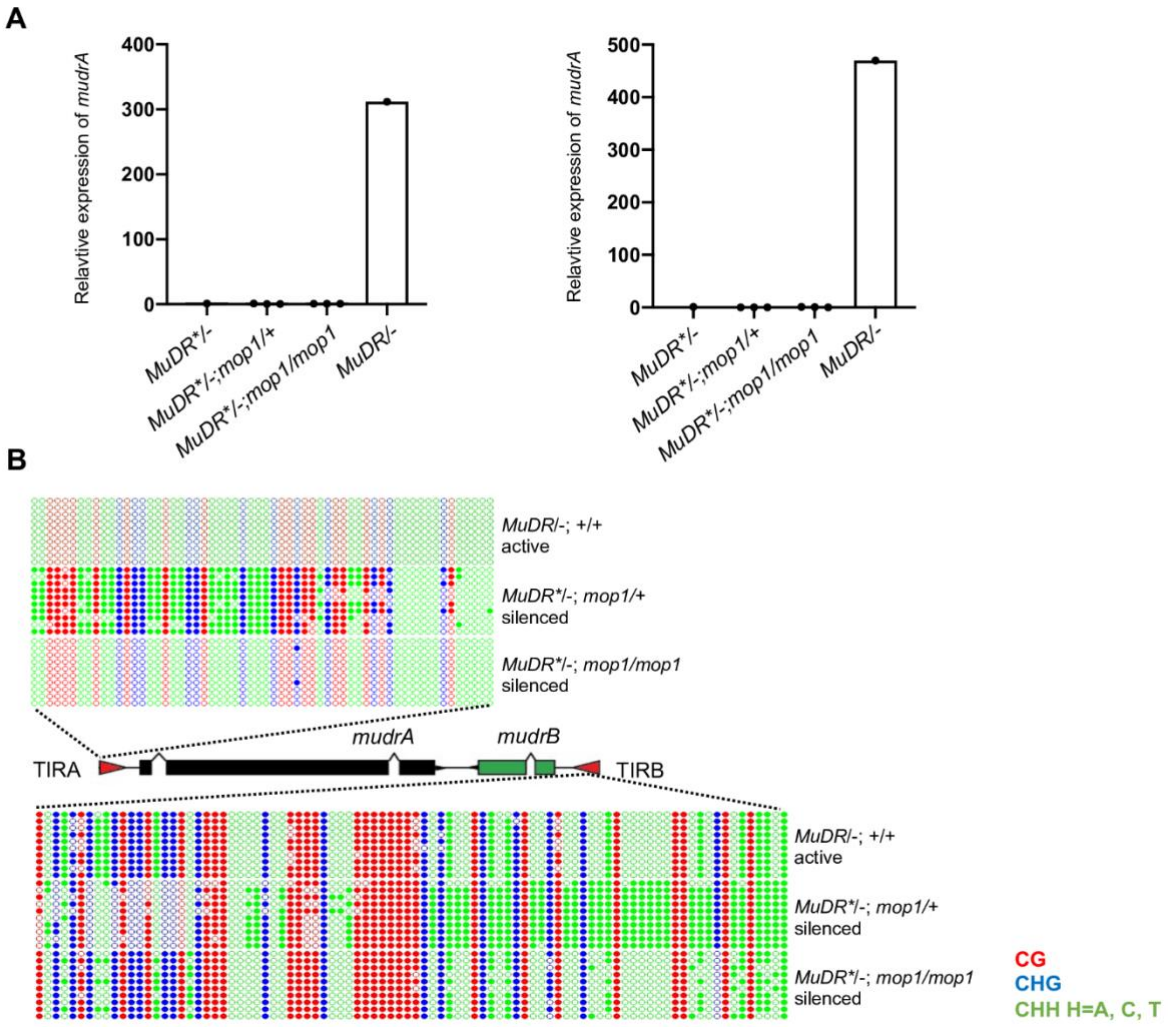

Figure 2.2 DNA methylation patterns at TIRA and TIRB of stably silenced F2 plants. (A) qPCR anlaysis of *mudrA* and *mudrB* expression from *MuDR*/-; mop1/+* and *MuDR*/-;mop1/mop1* plants. *Tub2* is used as an internal control gene. Six biological replicates are used for each experiment; two of six biological replicates are pooled together for each amplification. Error bars indicate mean ± standard deviation (SD) of three individuals. (B) DNA methylation patterns at TIRA and TIRB. Ten individual clones were sequenced from amplification of bisulfite-treated samples of the indicated genotypes. The cytosines in different sequence contexts are represented by different colors (red, CG; blue, CHG; green, CHH, where H=A, C, or T). For each genotype, DNA from six biological replicates were pooled.

2.3.2 *Mop1* enhances enrichment of H3K9me2 and H3K27me3

Transposon silencing is often associated with H3K9 and H3K27 dimethylation, two hallmarks of transcriptional silencing in plants. DNA methylation, particularly in the CHG context, is linked with H3K9 dimethylation through a self-reinforcing loop, and these two epigenetic marks often colocalize at TEs and, sometimes, adjacent genes (Matzke & Mosher, 2014). I had previously demonstrated that these two repressive histone modifications corresponded well with DNA methylation of silenced *MuDR* elements at TIRA (H. Li et al., 2010). However, our observation that silencing of *mudrA* can be maintained in the absence of DNA methylation in *mop1* mutants suggests that additional repressive histone modifications may be responsible for maintaining the silenced state of *mudrA* (Woodhouse et al., 2006). To test this hypothesis, I examined the enrichment of H3K9me2 at TIRA in individuals in a family that segregated for silenced *MuDR* and for *mop1* homozygotes and heterozygotes (Fig 2.3A) by performing a chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) assay. As controls, I also examined these two histone modifications in leaf tissue from plants carrying active and deeply silenced *MuDR* elements in a wild type background. Compared with active *MuDR/-;* +/+ plants, H3K9me2 and levels were significantly enriched at TIRA in the *MuDR**/-; +/+ plants (Fig 2.3A), suggesting that the loss of DNA methylation that resulted from the loss of MOP1 in these mutants actually resulted in an increase in a repressive chromatin mark, H3K9me2.

Like *mudrA*, *mudrB* is silenced by *Muk*, but maintenance of *mudrB* silencing has distinct requirements. Unlike *mudrA*, which is eventually reactivated in a *mop1* mutant background under normal conditions, *mudrB* remains silenced, suggesting that maintenance of silencing of this gene is independent of *MOP1*. ChIP-qPCR revealed that silencing of *mudrB* is not associated with H3K9me2 methylation. Instead, heritably silenced TIRB is enriched for H3K27me3, a modification normally associated with somatically silenced genes rather than transposable elements (Fig 2.3B). The *mop1* mutant appears to enhance H3K27me3 at TIRB relative to the *mop1* heterozygous siblings, although the enrichment is no greater that observed in the *MuDR**/-;* +/+ controls.

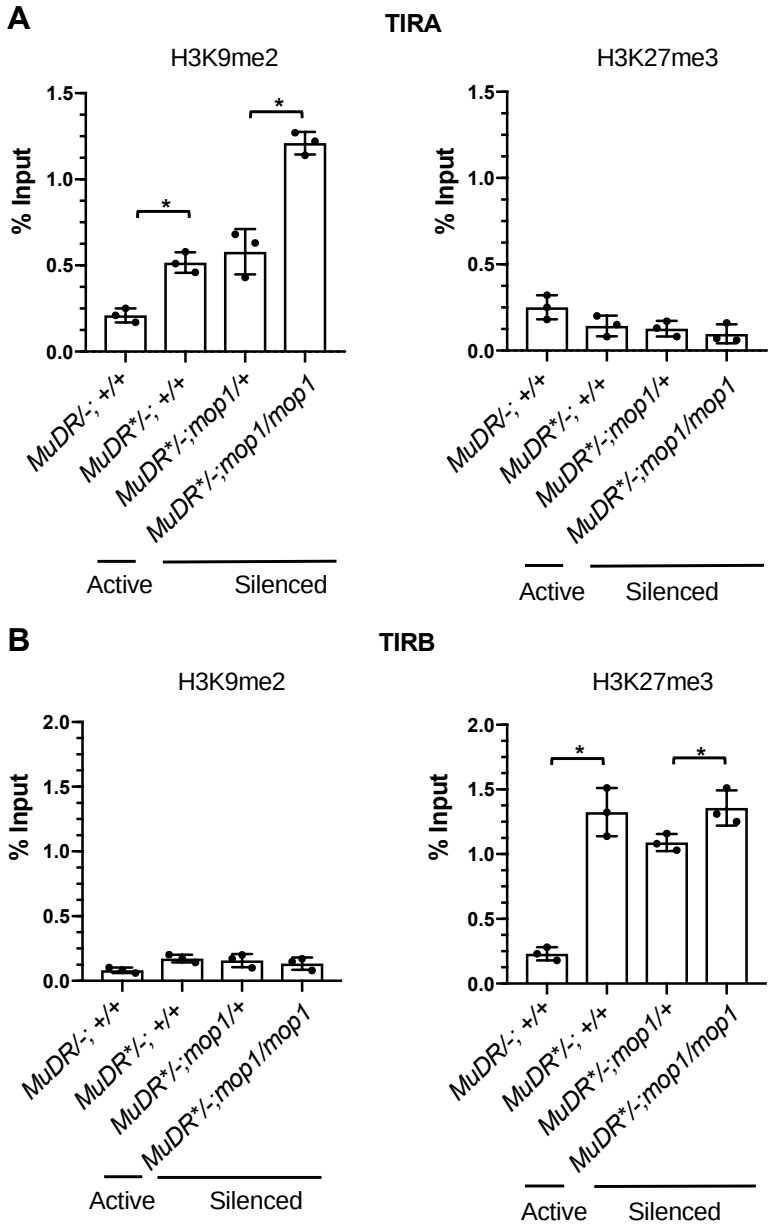

Figure 2.3 ChIP-qPCR analysis of enrichment of histone marks H3K9me2 and H3K27me3 at TIRA and TIRB in *mop1* mutants. ChIP-qPCR analysis of enrichment of histone marks, H3K9me2 and H3K27me3 at TIRA and TIRB. (A) Relative enrichment of H3K9me2 and H3K27me3 in leaf 3 of plants of the indicated genotypes. *MuDR*: active element. *MuDR**: inactive element. (B) Relative enrichment of H3K9me2 and H3K27me3 in leaf 3 of plants of the indicated genotypes. qPCR signal was normalized to Copia and then to the value of input sample. An unpaired t-test was performed. Error bars indicate mean ± standard deviation (SD) of the three biological replicates. *P<0.05; **P < **0.0**1

2.4 Discussion

2.4.1 DNA methylation is not necessary for the maintenance of silencing at TIRA or TIRB

My results demonstrating that methylation is not necessary for maintenance of epigenetic silencing in *mop1* mutant plants (Fig 2.2) suggest that at this particular locus, DNA methylation is not the key determinative factor with respect to either silencing or its reversal. In contrast, changes in H3K9me2 are closely correlated with changes in TIRA activity, suggesting that it is this modification, rather than DNA methylation, that mediates both activity and heritable transmission of silencing of *mudrA*. Given that H3K9me2 is normally tightly associated with cytosine methylation, particularly in the CHG context, this result is unexpected. However, our results clearly demonstrate that this modification can be heritably propagated in the absence of DNA methylation and in the absence of the original trigger for silencing, *Muk* (Burgess et al., 2020; Woodhouse et al., 2006). Even more unexpected is our observation that, once *mudrA* becomes silenced, in *mop1* mutants there appears to be reciprocal relationship between DNA methylation of TIRA and H3K9me2 enrichment. Based on previous experiments, our expectation was that *mop1* would eliminate cytosine methylation in the 5' end of TIRA, which is unrelated to transcriptional gene silencing of *mudrA*, but that it would not elimination of DNA methylation in the 3' portion of TIRA, which is primarily in the CG and CHG contexts and is specifically associated with silencing of this gene. In fact, I find that methylation in all three sequence context is eliminated throughout TIRA in *mop1* mutants, but this does not result in reactivation of *mudrA*. Instead, H3K9me2 actually significantly *increases* in the *mop1* mutant (Burgess et al., 2020; Woodhouse et al., 2006). This suggests that silencing at this locus is maintained via a balance between DNA and histone methylation, such that a loss of DNA methylation actually triggers an increase in histone modification. This in turn suggests that the state of activity of *mudrA* in some way determines the balance between histone and DNA modification, since neither modification by itself appears to be determinative.

There are other instances in which silencing can be reversed without a loss of methylation. For instance, mutations in the putative chromatin remodeler *MOTHER OF MORPHEOUS1*(*MOM1*) can result in activation of silenced transgenes and some endogenous loci in the absence of a loss of DNA methylation (Heard & Martienssen, 2014; Lang-Mladek et al.,

2010; Quadrana & Colot, 2016). Similarly, MICRORDIA (MORC) ATPase proteins, as well the H3K27 monomethyltransferases ATXR5 and ATXR6 in *Arabidopsis*, are required for heterochromatin condensation and TE silencing but not for DNA methylation or histone modification associated with that silencing (Rigal et al., 2016). However, unlike reactivated *MuDR* elements in our experiments, reintroduction of the wild type MOM1 or MORC alleles result in immediate re-silencing (Heard & Martienssen, 2014; Lang-Mladek et al., 2010; Quadrana & Colot, 2016). Finally, mutations in two closely related *Arabidopsis* genes, MAIL1 and MAIN, can also result in activation of a subset of *Arabidopsis* TEs in the absence of a loss of methylation (Matzke et al., 2015).

2.4.2 Heritably transmitted silencing of TIRB is associated with H3K27me3

My observation that transgenerationally heritable silencing of *mudrB* is associated with H3K27me3 was surprising, given that this mark is generally associated with somatic silencing of genes that is reset each generation. However, in the absence of that resetting, silencing can be heritably transmitted to the next generation. My data clearly shows that this is the case for *TIRB*, whose H3K27me3 enrichment can be heritably transmitted following the loss of *Mu killer* through at least two rounds of meiosis, and I have evidence that *mudrB* remains stably silenced for at least eight generations. Given that there is no selective pressure to reset TE silencing mediated by H3K27me3, this is not surprising.

There is evidence that heat stress can heritably reverse H3K27me3 at specific loci. H3K27 trimethylation can be reversed by the H3K27me3 demethylase REF6, which acts in conjunction the chromatin remodeler BRAHMA (BRM) to relax silencing at loci containing CTCTGYTY motifs (Cui et al., 2016; C. Li et al., 2016). In *Arabidopsis*, under heat stress, HEAT SHOCK TRANSCRIPTION FACTOR A2 (HSFA2) activates REF6, which can in turn de-repress HSFA2 by reducing H3K27me3 at this gene. This feedback loop can extend to the progeny of heat stressed plants, resulting in a heritable reduction in levels of H3K27me3 at *REF6* target genes (Cui et al., 2016; C. Li et al., 2016). However, as in the case for all transgenerational shifts in gene expression, the effect is temporary, and both H3K27me3 and gene expression levels are restored to their original state after two generations.

CHAPTER 3. GENETIC, MOLECULAR, AND BIOCHEMICAL CHARACTERIZATION OF A HEAT-REACTIVATED MUDR GENES IN *MOP1* MUTANTS

3.1 Introduction

Transposable elements were firstly described as 'controlling elements' by Barbara McClintock, who proposed that TEs can be reactivated by biotic and abiotic stress. Indeed, in rice, DNA transposon mPing can be reactivated in response to salt and cold stress. Similarly, expression of the tobacco Tnt1 transposon can be induced by both biotic and abiotic stress treatments (Hirsch & Springer, 2017). TEs account for 85% of the maize genome, and there are many TE insertions interspersed between maize genes, which makes maize a good system for studying the potential effect of TEs on regulation of nearby genes. Indeed, the majority of maize genes are located within 1 kb of an annotated transposon (R. K. Slotkin & Martienssen, 2007; M. Zhao, Zhang, Lisch, & Ma, 2017). Transposons are highly polymorphic from different background of maize inbred lines, and allelic variation for the presence of TE insertions near genes is high (Makarevitch et al., 2015). In plants, *de novo* DNA methylation is mediated through the RNA-directed DNA methylation (RdDM) pathway. It has been reported that loss of CHH methylation can lead to transcription reactivation of some TEs near genes, which is also accompanied by CG and CHG hypomethylation, suggesting that RdDM is required to prevent silenced TEs from being activated by active nearby genes (Q. Li et al., 2015). Under heat stress conditions, *nrpd1* mutants, which are defective in RdDM, exhibited more frequent transposition of the *Arabidopsis* retrotransposon, *ONSEN*, which is transcriptionally activated by heat stress (Vladimir V Cavrak et al., 2014). Many alleles containing ONSEN insertions show heat-responsive regulation by heat stress (Sun et al., 2020; X. Wang et al., 2016). This observation is also seen in maize system under stress conditions. These observations suggest that TE insertions near genes may influence gene regulation that may act as enhancers or alter the chromatin state of gene promoter regions (Hirsch & Springer, 2017; Makarevitch et al., 2015). Because of its dramatic and global effects on both gene expression and protein stability, heat stress has attracted considerable attention, particularly with respect to heritable transmission of TE activity (J. Liu et al., 2019). For both genes and TEs, although heat stress can trigger somatically heritable changes in gene expression, there appear to be a variety of mechanisms to prevent or gradually ameliorate transgenerational transmission of those

changes(Heard & Martienssen, 2014; Lang-Mladek et al., 2010). Thus, for instance, although the Onsen retrotransposon is sensitive to heat, it is only in mutants in the RdDM pathway that transposed elements are transmitted to the next generation (Matsunaga et al., 2015). Given that both TEs and various components of regulatory pathways that have evolved to regulated them are up-regulated in germinal lineages, this is not surprising (Lanciano & Mirouze, 2018). The observation that it is the combination of both heat and components of the RdDM pathway results in reactivation of TEs, rather than each by itself has led to the suggestion that a key role of RdDM is to prevent TE activation specifically under conditions of stress (H. C. Liu et al., 2018). Similar experiments using silenced transgenes have demonstrated that double mutants of *mom1* and *ddm1* cause these transgenes as well as several TEs to be highly responsive to heat stress, and the observed reversal of silencing can be passed on to a subsequent generation, but only in mutant progeny (Crisp. et al., 2016; Quadrana & Colot, 2016). It is also worth noting that in many cases of TE reactivation, silencing is rapidly re-established in wild type progeny (Hidetaka Ito et al., 2016; Matsunaga et al., 2015). The degree to which this is the case likely depends on a variety of factors, from the copy number of a given element, its position within the genome, its mode of transposition and the presence or absence of trans-acting small RNAs targeting that TE (Matsunaga et al., 2015; Wheeler, 2013).

Our lab previously found that *MuDR* elements can become transcriptionally reactivated after multiple generations in *mop1* mutants (Woodhouse et al., 2006). Given that a loss of methylation by itself is not sufficient to reactivate silenced *MuDR* elements, I decided to apply a heat stress treatment in order to determine if that stress can reverse silencing and to study the mechanisms of reversal of silencing. To do that, I heat-stressed mutant and wild type plants and examine transcriptional levels of *mudrA and mudrB* by performing qPCR. To further understand the mechanisms by which *MuDR* elements are reactivated, I then performed locus-specific bisulfite sequencing and ChIP assays to examine DNA methylation and histone modifications at TIRA and TIRB.

3.2 Materials and Methods

3.2.1 Plant materials and growth conditions

Maize seedlings and adult plants were grown in MetroMix under standard long-day greenhouse conditions at 26°C unless otherwise noted. All of the crosses used to generate the materials examined in this paper are depicted in Fig S1. Active *MuDR/-;mop1/mop1* plants were crossed to *Muk/-;mop1/+* plants. The resulting progeny plants were genotyped to screen for plants that carried *MuDR*, *Muk* and that were homozygous for *mop1*, which were designated F1 plants. F1 plants were then crossed to *mop1* heterozygotes. Progeny plants lacking *Muk* but carrying silenced *MuDR* elements, designated *MuDR**, were designated F2 *MuDR** progeny. F2 *MuDR** progeny that were homozygous for *mop1* were crossed to *mop1* heterozygotes. The resulting F3 plants were genotyped for the presence of *MuDR*. These plants were either homozygous or heterozygous for in *mop1*. These F3 plants were those that were used for the heat stress experiments. H1 refers to the first generation of these F3 plants that were subjected to heat stress, with successive generations designated H2, H3, etc. *MuDR* was genotyped using primers Ex1 and RLTIR2. Because Ex1 is complementary to sequences flanking *MuDR* in these families, this primer combination is specific to the single *MuDR* element segregating in these families. *Muk* was genotyped using primers TIRAout and 12-4R3. The mop1 mutation was genotyped using primers ZmRDR2F, ZmRDR2R and TIR6. All primer sequences are provided in Table S1.

Plants used in all experiments were genotyped individually. The visible portion of each developing leaf blade, when it was ≈10 cm, was harvested when it emerged from the leaf whorl. Only leaf blades of mature leaves were harvested. For the heat reactivation experiment, seedlings were grown at 26 °C for 14 days with a 12-12 light dark cycle. Seedlings were incubated at 42 °C for 4 hours and leaf 3 was harvested immediately after stress treatment. As a control, leaf 3 was also collected from sibling seedlings grown at 26 °C. For each genotype and treatment, 12 biological replicates were used, all of which were siblings. Samples were stored in -80 °C. After sample collection, all seedlings were transferred to a greenhouse at 26 °C. In order to determine if reactivation could be propagated to new emerging tissues, leaf 10 at a similar stage of development (~10 cm, as it emerged from the leaf whorl) and the immature tassel (~20 cm) were collected from each individual. For the bisulfite sequencing experiment, leaf 3 was collected from each individual, when it was ≈10 cm, as it emerged from the leaf whorl. In order to minimize potential variation

among different individuals, leaves from 6 individuals with the same genotype and treatment were pooled together. For the ChIP assays, a total of ~ 2 g of leaves from leaf 3 of 6 sibling plants with the indicated genotypes was harvested. Three independent sets of these sample collections were colected and analyzed for each genotype and treatment. Leaf samples were fixed with 1% methanol-free formaldehyde and then stored in -80 °C.

3.2.2 RNA isolation and qRT-PCR analysis

Total RNA was extracted using TRIzol reagent (Invitrogen) and purified by Zymo Direct-zolTM RNA Miniprep Plus kit. 2 µl of total RNA was first loaded on a 1% agarose gel to check for good quality. Then, RNA was quantified by a NanoDropTM spectrophotometer (Thermo Fisher Scientific) and reverse transcribed using an oligo-dT primer and GoScriptTM Reverse Transcriptase (Promega). Quantitative RT-PCR was performed by using SYBR Premix Ex TaqTM (TaKaRa Bio) on a ABI StepOnePlusTM Real-Time PCR thermocycler (Thermo Fisher Scientific) according to the manufacturer's instructions. Expression of *ZmHsp90* shown in Fig S3 was measured using primers HSP90-qPCR_F and HSP90-qPCR_F. Relative expression values for all experiments were calculated based on the expression of the reference gene, *ZmTub2* using primers TUB2-qPCR_F and TUB2-qPCR_R and determined by using the comparative CT method. Sequences for all primers used for qRT-PCR are available in Table S1.

For RNAseq experiment, total RNA was extracted using TRIzol reagent (Invitrogen) and purified by Zymo Direct-zolTM RNA Miniprep Plus kit. 2 µl of total RNA was first loaded on a 1% agarose gel to check for good quality. Then, RNA was quantified by a NanoDropTM spectrophotometer (Thermo Fisher Scientific). To validate if the heat treatment worked, 500 ng of RNA from each sample was reverse-transcribed into cDNA, and a semi-RT-PCR was performed using primers of *Aat* and *Hsp90*. RT-PCR products were electrophoresed on a 1.2% agarose gel.

3.2.3 Genomic Bisulfite Sequencing

These experiments were performed as previously described. In brief, genomic DNA was isolated and digested with RNase A (Thermo Fisher Scientific). 2 µl of this DNA was loaded on a 1% agarose gel to check for good quality and then quantified using a Qubit fluorometer (Thermo Fisher Scientific). 0.5-1 µg of genomic DNA from each genotype and treatment were used for

bisulfite conversion. The EZ DNA Methylation-Gold kit (Zymo Research) was used to perform this conversion. Fragments from TIRA and TIRB were PCR-amplified using EpiMark Hot Start Taq DNA Polymerase (New England BioLabs). For TIRA, the first amplification was for 20 cycles using p1bis2f and TIRAbis2R with an annealing temperature of 48 °C, followed by re-amplification for 17 cycles using TIRAbis2R and TIRAmF6 with an annealing temperature of 50 °C. Amplicons from TIRB were amplified for 30 cycles using methy_TIRBF and methy_TIRBR with an annealing temperature of 55 °C. The resulting fragments were purified and cloned into pGEM®-T Easy Vector (Promega). Ligations and transformations were performed as directed by the manufacturer's instructions. The resulting colonies were screened for the presence of insertions by performing a colony-based PCR using primers of pGEMF and pGEMTR with an annealing temperature of 52 °C. The sequences of all primers are provided in Table S1. Plasmid was extracted from positive colonies using the Zyppy Plasmid Kit (Zymo Research). Plasmid from at least 10 independent clones were sequenced at Purdue Genomics Core Facility. The sequences were analyzed using kismeth (http://katahdin.mssm.edu/kismeth/revpage.pl). All data are available in Table 3.

3.2.4 Chromatin Immunoprecipitation (ChIP)-qPCR assay

The ChIP assay was performed as described previously with some modifications (H. Li et al., 2010). Briefly, leaf samples were treated with 1% methanol-free formaldehyde for 15 minutes under vacuum. Glycine was added to a final concentration of 125 mM, and incubation was continued for 5 additional minutes. Plant tissues were then washed with distilled water and homogenized in liquid nitrogen. Nuclei were isolated and resuspended in 1 mL nuclei lysis buffer (50 Mm tris-HCl pH8, 10 mM EDTA, 0.25% SDS, protease inhibitor). 50 µl of nuclei lysis was harvested for a quality check. DNA was sheared by sonication (BioruptorTM UCD-200 sonicator) sufficiently to produce 300 to 500 bp fragments. After centrifugation, the supernatants were diluted to a volume of 3 mL in dilution buffer (1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl pH8, 167mM NaCl). Each sample of supernatant was sufficient to make 6 immunoprecipitation (IP) reactions. Every 500 µl sample was precleared with 25 µl protein A/G magnetic beads (Thermo Fisher Scientific) for 1 hour at 4 °C. After the beads were removed using a magnet, the supernatant was removed to a new pre-chilled tube. 50 µl from each sample was used to check for sonication efficiency and set aside to serve as the 10% input control. Antibodies used were anti-H3K9me2

(Millipore), H3K27me2 (Millipore), H3K27me3 (Active Motif), H3K4me3 (Millipore) and H3KAc (Millipore). After incubation overnight with rotation at 4°C, 30 µl of protein A/G magnetic beads was added and incubation continued for 1.5 hours. The beads were then sequentially washed with 0.5 mL of the following: low salt wash buffer (20 mM Tris (pH 8), 150 mM NaCl, 0.1% (wt/vol) SDS, 1% (vol/vol) Triton X-100, 2 mM EDTA), high salt wash buffer (20 mM Tris (pH 8), 500 mM NaCl, 0.1% (wt/vol) SDS, 1% (vol/vol) Triton X-100, 2 mM EDTA), LiCl wash buffer (10 mM Tris (pH 8), 250 mM LiCl, 1% (wt/vol) sodium deoxycholate, 1% (vol/vol) NP-40 substitute, 1 mM EDTA), TE wash buffer (10 mM Tris (pH 8), 1 mM EDTA). After the final wash, the beads were collected using a magnet and resuspended with 200 µl X-ChIP elution buffer (100 mM NaHCO3, 1% (wt/vol) SDS). A total of 20 µl 5M NaCl was then added to each tube including those samples used for quality checks. Cross-links were reversed by incubation at 65 °C for 6 hours. Residual protein was digested by incubating with 20 µg protease K (Thermo Fisher Scientific) at 55 °C for 1 hour, followed by phenol/chloroform/isoamyl alcohol extraction and DNA precipitation. Final precipitated DNA was dissolved in 50 µl TE. Quantitative RT-PCR was performed by using SYBR Premix Ex TaqTM (TaKaRa Bio) on an ABI StepOnePlusTM Real-Time PCR thermocycler (Thermo Fisher Scientific) according to the manufacturer's instructions. The primers used in this study are listed in Table S2. The primers used to detect H3K9 and H3K27 dimethylation of *Copia* retrotransposons and H3K4 trimethylation of actin that were used as internal controls in this study have been validated previously. Primers used for TIRA (TIRAR and TIRAUTRR) and TIRB (Ex1 and RLTIR2) were those used previously to detect changes in chromatin at these TIRs. Expression values were normalized to the input sample that had been collected earlier using the comparative CT method.

3.3 Results

3.3.1 Application of heat stress specifically in the early stage of growth can promote the reactivation of the silenced *MuDR* elements in *mop1* mutants

There is ample evidence that a variety of stresses can reactivate epigenetically silenced TEs. One particularly effective treatment is heat stress. Given that a loss of methylation by itself is not sufficient to reactivate silenced *MuDR* elements, I subjected *mop1* mutant and *mop1* heterozygous sibling seedlings carrying silenced *MuDR* elements (*MuDR**) to heat stress. Fourteen-day-old

MuDR/-; mop1/mop1* and *MuDR*/-; mop1/+* sibling seedlings were heated at 42 °C for four hours and leaf samples were collected immediately after that treatment (Fig 3.1A). RT-PCR for the heat response factor *Hsp90* (Zm00001d024903) confirmed that the seedlings were responding to the heat treatment (Fig 3.1B). I then examined *MuDR* transcription by performing qRT-PCR on RNA from leaf three immediately after the plants had been removed from heat and from control plants that had not been subjected to heat stress. In the *mop1* mutants, both *mudrA* and *mudrB* became transcriptionally reactivated upon heat treatment (Fig 3.1C). *MuDR* elements in plants that were *mop1* mutant that were not heat stressed and were those that were wild type and that were heat stressed were not reactivated, demonstrating that both a mutant background and heat stress are required for efficient reactivation. To determine if the application of heat stress at a later stage of plant development can also promote reactivation, I heat-stressed 28-day-old plants and examined *MuDR* transcription in leaf seven at a similar stage of development (~10 cm) as had been examined in heat stressed leaf three in the previous experiment. In these plants, I saw no evidence of reactivation, indicating that *MuDR* responsiveness to heat shifts over developmental time (Fig 3.1C), despite the fact that *Hsp90* expression was induced in these leaves (Fig 3.1B). Taken together, these data suggest that the application of heat stress specifically at an early stage of plant development can promote the reactivation of a silenced TE in a mutant that is deficient in the RdDM pathway.

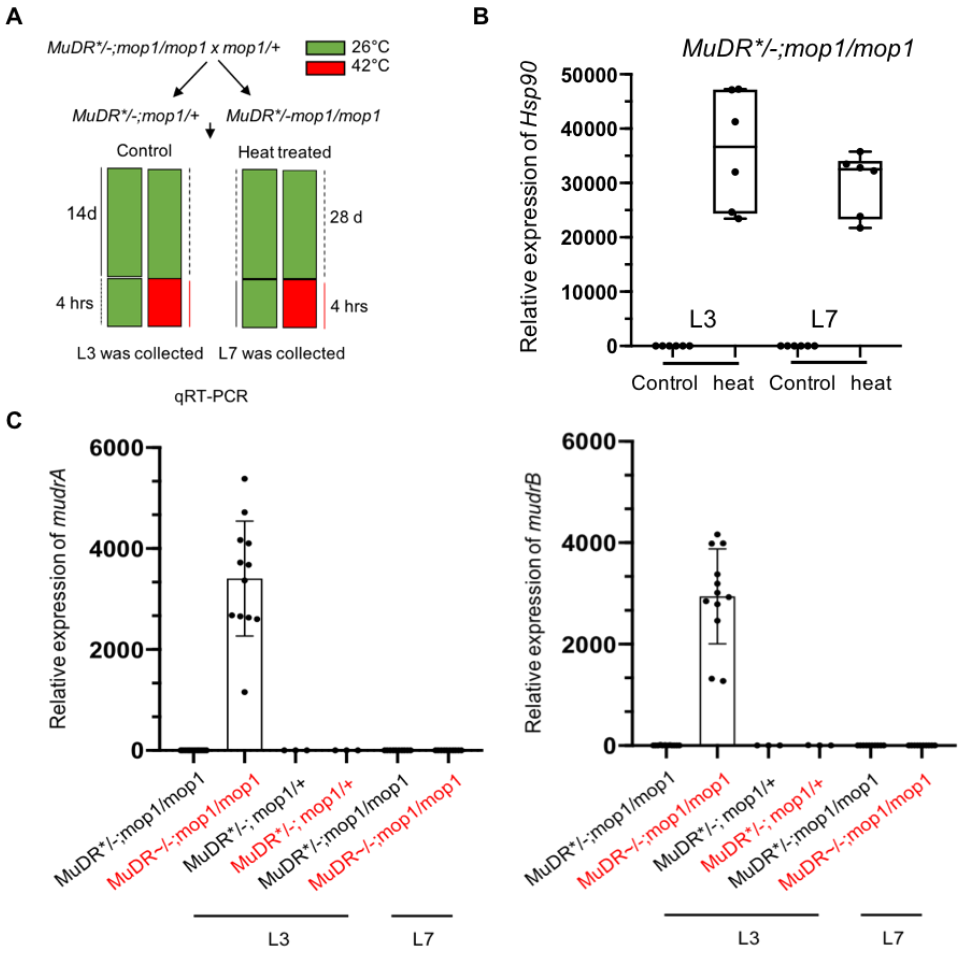

Figure 3.1 Expression of *mudrA* and *mudrB* in plants under heat stress. (A) Schematic diagram of the heat-reactivation experiment. (B) qRT-PCR of *Hsp90* in leaves of plants of the indicated genotypes and ages. (C) qRT-PCR of *mudrA* and *mudrB* of leaf 7 of heat-treated F2 plants. *Aat* is a housekeeping gene that was used as a positive expression control. Additional controls for each experiment included pools of ten *MuDR/-; mop1/+* heated and ten unheated plants, as well as plants that lacked *MuDR* and were wild type for *mop1* (*-/-; +/+*), samples with water or with no reverse transcriptase as negative controls, active *MuDR* as well as genomic DNA (gDNA) as positive controls for the *MuDR*-specific PCR primers.

3.3.2 The reactivation state is transmitted to the new emerging tissues

I next sought to determine whether or not the reactivated state can be maintained following a restoration of normal temperatures, and whether that active state can be propagated to progeny cells that had not experienced the heat stress. To do this, I performed quantitative RT-PCR to detect *mudrA* and *mudrB* transcripts in mature leaf ten of plants 35 days after the heat stress and

in immature tassels ten days after that. At V2, when the heat stress was applied and leaf three was assayed, cells within leaf 10 primordia are present and may have experienced the heat stress. In contrast, because the tassel primordia are not formed until V5, the cells of the tassel could not have experienced the heat stress directly. I found that both genes stayed active in both tissues, indicating heat-induced reactivation is stably transmitted to new emerging cells and tissues (Fig 3.2B).

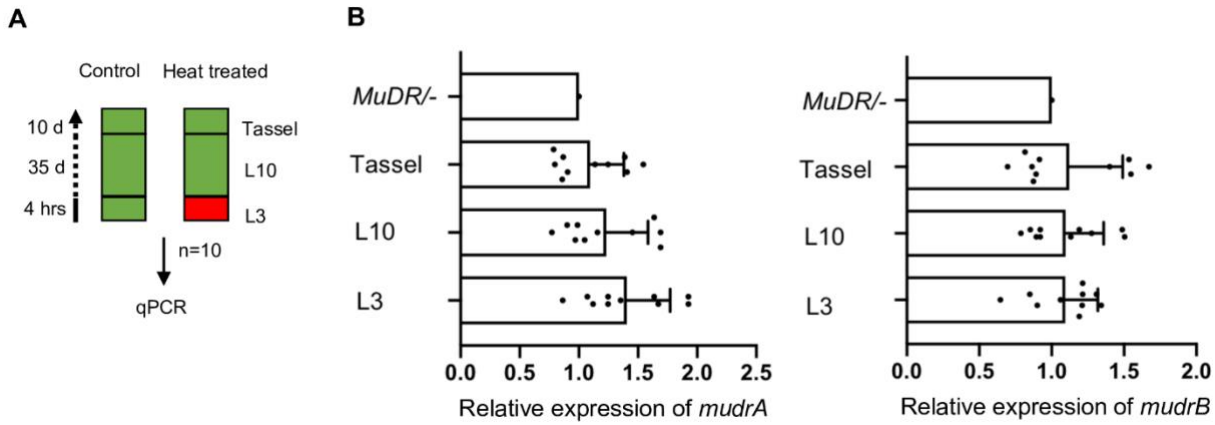

Figure 3.2 Expression of *mudrA* and *mudrB* in new emerging tissues following heat stress. (A) Diagram of the experiment. (B) qPCR was performed to measure transcript levels of *mudrA* and *mudrB* using expression of *Tub2* as an internal control. Expression levels were normalized to that of an active *MuDR* element. Error bars indicate mean ± standard deviation (SD) of the ten biological replicates.

3.3.3 *MuDR* activity is heritably transmitted to subsequent generations

Our previous work had demonstrated that silenced *mudrA* (but not *mudrB*) can be progressively and heritably reactivated only after multiple generations of exposure to the *mop1* mutation under normal conditions. Only after eight generations could this activity could be stably transmitted to subsequent generations in the absence of the *Mop1* mutation. To determine if the somatic activity I observed after heat stress can be transmitted to the next generation, I crossed the heat-treated *mop1* homozygous plants that carried transcriptionally reactivated *MuDR* (designated *MuDR*˜) and the sibling *mop1* homozygous *MuDR** control plants, to a tester that was homozygous wild type for *mop1* and that lacked *MuDR* (Fig 3.3A). MURA, the protein encoded by *mudrA* causes excision of a reporter element at the *a1-mum2* allele of the *A1* gene, resulting pale kernels with spots of colored revertant tissue. All plants used in these experiments were homozygous for

a1-mum2. If *mudrA* were fully heritably reactivated, a cross between a *MuDR˜/-; mop1/mop1* plant and a tester would be expected to give rise to 50% spotted kernels, and this phenotype would be expected to cosegregate with the reactivated *MuDR* element. The progeny of ten independent heat-reactivated individuals gave a total of 45% spotted kernels. In contrast, ten *mop1* homozygous siblings that carried *MuDR** and that had not been heat treated gave rise to an average of only 0.7% spotted kernels after test crossing (Table 2). These results show that *MuDR* activity induced by heat treatment was transmitted to the next generation. I employed a similar strategy to test stability of heritability. I crossed three subsequent generations to testers and counted the spotted kernels. I observed that the progeny of heat-reactivated individuals gave a total of 51%, 48% and 47% spotted kernels in the three subsequent generations. In contrast, subsequent generations of the lineage carrying *MuDR** that had not been heat treated gave rise to only a small number of weakly spotted kernels (Fig 3.3C, D, Table 2). These results demonstrate that heat reactivation is stable over multiple generations in a non-mutant genetic background, as is silencing in the absence of heat stress.

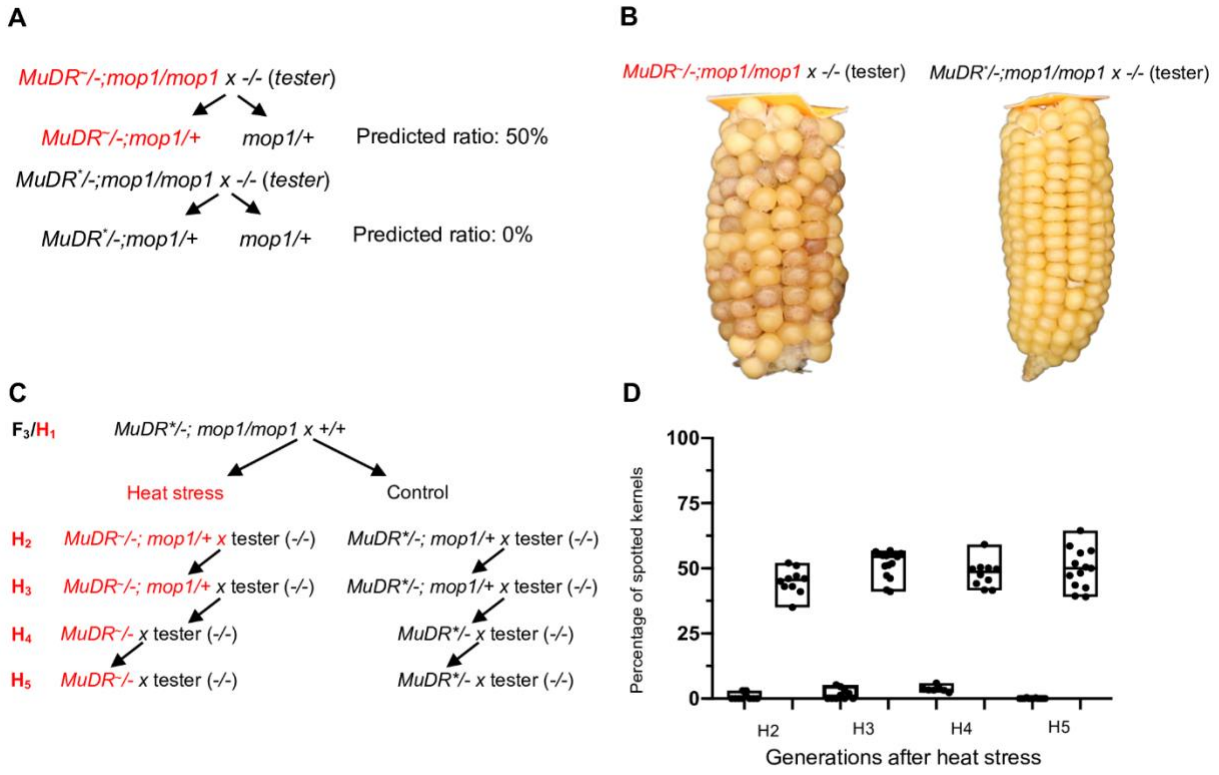

Figure 3.3 Testing transgenerational inheritance. (A) A schematic diagram showing the crosses used to determine transgenerational inheritance. (B) Ears derived from heat treated and control individuals. (C) Crosses done in the generations following heat stress. (D) Ratios of spotted kernels in generations of wild type plants following the heat stress (H1) generation.

3.3.4 Heat stress has no effect on DNA methylation

TIRA in a *mop1* mutant background already lacks any DNA methylation prior to heat treatment and thus heat would not be expected to reduce TIRA methylation. However in *mop1* mutants TIRB retained CG and CHG methylation and also remained inactive (Fig 2.2A). To determine if reactivation after heat treatment is associated with a loss of this methylation, we examined DNA methylation at TIRB in *mop1* mutants in the presence or absence of heat treatment. This assay was performed on the same tissues that we collected for *MuDR* expression reactivation analysis. We found that the DNA methylation pattern was the same for both the heat treated and the control *mop1* mutant plants, indicating that heat stress does not alter TIRB methylation and that a further loss of DNA methylation is not the cause of *mudrB* reactivation in this tissue (Fig 3.4).

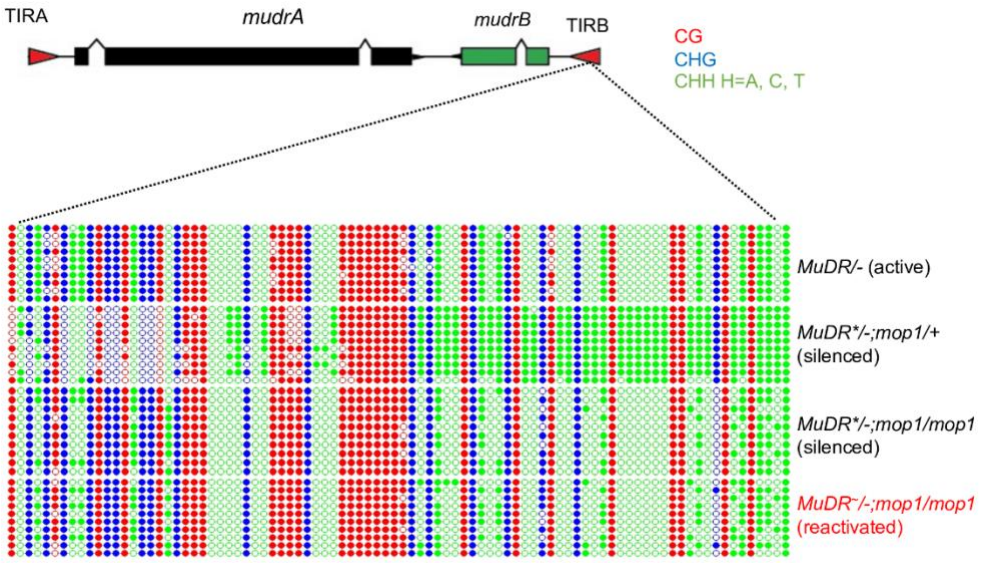

Figure 3.4 DNA methylation patterns at TIRB of heat-treated H1 *mop1/mop1* plants. Ten individual clones were sequenced from each amplification of bisulfite-treated samples with the indicated genotypes. The cytosines in different sequence contexts are represented by different colors (red, CG; blue, CHG; green, CHH, where H=A, C, or T). For each sample, six independent samples were pooled together.

3.3.5 Heat stress reverses TE silencing by affecting histone modifications

Under normal conditions, I found that H3K9me2 at TIRA is associated with silencing, and H3K9me2 is actually enriched when TIRA methylation is lost in *mop1* mutants (Fig 2.3A). In contrast, I find that H3K27me3, rather than H3K9me2, is enriched at TIRB and is maintained at similar or slightly elevated levels in *mop1* mutant relative to *mop1* heterozygous siblings (Fig 2.3B). Given these observations, I hypothesized that heat stress may reverse H3K9me2 enrichment at TIRA and H3K27me3 enrichment at TIRB. To test this hypothesis, I determined the level of H3K9me2 and H3K27me3 at TIRA and TIRB under normal and stressed conditions using ChIP-qPCR. Upon heat stress, the level of H3K9me2 at TIRA was significantly decreased in *mop1* mutants compared to that of non-treated *mop1/mop1* mutant siblings (Fig 3.5A). Interestingly, however, H3K9me2 enrichment only decreased to the level observed at TIRA in silenced *MuDR*/-*; +/+ plants, and it remained significantly higher than that of TIRA in the naturally active *MuDR/-;* +/+ plants. In contrast, I observed no changes in H3K27me3 at TIRA. At TIRB, I observed no changes in H3K9me2 enrichment in any of our samples. Instead, I found that heat treatment reversed previously established H3K27me3 at TIRB, supporting the hypothesis that this

modification, rather than H3K9me2, mediates heritable silencing of *mudrB* (Fig 3.5B). Consistent with evidence for transcriptional activation of both *mudrA* and *mudrB*, I observed enrichment of the active mark H3K4me3 in reactivated TIRA and TIRB (Fig 3.5C,D). Taken together, these data demonstrate that heat stress can simultaneously reduce two often mutually exclusive repressive histone modifications, H3K9me2 and H3K27me3 at the two ends of a single TE.

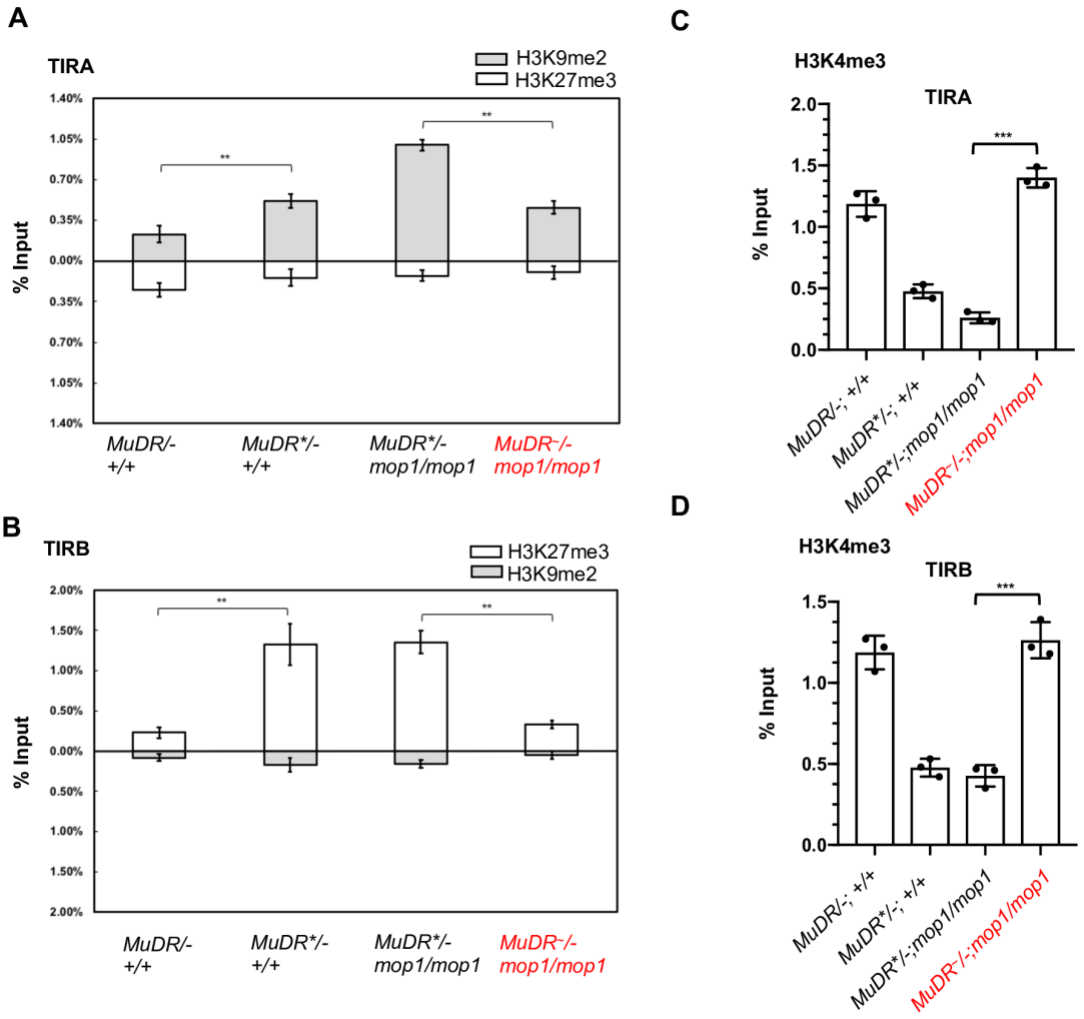

Figure 3.5 ChIP-qPCR analysis of histone marks TIRA and TIRB under heat stress. Relative enrichment of H3K9me2 and H3K27me3 at TIRA (A) and TIRB (B) in leaf 3 of plants of the indicated genotypes. (Relative enrichment of H3K4me3 at TIRA (C) and TIRB (D) in leaf 3 of plants of the indicated genotypes. qPCR signals were normalized to *Copia* and then to the value of input samples. *MuDR** refers to a silenced *MuDR* element. *MuDR~* refers to a reactivated element. Red text indicates a sample that has been heat treated. Error bars indicate mean ± standard deviation (SD) of the three biological replicates. ******P < **0.0**1; *******P < **0.0**01

3.3.6 DNA hypomethylation is not associated with transgenerational inheritance of activity

I have shown that DNA methylation is not reduced under heat stress at TIRB, and that even a complete absence of methylation of TIRA under normal conditions does not result in transcriptional activation. These results suggest that, at least under normal conditions, DNA methylation of *MuDR* is neither necessary nor sufficient to mediate silencing. However, only plants that were *mop1* mutant and whose TIRs were missing either methylation of cytosines in all sequence contexts in the case of TIRA or those in the CHH sequence context in the case of TIRB were reactivated under heat stress. This suggests that a loss of methylation may be a precondition for initiation, and perhaps propagation, of continued activity after that stress. To test the later possibility, I examined DNA methylation at TIRA and TIRB in the *mop1* heterozygous H2 progenies of heat-reactivated *mop1* mutant plants and those of their unheated *mop1* mutant sibling controls. Surprisingly, I found that both TIRA and TIRB were extensively methylated in all three sequence contexts in all progeny examined regardless of their activity status (Fig 3.6). Indeed, their methylation was indistinguishable from that observed at silenced *MuDR* elements. This suggests that after reactivation, although the restoration of MOP1 does result in the restoration of methylation at both TIRA and TIRB, this methylation is not sufficient for reestablishment of silencing at either of these TIRs. In order to determine whether DNA methylation I observed in these wild type H2 plants was stable, I examined TIRA and TIRB methylation in plants four generation removed from the initial heat stress. Surprisingly, I found that the observed patterns of methylation in this generation at both TIRs closely resembled that of fully active *MuDR* elements (Fig 3.6). This suggests that patterns of methylation consistent with activity are in fact restored in the heat stressed lineage, but only after multiple rounds of meiosis in a non-mutant genetic background.

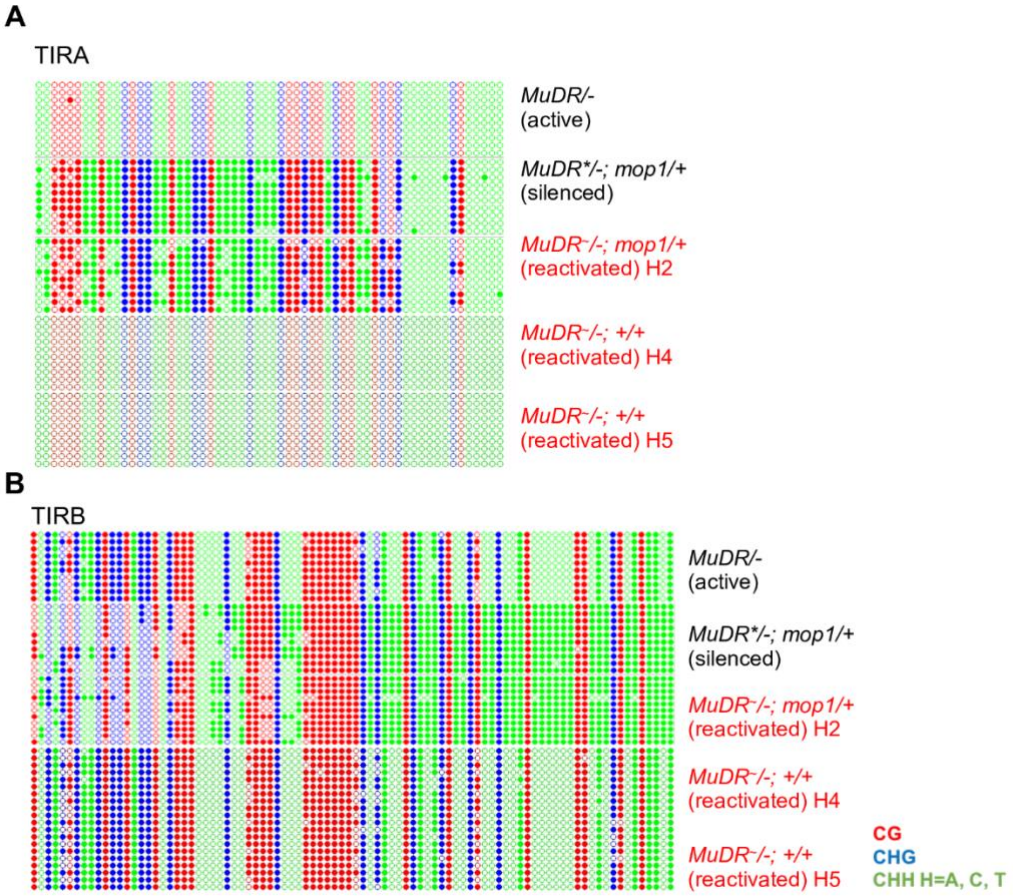

Figure 3.6 DNA methylation patterns at TIRA and TIRB of progeny of heat-treated H2 and H5 plants. (A) DNA methylation patterns at TIRA. (B) DNA methylation patterns at TIRB. Ten individual clones were sequenced from each amplification of bisulfite-treated sample. The cytosines in different sequence contexts are represented by different colors (red, CG; blue, CHG; green, CHH, where H=A, C, or T). For each assay, six independent samples were pooled together.

3.3.7 Transgenerational heritability is maintained through histone modifications

DNA hypomethylation is not associated with transgenerational inheritance of *MuDR* activity, and DNA hypermethylation does not result in a restoration of silencing in wild type progeny of heat reactivated mutants. A plausible alternative is that the observed changes in histone marks mediate heritable propagation of activity of both *mudrA* and *mudrB* independent of methylation status. To test this hypothesis, I determined the levels of H3K9me2, H3K27me3 and H3K4me3 at TIRA and TIRB in the *mop1* heterozygous H_2 progenies of heat-reactivated *MuDR˜/-; mop1/mop1* plants and those of their sibling untreated *MuDR*/-; mop1/mop1* sibling controls. Consistent with the continued activity of *mudrB* in the progeny of the heat stressed plants, relative

levels of H3K27me3 levels remained low and H3K4me3 remained high at TIRB in these plants, suggesting that heritable propagation of H3K27me3 is responsible for that continued activity (Fig 3.7). Similarly, at TIRA, H3K9me2 remained low and H3K4me3 remained high in these progenies. Interestingly, the increase in DNA methylation in these *MuDR* active *mop1* heterozygous plants was associated with a further decrease in levels of H3K9me2 at TIRA relative to that of their heat stressed *mop1* homozygous parents, down to the levels of the active *MuDR* control. This suggests that an increase in methylation of these active elements in the wild type background resulted in a concomitant decrease in H3K9me2 at TIRA.

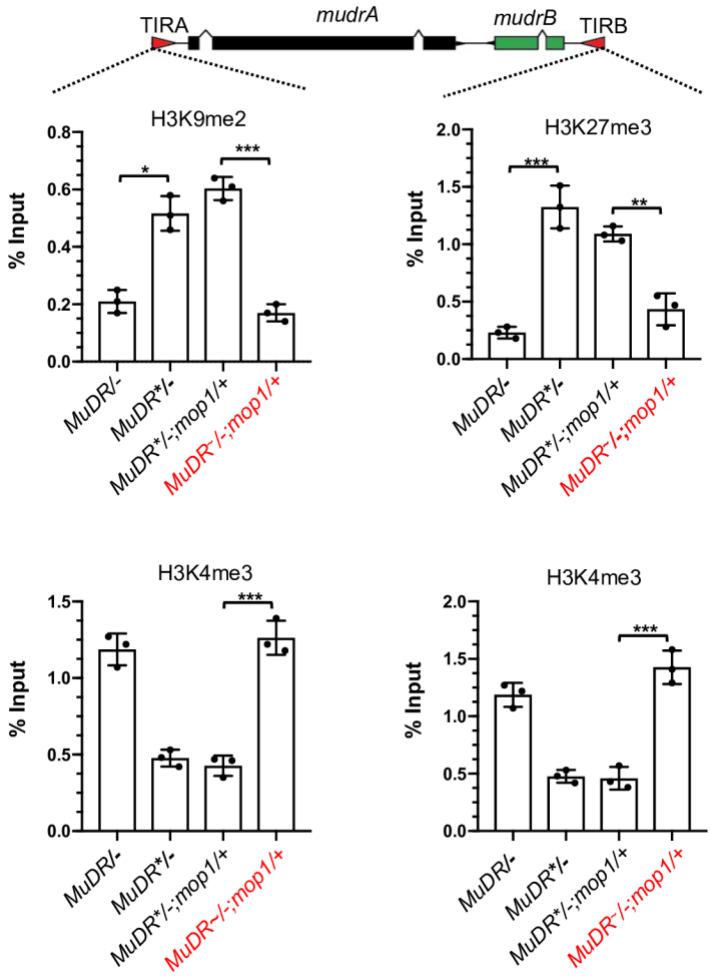

Figure 3.7 ChIP-qPCR analysis of enrichment of histone marks, H3K9me2, H3K27me3 and H3K4me3 at TIRA and TIRB. Relative enrichment of H3K9me2, H3K27me3 and H3K4me3 at TIRA and TIRB in leaf 3 of plants of the indicated genotypes. qPCR signals were normalized to *Copia* and then to the value of input samples. An unpaired t-test was performed. Error bars indicate mean ± standard deviation (SD) of the three biological replicates. *P<0.05; **P < **0.0**1; ***P<0.001

3.4 Discussion

3.4.1 The effects of heat on *mop1* mutants are dependent on the stage of development

My heat stress experiments demonstrated that although heat exposure has a rapid and dramatic effect on *MuDR* activity in juvenile leaves, heat stress later during adult growth has no effect on this element. Expression analysis of *Hsp90*, a key marker of heat stress in maize, suggests that the older maize *mop1* mutant plants are in fact responding to the heat, but the response does not include reactivation of *MuDR*. The reason for this difference is not clear. Presumably there are factors expressed later during development that can compensate for the lack of MOP1 in these later leaves. Expression analysis shows dramatic differences between juvenile and adult leaves, including differences in a large number of genes related to stress response. Further, the transition from juvenile to adult growth in maize is associated with a transient loss of *mudrA* silencing in F1 plants carrying both *MuDR* and *Muk*, suggesting that this transition represents an important stage of development with respect to silencing pathways. Future experiments will focus on mutations that affect the time of the juvenile to adult transition that are known to affect the transient loss of *MuDR* silencing.

3.4.2 The RdDM pathway buffers the effects of heat stress on silenced *MuDR* elements.

Heat stress rapidly reverses silencing and is associated with a reduction of H3K9me2, but only in a *mop1* mutant background. This suggests that although DNA methylation is not required for the maintenance of silencing of *mudrA* and is not sufficient to trigger *de novo* silencing of this gene, it is required to prevent a response to heat stress. Thus, I suggest that the primary role of DNA methylation in this instance is to buffer the effects of heat. I note that this observation is similar but distinct from what has been observed for the ONSEN retrotransposon in *Arabidopsis*. In that case, although heat stress by itself can induce transcription of ONSEN, it is only when the RdDM pathway is deficient that new insertions are transmitted to the next generation (V. V. Cavrak et al., 2014; Ito et al., 2011; Matsunaga et al., 2015). However, in wild type progenies of heat stressed mutants, ONSEN elements are rapidly re-silenced (Ito et al., 2011). In contrast, reactivated *MuDR* elements remain active for at least five generations, despite the fact that the RdDM pathway rapidly restores DNA methylation at both TIRA and TIRB (Woodhouse et al., 2006). This is likely due to differences between these two elements with respect to the means by

which the two elements are maintained in a silenced state (Burgess et al., 2020; Woodhouse et al., 2006). In the absence of *Muk*, *MuDR* elements are stably active over multiple generations. This suggests that silencing of *MuDR* requires aberrant transcripts that are distinct from those produced by *MuDR* that are not present in the minimal *Mutator* line. Experiments involving some low copy number elements in *Arabidopsis* that are activated in the DNA methylation deficient *ddm1* mutant background suggest that the same is true for these elements as well (Jeddeloh, Bender, & Richards, 1998; Singer et al., 2001); once activated, these elements remain active even in wild type progeny plants. In contrast, evidence from other TEs suggests that transcripts from these elements or their derivatives contribute to their own silencing.

CHAPTER 4. TRANSCRIPTOME PROFILING OF *MOP1* MUTANTS UNDER HEAT STRESS

4.1 Introduction

Because of its dramatic and global effects on both gene expression and protein stability, heat stress has attracted considerable attention, particularly with respect to heritable transmission of TE activity (Ito et al., 2011; Matsunaga et al., 2015; Sun et al., 2020). For both genes and TEs, although heat stress can trigger somatically heritable changes in gene expression, there appear to be a variety of mechanisms to prevent or gradually ameliorate transgenerational transmission of those changes (Ito et al., 2011; H. Ito et al., 2016; Matsunaga et al., 2015). Thus, for instance, although the Onsen retrotransposon is sensitive to heat, it is only in mutants in the RdDM pathway that transposed elements are transmitted to the next generation (Ito et al., 2011; Hidetaka Ito et al., 2016; H. Ito et al., 2016; Matsunaga et al., 2015). Given that both TEs and various components of regulatory pathways that have evolved to regulated them are up-regulated in germinal lineages, this is not surprising. Similar experiments using silenced transgenes have demonstrated that double mutants of *mom1* and *ddm1* cause these transgenes as well as several TEs to be highly responsive to heat stress, and the observed reversal of silencing can be passed on to a subsequent generation, but only in mutant progeny (Heard & Martienssen, 2014; Lang-Mladek et al., 2010; Sanchez & Paszkowski, 2014). It is also worth noting that in many cases of TE reactivation, silencing is rapidly re-established in wild type progeny (Ito et al., 2011; Matsunaga et al., 2015). The degree to which this is the case likely depends on a variety of factors, from the copy number of a given element, its position within the genome, its mode of transposition and the presence or absence of trans-acting small RNAs targeting that TE (Ito et al., 2011; Matsunaga et al., 2015; Okamoto & Hirochika, 2001; Wheeler, 2013). In maize, our observation that heat stress can reactivate a silenced *MuDR* element in *mop1* mutants suggests the maize RdDM pathway may play an essential role in genome integrity under stress conditions. To leverage the specificity of this observation, I also decided to perform a transcriptome analysis in *mop1* mutants under heat stress to examine a global behavior of genes, and TE's influence on gene regulation.

4.2 Materials and Methods

4.2.1 Plant materials and growth conditions

Seeds from *mop1* mutants and their wild-type siblings in B73 were imbibed overnight and then planted in MetroMix under standard long-day greenhouse conditions. For the heat reactivation experiment, seedlings were grown at 26 °C for 14 days with a 12-12 light dark cycle. Seedlings were incubated at 42 °C for 4 hours and leaf 3 was harvested immediately after stress treatment. As a control, leaf 3 was also collected from sibling seedlings grown at 26 °C. For each genotype and treatment, three biological replicates were used, all of which were siblings. Samples were stored in -80 °C.

4.2.2 RNAseq data processing

RNA samples were sent to Novogene Co. Ltd. for sequencing. The reads were generated in the paired-end 150-bp mode. Raw reads per sample were trimmed and preprocessed using fastq using default settings. Preprocessed reads per sample were aligned to their own indexed reference genome (B73 RefGen_v4 and Mo17 RefGen_CAU-1.0) using HISAT2 (version 2.1.0) with up to 20 multi-mapping positions. Alignment files were converted to BAM format using Samtools (version 1.9)

4.2.3 Differentially expressed genes (DEGs) and Gene Ontology (GO) enrichment analysis

Gene annotation file of B73 was downloaded from MaizeGDB. Unique read counts were used for gene expression analysis using Cufflinks (version2.2.1). Aggregate GO terms without duplication and redundancy for B73 were downloaded from the maize GAMER project (https://datacommons.cyverse.org/browse/iplant/home/shared/commons_repo/curated). GO enrichment analysis was performed using ClusterProfiler (version 3.12) for both up-regulated genes. P-adjusted values per GO term were corrected using Bonferroni multiple testing correction methods. GO terms with P-adjusted value of less than 0.05 were reported.

4.2.4 TE quantifications

TE annotations of B73 were downloaded from MaizeGDB. Those reads that mapped on more than 10 different positions were discarded for TE analysis. The newly generated BAM files were used for TE quantification using a custom script and the TE annotation file (B73 RefGen_v4).

4.3 Results

4.3.1 Identification of differentially expressed genes (DEGs) in *mop1* mutants under heat stress conditions

Maize (*Zea mays)* inbred line B73 wild type and *mop1* mutant plants grown at 28 degrees were exposed to heat stress (42 degrees) for 4 hrs at the V3 stage. Three biological replicates were sent for RNA sequencing and analysis. Overall, an average of over 40 millions raw reads were received for each samples, and roughly, an average of 90.23% of unique mapping ratio was observed for each sample (Fig 4.1A). To examine the variance among individual genotype and treatment, a principal component analysis (PCA) was performed, which shows that the degree of heat-responsiveness for each sample is quite consistent. (Figure 4.1B). Upon heat stress, thousands of genes are differentially expressed (Table 4, Fig 4.1C)

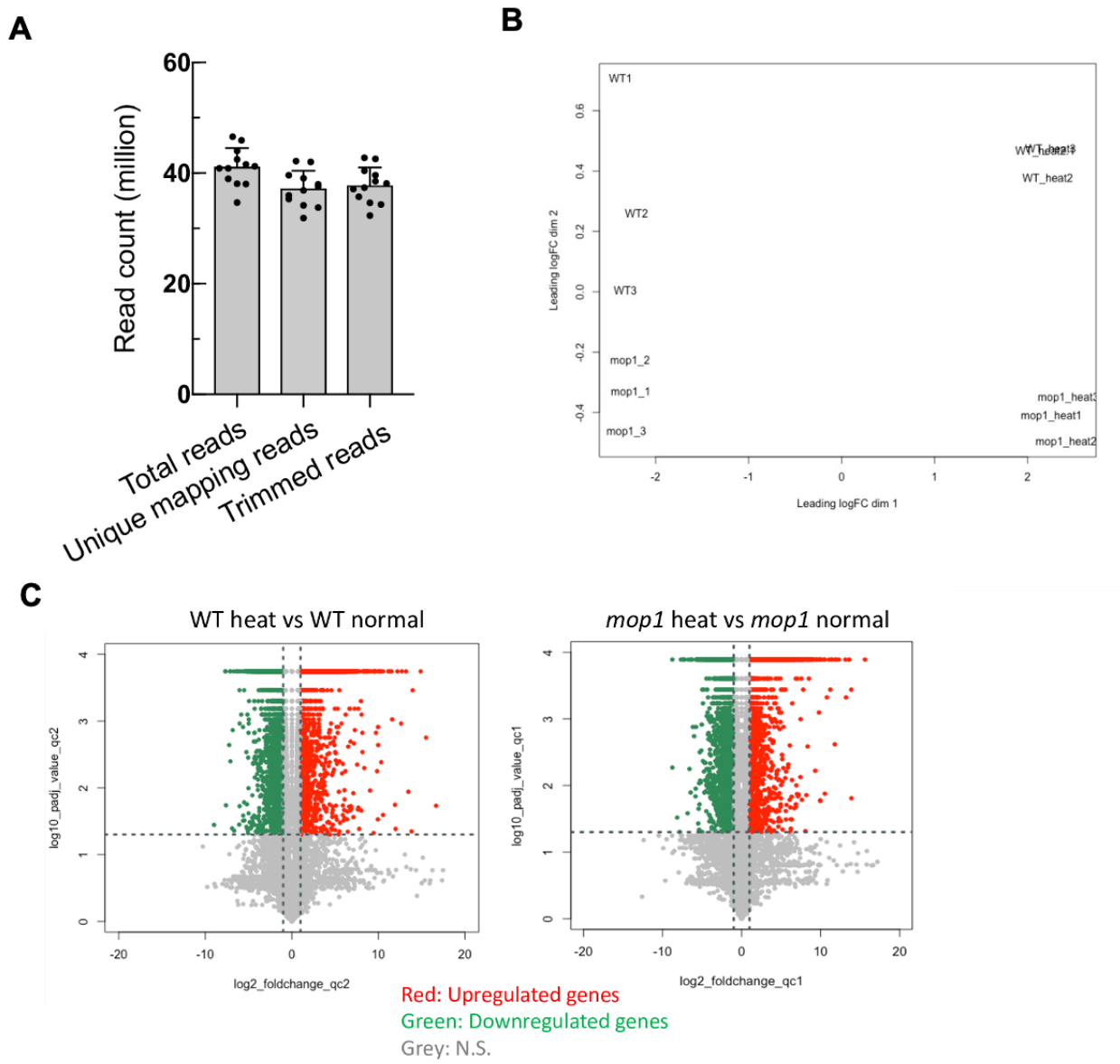

Figure 4.1 The basic statistics of my RNAseq datasets. (A) The number of mapped reads from each class. (B) Principal component analysis of each dataset. (C) Volcano plot for each genotype.

To determine if *Mop1* plays an essential role in heat stress response, a comparison of the list of DEGs between wild type and *mop1* mutants was conducted. Overall, there are more than 1000 DEGs that are either up- or down- regulated in *mop1* heat stressed mutants relative to heat stressed wild type plants, suggesting that *Mop1* plays a role in global gene regulation under heat

stress. In order to identify potential pathways downstream of RdDM in *mop1* mutants under heat stress, *mop1*-specific up- or down- regulated genes were extracted from the treated samples in each genotype. Venn diagrams were created. As shown in Fig 4.2A, there were over one thousand *mop1*-specific up regulated DEGs in response to heat. To determine if these *mop1*-specific heat responsive genes exhibit a similar pattern in wild type plants under heat stress, a heatmap was generated for those DEGs. Interestingly, most of those genes that are upregulated in *mop1* mutants under heat stress are actually are down-regulated in wild type plants under heat stress (Fig 4.2B), suggesting that the *mop1*-specific up-regulated DEGs are special in terms of heat-responsiveness.

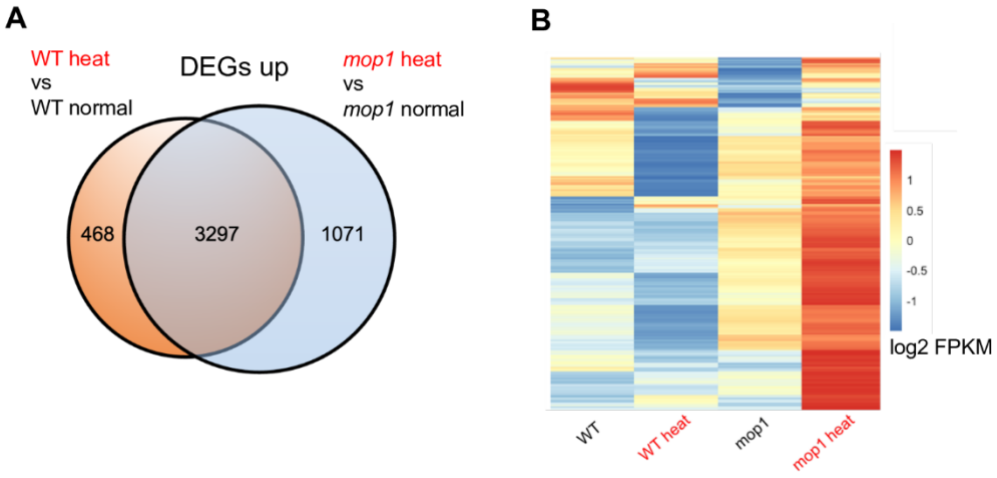

Figure 4.2 Venn diagram and heatmap of up-regulated DEGs in wild type and *mop1* mutants under heat. (A) Venn diagram of all the up-regulated DEGs under heat in wild type and *mop1* mutants. (B) Heapmap of all the *mop1*-specific DEGs in each genotype and treatment.

To determine which pathways this unique set of DEGs belong to, a GO term analysis was performed. It's not uncommon to see that some of these genes belong to the hormone signaling pathway (Fig 4.3), which has been seen frequently that is involved in stress response (Bita & Gerats, 2013; J. Liu et al., 2015; J. Zhao et al., 2021). However, interestingly, a major portion of genes belong to protein phosphorylation and protein ubiquitination pathways, suggesting protein degradation and phosphorylation may be important for sensing heat stress in the absence of a functioning RdDM pathway.

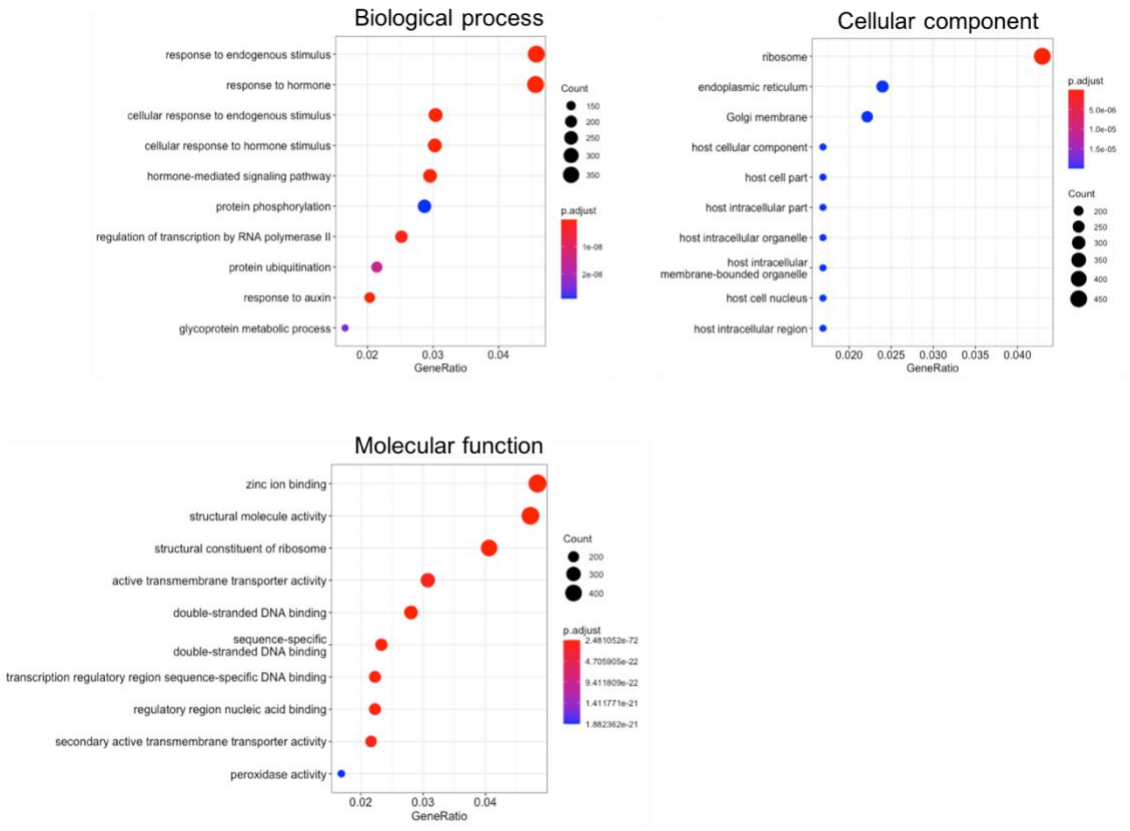

Figure 4.3 GO term analysis of the *mop1*-specific DEGs under heat. GO enrichment analysis for 'Biological Process', 'Cellular Component', and 'Molecular Function' was performed for *mop1*-specific up-regulated genes. P-adjusted values per GO term were corrected using Bonferroni multiple testing correction methods. GO terms with P-adjusted value of less than 0.05 were reported.

4.3.2 Global TE reactivation in *mop1* mutants under heat stress conditions

Maize (*Zea mays)* inbred line B73 plants grown at 28 degrees were exposed to heat stress (42 degrees) for 4 hrs at the V3 stage. Three biological replicates were sent for RNA sequencing and analysis. Unique mapping reads were used for DEGs analysis using Cufflinks at default settings. Overall, the unique mapping ratio for each sample is high, with an average value of 90.5% (Fig 4.1A, Table 4).

Raw reads from my RNAseq samples being mapped to less that 10 different positions were retained for TE quantification, which simplifies analysis but does not result in a significant loss of reads for downstream analysis (Fig 4.1A, Table 4). The newly generated BAM files were used for TE quantification using the TE annotation file for B73 and a custom script. To determine TE

reactivation upon a stress treatment in each genotype, the value of fragments per kilobase (FPKM) was determined for each annotated TE element. For each TE in each genotype or treatment, TEs were considered to be expressed when the mean FPKM was higher than 1, and not expressed when the mean FPKM was lower than 0.5. To detect reactivated TEs after a treatment in a given genotype, a baseline set of not expressed TEs was extracted. Then, a FPKM value of greater than one was used to determine which TEs were reactivated in the treated samples. To determine the portion of reactivated TEs in general, TEs were profiled in wild type and *mop1* mutants under normal and heat stress. This analysis revealed that hundreds of TEs became transcriptionally reactivated upon heat stress in both genotypes (Fig 4.4A). Among all those reactivated TEs under heat stress, over one hundred TEs were found to be the *Mop1* genotype specific, suggesting that those TEs are subject to be under *Mop1*-regulation under heat stress (Figure 4.4B). However, more TEs became reactivated in *mop1* mutants under heat than under wild type, suggesting *Mop1* does play a role in regulation of at least some TEs under heat stress. To determine the specificity of TE reactivation under heat stress, I classified these TEs. Among the reactivated TEs, most belong to two TE families, Helitron and TIR (Fig 4.4C, D), indicating that these TEs may have unique sequence features that make them more sensitive to heat stress.

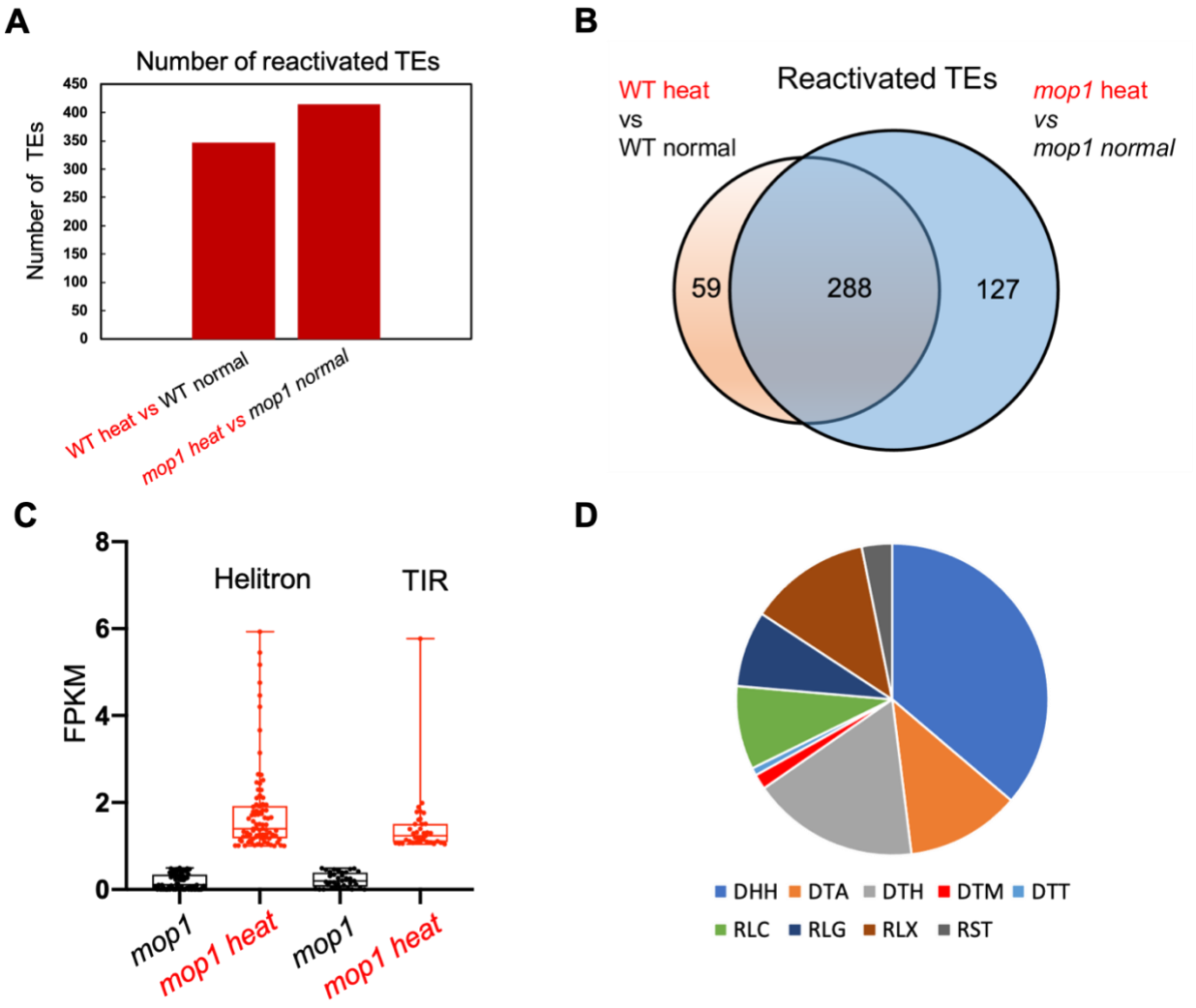

Figure 4.4 The number of reactivated TEs and their classifications in wild type and *mop1* mutants under heat stress. (A) The number of reactivated TEs under heat in each genotype. (B) Venn diagram of all the reactivated TEs under heat. (C) Classification of all the reactivated TEs in mop1 mutants under heat. (D) A sub-classification of all the reactivated TEs in *mop1* mutants under heat.

4.3.3 Identification of *mop1*-specific TE-adjacent gene expression under heat

Because TE insertions near genes may influence gene regulation, I sought to determine if the *mop1*-specific heat reactivated TEs are associated with changes in expression of nearby genes. To do this, 127 *mop1*-specific reactivated TEs were extracted (Figure 4.5A), genes inserted 1 kb upstream of the transcription start site or 1 kb downstream of the transcription termination site were extracted from those *mop1*-specific reactivated TEs. The FPKM values of these genes were

then determined from the Cufflinks results. A heatmap was generated to examine their expression pattern. Remarkably, most of those genes were up-regulated as well (Fig 4.5B), suggesting that TEs may act as local heat-responsive enhancers of nearby gene expression in the absence of RdDM.

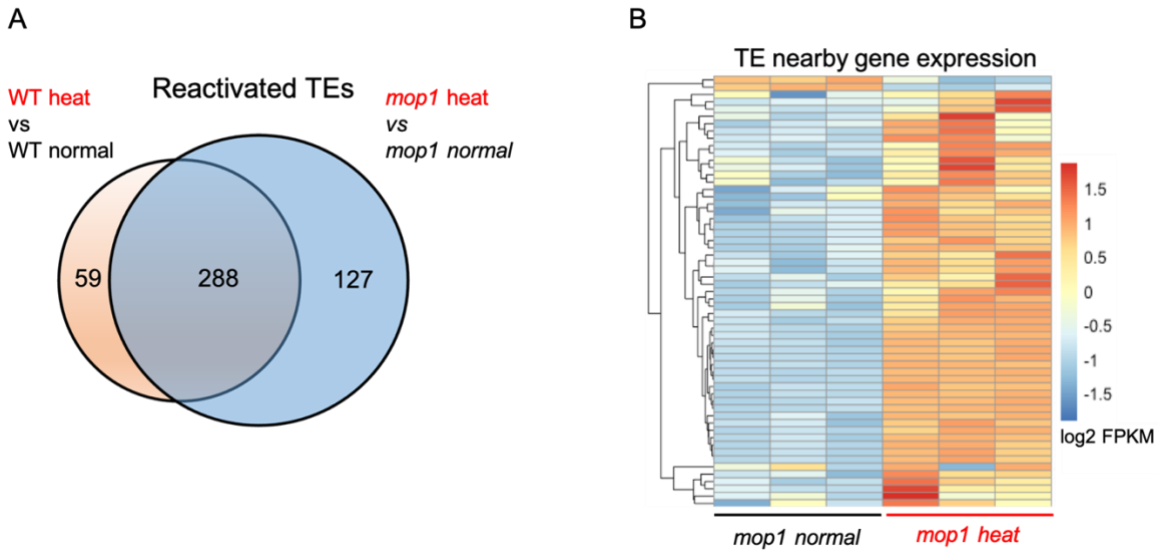

Figure 4.5. Venn diagram and heatmap of the *mop1*-specific TE-adjacent gene expression before and after heat stress. (A) Venn diagram of all the reactivated TEs under heat. (B) Heatmap of all the *mop1*-specific reactivated TE-adjacent genes in control and heat treated *mop1* mutants.

To determine if specific classes of genes are affected by this combination of TE presence, RdDM and heat stress, a GO analysis was performed for those TE-adjacent genes. Surprisingly, most genes belong to a pathway that is involved in the metabolic transportation and sensing (Fig 4.6), suggesting *Mop1* specifically prevents inappropriate expression of genes that are close to TEs and that are involved in these processes.

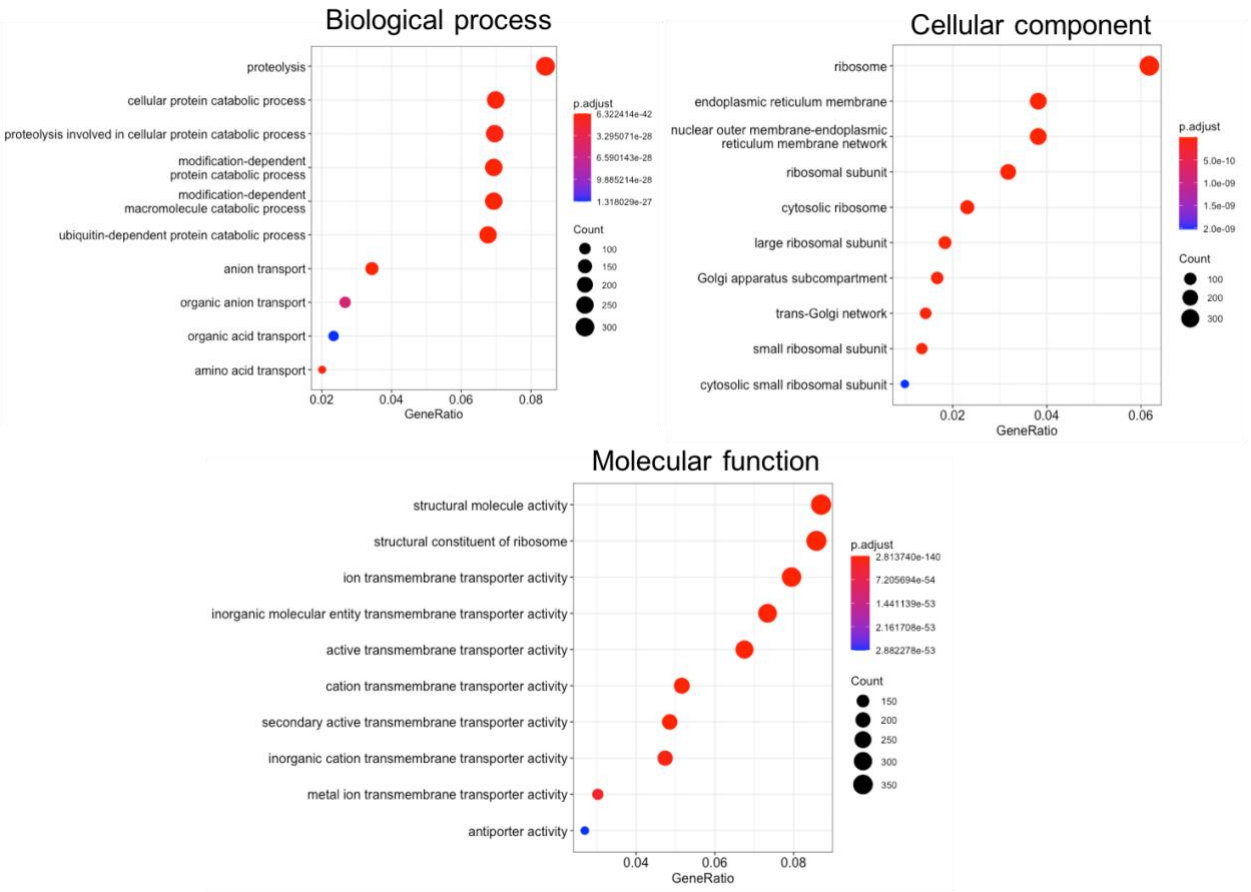

Figure 4.6. GO term analysis of the *mop1*-specific reactivated TE-adjacent DEGs under heat. GO enrichment analysis for 'Biological Process', 'Cellular Component', and 'Molecular Function' was performed for *mop1*-specific up-regulated genes. P-adjusted values per GO term were corrected using Bonferroni multiple testing correction methods. GO terms with P-adjusted value of less than 0.05 were reported.

4.4 Discussion

4.4.1 *Mop1* prevent TE activity under heat stress conditions

To leverage the specificity of my bench observation that heat can heritably stably reactivates a silenced *MuDR* element in *mop1* mutants under heat, I prepared and analyzed my own RNAseq datasets focusing on global TE reactivation from heat-treated *mop1* mutants in B73 background. I found that heat can reactivate hundreds of silenced TE elements in both wild type and *mop1* mutant plants treated with heat. This is not surprising because it has been proposed or observed frequently that TE can become transcriptionally reactivated upon various types of abiotic and biotic stress treatments, and the number of such reactivated TE elements are similarly only

around three hundred respectively (Liang et al., 2020; Makarevitch et al., 2015; McCue, Nuthikattu, Reeder, & Slotkin, 2012). However, from looking at a comparison of the number of heat-reactivated TEs between wild type and *mop1* mutants, I found that there are over one hundred more reactivated TE elements in *mop1* mutants compared with that in wild type, suggesting that the *Mop1* does prevent TE activity under stress conditions. To test if there are TEs that are only subject to be under *Mop1*-regulation under heat, I extracted a set of the *mop1*-specific reactivated TEs and classified those. I found that most of those TEs belong to two TE families, Helitron and TIR, suggesting that these TEs may have unique features that make them more sensitive to heat stress in *mop1* mutants.

4.4.2 The *mop1*-specific reactivated TEs are associated with expression of a pathway involved in metabolic transportation and sensing

It has already been proposed that TEs could contribute to adjacent gene expression (Drongitis, Aniello, Fucci, & Donizetti, 2019; Feng et al., 2013; Horvath et al., 2017). My results support this proposal, which show that up-regulated TEs mostly are associated with up-regulated expression of genes close to those TEs under heat. One possible mechanism by which those TEs could regulate adjacent genes is that TEs may act as local enhancers under stress conditions (Makarevitch et al., 2015; McCue et al., 2012). This hypothesis highlights the importance of orientation of the TE relative to its adjacent gene, and it's possible that a novel transcript containing TE sequences fused to gene sequences would be generated under stress conditions (Makarevitch et al., 2015). It's also possible that those TE-adjacent genes are influenced by changes of the local epigenetic state caused by heat-reactivated TEs indirectly, which would suggest that changes in TE's epigenetic activity can modulate the stress response of the host organism. On that other hand, surprisingly, I found that most *mop1*-specific reactivated TE-adjacent genes belong to a pathway that is involved in the metabolic transportation and sensing. This indicates that this pathway is unique to a heat stress response potentially influenced by TE activity that is downstream of RdDM.

Table 1. Primers used in this study

Primer	**Sequence (5' to 3')**
Ex1	ACATCCACGCTGTCTCAGCC
RLTIR2	ATGTCGACCCCTAGAGCA
TIRAout	GCTGTCACCTTTCTGTTTTGGCGAT
12-4R3	CGGTATGGCGGCAGTGACA
ZmRDR2_F	TCTCCACCGCCCACTTGAT
ZmRDR2_R	ATGGCCAGCAGGGTGTCGCAGAT
TIR6	AGAGAAGCCAACGCCAWCGCCTCYATTTCGTC
HSP90-qPCR_F	CTCAGAGAGCTCATCTCCAACGC
HSP90-qPCR_R	GATGATGGACAGCGTCTTGCTGG
mudrA-qPCR_F	ATTGTGGTGAATTGGGACACC
mudrA-qPCR_R	TCCTCTGCTACGTCTGGCTGT
mudrB-qPCR_F	ATCTCAACGAATTCTTTATGCT
mudrB-qPCR_R	TTCAGTTCGCCGCCATT
Tub2-qPCR_F	CTACCTCACGGCATCTGCTATGT
Tub2-qPCR_R	GTCACACACACTCGACTTCACG
p1bis2f	GTTGGYGAGGAGGAGYAYGAGGTG
TIRAbis2R	CARCTCCCRARAACACTCCAATTC
TIRAmF6	TYAGGGAAYTGGAGYGAYGGGTG
methy_TIRBF	GTTAACCAAACAAGGCAGAGAT
methy_TIRBR	CAGTCGTCCGTTTTATGATTC
pGEM_F	TGTAATACGACTCACTATAGGGCG
pGEMT_R	ATTTAGGTGACACTATAGAATACTC
copia_F	CGATGTGAAGACAGCATTCCT
copia_R	CTCAAGTGACATCCCATGTGT
TIRAR	AGGAGAGACGGTGACAAGAGGAGTA
TIRAUTRR	ACTGGAGCGACGGGTGCTCGGCT

Table 2. Number of spotted progenies from test crosse

Cross(H2)	**hm**	**wk**	**t sp**	**pale**	**total**	**% spotted**	**P Ch sq 1:1**	**%hm**
***MuDR˜/-; mop1/mop1* x -/-; +/+**								
1	6	0	6	7	13	46.2%	0.782	46.2%
2	32	3	35	40	75	46.7%	0.564	42.7%
3	4	12	16	21	37	43.2%	0.411	10.8%
4	61	11	72	66	138	52.2%	0.610	44.2%
5	9	3	12	16	28	42.9%	0.450	32.1%
6	19	19	38	52	90	42.2%	0.140	21.1%
7	16	6	22	46	68	32.4%	0.004	23.5%
8	2	5	7	10	17	41.2%	0.467	11.8%
9	41	11	52	49	101	51.5%	0.765	40.6%
10	15	4	19	22	41	46.3%	0.639	36.6%
Total	205	74	279	329	608	45.9%	0.043	33.7%
***MuDR*/-;mop1/mop1* x -/-; +/+**								
1	0	0	0	113	113	0.0%	0.000	0.0%
2	0	0	0	14	14	0.0%	0.000	0.0%
3	0	1	1	26	27	3.7%	0.000	0.0%
4	0	0	0	97	97	0.0%	0.000	0.0%
5	0	0	0	77	77	0.0%	0.000	0.0%
6	0	0	0	35	35	0.0%	0.000	0.0%
7	0	0	0	45	45	0.0%	0.000	0.0%
8	0	2	2	63	65	3.1%	0.000	0.0%
Total	0	3	3	470	473	0.6%	0.000	0.0%
Cross (H3)								
***MuDR˜/-; mop1/+* x -/-; +/+**								
1	128	28	156	138	294	53.1%	0.294	43.5%
2	7	0	7	10	17	41.2%	0.467	41.2%
3	39	16	55	53	108	50.9%	0.847	36.1%
4	142	30	172	164	336	51.2%	0.663	42.3%
5	52	27	79	69	148	53.4%	0.411	35.1%
6	18	5	23	22	45	51.1%	0.881	40.0%
7	74	35	109	101	210	51.9%	0.581	35.2%
8	47	15	62	48	110	56.4%	0.182	42.7%
9	35	15	50	38	88	56.8%	0.201	39.8%

Table 2 continued

10	27	7	34	38	72	47.2%	0.637	37.5%
11	12	9	21	17	38	55.3%	0.516	31.6%
12	5	0	5	7	12	41.7%	0.564	41.7%
13	11	4	15	12	27	55.6%	0.564	40.7%
14	36	12	48	56	104	46.2%	0.433	34.6%
15	59	21	80	67	147	54.4%	0.284	40.1%
Total	564	196	760	702	1462	52.0%	0.129	38.6%
***MuDR*/-;mop1/+* x -/-; +/+**								
1	0	2	2	95	97	2.1%	0.000	0.0%
2	0	5	5	154	159	3.1%	0.000	0.0%
3	0	7	7	145	152	4.6%	0.000	0.0%
4	0	3	3	54	57	5.3%	0.000	0.0%
5	0	0	0	288	288	0.0%	0.000	0.0%
6	0	0	0	197	197	0.0%	0.000	0.0%
7	0	0	0	142	142	0.0%	0.000	0.0%
8	0	0	0	97	97	0.0%	0.000	0.0%
9	0	2	2	153	155	1.3%	0.000	0.0%
10	0	0	0	218	218	0.0%	0.000	0.0%
Total	0	19	19	1543	1562	1.2%	0.000	0.0%
Cross (H4)								
***MuDR˜/-* x -/-; +/+**								
1	106	0	106	148	254	41.7%	0.008	41.7%
2	91	17	108	119	227	47.6%	0.465	40.1%
3	96	5	101	128	229	44.1%	0.074	41.9%
4	72	20	92	93	185	49.7%	0.941	38.9%
5	114	21	135	119	254	53.1%	0.315	44.9%
6	134	0	134	160	294	45.6%	0.129	45.6%
7	96	32	128	180	308	41.6%	0.003	31.2%
8	84	7	91	63	154	59.1%	0.024	54.5%
9	162	8	170	168	338	50.3%	0.913	47.9%
10	153	13	166	165	331	50.2%	0.956	46.2%
Total	1108	123	1231	1343	2574	47.8%	0.027	43.0%
***MuDR*/-* x -/-; +/+**								
1	0	6	6	182	188	3.2%	0.000	0.0%
2	2	7	9	239	248	3.6%	0.000	0.8%
3	0	11	11	320	331	3.3%	0.000	0.0%

Table 2 continued

4	0	4	4	172	176	2.3%	0.000	0.0%
5	1	0	1	298	299	0.3%	0.000	0.3%
6	0	8	8	188	196	4.1%	0.000	0.0%
7	0	15	15	238	253	5.9%	0.000	0.0%
Total	3	51	54	1637	1691	3.2%	0.000	0.2%
Cross (H5)								
MuDR˜/-* x *-/-; +/+								
1	74	1	75	113	188	39.9%	0.006	39.4%
2	37	2	39	46	85	45.9%	0.448	43.5%
3	16	0	16	25	41	39.0%	0.160	39.0%
4	135	0	135	103	238	56.7%	0.038	56.7%
5	60	0	60	59	119	50.4%	0.927	50.4%
6	205	2	207	143	350	59.1%	0.001	58.6%
7	167	1	168	154	322	52.2%	0.435	51.9%
8	152	2	154	168	322	47.8%	0.435	47.2%
10	162	1	163	173	336	48.5%	0.585	48.2%
11	204	7	211	269	480	44.0%	0.008	42.5%
12	91	1	92	104	196	46.9%	0.391	46.4%
13	161	0	161	127	288	55.9%	0.045	55.9%
14	90	0	90	90	180	50.0%	1.000	50.0%
Total	1554	17	1571	1574	3145	50.0%	0.957	49.4%
MuDR*/- x -/-; +/+					0			
1	0	0	0	86	86	0.0%	0.000	0.0%
2	1	7	8	256	264	3.0%	0.000	0.4%
3	0	0	0	216	216	0.0%	0.000	0.0%
4	0	0	0	103	103	0.0%	0.000	0.0%
5	0	0	0	240	240	0.0%	0.000	0.0%
6	0	0	0	104	104	0.0%	0.000	0.0%
7	1	9	10	326	336	3.0%	0.000	0.3%
8	0	3	3	357	360	0.8%	0.000	0.0%
9	0	0	0	26	26	0.0%	0.000	0.0%
10	0	0	0	100	100	0.0%	0.000	0.0%
11	0	0	0	288	288	0.0%	0.000	0.0%
12	0	0	0	240	240	0.0%	0.000	0.0%
13	0	0	0	113	113	0.0%	0.000	0.0%
14	0	0	0	192	192	0.0%	0.000	0.0%
15	1	1	2	298	300	0.7%	0.000	0.3%

Table 2 continued

Total	3	20	23	2945	2968	0.8%	0.000	0.1%
hm - heavily and medium spotted								
wk - weakly spotted								
pale - no spots								
***MuDR*~ - heat reactivated *MuDR* element**								
MuDR* **- control *MuDR* element**								
t sp – total spotted								

Table 3. Summary of all bisulfite sequencing results

Bisulfite sequencing results for Figure 2.2					
TIRA in F2					
Samples	**CG%**	**CHG%**	**CHH%**	**Total%**	
MuDR/-;mop1/+*	88	82.35	47.61	66.666	
MuDR/-;mop1/+*	92	70.58	40.47	61.904	
MuDR/-;mop1/+*	88	82.35	45.23	65.476	
MuDR/-;mop1/+*	80	64.7	42.88	58.333	
MuDR/-;mop1/+*	76	58.82	40.47	54.761	
MuDR/-;mop1/+*	92	76.47	40.47	63.095	
MuDR/-;mop1/+*	88	82.35	45.23	66.666	
MuDR/-;mop1/+*	68	47.05	47.61	46.428	
MuDR/-;mop1/+*	72	82.35	33.33	53.571	
MuDR/-;mop1/+*	76	82.35	30.95	61.904	
MuDR/-;mop1/mop1*	0	0	0	0	
MuDR/-;mop1/mop1*	0	5.88	0	1.19	
MuDR/-;mop1/mop1*	0	0	0	0	
MuDR/-;mop1/mop1*	0	0	0	0	
MuDR/-;mop1/mop1*	0	0	0	0	
MuDR/-;mop1/mop1*	0	0	0	0	
MuDR/-;mop1/mop1*	0	5.88	0	1.19	
MuDR/-;mop1/mop1*	0	0	0	0	
MuDR/-;mop1/mop1*	0	0	0	0	
MuDR/-;mop1/mop1*	0	0	0	0	
TIRB in F2					
Samples	**CG%**	**CHG%**	**CHH%**	**Total%**	
MuDR/-;mop1/+*	14.3	33.33	25	23.333	
MuDR/-;mop1/+*	21.4	38.88	25	26.666	
MuDR/-;mop1/+*	14.3	33.33	22.72	22.222	
MuDR/-;mop1/+*	14.3	33.33	22.72	22.222	
MuDR/-;mop1/+*	14.3	38.88	22.72	23.333	
MuDR/-;mop1/+*	14.3	55.55	25	27.777	
MuDR/-;mop1/+*	14.3	38.88	25	24.444	
MuDR/-;mop1/+*	17.9	55.55	25	28.888	
MuDR/-;mop1/+*	17.9	38.88	22.72	24.444	
MuDR/-;mop1/+*	3.57	5.55	72.72	37.777	
MuDR/-;mop1/mop1*	0	11.11	77.27	40	
MuDR/-;mop1/mop1*	0	5.55	70.45	35.555	
MuDR/-;mop1/mop1*	0	11.11	75	38.888	

Table 3 continued

MuDR/-;mop1/mop1*	3.57	5.55	77.27	40	
MuDR/-;mop1/mop1*	3.57	5.55	86.36	44.444	
MuDR/-;mop1/mop1*	0	5.55	70.45	35.555	
MuDR/-;mop1/mop1*	3.57	5.55	86.36	44.444	
MuDR/-;mop1/mop1*	3.57	11.11	79.54	42.222	
MuDR/-;mop1/mop1*	0	11.11	70.45	36.666	
MuDR/-;mop1/mop1*	3.57	5.55	77.27	40	
Bisulfite sequencing results for Figure 4.8					
TIRB in H1					
Samples	**CG%**	**CHG%**	**CHH%**	**Total%**	
MuDR/-;mop1/mop1*	85.71	66.66	75	76.666	
MuDR/-;mop1/mop1*	78.57	61.11	75	73.333	
MuDR/-;mop1/mop1*	85.71	66.66	77.27	77.777	
MuDR/-;mop1/mop1*	85.71	61.11	77.27	76.666	
MuDR/-;mop1/mop1*	85.71	44.44	75	72.222	
MuDR/-;mop1/mop1*	85.71	61.11	75	75.555	
MuDR/-;mop1/mop1*	72.14	4.44	75	71.111	
MuDR/-;mop1/mop1*	82.14	61.11	77.27	75.555	
MuDR/-;mop1/mop1*	75	55.55	45.45	56.666	
MuDR/-;mop1/mop1*	75	55.55	45.45	56.666	
MuDR~/-;mop1/mop1_heat	82.14	61.11	81.81	77.77	
MuDR~/-;mop1/mop1_heat	82.14	61.11	81.81	77.77	
MuDR~/-;mop1/mop1_heat	89.28	50	81.81	77.77	
MuDR~/-;mop1/mop1_heat	82.14	61.11	77.27	75.555	
MuDR~/-;mop1/mop1_heat	82.14	55.55	77.27	74.444	
MuDR~/-;mop1/mop1_heat	82.14	44.44	75	71.111	
MuDR~/-;mop1/mop1_heat	82.14	55.55	75	73.333	
MuDR~/-;mop1/mop1_heat	78.57	44.44	72.72	68.888	
MuDR~/-;mop1/mop1_heat	78.57	50	77.27	72.222	
MuDR~/-;mop1/mop1_heat	82.14	61.11	81.81	77.777	
Bisulfite sequencing results for Figure 4.10					
TIRA in H2, H4, H5					
Samples	**CG%**	**CHG%**	**CHH%**	**Total%**	**Generation**
MuDR~/-;mop1/+	48	76.74	40.47	50	H2
MuDR~/-;mop1/+	72	82.35	33.33	54.761	H2

Table 3 continued

MuDR~/-;mop1/+	84	82.35	35.71	59.523	H2
MuDR~/-;mop1/+	72	82.35	36.58	56.626	H2
MuDR~/-;mop1/+	88	94.11	52.38	71.428	H2
MuDR~/-;mop1/+	88	82.35	38.09	61.904	H2
MuDR~/-;mop1/+	84	82.35	35.71	59.523	H2
MuDR~/-;mop1/+	84	94.11	45.23	66.666	H2
MuDR~/-;mop1/+	72	88.23	24.39	51.807	H2
MuDR~/-;mop1/+	80	82.35	38.09	59.523	H2
MuDR~/-	0	0	0	0	H4
MuDR~/-	0	0	0	0	H4
MuDR~/-	0	0	0	0	H4
MuDR~/-	0	0	0	0	H4
MuDR~/-	0	0	0	0	H4
MuDR~/-	0	0	0	0	H4
MuDR~/-	0	0	0	0	H4
MuDR~/-	0	0	0	0	H4
MuDR~/-	0	0	0	0	H4
MuDR~/-	0	0	0	0	H4
MuDR~/-	0	0	0	0	H5
MuDR~/-	0	0	0	0	H5
MuDR~/-	0	0	0	0	H5
MuDR~/-	0	0	0	0	H5
MuDR~/-	0	0	0	0	H5
MuDR~/-	0	0	0	0	H5
MuDR~/-	0	0	0	0	H5
MuDR~/-	0	0	0	0	H5
MuDR~/-	0	0	0	0	H5
MuDR~/-	0	0	0	0	H5
TIRB in H2, H4, H5					
Samples	**CG%**	**CHG%**	**CHH%**	**Total%**	
MuDR~/-;mop1/+	85.71	66.66	75	76.666	H2
MuDR~/-;mop1/+	78.57	61.11	75	73.333	H2
MuDR~/-;mop1/+	85.71	66.66	77.27	77.777	H2
MuDR~/-;mop1/+	75	72.22	47.72	61.111	H2
MuDR~/-;mop1/+	85.71	66.66	77.27	77.777	H2
MuDR~/-;mop1/+	85.71	61.11	77.27	76.666	H2
MuDR~/-;mop1/+	85.71	44.44	75	72.222	H2

Table 3 continued

MuDR~/-;mop1/+	85.71	61.11	75	75.555	H2
MuDR~/-;mop1/+	82.14	44.44	75	71.111	H2
MuDR~/-;mop1/+	82.14	61.11	77.27	75.555	H2
MuDR~/-	14.28	11.11	72.72	42.222	H4
MuDR~/-	14.28	5.55	70.45	40	H4
MuDR~/-	7.14	5.55	70.45	37.777	H4
MuDR~/-	14.28	16.66	72.72	43.333	H4
MuDR~/-	0	0	75	36.666	H4
MuDR~/-	3.57	0	75	37.777	H4
MuDR~/-	14.28	16.66	72.72	43.333	H4
MuDR~/-	7.14	11.11	70.45	38.888	H4
MuDR~/-	3.57	5.55	72.72	37.777	H4
MuDR~/-	10.71	5.55	70.45	38.888	H4
MuDR~/-	10.71	5.55	70.45	38.888	H5
MuDR~/-	10.71	16.66	70.45	41.111	H5
MuDR~/-	3.57	5.55	72.72	37.777	H5
MuDR~/-	10.71	11.11	70.45	40	H5
MuDR~/-	3.57	0	70.45	35.555	H5
MuDR~/-	14.28	16.66	70.45	42.222	H5
MuDR~/-	10.71	5.55	70.45	38.888	H5
MuDR~/-	0	0	70.45	34.444	H5
MuDR~/-	14.28	5.55	70.45	40	H5
MuDR~/-	7.14	5.55	70.45	37.777	H5

Table 4. Mapping statistics of my RNAseq datasets

Sample IDs	**Total reads**	**Unique mapping reads**	**%unique mapping rate**	**After 10omitted**	**Overall alignment rate**	**Inbred**
WT normal	41579303	35967113	86.5	37104937	95.59	B73
WT normal	44003517	39627377	90.06	40410281	97.92	B73
WT normal	46590169	42018775	90.19	42580266	98.16	B73
WT heat	38079631	33794485	88.75	34301121	97.99	B73
WT heat	40864103	37579010	91.96	38115356	97.98	B73
WT heat	45943539	42188106	91.83	42770263	98	B73
mop1 normal	38018102	34193747	89.94	34642098	98.12	B73
mop1 normal	38986733	35324771	90.61	35745188	98.2	B73
mop1 normal	40872181	36880211	90.23	37386678	98.14	B73
mop1 heat	34691175	31854576	91.82	32300202	97.85	B73
mop1 heat	41224665	37951763	92.06	38521059	97.85	B73
mop1 heat	42459698	39067765	92.01	39636120	97.95	B73

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