**Mu killer**-mediated and spontaneous silencing of *Zea mays* mutator family transposable elements define distinctive paths of epigenetic inactivation

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Mu killer contains a partial inverted duplication of the *mudrA* transposase gene and two copies of the terminal inverted repeat A (TIRA) region of the master *MuDR* element of maize. *Mu killer* can effectively silence single copy *MuDR/Mu* lines, and it is proposed that a ∼4 kb hairpin RNA is generated by read through transcription from a flanking gene and that this transcript serves as a substrate for siRNA production. *Mu killer* was sequenced, except for a recalcitrant portion in the center of the locus, and shown to be co-linear with *mudrA* as originally proposed. The ability of the dominant *Mu killer* locus to silence a standard high copy number *MuDR/Mu* transposon line was evaluated. After two generations of exposure, about three quarters of individuals were silenced indicating reasonable effectiveness as measured by the absence of *mudrA* transposase transcripts. *Mu killer* individuals that effectively silenced *MuDR* expressed two short antisense transcripts. In contrast, *Mu killer* individuals that failed to silence *MuDR* expressed multiple sense transcripts, derived from read through transcription initiating in a flanking gene, but no antisense transcripts were detected.

**Keywords:** transposon, mutator, *Mu killer*, anther, maize, splicing

**INTRODUCTION**

*MuDR/Mu* comprise the mutator family of maize Class II transposable elements (reviewed in Lisch, 2002; Walbot and Rudenko, 2002). These high copy number elements are very effective mutagens, because they target RNA Polymerase II transcription units (Fernandes et al., 2004); *MuDR/Mu* in active mutator lines increase the forward mutation frequency 50–100-fold per locus (Walbot and Rudenko, 2002). *MuDR* contains two convergently transcribed genes: *mudrA* encodes transposase and *mudrB* encodes a protein of unknown function. Transcripts of both genes are subject to alternative splicing, which results in multiple protein isoforms (Walbot and Rudenko, 2002). The diverse elements share ∼215 bp terminal inverted repeats (TIRs) within which there is a highly conserved MURA transposase binding site in all mobile *Mu* (Benito and Walbot, 1997) and the gene promoters (Raizada et al., 2001a,b). A key feature of the family is the high copy number of ∼50–100 mobile *Mu* elements in active mutator plants. Utilizing replicative transposition in which parental transposon locations are preserved, *MuDR/Mu* elements double in copy number late in the lifecycle by inserting throughout the genome. Consequently, progeny of an outcross to a non-mutator line have about the same number of elements as the mutator parent (Alleman and Freeling, 1986; Walbot and Warren, 1988).

When *MuDR/Mu* copy number increases substantially, as after self-pollination, the likelihood of transposon silencing increases (Robertson, 1986; Walbot, 1986). Historically *Mu* element silencing was followed using visible phenotypes, either loss of somatic excision from mutable alleles, for example the switch from spotted to colorless kernels (Robertson, 1986; Walbot, 1986), or by activation of gene expression at a locus suppressed by a nearby *Mu* insertion, for example converting pale green to darker leaf pigmentation (Martienssen et al., 1990). Molecular assays for silencing include DNA methylation within *Mu* elements (Chandler and Walbot, 1986) and decreased *mudrA* transposase transcripts (Rudenko et al., 2003). Although some individuals switch abruptly from an active to inactive status, in many cases loss of *Mu* element insertion and excision occur over a span of time. Silencing is progressive, both within the sequential organs of a developing plant (Martienssen et al., 1990) and over several generations (Takumi and Walbot, 2007). The trigger for silencing other than the correlation with high *MuDR/Mu* copy number has been difficult to define, although there are clearly separable steps for imposition and maintenance of silencing (Woodhouse et al., 2006).

The stochastic appearance and incomplete penetrance of silencing have been barriers to establishing the specific components of the silencing machinery. All maize lines contain defective *hMuDR* elements (Rudenko and Walbot, 2001) that generate both sense and antisense transcripts, however, these elements are insufficient to direct silencing efficiently in crosses with active mutator individuals. Similarly introduction of antisense transgenes is not
an efficient silencing method (Kim and Walbot, 2003). In an unusual single copy Mu1 plus single copy MuDR line, called minimal mutator (Lisch et al., 1995), spontaneous silencing is exceedingly rare (Lisch and Freeling, 1994). This permitted detection of a newly arisen dominant factor, Mu killer (GenBank accession: DQ011286), that could swiftly silence the minimal mutator system (Slotkin et al., 2003). The Mu killer locus has an internally deleted and partially duplicated mudrA element (Figure 1); the two TIRs are both derived from the mudrA adjacent element end and there are two partial copies of mudrA that terminate in an inverted duplication. The precise DNA sequence of Mu killer has not been reported. It is hypothesized that transcripts containing the inverted mudrA duplicated region form fold back RNA that is processed into small interfering RNAs that direct silencing of the single MuDR in the line (Slotkin et al., 2005).

We have reported a transcriptomic and proteomic comparison of anthers from sister lines that remained active or were recently fully silenced. In standard, high copy number active mutator lines the transcriptome is altered ~25% in comparison to the silenced line, including activation of stress pathways (Skibbe et al., 2009), indicating that mutator activity has a major impact on the host without disrupting normal anther development. In the current study we sought to determine if Mu killer could silence a standard high element copy number mutator line, because these lines are non-responsive to ectopically expressed antisense RNA (Kim and Walbot, 2003). Second, if Mu killer can invoke silencing in such lines is the impact on the transcriptome similar to what occurs during spontaneous silencing? Third, we wished to better define the structure of Mu killer as an aid to understanding the structure of predicted RNA transcripts that might be mechanistically involved in MuDR/Mu silencing.

MATERIALS AND METHODS

GENETIC MATERIALS

A Mu killer, a1-mu1/a1 single copy MuDR minimal mutator stock was obtained from Damon Lisch. This line was maintained by crossing to an a1 minimal mutator line or to an a1 tester grown at Stanford CA or on Moloka’i HI. High copy MuDR/Mu lines with bz2-mutable alleles were described previously (Skibbe et al., 2009).

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SEQUENCING Mu killer

The Mu killer locus was PCR amplified in two halves (Figure 1). A 1.8 kb amplicon was obtained for the “left” half using the

| Cross 1 | Cross 2a | Cross 2b | Cross 2c | Cross 2d |
|---------|---------|---------|---------|---------|
| bz2-mu2 | Bz2    | Bz2    | Bz2    | Bz2    |
| bz2-ref | MuK    | MuK    | MuK    | MuK    |
| +       | +       | +       | +       | +       |
| Bz2     | bz2-ref | bz2-ref | bz2-ref | bz2-ref |
| purple  | 1 purple: 1 spotted | 1 purple: 1 spotted | 1 purple: 1 bronze | 1 purple: 1 bronze |

FIGURE 1 | Schematic of the Mu killer locus and the two flanking maize genes amy4 and acm1. The triangles indicate the TIRA sequences. Primers used in the analysis of Mu killer genomic DNA and RT-PCR products are indicated.

FIGURE 2 | Crossing scheme to test for Mu killer-induced silencing of a high copy mutator line. Half of the progeny ears contain the bz2-mu2 reporter allele and are expected to segregate 1:1 purple:spotted (Crosses 2a and 2b); the other half (Crosses 2c and 2d) lack the reporter allele and are expected to segregate 1:1 purple:bronze. Mu killer is segregating 1:1 in Crosses 2a and 2c.
muk-flank (5′ CCTTTGCTAGCTTGGTATCTTCG 3′) and mudrA-1298r (5′ GCACCATTGTTGGTTTCTCT 3′) primers. A 1.3 kb amplicon was obtained for the “right” half using the acm1-Mu killer chimeric primer acm1-muk_86r (5′ CCCC-TACCTTTGGTGGATATAATG 3′) and mudrA-1298r primer. Genomic DNA targets were amplified in a 25 µl reaction using 20 ng genomic DNA as template, 0.5 µM of each primer, 2 mM MgSO4, 0.2 mM dNTPs, 1× HiFi Buffer, and 1 unit HiFi Platinum Taq (Invitrogen Life Science, Carlsbad CA, USA) per reaction under the following conditions: 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 1.5 min; there was a final extension at 72°C for 10 min. The left and right Mu killer amplicons were purified using the Qiagen (Valencia, CA, USA) PCR Purification Kit, TOP10 subcloned, and sequenced using M13 forward and M13 reverse primers. The Mu killer sequence segments were assembled and validated by replicate sequencing and are available under GenBank numbers JX067398 (left half) and JX067399 (right half).

**GENOTYPING**

Genomic DNA was extracted from adult leaf tissue using the CTAB method of Rogers and Bendich (1985). Genotyping was performed using the PCR parameters described above. Target, amplicon size, and corresponding primer pairs are: Mu killer, 0.7 kb amplicon with the muk-flank (5′ CTTTTGCTAGCTTGTTATCTTCG 3′) and mudr-219r (5′ AGGAGAGACCTTTGAAACACAGGTTA 3′) primers; bz2-mu2: 0.2 kb amplicon with the bz2-637f (5′ CAGAGAGTTGCCAAGAAGT 3′) and Mu-TIR (5′ AGAGAAGCAGAGCCAWCGCTATTTGC 3′) primers.

**RNA ANALYSIS**

Total RNA was extracted from either whole spikelets containing 1 mm (pre-meiotic) to ~2 mm (late prephase I meiotic stage) anthers or dissected 1–2 mm anthers using Trizol reagent (Invitrogen). RNA quantity and quality were assessed with the Quant-iT RiboGreen RNA Assay Kit (Invitrogen) and agarose gel electrophoresis, respectively. Approximately 1 µg of total RNA was treated with DNase I and reverse transcribed into cDNA with an oligo(dT) primer using the SuperScript III Reverse Transcription system (Invitrogen) as per manufacturer’s instructions. Approximately 20 ng cDNA was used as template for RT-PCR. Target, expected amplicon size, and corresponding primer pairs are: Mu killer, 0.5 and 0.7 kb amplicons with the muk-flank (5′ CTTTTGCTAGCTTGTTATCTTCG 3′) and mudr-219r (5′ AGGAGAGACCTTTGAAACACAGGTTA 3′) primers; 0.5 kb (spliced) and 0.6 kb (alternatively spliced) mudrA with the mudrA-2345f (5′ AGGAGAGACCTTTGAAACACAGGTTA 3′) and mudrA-2938r (5′ TTCAATGACCTTTGCAAGAAGTACCTTCG 3′) primers; and tubulin6 (a DNase I control transcript), 0.5 kb (cDNA) with the tub6-475f (5′ AGATATGGCATCTCCTCCTGGTTGTA 3′) and tub6-979r (5′ GGCCACATCGTTGTTCTGGTA 3′) primers. Other primers used to amplify Mu killer were: amy-muk512f (5′ ATCTCGTGGAGAGACTTTGC 3′) and mudrA-899r (5′ TTGGTGGATTTAGAAGACTCTCCT 3′). PCR amplification parameters were identical to those described above. PCR products were resolved on a 2% agarose/TBE gel stained with ethidium bromide.

**TRANSCRIPTOME PROFILING**

Individuals from ears segregating for mutant active and siblings silenced by Mu killer were used as generated from Cross 2 (Figure 2). Spotted (mutator active) or bronze (Mu killer) kernels were selected and grown in Stanford, California in summer 2008, and the presence or absence of Mu killer was confirmed via PCR on genomic DNA. Anthers were staged from the upper florets and then dissected onto dry ice and stored at −80°C. Total RNA was extracted from four biological replicates of mutant active and Mu killer anthers (15–20 anther sample size) and quantified and qualitatively assessed as described above. DNase I-treated total RNA (1 µg) was amplified and labeled with either Cy3 or Cy5 using a Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies, Santa Clara, CA, USA). Eight hundred twenty-five nanograms of each labeled cRNA was hybridized for 17 h at 60°C on a 4 K × 44 K Agilent in situ synthesized oligonucleotide platform along with spike-in controls for calculating target RNA concentrations. Data extraction, image processing, spot flagging and removal, and normalization were performed as described in Skibbe et al., 2009. Probes were considered “on” if fluorescence values were greater than a cutoff of 2.6 SD above the average background for all four replicates. Probes with only three replicates above the cutoff were also considered “on” if the average intensity was above the median for the set of probes with only three replicates above the cutoff. All microarray data associated with these experiments are available at GEO1 under the series accession number GSE38314.

**RESULTS**

**Mu killer TRANSCRIPTIONAL SILENCING OF MUDR IN HIGH COPY LINES IS RELATIVELY EFFICIENT BUT INCOMPLETE**

*Mu killer* acts as a dominant locus that effectively silences the minimal mutator system (Slotkin et al., 2003); based on the presence of a large inverted duplication of ~1300 bp of the *mudrA* gene (Figure 1), it is hypothesized that *Mu killer* encodes a large fold back, double-stranded RNA transcript directing *MuDR* silencing by targeting transposase-encoding *mudrA* transcripts. *Mu killer* effectiveness in standard, high copy number mutator lines has not been reported. To address this question, a line with a single copy of *Mu killer* in a *Bz2* line was cross (Cross 1) to a high copy mutator *bz2-mu2*/bz2-ref spotted kernel line that has been demonstrated to have both a high forward mutation frequency and a large impact on the transcriptome of developing maize anthers (Skibbe et al., 2009). When the mutator system is active, late somatic excision from the *bz2-mu2* reporter allele results in fine purple spots on a beige (bronze) background; when mutator activity is silenced, kernels are uniformly bronze. After Cross 1 all progeny have purple seed, requiring genotyping to identify the *Mu killer* carriers. As expected, all these individuals were also carriers of the *MuDR/Mu* transposon system. Because *Mu killer*-mediated silencing occurs most effectively when *Mu killer* is inherited through the female parent, an additional cross was performed by a *Bz2* tester strain. If *Mu killer* is highly effective, then the expectation was that half of the progeny ears would segregate 1:1 purple:bronze and half of...

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1 [http://www.ncbi.nlm.nih.gov/geo/](http://www.ncbi.nlm.nih.gov/geo/)
the progeny ears would be fully bronze. Among the fully bronze cohort, there are four classes. Two of the classes lack the bz2-mu2 reporter gene and will be bronze only, independent of Mu killer. Classes 2a and 2b (Figure 2) inherit the bz2-mu2 reporter allele; both of these classes were exposed to Mu killer in the previous generation and one class retains Mu killer (Class 2a, segregating 1:1 in the progeny).

In Crosses 2a and 2b, ears contained the expected 50% purple kernels, but the remaining kernels were a mixture of spotted (active mutator) and bronze (loss of mutator activity). The mutator system can silence spontaneously, and there is a very wide variation in this frequency and in the percentage of spotted kernels during progressive spontaneous silencing (Takumi and Walbot, 2007). Therefore, interpreting loss of the somatic kernel phenotype is problematic. Instead, we conducted a molecular test independent of the spotted kernel phenotype to assess the ability of Mu killer to eliminate mudrA transcripts. Plants were grown from bronze kernels of Cross 2a (Mu killer carrier parent), using genotyping to pick plants that inherited Mu killer, to compare plants derived from spotted kernels of the parental bz2-mu2/bz2 active mutator line. Anthers were chosen for analysis because they are the final organs differentiated and thus represent the location most likely to experience silencing.

The selected primers span the third intron of mudrA; this region of MudDR is absent from the Mu killer locus. The primers amplify a single genomic DNA band (Figure 3, lane g); however, near-complete splicing of the third intron, as expected (Hershberger et al., 1995), generated a smaller cDNA product with a faint larger band in active mutator samples. RT-PCR confirmed that all spotted kernels from the standard mutator line resulted in plants that were the final organs differentiated and thus represent the location most likely to experience silencing.

UNEXPECTED TRANSCRIPTS ENCODED BY Mu killer

In their analysis of the Mu killer locus, Slotkin et al. (2005) proposed novel transcription regulation in which the promoter of one of the flanking genes drives expression of Mu killer transcripts proposed to cause mutator silencing. This model invokes the unexpected feature that both TIRA promoters are inactive and that the TIRs fail to “insulate” the element from read through transcription. To test these aspects of the model, progeny from Cross 1 were classified as active (gDNA “+”) or inactive (gDNA “−”) in silencing based on PCR confirmation of Mu killer using genomic DNA. In an effort to identify Mu killer transcripts associated with silencing activity, five gDNA “−” and five gDNA “+” individuals were tested via RT-PCR for accumulation of MudDR transcripts in whole spikelets. As expected, all of the gDNA “−” individuals accumulated MudDR-derived transcript but failed to amplify detectable levels of Mukiller transcript (Figure 4, lanes 6–10). Surprisingly, only three of the five gDNA “+” individuals (Figure 4, lanes 1, 3, and 4, top row) amplified Mu killer transcript types. Furthermore, although only a 0.7 kb transcript was previously reported, an additional type was detected here. The remaining two gDNA “+” individuals (Figure 4, lanes 2 and 5, top row), failed to amplify detectable levels of Mukiller transcript. The MudDR and tubulin 6 control primers amplified as expected for each individual.
The two individuals that contained Mu killer but lacked Mu killer cDNA, along with two individuals that did accumulate mudrA cDNA were selected for further analysis along with positive and negative controls. The transcript accumulation in these four individuals was interrogated using primers positioned at four locations: (1) ~500 bp upstream of Mukiller (amy4-flank) (2) at the junction of the amy4/Mu killer (amy4-muk_512f), (3) ~900 bp into the Mu killer gene (mudrA-899r), and (4) ~1300 bp into the Mu killer gene (mudrA-1298r). When the amy4-flank primer was paired with the two internal Mu killer primers, amplicons of the expected size (plus a splice variant ~100 bp smaller) accumulated in the two Mu killer transcript “+” individuals but no transcripts were detected in either of the Mu killer transcript “−” individuals. When the amy4/Mu killer chimeric primer was paired with the two internal Mukiller primers, amplicons of the expected size (without any splice variants) accumulated in the two Mu killer transcript “+” individuals. In the Mu killer transcript “−” individuals, amplicons of an unexpected size were detected (Figure A1 in Appendix for the RT-PCR results). All amplicons were purified, sequenced, and the results are diagrammed in Figure 5.

Several unexpected molecular observations were made while assessing accumulation of Mu killer and mudrA transcripts in whole spikelets and anthers of these effective silencing cases. First, two families of antisense transcripts initiating within Mu killer and proceeding toward the amy4 flank accumulated in whole spikelets (Figure 5). A 0.74 kb amplicon was reported previously (Slotkin et al., 2005); however, none of the larger amplicons or the smaller 0.65 kb size class has been described. Approximately 80% of total transcript is in the 0.65 kb class, therefore, the 0.65 and 0.74 kb transcripts were cloned and sequenced to elucidate their structures. The 0.74 kb transcript type is antisense to mudrA. It is co-linear with an internal region contained in both Mu killer and MudR (Hershberger et al., 1995) and with TIRA; the transcript terminates within amy4 (at position 1, Figure 5). Therefore, we conclude that the 0.74 kb transcripts initiate at a previously unreported antisense promoter within the mudrA sequence (within the sense intron 1) shared by Mu killer and MudR. Additional analysis using RT-PCR amplification employing oligonucleotides further into Mu killer (mudrA-899r and mudrA-1298r) demonstrated that longer transcripts (Figure 5) can originate at least 1.298 kb into the Mu killer locus, starting within or beyond the region of Mu killer that could not be sequenced; it is possible that some transcripts initiate within the right side TIRA of Mu killer producing sense mudrA-containing RNA (5’ half of the transcripts) plus antisense mudrA (3’ half) in the region shared with the 0.74 kb transcript type.

Interestingly, the major 0.65 kb transcript results from splicing of a short intron with canonical 5’ donor and 3’ acceptor sites in the amy4 flanking region. Similarly, the minor 1.8 and 1.4 kb transcripts diagrammed in Figure 5 are inferred to be unspliced forms, and the minor 1.7 and 1.3 kb forms involve removal of the same intron documented in the 0.65 kb transcripts. Tissue-specific variation in the ratio of the 0.74 kb and alternatively spliced 0.65 kb transcripts was observed in whole spikelets (containing the vegetative glumes, lemma, and palea plus anthers), in anthers, and in adult leaves but the 0.65 kb form is always predominant.

Next, transcripts were analyzed from two individuals that failed to silence MudR: they contained Mu killer but generated mudrA cDNA. In these individuals, no antisense transcript types were identified that extended into the amy4 flanking region, that is, both the 0.74 and 0.65 kb transcript types were missing. We therefore propose that production of the antisense 0.74 and 0.65 kb transcripts is required for Mu killer-mediated silencing of mudrA. In individuals that failed to silence mutator, RT-PCR amplification products were only identified for the combination of the chimeric amy4/Mu killer primer paired with the two internal mudrA oligonucleotides (Figure 6). These amplicons are in the sense orientation relative to mudrA but were shorter in length than canonical mudrA transcripts. Sequence analysis demonstrated that several alternatively spliced transcript types are present; analysis of the acceptor and donor sites confirmed that these transcripts are in the sense orientation with respect to the normal mudrA transcript. The introns identified are not spliced from normal mudrA transcripts, although alternative splicing does occur in master element transcripts (Hershberger et al., 1995).

Collectively, transcript analyses demonstrated that Mu killer individuals that silence MudR accumulate mudrA antisense transcripts that extend into the amy4 flanking gene region; the promoter for these transcripts is not yet defined but it resides inside Mu killer. This result disproves the proposed Slotkin et al. (2005) model that antisense transcripts would originate from read through transcription from flanking maize genes. The results also demonstrate that TIRA lacks a transcription terminator in the context of the Mu killer structure at this locus. In contrast, Mu killer individuals that fail to silence MuDR accumulate sense transcript.
FIGURE 5 | Antisense transcripts initiating in Mu killer and continuing into amy4 occur in plants with Mu killer-induced mutator silencing. Individuals were scored as having experienced Mu killer-induced silencing based on having genomic DNA positive for Mukiller but cDNA that lacked mudrA 3′ transcripts indicative of mutator activity (Figure 1).

FIGURE 6 | Sense transcripts contained within Mu killer occur in lines failing to silence MuDR. The two plants analyzed were genotyped as Mu killer carriers, however, the cDNA is negative for Mu killer antisense transcripts and positive for mudrA sense transcripts covering the 3′ end of the gene characteristic of mudrA (Figure 1).
types that are predominately alternatively spliced compared to authentic *muA* transcripts. These sense transcripts appear to arise from transcriptional read through from the *amy4* locus, as opposed to the Slotkin et al. (2005) model where antisense transcripts derive from flanking gene promoters. In our analysis such transcripts were determined to be in the sense orientation and not involved in mutator silencing. The existence of read through transcripts indicates that the TIRA in the context of *Mu killer* fails to insulate the *Mu* element from flanking gene transcriptional activity.

**Mu killer** AND **SPONTANEOUS SILENCING RESULT IN DISTINCTIVE GENE EXPRESSION CHANGES IN ANTHERS

A third question we addressed is how similar the consequences are of *Mu killer*-mediated compared to spontaneous epigenetic silencing in anther development. In a previous study, 1 and 2 mm anthers were shown to express more than 30,000 transcripts (Skibbe et al., 2009). In the current study the same criteria to be considered expressed or “on” were employed after analyzing four biological replicates of the mitotic (1 mm) and meiotic (2 mm) anther stages in *Mu* active and *Mu killer* silenced lines (Figure 7).

We found that the pre-meiotic 1 mm anther transcriptome contains 34,338 transcripts in an active mutator background and slightly more (35,056) transcript types in *Mu killer*. The 2 mm meiotic anther samples expressed a similar number of transcripts with 34,508 (*Mu* active) and 33,700 (*Mu killer*). There were many examples of stage-specific transcripts in both biological sample types. Two types of differences in transcript expression comparing *Mu* active to *Mu killer* silenced lines were analyzed: (1) “Off/On” differences represent expression that was detected in one sample but not in the other and (2) “Up/Down” differences are the differential expression class and include probes where expression was at least 1.5-fold higher in one sample than the other (*p*-value < 0.05). Venn diagrams summarizing On/Off differences for *Mu* active vs. *Mu killer* backgrounds for both anther stages are shown in Figure 8. Both stages have distinct sets of more than 1500 transcripts not detected in the *Mu* active background but expressed in *Mu killer* (green numbers).

The ectopic activation of gene expression indicates that *Mu killer* has a major impact on the anther transcriptome compared to an active mutator line. *Mu killer* impact is dynamic, because only 381 transcript types were OFF in *Mu* active anthers at both developmental stages. In the reverse comparison (present in *Mu* active but absent in *Mu killer*) there are 1156 transcripts at 1 mm and 2425 at 2 mm with 301 transcripts absent in *Mu killer* for both anther lengths. Collectively, the differences between the two lines are more than 10% of the anther transcriptome. It is already established that high copy mutator lines express a diverse suite of stress response genes, compared to silenced sister lines (or to similar stage fertile anthers in a standard inbred line; Skibbe et al., 2009). Although not the largest GO category, 29 of the 1457 transcript types present in *Mu* active but missing from *Mu killer* at 1 mm and 47 of the 2726 transcript types at 2 mm are stress-associated. Surprisingly, there were far fewer differentially expressed transcript types than transcripts present in the On/Off category (Figures 8A,B). At 1 mm less than 1% of the transcriptome was impacted in the differential gene expression analysis, with only 2% altered at the 2 mm stage. It appears, therefore, that most transcriptome changes in *Mu* active compared to *Mu killer* lines involve complete suppression or *de novo* activation of suites of genes rather than modulation of transcript levels.

The transcriptome data was next analyzed with regard to how similar epigenetic silencing is to the silencing directed by *Mu killer*. We compared the differentially expressed transcripts determined from the previous study of *Mu* active vs. *Mu* inactive lines (Skibbe et al., 2009) with the differentially expressed transcripts found here in the *Mu* active vs. *Mu killer* lines. For both On/Off and Up/Down differences (Figures 8C + E,D + F) distinctive sets of transcripts are affected at each stage with a much smaller number of transcripts affected in common. These data suggest that *Mu killer* inactivation reprograms gene expression using a different route and perhaps involving different mechanisms that result in such distinctive consequences compared to spontaneous epigenetic silencing.

The transcripts that were “on” in *Mu killer* but “off” in *Mu* active anthers at the 2 mm stage, as well as transcripts in the reverse situation, were mapped to version 2 of the MaizeCyc metabolic pathways database2 using the Omics Viewer feature of the Pathway Tools application3. The Omics Viewer displays metabolic pathways grouped into pathway classes (e.g., carbohydrate cycles or secondary metabolites biosynthesis) to acquire a general picture of the experiment effects. The On/Off status of the *Mu killer* line as compared to the *Mu* inactive line was used to color-code the affected reactions in each pathway; an opposite status is indicated by blue (“off” *Mu killer*, “on” *Mu inactive*) or red (“on” *Mu killer*, “off” *Mu inactive*). The combined “different” effects (27, blue and red) is more than the combined “same” effects (20, teal and orange), again indicating that *Mu killer* has distinctive aspects to its silencing mechanism. The many pathways affected in the “Carbohydrate biosynthesis”

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2http://maizecyc.maizegdb.org/overviewWeb/celOv.shtml
3http://bioinformatics.ai.sri.com/ptools/
FIGURE 8 | Venn diagrams showing unique sets of differentially expressed transcripts result from epigenetic and genetic inactivation of the mutator transposon family.

Table 1 | Five metabolic pathway classes showing affects by Mu killer in common or different from Mu inactive lines.

| Pathway class                              | One-color pathways | Two-color pathways |
|--------------------------------------------|--------------------|--------------------|
|                                            | Blue | Teal | Red | Orange | BT | RO | BR | BO |
| Secondary metabolites biosynthesis         | 10   | 1    | 0   | 1      | 0  | 0  | 0  | 0  |
| Cofactors, prosthetic groups, electron carriers | 2    | 3    | 0   | 2      | 1  | 1  | 1  | 0  |
| Amino acid biosynthesis                    | 3    | 1    | 1   | 2      | 0  | 0  | 1  | 1  |
| Carbohydrate biosynthesis                  | 5    | 3    | 4   | 1      | 1  | 0  | 1  | 1  |
| Hormone biosynthesis                       | 2    | 4    | 0   | 2      | 0  | 0  | 0  | 0  |
| Totals                                     | 22   | 12   | 5   | 8      | 2  | 1  | 3  | 2  |

pathway class are displayed in Figure 9; five pathways have “off” reactions only with Mu killer (blue) and four pathways have “on” reactions only in Mu killer (red).

Interestingly, three of the pathway classes in Table 1 had pathways with multiple reactions affected in different ways; these are shown by non-zero counts in the last
DISCUSSION

This study was formulated to examine three aspects of Mu killer: effectiveness in silencing standard high Mu copy number lines, molecular organization and expression of the locus, and impact on the anther transcriptome. After two generations of Mu killer exposure, 77% (10/13) high copy number MuDR/Mu individuals tested were silenced based on loss of transcripts characteristic of mudrA. Thus Mu killer-mediated silencing is considerably more frequent than the spontaneous ~10–20% loss of mutator activity generally observed (Robertson, 1986; Walbot, 1986) and is a useful practical tool to accelerate mutator silencing. Second, sequencing Mu killer confirmed the overall structure originally proposed without resolving the nature of the element center, which failed to amplify in any PCR strategy attempted. On the other hand, detailed analysis of transcript types documented numerous additional transcript types, clarified the strandedness of these and a previously documented transcript, discovered an association between two short antisense transcripts and mutator silencing, and found accumulation of sense transcripts in Mu killer individuals that failed to silence MuDR elements. Third, the impact of Mu killer silencing activity on the anther transcriptome is different from the impact of spontaneous silencing.

Standard MuDR elements contain promoters and transcription start sites within the TIRs (at about +165 and at sequences just internal to TIRA) that generate sense mudrA and mudrB RNA while antisense transcripts result from termination failure in the region separating these two genes (Walbot and Rudenko, 2002). Transgenic maize expressing additional antisense RNA failed to suppress MuDR sense transcript accumulation (Kim and Walbot, 2003); nonetheless, in this study a single copy Mu killer element producing 0.74 and 0.65 kb antisense mudrA transcripts was associated with effective silencing. Because these transcripts terminate in the flanking amy4 locus, it is possible that antisense RNA stability or effectiveness depends on the sequences contributed by the flanking gene. Both antisense transcripts are complementary to a short region of about 250 bases in authentic mudrA transcripts, including part of the second exon and the first exon including the 5′ untranslated region. Because the 0.65 and 0.74 kb RNAs cannot form large hairpin structures, it is presumed that small RNAs derived from them target either translation or transcription of mudrA. This is a new model to explain how Mu killer silences MuDR. It is possible that the minor longer transcript types (Figure 6) also contribute through the previously proposed mechanism of long hairpin formation (Slotkin et al., 2005).

In Mu killer individuals that failed to silence MuDR, we found sense rather than antisense transcripts derived from Mu killer. These read through transcripts initiated in the amy4 flanking locus, and also included the non-transcribed portion of TIRA, and mudrA-homologous sequences. A complex splicing pattern was observed, utilizing canonical 5′ donor and 3′ acceptor sites that are not used in authentic mudrA transcripts (Figure 7).
Because MuDR is transcribed independent of its insertion sites, it is thought that transcription at MuDR loci is driven primarily by the enhancer elements located within the element TIRs; there is no evidence for read through transcripts when MuDR is inserted within the coding region of bz2-mu4 (Hershberger et al., 1995) although the TIR functions as promoters when assayed in heterologous constructs (Benito and Walbot, 1994; Raizada et al., 2001a,b). Thus the unusual structure of Mu killer and the flanking sequence context unexpectedly highlight that both MuDR promoters and the insulator functions of the TIRs can be suppressed.

If there is an “arms race” between transposon and host mechanisms to suppress them, aberrant transposon forms could be a potent weapon in host-mediated defenses as originally proposed for Mu killer. Endogenous hMuDR elements reside in every maize line tested; they are co-linear with MuDR but contain point mutations and other small defects that make them incompetent to program Mu element activities. They are also impotent in regulating MuDR (Rudenko and Walbot, 2001) suggesting that in this case MuDR persisted, or won the contest. The newly arisen Mu killer element can silence MuDR with reasonable frequency; however, we also found that when Mu killer fails to silence MuDR it switches from producing antisense to producing sense mudaR transcripts. In this case too, it appears that MuDR is victorious. The stability of the Mu killer transcription states is unknown, but it will be interesting in future studies to assess the persistence of Mu killer over a multiple generation time scale to determine if, like hMuDR elements, it ultimately fails to contain MuDR/Mu elements.

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**APPENDIX**

**FIGURE A1 | Mu killer individuals undergoing Mu killer silencing accumulate aberrant Mu killer transcript types.** $S = 1 \text{ kb plus DNA ladder}$; Lanes 1–4 = Mu killer gDNA “+” individuals; Lanes 1 and 3 accumulate expected transcript types; Lanes 2 and 4 accumulate aberrant transcript types; Lane 5 = mudrA control DNA; Lane 6 = Mu killer control DNA; Primer Pair 1: amy4-flank + mudrA-899r (expected size $= 1400 \text{ bp}$); Primer Pair 2: amy4-flank + mudrA-1298r (expected size $= 1819 \text{ bp}$); Primer Pair 3: amy4-muk_512f + mudrA-899r (expected size $= 910 \text{ bp}$); Primer Pair 4: amy4-muk_512f + mudrA-1298r (expected size $= 1308 \text{ bp}$). The amy4-muk_512f primer is a chimeric primer that is expected to amplify both Mu killer and MuDR loci.