Initiation, Establishment, and Maintenance of Heritable MuDR Transposon Silencing in Maize Are Mediated by Distinct Factors

Margaret Roth Woodhouse, Michael Freeling, Damon Lisch*
Department of Plant and Microbial Biology, University of California Berkeley, Berkeley, California, United States of America

Paramutation and transposon silencing are two epigenetic phenomena that have intrigued and puzzled geneticists for decades. Each involves heritable changes in gene activity without changes in DNA sequence. Here we report the cloning of a gene whose activity is required for the maintenance of both silenced transposons and paramutated color genes in maize. We show that this gene, Mop1 (Mediator of paramutation1), codes for a putative RNA-dependent RNA polymerase, whose activity is required for the production of small RNAs that correspond to the MuDR transposon sequence. We also demonstrate that although Mop1 is required to maintain MuDR methylation and silencing, it is not required for the initiation of heritable silencing. In contrast, we present evidence that a reduction in the transcript level of a maize homolog of the nucleosome assembly protein 1 histone chaperone can reduce the heritability of MuDR silencing. Together, these data suggest that the establishment and maintenance of MuDR silencing have distinct requirements.

Introduction

Epigenetic variation involves heritable changes in gene activity in the absence of changes in DNA sequence. These changes are a characteristic feature of some developmental programs, where epigenetic states of gene activity can be maintained through mitotic cell divisions [1,2]. Variations in epigenetic states can also persist through meiosis, resulting in non-Mendelian patterns of inheritance. This form of epigenetic variation has been well documented over the years, particularly in maize, but it is only recently that we have begun to understand the mechanism that makes it possible. This knowledge is now informing our understanding of two phenomena that have intrigued geneticists for decades: paramutation and transposon silencing.

In paramutation, a paramutagenic allele of a gene can heritably alter the expression of a second paramutable allele of the same gene. In many cases, the altered allele can then itself become paramutagenic [3]. This phenomenon, which does not involve changes in DNA sequence, has been best studied in maize through the use of alleles of various color genes that undergo paramutation, including r1, b1, p1, and p1 [4]. The molecular mechanism that makes paramutation possible has been enigmatic. However, in each case where paramutagenic activity can be mapped to a specific region, it is associated with repeated sequences whose copy number has a direct effect on the degree of that activity [5–7]. To date, no evidence for RNAs that can trigger paramutation has been found, and it has been an open question as to whether RNA is directing this process.

Like paramutagenic alleles, most transposons contain tandem or inverted repeats and can trigger heritably silencing [8]. Indeed, Barbara McClintock, who discovered transposons in maize in the 1950s, spent several decades exploring the phenomenology of transposon silencing and reactivation [9]. It is clear from those and subsequent experiments in a number of plant and animal species that transposons and other repetitive elements are particularly prone to epigenetic silencing [10]. In fact, it has been hypothesized that epigenetic silencing arose as a mechanism to inactivate invasive DNA [11]. Certainly, given the mutagenic potential of transposable elements, it is clear that transposon inactivation has become a primary function of gene silencing in eukaryotes [12].

Repeated elements, whether in paramutable alleles or in transposons, can potentially trigger silencing if transcription of those repeats results in the production of double-stranded RNA (dsRNA), which can then be processed into small interfering RNAs (siRNAs) [13]. Mutations in a number of the genes involved in RNA interference (RNAi) in both plants and animals are associated with transposon activation [14–16]. Not only are siRNAs resulting from the processing of dsRNA associated with post-transcriptional degradation of target mRNAs, but they are also implicated in transcriptional...

* To whom correspondence should be addressed. E-mail: dlisch@berkeley.edu
silencing of target genes via DNA methylation and histone modification [17,18].

In plants, it appears that RNA silencing pathways have become functionally diversified, such that there are distinct (albeit overlapping) mechanisms for recognition and processing of various aberrant RNAs [19]. The RNAi pathway that appears to be specifically associated with transposon and heterochromatin repeat silencing involves several components. In Arabidopsis, these include (but are certainly not limited to) DICER-LIKE3 (DCL3), ARGONAUTE 4 (AGO4), RNA-dependent RNA polymerase 2 (RDR2), and the components of RNA polymerase IV [19–22]. This pathway appears to be functionally distinct from that which is involved in microRNA processing, which also centers around the production and use of small RNAs. Thus, although mutations in DCL3 and RDR2 eliminate small RNAs from some transposons [19], they have no effect on microRNA accumulation; the same is true for Ago4 [22,23]. The available evidence suggests that these factors cooperate to maintain and/or initiate heterochromatic silencing of many endogenous repeated elements [21,24].

One example of an inverted duplication that can reliably and heritably silence a transposon has been identified. This locus, called Mu killer (Muk), is a variant of the MuDR autonomous transposon in maize that has duplicate portions of that element joined in an inverted repeat orientation [25]. Muk produces a long dsRNA that triggers the rapid processing of normal MuDR transposon transcript into small RNAs. This is followed by methylation and transcriptional inactivation of MuDR. The two genes encoded by MuDR appear to be silenced via distinct mechanisms: the Muk transcript only shares homology with the transposase mudraA, and during silencing, it is mudraA small RNAs that are amplified, and it is the full-length mudraA transcript that is targeted for degradation [25]. By the immature ear stage of F1 plants carrying both MuDR and Muk, mudraA is transcriptionally silenced [8].

The second gene encoded by MuDR, mudrB, is required for transposon insertion activity [26,27]. When Muk initiates the heritable silencing of an active MuDR element, the silencing begins at mudraA and eventually spreads in cis to the mudrB gene, which is not directly targeted by Muk [25]. Silencing of mudrB begins with the production of only nonpolyadenylated transcript in plants that carry both Muk and MuDR. In subsequent generations, all transcript from mudrB is lost [8], and its terminal inverted repeat (TIR) becomes methylated [27].

Both Muk-induced silencing of MuDR and paramutation involve directed and heritable epigenetic changes in gene expression, raising the possibility that these phenomena have common requirements. This idea was confirmed with the discovery of a gene that is required for both paramutation and Mu transposon methylation [28]. This mutation, mop1 (mediator of paramutation1), causes paramutated alleles of the b1 gene to express at a high level, and it prevents the process of paramutation at this and several other paramutable loci in maize [29]. mop1 mutations also reverse Muk-induced silencing of mudraA after several generations in a mop1 mutant background [27], and these mutants can also reactivate silenced transgenes [30]. The Mop1 wild-type allele is required for the default methylation that occurs at nonautonomous elements in the absence of the transposase and is also required to maintain methylation of the TIR adjacent to the mudraA gene in Muk-silenced MuDR elements. It is not required for methylation of restriction sites at the mudrB TIR, and after multiple generations in a mop1 mutant background, mudraA, but not mudrB, becomes transcriptionally active [27].

In addition to containing MuDR, all maize lines examined contain hMuDR, or heterologous MuDR elements, which are paralogs of the autonomous MuDR element. These elements appear largely inactive; they express only small amounts of largely nuclear localized transcript [31], they do not trans- pose, and they do not cause transposition of nonautonomous elements [32]. Relative to an active MuDR element, these elements exhibit decreased DNaseI hypersensitivity (Lisch D, unpublished data). Because many of these elements share high homology to MuDR, including promoter regions, it is likely that these elements were once active and have become epigenetically silenced. Thus, in addition to any active MuDR elements a given maize line has, the line also contains multiple previously active elements that have presumably become permanently silenced. Given the vast numbers of transposons resident in most genomes and the relatively modest rate of naturally occurring insertional mutations, the silenced state is probably the normal condition for the majority of elements.

Here we describe the cloning and characterization of mop1 and its role in Mutator silencing. We show that Mop1 is an ortholog of RDR2 in Arabidopsis, confirming recent work by the Chandler laboratory [33]. We find that in mop1 mutants, small RNAs homologous to MuDR transcripts are lost and Mu transposon terminal inverted repeats are hypomethylated, consistent with a requirement for rdr2 activity in the production of small RNAs and subsequent DNA methylation of transposon or transposon-derived target sequences [19,20]. However, mop1 mutants do not prevent Muk silencing of MuDR, consistent with the observation that Muk produces a double-stranded transcript and has no requirement for RDR activity. Conversely, we provide evidence that maize homologs of NAP1 (nucleosome assembly protein 1) genes are specifically involved in the establishment of heritable MuDR silencing, but may not be involved in the maintenance of silencing once it has been established.

By using a genetic approach, we demonstrated that two genes, Mop1 and NAP1, play distinct roles in Mutator silencing by Muk. The mode of action of each of these mutants, along with what is known about Muk, make it possible to propose a tentative model for the initiation, establishment, and maintenance of Mutator transposable element silencing in maize.

**Results**

**Mop1** is an RDR

The mop1–1 allele is the result of a Mu transposon insertion. While examining families segregating for mop1–1/4 and homozygous wild-type plants, we observed two Mu1.7-homologous fragments that were only present in mop1–1/4 individuals (as determined by simple sequence repeat [SSR] mapping; see Materials and Methods) (Figure 1A). Given that the mop1–1 allele arose in a Mu-active line [29], we hypothesized that the mop1–1 allele may contain a Mu1.7 insertion. Because mop1 had been mapped by the Chandler laboratory [33] by using SSRs umc1541 and bnlg1018 from
the maize putative homolog of this gene (designated here as ZmRDR2) by BLASTing the rice DNA sequence against the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/) and ChromDB (http://www.chromdb.org/) and found a maize express sequence tab (CL3242_1) and a contig sequence (RDR101), which share 99% sequence identity to each other in the coding region. From these sequences, we designed a pair of primers, one from ZmRDR2 and one from the Mu1.7 sequence (Figure 1B, primers 1 and 2), to look for an amplification product that was only present in individuals that were either homozygous (as evidenced by demethylation of Mutator TIRs and high levels of B’ expression) or heterozygous (as evidenced by SSR genotyping) for the mop1–1 allele, which was detected (Figure 1C). Sequencing of the amplification product revealed that the insertion was in exon 4 of ZmRDR2 (Figure 1 and Figure S1). Restriction digests with several different enzymes resulted in two cosegregating fragments on Southern blots, whereas others resulted in a single, larger fragment that hybridized to our probe with greater intensity (unpublished data). Based on these observations, we hypothesize that the insertion is a compound element that consists of two Mu1.7-homologous elements, although we have been unable to amplify the entire element, presumably because of its complex structure. However, we have sequenced both ends of the element along with flanking sequence. As expected, the insertion is flanked by Mu terminal inverted repeats and a 9–base pair (bp) target site duplication, consistent with a genuine Mutator insertion allele (Figure S1).

We have sequenced a portion of a second ethylmethane sulphonate (EMS) allele of mop1, mop1–2 [28,29]. Our sequencing revealed that this allele has a substitution of a G to an A, resulting in a stop codon at amino acid 494 of the deduced protein sequence for the maize RDR2 gene (Figure S1). This polymorphism is unique to mop1–2; neither the mop1–1 allele nor the wild-type Mop1 allele in our minimal line has this stop codon (unpublished data). Our sequencing data is identical to that obtained by the Chandler laboratory for the mop1–2 allele. We concluded that the mop1–2 EMS allele, like mop1–1, has a lesion in ZmRDR2 that would be unlikely to make a functional product. The finding that two independently derived alleles of mop1 contain unique lesions in ZmRDR2 demonstrates that mop1 is indeed an RDR homolog.

Many organisms carry several different classes of RDR genes. In Arabidopsis, these genes have duplicated and diversified in function, with RDR2 being most closely associated with transposon silencing [19]. To confirm that Mop1 encodes an ortholog of AtRDR2, and not an ortholog of a different RDR gene, we obtained partial sequences of orthologs of AtRDR2, AtRDR1, and AtRDR6 from rice and maize (Figure S2). These sequences correspond to those flanking the Mu insertion depicted in Figure 1A and shown in Figure S1. Phylogenetic analysis of the deduced amino acid sequence of all of these sequences reveals that ZmRDR2 is more closely related to rice and Arabidopsis RDR2 than it is to maize, rice, or Arabidopsis RDR1 or RDR6 (Figure S3). Thus we conclude that ZmRDR2/Mop1 is a true ortholog of Arabidopsis RDR2.

The mop1 homozygous mutant produces aberrant mRNA transcripts. Reverse transcriptase (RT)-PCR using primers that span the Mu insertion in ZmRDR2 failed to amplify in the

Figure 1. Cloning of the Mop1 Gene
(A) Southern blot depicting a family segregating for either wild-type (WT) or mop1–1 heterozygous individuals (m/+). Individuals were genotyped using the SSR marker umc1541, which has been previously mapped to within one centimorgan from the mop1 locus. Arrows denote both Mu1.7 transposon elements that are only present in individuals containing the mop1–1 allele. The two other bands in the middle of the blot found in all lanes denote the Mu1 element in the a1-num2 allele that is present in all individuals in this family. DNA was digested with NcoI restriction enzyme.
(B) Depicted is a map of ZmRDR2 (not to scale). The Mu insertion is depicted here as a gray inverted triangle. Primers (indicated as arrows) correspond to the canonical sequence of ZmRDR2/RDR101 exon 4 (primer 1) and the Mu1.7 terminal inverted repeat sequence (primer 2). Primers flanking the insertion used for RT-PCR portrayed in figure 2 are designated primer 3 and primer 4. The indicated sizes are those obtained from amplification and sequencing of these products. Primers used to amplify sequences 3’ of the insertion were primer 4 and primer 5. The region sequenced in mop1–1, mop1–2, and minimal line wild-type Mop1 is indicated by the black bar above exon 2.
(C) PCR of a family segregating for mop1–1 homozygote (m/m), heterozygote (m/+), and WT individuals using primers corresponding to ZmRDR2 exon 4 and Mu1.7 as depicted in Figure 1B. Individuals carrying the mop1–1 allele (m/m and m/+1) give rise to the expected 470-bp amplicon, indicating the presence of a Mu1.7 insertion at the mutant allele. Conversely, the WT individuals do not give rise to this amplicon, suggesting that the mop1–1 allele is the result of a Mu1.7 insertion in exon 4 of ZmRDR2.

DOI: 10.1371/journal.pbio.0040339.g001
mop1–1 homozygous mutants (Figure 2A). However, expression in the mop1–1 homozygotes was seen when RT-PCR was performed using PCR primers corresponding to exon 3 and the Mu1.7 TIR, indicating that the produced transcript is aberrant because it contains an unspliced insertion (Figure 2B). The insertion is located in a conserved portion of the gene approximately 220-bp downstream of the RdRP domain (pfam05183) [34] (Figure S2). BLAST searches reveal that sequences flanking the insertion are conserved among RDRs in most species (Figure S2), suggesting that the mop1–1 mutant allele is unlikely to produce a functional protein.

**MuDR Small RNAs Are Not Seen in mop1–1 Homozygous Mutants**

RDR2 has a role in *Arabidopsis* in the maintenance of silencing; when mutated, small RNA transcripts for one SINE retrotransposon, *Arabidopsis thaliana* short interspersed element 1 (AtSNE), as well as small RNAs for some other transscripts, are lost [19]. We looked at small RNAs corresponding to *mudrA* and *mudrB* in immature ears of mop1 homozygous mutants and closely related wild-type plants. We focused on immature ears because the expression of *mudrA* and *mudrB* is normally highest in this tissue. We found small RNAs homologous to both *mudrA* (24 and 26 nucleotides [nt]) and *mudrB* (24 nt) in all mop1 heterozygous and homozygous wild-type individuals, including those that carried an active *MuDR* element (Figure 3A) as well as those that lacked *MuDR* (unpublished data). In contrast, *mop1* homozygous mutant plants lacked small RNAs corresponding to either *mudrA* or *mudrB* (Figure 3A). All maize lines contain at least some sequences that are homologous to *MuDR*, known as h*MuDR*s. These sequences do not appear to contribute to *Mutator* activity, but they do produce some largely nuclear localized transcript [31]. Given that we see small *MuDR*-hybridizing RNAs in plants lacking a functional *MuDR* element, it is likely that these small RNAs are the result of *hMuDR* transcript processing, although silenced *MuDR* elements could also be the source of these small RNAs in plants that carry those elements as well.

Small 24- to 26-nt RNAs that we previously reported [25] as being associated with *Muk*-induced silencing are specific to the first few emerging leaves of plants that carry both *MuDR* and *Muk*. In wild-type plants not undergoing *MuDR* silencing, this young leaf tissue, unlike immature ears, lacks detectable quantities of *MuDR*-homologous 24- to 26-nt small RNAs; in
young leaves, these small RNAs are only detected in plants carrying Muk or Muk with MuDR [25]. ZmRDR2 transcript is present in much lower quantities in leaf tissue compared to either immature ears or embryos (Figure 3B), where we also see small hMuDR RNAs (unpublished data), suggesting that the processing of hMuDR transcripts may be a function of the availability of ZmRDR2 gene product in any given tissue. Together with the observation that MuDr/hMuDr small RNAs are missing in mop1 mutants, these data suggest that the production of MuDr/hMuDr small RNAs is dependent on the synthesis of dsRNA by the ZmRDR2 RDR.

The mop1 Mutation Does Not Prevent Silencing of MuDR by Muk

The mop1 homozygous mutant prevents B'/B-1 paramutation in maize [29]. Because the mop1 mutant reverses both Mutator element methylation and mud1/a silencing, we wanted to know if it could also prevent the initiation of MuDR silencing by Muk. To test this, we used genetic analysis to combine MuDr with the presence or absence of the mop1 mutation (Figure 4A). Plants that were homozygous for mop1 and that carried a single MuDr element were crossed reciprocally to and by plants that were heterozygous for both mop1 and Muk. The resulting plants were genotyped for mop1, Muk, and MuDr (see Materials and Methods) and then tested to wild-type testers that lacked MuDr and Muk. Mutator activity was monitored in the kernels of the next generation by the presence of somatic excisions of the nonautonomous M1 element from a1-mum2, which are visualized as spots of color on a pale background [32].

If Mop1 were required for the initiation of a heritable silenced state at MuDr, we would expect that MuDr would remain active in the progeny of mop1 mutant plants, which would result in the transmission of roughly 25% heavily spotted kernels (progeny that inherited MuDr but not Muk). In the presence of the wild-type Mop1 allele, very few of the progeny kernels of plants that carried Muk would be expected to be spotted [8]. We could also examine the plants themselves for evidence of transposase activity by examining a1-mum2 suppressibility. In the presence of the transposase, a1-mum2 expression is largely suppressed, resulting in a pale red plant with spots of color due to excision of M1 from a1-mum2. In the absence of the transposase, a1-mum2 expresses and plants are darker red [32]. Plants with silenced MuDr elements are consistently darker than plants that carry active MuDr due to a lack of suppression of the a1-mum2 allele (Figure 4A). Thus, it was possible to examine these F1 plants for evidence of MuDr activity before analyzing their progeny.

We did not see transmission of active MuDr elements from any of the plants that carried Muk in this experiment (Table 1). Nine plants that were mop1 mutants and that carried both MuDr and Muk gave rise to a total of 2% (8/436) spotted kernels, a percentage consistent with Muk successfully silencing MuDr [8]. Five sibling plants that were heterozygous for mop1 and carried Muk and MuDr gave rise to a total of 4% (37/836) spotted kernels. In contrast, plants that carried MuDr but that lacked Muk gave rise to 53% (932/1770) spotted kernels, consistent with the segregation of a single MuDr element in these families. None of the mop1 mutant plants that carried Muk and MuDr showed evidence of a1-mum2 suppression, consistent with a lack of MuDr activity in these plants.

Clearly, mop1 did not prevent either the initiation of silencing of MuDr in these families, as evidenced by the lack of a1-mum2 suppression in the F1 plants, or in the establishment of a heritable silenced state, as evidenced by the lack of spotted progeny kernels. This was true regardless of the direction of the original Muk cross (Table S1). Because of this, we predicted that the leaf 2–specific small RNA associated with the initiation of silencing of MuDr by Muk would be still present in mop1/mop1; Muk; MuDr leaves, and this is what we observed (Figure 4B). Based on these data, we conclude that ZmRDR2 is not required for the production of these small RNAs or for the initiation of heritable Muk-induced silencing of MuDr.

NAP1 Homolog Knockdown Mutants Prevent Heritable Muk Silencing

We obtained a number of publicly available transgenic maize lines from the Chromatin Group at the University of Arizona (ChromDB). These maize lines contain constructs that express inverted repeat portions of genes involved in chromatin remodeling; aside from the inverted repeat sequence that is specific for a particular target gene, these
constructs are otherwise identical. Expression of these constructs results in a reduction in the amount of endogenous mRNA from the target gene present in the transgenic plants [35].

We wanted to know if these mutations would affect the process of Muk-induced silencing of MuDR. To test this, we crossed a selection of plants carrying transgenes targeting one of four different genes to active heterozygous MuDR lines: SRT101 (a SIR2-like histone deacetylase), CHR101 (a SWI2/SNF2 chromatin remodeling complex unit), and nucleosome/chromatin assembly factor group A (NFA101 and NFA104, both of which are orthologs of NAP1 nucleosome assembly proteins). We then crossed progeny plants that carried both the transgene and MuDR to lines homozygous for Muk. Transgenic and nontransgenic individuals that were heterozygous for both Muk and MuDR were then crossed to wild-type tester lines carrying the a1-mum2 reporter gene to test for transposon activity (Figure 5A).

One transgene targeting NFA104 had a pronounced effect on the heritability of Muk-induced silencing of MuDR. Of the 30 parent individuals of the F1 cross that had both Muk and MuDR, 16 carried the NFA104 transgene (Figure 5B). When crossed to a1-mum2 testers, nine out of the 16 transgenic individuals gave rise to a large number of heavily spotted kernels (between 14% and 41%). In contrast, the 14 individuals that were not transgenic gave rise to a much lower proportion of spotted kernels (0% to 8%), most of which were very weakly spotted (Table S2). Kernel spotting is directly correlated with MuDR activity; therefore this finding demonstrates that silencing of the NFA104 endogenous gene prevents full heritable MuDR silencing by Muk. Transgenic individuals that did not prevent Muk silencing also failed to exhibit a loss of endogenous NFA104 gene transcript, as demonstrated by RT-PCR (Figure 6), and all of the plants that had a confirmed knockdown of the target gene gave rise to a high frequency (17% to 37%) of spotted progeny kernels. Thus, the variability we see in the heritability of activity in the transgenic class of crosses is likely due to variations in the degree to which the target gene is down-regulated by the transgene.

In contrast to this finding, the two other transgenes, SRT101 and CHR101, which carry identical constructs to NFA104 except for the target sequence, had no effect on Muk silencing (Table S3). Because we have not established that SRT101 or CHR101 exhibited successful knockdown of their target genes, we cannot conclude that these targets are not required for silencing. However, these results do demonstrate that the

Table 1. The mop1–1 Mutant Does Not Prevent Mutator Silencing by Muk

<table>
<thead>
<tr>
<th>Genotype#</th>
<th>Number</th>
<th>Spot</th>
<th>Pale</th>
<th>Total</th>
<th>Percent Spotted</th>
<th>Mukb</th>
<th>mop1c</th>
<th>Phenotyped</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/+; no Muk</td>
<td>1 84 89 173 49% no het B’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 77 63 140 55% no het B’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 40 43 83 48% no het B’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 45 27 72 63% no het B’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 49 52 101 49% no het B’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 69 77 146 47% no het B’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 153 69 222 69% no het B’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 73 62 135 54% no het B’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 68 67 135 50% no het B’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total 658 549 1,207 55%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m/m; no Muk</td>
<td>1 63 62 125 50% no ho sup</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 61 54 115 53% no ho sup</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 25 63 88 28% no ho sup</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 31 34 65 48% no ho sup</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 94 76 170 55% no ho sup</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total 274 289 563 49%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m/+; Muk</td>
<td>1 13 163 176 7% yes het B’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 4 76 80 5% yes het B’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 8 179 187 4% yes ho B’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 2 217 219 1% yes het B’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 10 164 174 6% yes het B’</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total 37 799 836 4%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m/m; Muk</td>
<td>1 0 133 133 0% yes ho dk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 0 26 26 0% yes ho dk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 0 70 70 0% yes ho dk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 0 29 29 0% yes ho dk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 0 77 77 0% yes ho dk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 0 42 42 0% yes ho dk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 4 98 102 4% yes ho dk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 4 108 112 4% yes ho dk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 0 74 74 0% yes ho dk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total 8 428 436 2%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#Genotype of plants that were test crossed to yield the indicated numbers of spotted and pale kernels. m, mop1–1. All plants examined carried MuDR.

bPresence or absence of Muk as determined using PCR primers inside of and flanking the Muk insertion.

cPlants were heterozygous (het) or homozygous (ho) for the mop1–1 mutant allele based on both PCR genotyping for ZmRDR2 and plant phenotype.

dPhenotype of the plants; green with streaks of red (B’), light red due to suppression (sup), or darker red (dk).

DOI: 10.1371/journal.pbio.0040339.t001

Transposon Silencing in Maize
Figure 5. The NAP1 RNAi Mutants Prevent Mutator Silencing by Muk
(A) Diagram of the crosses performed to generate the prophylactic experiment to examine the effects of various RNAi knockdown mutants on the process of Mutator silencing by Muk. The images are examples of ears derived from plants either lacking (left) or carrying the NFA104 transgene (right).
(B) Percent spotted progeny kernels from individuals either carrying the NFA104 transgene (left) or not (right). All individuals carry both MuDR and Muk. Individuals that carry the NFA104 transgene on average have a higher percentage of spotted kernels compared to individuals that do not, suggesting that the NFA104 transgene can prevent MuDR silencing by Muk.
(C) Percent spotted progeny kernels from individuals either carrying (left) or lacking (right) the NFA101 transgene. All individuals carry both MuDR and Muk. Individuals that carry the transgene on average have a higher percentage of kernel spotting versus individuals that do not carry the transgene.

DOI: 10.1371/journal.pbio.0040339.g005

Figure 6. Knockdown of the Endogenous NFA104 Transcript by the NFA104 Transgene Is Correlated with Kernel Spotting in MuDR/Muk F1 Progeny
RT-PCR of the endogenous NFA104 transcript in individual progeny from the cross NFA104; MuDR × Muk, NFA104 transgenic individuals (T) (lanes 1–5) in which the endogenous NFA104 transcript has been lost give rise to a significant percentage of spotted kernels (% spotting) when outcrossed to a1-mum2. Transgenic individuals where the endogenous NFA104 transcript is present (lanes 6 and 7) give rise to few spotted kernels when crossed to a1-mum2. Individuals not carrying the transgene (lanes 8–10) express the endogenous NFA104 transcript and give rise to few spotted kernels when crossed to a1-mum2. All individuals carry both MuDR (except for lane 11) and Muk.

DOI: 10.1371/journal.pbio.0040339.g006

prevention of Muk silencing by NFA104 is not due to the generic presence of the construct but rather due to the effect of the NFA104 knockdown on the endogenous NFA104 gene.

NFA104 Does Not Prevent Loss of mudrA Activity in F1 Plants
To study the possible effects of NFA104 knockdown on the initiation of MuDR silencing, we examined 85 individual F1 parents segregating for MuDR, Muk, and the NFA104 transgene for evidence of Mu1 methylation. Mu1 methylation is a reliable indicator of the absence of MuDR transposase.

Of the five transgenes we examined, only one besides NFA104 prevented MuDR silencing by Muk; this transgene was another NAP1 homolog, NFA101. Although both NFA101 and NFA104 are homologous to NAP1, they are not the result of a recent duplication; their amino acid sequences are only 27% identical and 48% similar. The results of the F1 cross were similar to those using NFA104: individuals with the endogenous NFA104 transcript gave rise to a much higher number of spotted kernels than those that lacked the transgene (Figure 5C and Table S4). The fact that both NFA/NAP1 transgenes were capable of preventing heritable MuDR silencing by Muk suggests that this class of genes plays a role in the establishment of Mutator silencing in maize.

Maize NAP1 Homologs Do Not Reactivate Previously Silenced MuDR Elements
Because NAP1 mutants reactivate silenced transposons in Caenorhabditis elegans [38], we wanted to see whether knockdown of the NAP1 endogenous genes would reactivate MuDR elements silenced by Muk. To do this, plants that carried silenced MuDR that had lost Muk due to genetic segregation were crossed to NFA104 and NFA101 transgenic plants. Plants carrying the transgenes and a silenced MuDR element were then test crossed and the progeny kernels examined for somatic activity. Of four individuals carrying both silenced MuDR and the NFA104 transgene and six individuals carrying (mudrA); in active MuDR lines, Mu1 TIRs are not methylated [36]. When mudrA is lost via genetic segregation or deletions within MuDR, Mu1 TIRs are invariably methylated [32,37]. All 54 individuals that carried both MuDR and Muk exhibited methylation of the Mu1 TIRs, whether the NFA104 transgene was present (38/54) or absent (16/54) (Figure 7). Together with the test cross data from these plants, this finding suggests that although NFA104 prevents the establishment of a heritably transmitted silenced chromatin state, it does not prevent the initiation of silencing of MuDR by Muk.

A Second NFA Transgene Prevents the Full Establishment of Muk-Induced Silencing of MuDR

Because NAP1 mutants reactivate silenced transposons in Caenorhabditis elegans [38], we wanted to see whether knockdown of the NAP1 endogenous genes would reactivate MuDR elements silenced by Muk. To do this, plants that carried silenced MuDR that had lost Muk due to genetic segregation were crossed to NFA104 and NFA101 transgenic plants. Plants carrying the transgenes and a silenced MuDR element were then test crossed and the progeny kernels examined for somatic activity. Of four individuals carrying both silenced MuDR and the NFA104 transgene and six individuals carrying (mudrA); in active MuDR lines, Mu1 TIRs are not methylated [36]. When mudrA is lost via genetic segregation or deletions within MuDR, Mu1 TIRs are invariably methylated [32,37]. All 54 individuals that carried both MuDR and Muk exhibited methylation of the Mu1 TIRs, whether the NFA104 transgene was present (38/54) or absent (16/54) (Figure 7). Together with the test cross data from these plants, this finding suggests that although NFA104 prevents the establishment of a heritably transmitted silenced chromatin state, it does not prevent the initiation of silencing of MuDR by Muk.

A Second NFA Transgene Prevents the Full Establishment of Muk-Induced Silencing of MuDR

Of the five transgenes we examined, only one besides NFA104 prevented MuDR silencing by Muk; this transgene was another NAP1 homolog, NFA101. Although both NFA101 and NFA104 are homologous to NAP1, they are not the result of a recent duplication; their amino acid sequences are only 27% identical and 48% similar. The results of the F1 cross were similar to those using NFA104: individuals with the endogenous NFA104 transcript gave rise to a much higher number of spotted kernels than those that lacked the transgene (Figure 5C and Table S4). The fact that both NFA/NAP1 transgenes were capable of preventing heritable MuDR silencing by Muk suggests that this class of genes plays a role in the establishment of Mutator silencing in maize.
methylation of mop1 plants carried hypomethylated maintenance of rise to any spotted kernels (0/792 and 0/980, respectively), unnecessary. renders the need for RDR2 in the RDR2 protein. Conversely, allele does not produce a dsRNA hairpin product. In that case, Discussion both silenced MuDR and the NFA101 transgene, none gave rise to any spotted kernels (0/792 and 0/980, respectively), indicating that the NAP1 genes are not involved in the maintenance of Mutator silencing in maize but only in its establishment. Although it is possible that prolonged exposure to the NAP1 transgene would eventually affect MuDR silencing (as was seen in mop1 mutants), none of these mutant plants carried hypomethylated Mu1 elements (unpublished data), unlike mop1 mutants, which cause immediate hypomethylation of Mu1 TIRs whether or not MuDR is present.

Discussion

Mop1 is the first gene to be cloned in a series of genes implicated in both paramutation [29,33,39] and Mutator silencing [27,28]. The discovery that Mop1 is an RDR2 ortholog demonstrates that both processes are regulated at least in part by RNA processing [33]. However, there are differences between the silencing mechanisms of Mutator and paramutation. For instance, whereas the mop1 mutation does not prevent silencing of Mutator transposons, it does prevent paramutagenic silencing of B-I by B' [29]. This may be explained if the B' allele does not produce a dsRNA hairpin product. In that case, dsRNA production in B-I silencing initiation would depend on the RDR2 protein. Conversely, Muk’s dsRNA hairpin transcript renders the need for RDR2 in Mutator silencing initiation unnecessary. rdr2 mutants in Arabidopsis also prevent FWA transgene silencing, which, like B' paramutation, does not include a dsRNA hairpin [20]. Therefore it appears that the RDR2 protein is necessary for silencing initiation only in circumstances where a dsRNA hairpin is lacking, and RDR activity is therefore required to produce a dsRNA.

In addition to the role of RDR2 in initiation of silencing at loci that do not appear to produce dsRNA on their own, RDR2 is also required for the stable inheritance of the silenced state at several loci in Arabidopsis [19]. Similarly, Mop1 (ZmRDR2) is required to maintain the silenced state at several paramutant loci in maize as well as transcriptionally silenced transgenes [30], and it is also required to maintain the default methylation at nonautonomous Mu element TIRs in the absence of the transposase [28]. However, although mop1 reverses methylation at the TIR adjacent to the mudrA gene, it takes at least five generations in a mop1 homozygous mutant background to reactivate mudrA expression to near-normal levels [27]; a cumulative effect can also be observed with transcriptionally silenced transgenes in the presence of the mop1 mutation [30]. Thus, the loss of MuDR-homologous siRNAs in a mop1 background causes only a slight destabilization of the silenced chromatin conformation at mudrA, but this conformation becomes less stable through multiple cycles of meiosis in the continued absence of siRNAs. It is worth noting that, with the exception of AtSN1, most silenced transposons are not immediately reactivated in an rdr2 mutant background in Arabidopsis [40]. It would be interesting to see if, like MuDR in a mop1 background, these Arabidopsis transposons would become reactivated after several generations in the absence of RDR2 product.

The presence of small RNAs that hybridize to both mudrA and mudrB probes in all wild-type individuals suggests that hMuDR and/or Mudr transcripts are being processed regardless of the Mudr activity. The absence of these small RNAs in mop1 mutant individuals demonstrates that this processing is dependent on ZmRDR2. We suggest that these small RNAs are the result of the maintenance of hMuDR silencing, which continues to occur even in the presence of mudrA transposase. Although these small RNAs may be important in the maintenance of hMuDR silencing, they appear to have no effect on Mudr activity in the absence of Muk. This may be because these small RNAs only play a role in reinforcing a preexisting chromatin state that does not exist at an active Mudr element. Once Muk has silenced MuDR, small RNAs from either hMuDRs or from the silenced element itself may be required for the stability of silencing.

The presence of small RNAs homologous to mudrB is somewhat surprising, given that only mudrA from a silenced MuDR element becomes active in a mop1 mutant background and that hypomethylation of MuDR in a mop1 mutant background is restricted to the TIR adjacent to mudrA [27]. It is likely that another silencing pathway exists for mudrB and that RDR2 expression is much higher in floral tissue than it is in leaves in Arabidopsis [19]. Similarly, we see high levels of both ZmRDR2 expression and hMuDR small RNAs in the embryo and immature ears. Because DNA damage caused by transposon activity would be particularly severe and the changes would be heritable in these tissues, it is reasonable that the machinery for silencing transposons would be up-regulated in these tissues as well. It is possible that the high level of expression of ZmRDR2 in embryos and immature ears versus that of leaves represents a mechanism to reinforce silencing information specifically in undifferentiated cells, and thus may play a role in the regulation of epigenetic information before tissue differentiation. It will be interesting to see a more detailed analysis of the tissue specificity of RDR2 and what role it plays, if any, in cell differentiation.

![Figure 7. NFA104 Does Not Prevent Methylation of Mu1 TIRs in a Muk Background](image-url)
Although the mop1-1 mutant does not prevent the establishment of the heritable silencing of MuDR by Muk, the NAP1 knockdown mutant clearly does. The NAP1 gene has been implicated in chaperoning H2A-H2B histone dimers in yeast [41] and histone H1 in Drosophila [42]. Exchange of canonical H2A with variants such as H2AX during nucleosome assembly has been linked with heterochromatin formation [43], and modified forms of histone H1 are associated with the silenced chromatin state of mammals [44]. Further, in Drosophila, NAP1 interacts genetically with ACF1 (ATP-utilizing chromatin assembly factor 1) [45] to form repressive chromatin. Given these observations, it is reasonable that NAP1 should also be involved in the establishment of heritable transposon silencing in maize.

We have now identified factors that are necessary for the initiation, establishment, and maintenance of MuDR silencing. Muk initiates silencing of Mutator by targeting the 5′ region of mudrA through an RNAi pathway triggered by the hairpin structure of the Muk transcript, whereupon mudrA mRNA is lost and TIRs become methylated. This process involves the transient production of 24- to 26-m small RNAs [25], even in the absence of ZmRDR2 gene activity (Figure 4B). Silencing of mudrA in the first generation is also associated with the loss of polyadenylated mudrB [8]. It is not known whether alterations in the mudrB transcript require an RNA intermediate, but the fact that this only happens in cis argues for a chromatin-based spreading process akin to SW16-mediated spreading at the yeast mating type locus [46]. The establishment of a heritable silenced state does not require ZmRDR2, but it does require two maize NAP1 homologs: NFA101 and NFA104. This suggests that these NAP1 homologs are required to establish a form of heritable heterochromatin, perhaps by recruiting specific histone variants. Once established, that silenced state does not appear to require NFA101 or NFA104. Our observation that heritable silencing of MuDR by Muk does not require ZmRDR2 activity demonstrates that chromatin modification at MuDR that leads to heritable silencing is probably independent of the DCL3/RDR2/AGO4 pathway. Finally, maintenance of MuDR silencing is assisted by the Mop1/ZmRDR2 component via an siRNA pathway that is required for RNA-directed DNA methylation. The fact that the mop1 mutant only gradually reactivates silenced MuDR elements, despite the observation that mop1 mutant invariably and rapidly loses Mok TIR methylation and Mudr small RNAs, suggests that ZmRDR2 activity can act to reinforce a preexisting chromatin state, which can be destabilized if the small RNAs are lacking for several generations.

This model of the silencing mechanism of Mutator transposons, although certainly incomplete, provides a clear framework for understanding the progression of naturally occurring transposon silencing. It encompasses factors that specifically influence the initiation (Muk), the establishment (NAP1/NFA), and the maintenance (Mop1/ZmRDR2) of MuDR silencing. This analysis complements more global analysis of transposon silencing and reactivation, because it suggests that observed variation in epigenetic regulation of transposons [40] can be a function of silencing history as well as position and type of element. As other mutants implicated in both Mutator silencing and paramutation are cloned, it will be interesting to see the similarities and differences in these systems and what they can tell us about the process of gene silencing in maize and other organisms.

Materials and Methods

Plant materials. The generation of the mop1-1 mutant lines and MuDR lines were previously described in [27] and [8], respectively. Both lines are in the Minimal Mutator background, which consists of one MuDR element at position 1 (p1) on chromosome 2L and one Mul element at the A1 color gene (the al-mum2 allele) [32]. The mop1-1 line was provided by the Chandler laboratory and is derived from an EMS-treated W22 color-converted line [29]. The generation of transgenic lines is described in [35]. Upon reception of the transgenic lines, we crossed these individuals to Minimal Mutator line plants heterozygous for active MuDR. The progeny of this cross were then crossed to plants homozygous for Muk, and the resulting progeny crossed to the al-mum2 Minimal Mutator tester line. The al-mum2 tester lacks MuDR or Muk and is homozygous wild-type for mop1.

Southern blotting. DNA extraction and Southern blotting were performed on mature maize leaf tissue as described in [27]. Briefly, we probed blots of DNA from families segregating for mop1-1 and wild-type individuals that had been digested with either HindIII (Figure 7) or NeoI (Figure 1) with a Mul probe that hybridizes to both Muk and Mul.1 nonallelic elements.

PCR and genotyping. Primers used to identify the Mu insertion into ZmRDR2 were designed based on the Mul17 nonallelic element sequence and the ZmRDR2/ZmRDR1 canonical sequence of exon 4 as follows: RDR2 exon 4F (primer 1), 5′TCTCCACGGCCCACTTTGAT3′; Muk1.7 (primer 2), 5′GAAGAATGGTCACAGTGAGAT3′; PCR conditions were 94°C, 35 s; annealing temperature 59.5°C, 45 s; elongation temperature 72°C, 45 s, for 30 cycles, giving rise to a 470-bp amplicon. For Muk genotyping, primers used for genotyping for Mu spanned the 5′ region of Mu that also corresponds to a portion of MuDR TIR as well as the flanking sequence corresponding to the AC101 gene with which Muk resides. The primers were as follows: for a TIR, 5′AGAGAGACGGTGACCAAGAGAGAT3′; for dRDR, 5′GGGATGCGGCGGCTGCA3′, with the cycle 94°C, 38 s; annealing temperature 39.5°C, 45 s; elongation temperature 72°C, 1 min, 34 cycles. Muk genotyping was as per [27].

mop1 genotyping. All plants described as homozygous for mop1 showed high levels of expression of B′ and hypomethylation of Mu-element TIRs, two characteristic features of this mutation. In addition, all plants described as mop1 homozygous or heterozygous were genotyped using the SSR umc1541, which is tightly linked (1 cM) to the mop1 locus. For primer sequences and amplification conditions for umc1541, refer to http://www.maizegdb.org. Plants examined for the prophylactic experiment described in Figure 4 and Table 1 were also genotyped using primers 3 and 4 illustrated in Figure 1. These primers, which flank the Mu insertion, failed to amplify a product only in individuals that were determined to be heterozygous based on B′ expression levels (19 plants). In contrast, all heterozygotes (20 plants) gave rise to the expected 854-bp amplicon.

Amplification and sequencing of portions of the mop1-1 and mop1-2 alleles. Sequences at the 5′ end of the Mu insertion in the mop1-1 allele were obtained by amplifying using primers 1 and 2 as described above (Figure 1). Sequences at the 3′ end of the insertion were obtained by amplifying with primer 4 (RDR2 exon 4R 5′ATGGCCACGGAGGTGTTGGAGAT3′) and primer 5 (TIR3′out: 5′GTGGCGGTGCTGCCTCACAACCAG3′) (Figure 1A) using the same amplification conditions. These products were amplified and sequenced twice independently, and each product was sequenced on both strands. Sequences for the mop1-2 allele were obtained by amplification using nested primers located within the second exon of ZmRDR2. Primary amplification was with the following: RDRF12 5′CTCTTTGATTTATCCTGC3′ and RDRR12 5′ATCTTATATCCAGGGTAAGAT3′, with the cycle 94°C, 45 s; annealing temperature 54°C, 45 s; elongation temperature 72°C, 1 min, 35 cycles. The products were gel isolated and then reamplified using a following set of nested primers: RDRF14 5′AGGTTGCTGCTTGACAGAT3′ and RDRR14 5′TCCAATTTGTAATGTTCAG3′, with the cycle 94°C, 90 s; annealing temperature 59°C, 45 s; elongation temperature 72°C, 45 s, for 32 cycles, giving rise to a 415-bp amplicon.
region. All samples were subjected to 35 rounds of amplification, gel isolated, purified using the Qiagen Gel Extraction kit (Qiagen, Valencia, California, United States), and sequenced using an Applied Biosystems (Foster City, California, United States) sequencer at the University of California.

RT-PCR. RNA extraction from maize embryo, mature leaf, and immature ear tissue was performed using TRZol reagent (Invitrogen, Carlsbad, California, United States) via manufacturer's recommendations, and the reverse transcriptase (RT) procedure was carried out as described previously [27]. For mop1–1, primers spanned both intron 3 of the ZmRDR2 gene and the Mu1.7 insertion, giving rise to an 854-bp amplicon for mop1–1 and wild-type cDNA and a 1335-bp amplicon for wild-type DNA. RDR2 exon 3 (primer 3), 5’ATGCTCCGGGCGGACTTTGATTAG3’; RDR2 exon 4R (primer 4), 5’ATGCGCAAGCAGGTGTCGAGAT3’

(A) Partial sequence of the mop1–2 EMS allele. This sequence is identical to the mop1–1 W22 allele from nucleotides 304 to 1251. The mutation relative to the wild-type B73 sequence is at position 630 (G to A) in our sequence (in red), and at position 933 in the published sequence. The lesion is in exon 2 of the published ZmRDR2 gene.

(C) A translation of the mop1–2 EMS allele in the region of interest. Note the stop codon at amino acid 493 replacing a W with a termination codon.

Found at DOI: 10.1371/journal.pbio.0040339.sg001 (12 KB PDF).

Figure S2. An Alignment of a Portion of RDRs from Maize, Rice, and Arabidopsis

This particular region, which extends from amino acid 865 to 1106 in the published maize RDR sequence, was used because sequences were available for the maize and rice orthologs of AtRDR2, AtRDR1, and AtRDR6. This region includes amino acids that are conserved between all of these sequences as well as an RDR from Branchiostoma floridae, the Florida lancelet, which serves as an outgroup. The site of the Mu insertion in mop1–1 is indicated by a black triangle just after the first block of conserved amino acids.

Found at DOI: 10.1371/journal.pbio.0040339.sg002 (49 KB PDF).

Figure S3. A Phylogenetic Tree of the Sequences Presented in Figure 2

Multiple sequence alignments were performed using the CLUSTALW server available at European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw/) with default parameters. A parsimony tree was generated using PAUP 4.0b10 with default settings and 1000 bootstraps. Bootstrap values are as indicated.

Found at DOI: 10.1371/journal.pbio.0040339.sg003 (9 KB PDF).

Supporting Information

Figure S1. Sequences Flanking Mu Element Insertion into ZmRDR2 in the mop1–1 Allele

(A) Blue nucleotides represent the 9-bp target site duplication characteristic of a Mu insertion. Green nucleotides are TIR sequences. “n”s represent an unspecified number of nucleotides within the insertion. The 3’ end of this sequence, including the last 19 bp of the Mu TiR, the 5’ TiR, and the Banking ZmRDR sequences are identical to the mop1–2 allele with the exception of an additional A at position 33 in the published sequence and a G in place of an A at position 218 in the published sequence.

References