Transcriptionally Silenced Transgenes in Maize are Activated by Three Mutations Defective in Paramutation

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Plants with mutations in one of three maize genes, *mop1*, *rmr1* and *rmr2*, are defective in paramutation, an allele-specific interaction that leads to meiotically heritable chromatin changes. Experiments reported here demonstrate these genes are required to maintain the transcriptional silencing of two different transgenes, suggesting paramutation and transcriptional silencing of transgenes share mechanisms. We hypothesize the transgenes are silenced through an RNA-directed chromatin mechanism, because *mop1* encodes an RNA-dependent RNA polymerase. In all the mutants, DNA methylation was reduced in the active transgenes relative to the silent transgenes at all of the CNG sites monitored within the transgene promoter. However, asymmetrical methylation persisted at one site within the reactivated transgene in the *rmr1-1* mutant. With that one mutant, *rmr1-1*, the transgene was efficiently resiledenced upon outcrossing to reintroduce the wild type protein. In contrast, with the *mop1-1* and *rmr2-1* mutants, the transgene remained active in a subset of progeny even after the wild type proteins were reintroduced by outcrossing. Interestingly, this immunity to silencing increased as the generations progressed, consistent with a heritable chromatin state being formed at the transgene in plants carrying the *mop1-1* and *rmr2-1* mutations that becomes more resistant to silencing in subsequent generations.
INTRODUCTION

During the past twenty years extensive studies on transgene expression in plants have revealed the potential for transcriptional or post transcriptional silencing (BAULCOMBE 1996; FINNEGAN et al. 2001; FLAVELL 1994; MATZKE et al. 2002; VAUCHERET and FAGARD 2001). Transcriptional gene silencing (TGS) is mediated through repression of transcription, while post transcriptional gene silencing (PTGS) is mediated through mRNA degradation. TGS is a mitotically and often meiotically heritable phenomenon, while PTGS is usually meiotically reversible. Silencing can happen in cis or in trans, affecting unlinked sequences (including transgenes, endogenous genes, or viruses) that share high sequence similarity.

TGS at either endogenous or transgenic loci has been associated with a number of different epigenetic hallmarks. Examples of correlations with TGS include changes in DNA methylation, histone methylation, histone acetylation, and chromatin structure (reviewed in MEYER 2000). TGS is often associated with DNA methylation in the promoter regions and can be induced by production of a double stranded RNA (dsRNA) homologous to a targeted promoter (METTE et al. 2000).

In this report, mutants defective in paramutation were used to investigate whether paramutation is mechanistically related to transgene silencing. Paramutation involves trans-communication between homologous sequences resulting in heritable changes in chromatin (CHANDLER and STAM 2004) associated with transcriptional gene silencing (HOLLICK et al. 2000; PATTERSON et al. 1993).

The three mutations that prevent paramutation were originally isolated in genetic screens using two paramutation systems, b1 and pl1, both genes that control the production of purple anthocyanin pigment in seedlings and mature plant tissues. The recessive mop1-1 mutation
The mediator of paramutation prevents paramutation at three genes that undergo paramutation (Dorweiler et al. 2000) and increases the transcription rate of the two silenced paramutant alleles tested. Two mutations, rmr1-1 and rmr2-1 (required to maintain repression) were isolated based on their ability to disrupt the silencing associated with paramutation at pl1 (Hollick and Chandler 2001), but their effects on paramutation at other genes have not been reported. The mopl-1 (Dorweiler et al. 2000), rmr1-1 and rmr2-1 (Chandler and Lin, unpublished data) mutations do not affect global DNA methylation, as none of the mutants show altered DNA methylation levels within repeated sequences such as centromeres and ribosomal DNA genes, but Mutator transposable elements are hypomethylated in all three mutants (J. Hollick and D. Lisch, personal communication; Dorweiler et al. 2000). The mopl-1 mutation also reactivates Mutator transposable elements, but the reactivation takes multiple generations in the mopl-1 background (Lisch et al. 2002; Woodhouse et al. 2006). The experiments reported herein demonstrate that each of the mutants could reactivate the previously silent tran genes, but the mutants differed with respect to the associated DNA methylation changes and whether the transgene could remain active in subsequent generations.

MATERIALS AND METHODS

Constructs and transgenic lines: The silent transgenic lines used for this work include the genomic region of the B-I allele, which corresponds to nucleotides 22136 through 27972 of the b1 gene contained in GenBank AF466203 (Selingier et al. 1998). This portion of the gene includes the genomic sequence spanning the complete coding region and both the 5’ and 3’ UTRs of the b1 gene. The 35SBTG transgene also includes the 35S CaMV promoter, corresponding to nucleotides 7012-7435 of GenBank V00140, and the first intron of maize alcohol dehydrogenase1 (included as an enhancer of expression), corresponding to nucleotides...
1222 through 1774 of GenBank X04049. In the BBBS transgene, the B-Bolivia promoter was used to drive expression of the same genomic region of the bl gene as in the 35SBTG construct. B-Bolivia is an allele that does not undergo paramutation, and its promoter was previously characterized (SELINGER and CHANDLER 2001). The BBBS construct (SELINGER and CHANDLER 2001) and the protocol used to generate transgenic maize plants with biolistic particle bombardment have been described previously (SELINGER et al. 1998).

Both constructs were co-bombarded with a selectable marker for identifying transgenic plants, the bar gene driven by the 35S CaMV promoter, as described previously (SELINGER and CHANDLER 2001). The bar gene confers resistance to glufosinate-ammonium herbicides (DENNEHEY et al. 1994).

**Genetic stocks and genotyping:** Following transformation of the maize inbred line CG00526, the regenerated T₀ plants were outcrossed for two generations with b r-g stocks and transgenic lines were selected that were homozygous for b and r-g (SELINGER et al. 1998). The bl gene is highly homologous to rl, both encode functionally equivalent bHLH proteins (CHANDLER et al. 1989). The r-g allele is recessive and produces no pigment in either the plant or seed. The b allele is also recessive and produces no pigment. The stocks used for the mop1-1 and rmr1-1 experiments were either r-r/r-g or r-g; these genotypes result in colorless seeds and purple or yellow anthers, respectively. The recessive r allele in the seeds of these stocks enabled unambiguous identification of individuals carrying the transgenes, as bl expression in the aleurone layer results in purple kernels. The rmr1-1, rmr2-1 and some of the mop1-1 stocks also carried the Pl’ allele. The Pl’ allele allowed for scoring of heterozygous vs. homozygous mutants in some families, as all the mutants upregulate the Pl’ allele (DORWEILER et al. 2000;
Anthers of plants that are \( r \) and \( Pl' \) are lightly pigmented and sectored when heterozygous, and solid purple when homozygous, for each of the mutations.

For the experiments with \( rmr2-1 \) a dominant \( r1 \) allele (\( R-r \)) was segregating, which also conferred purple aleurone pigment. As \( R-r \) segregates independently of the transgene and \( rmr2 \), 1/3 of the plants derived from purple seed would be expected to receive the transgene and be homozygous for \( rmr2-1 \). Segregation analysis, test crosses, PCR with transgene specific primers and Southern blots with transgene specific probes were used to determine the genotype of progeny plants.

**Northern blot analysis:** Total RNA extractions, blotting and hybridizations were performed as described previously (Chandler et al. 1989). The probe for \( b1 \) corresponds to coordinates 1 to 1970 in Genbank accession X57276, and the \( bar \) probe corresponds to coordinates 317 to 767 in Genbank accession AY572837. Both probes were generated by PCR. All plasmid and primer sequences utilized in this work are available upon request. Northern blots were analyzed with a STORM PhosphorImager and ImageQuant software (GE Healthcare). Northern blot analysis on small RNA enriched samples was performed according to an unpublished protocol (Doetsch and Jorgensen, personal communication) derived from published work in other laboratories (Hutvagner et al. 2000; Mette et al. 2000).

**Nuclear run-on assays:** Transcription rates were determined using transcription assays on isolated nuclei as described (Dorweiler et al. 2000). Briefly, nuclei from 10-15 g of sheath or husk tissue from plants at anthesis were prepared using a modified chromatin isolation protocol. Denatured PCR product or linearized plasmid (100ng of DNA) of each gene listed below was slot blotted onto nitrocellulose filter membranes. With the exception of the linearized backbone
of the transgene (SK+), all probes were generated by PCR; the coordinates and relevant accession numbers are:

35S CaMV promoter: 3577 to 4096 in AY553053

b1 coding region: 1 to 1970 in X57276

bar gene: 317 to 767 in AY572837

c2 coding region: 156 to 791 in AY109395

ubi gene: 975 bp Ubi-2 specific probe (CHRISTENSEN et al. 1992)

a1 coding region: 1-1436 in AY105150

Data normalization was performed as previously described (DORWEILER et al. 2000).

**Analysis of transgene structure:** The structures of the transgene were determined using Southern blot analysis. DNA isolation, blotting and hybridization protocols have been described previously (DORWEILER et al. 2000). The b1 coding region and 35S CaMV promoter probes utilized for Southern blots are the same sequences described in the nuclear run-on assay methods.

**Analysis of DNA methylation:** Total genomic DNA was digested by a combination of methylation sensitive and insensitive restriction endonucleases. The methylation sensitive enzymes DdeI, PstI, and BstBI were each used together with BstNI, a methylation insensitive enzyme, to test for DNA methylation within the transgene. BstNI sites occur 5’ of the 35S promoter in the 35SBTG construct, and at a position 280 bp from the 5’ end of the adh1 intron; the other restriction sites utilized lie between these two BstNI sites. Following complete digestion according to manufacturer’s instructions for each enzyme, Southern blot analysis was performed. The 35S CaMV promoter probe is the same as described in the nuclear run-on assay methods. A probe specific for the adh1 intron was used to identify regions of the 35S CaMV
promoter that were adjacent to the adh1 intron and b1 transgene. The probe was amplified by PCR from pMCG161 (Genbank AY572837) with the probe sequence corresponding to coordinates 4837 to 5402 of that accession.

RESULTS

Two silent transgenes can be reactivated by three paramutation mutants: Previous work had generated two transgenic lines, each containing distinct b1 transgenes originally produced to study anthocyanin gene regulation. The b1 gene encodes a transcription factor that activates the anthocyanin biosynthetic pathway (CHANDLER et al. 1989). Expression of the b1 gene in a particular tissue will give rise to purple pigment in that tissue, if the other regulatory and biosynthetic proteins are present. The 35SBTG transgenic line carries the 35S CaMV promoter driving the B-I genomic sequences spanning the transcribed region, including all introns, exons, and 5’ and 3’ UTRs (Figure 1A). Active 35SBTG lines had darkly pigmented seeds and pigment throughout the vegetative and many of the floral parts of the plant. The only obvious above ground epidermal tissue that was not pigmented was the anthers (Figure 1A). The BBBS derived transgenic line carries the promoter from the B-Bolivia allele fused to the same B-I genomic sequence as the 35SBTG construct (Figure 1B). Active BBBS lines showed medium to dark pigment in the seeds, and had medium pigment in a variety of vegetative tissues [Figure 1B, and (SELINGER and CHANDLER 2001) for details on the pigment phenotype of plants with active BBBS transgenes].

To test whether the paramutation mutants could affect transgene silencing, lines were identified in which transgenes were silenced. To help ensure that the transgenes had the potential to be reactivated, lines were used in which the transgene was silenced in vegetative and reproductive plant tissues, yet remained active in the aleurone layer of the seed. The fact that the
transgenes were expressed in the seed indicated that there was an intact coding region present within the transgene array capable of activating anthocyanin biosynthesis. It is not known why these transgenic lines were completely silent in vegetative and floral tissue while expression was observed in the aleurone layer of the seed. A similar pattern of expression has been reported for other transgenes (Cocciolone et al. 2000), and one speculation is that the chromatin structure is distinct in endosperm and related aleurone tissues as compared to the embryo and resulting plant.

The 35SBTG line used for this work contained ~2 copies of the introduced \( b1 \) construct and ~3 copies of the selectable marker gene, \( \text{bar} \), all segregating as a single locus (data not shown). In this transgenic line, expression of the 35SBTG construct had never been detected in the plant. The BBBS transgenic locus was more complex, it contained ~15 copies of the introduced \( b1 \) construct, which segregated as a single locus (Selinger and Chandler 2001). This transgenic line originally produced vegetative and seed pigment (Selinger and Chandler 2001). Individual progeny plants that were green because the transgene had become silenced in plant tissues were observed at a frequency of ~1%.

Three mutations defective in paramutation (\( \text{mop1-1, rmr1-1 and rmr2-1} \)) were each separately combined with the two transgenic lines by genetic crosses (Figure 2). Resulting F\(_1\) plants heterozygous for each mutant and containing the transgene had the same low level of pigment as their non-transgenic siblings, indicating the transgene remained silent. The F\(_1\) plants were lightly pigmented in the \( \text{mop1-1} \) experiments, because the weakly expressed \( B' \) allele was present, or green in the \( \text{rmr} \) experiments, because a null \( b1 \) allele was present. Transgenic F\(_1\) plants were backcrossed (BC\(_1\)) to plants homozygous for the particular mutation yielding families segregating for the transgene and heterozygous or homozygous for the mutation being tested. In the \( \text{rmr2-1} \) families, \( R-r \) was segregating, producing purple or mottled seed color.
depending on the direction of the cross (KERMICLE and ALLEMAN 1990). Thus in the rmr2-1 families, not all purple seed is expected to result from the transgene (Figure 2C).

For all three mutants, reactivated transgenes (Tg-a) segregated in the BC₁ generation (Figure 3A-C); the frequencies observed were consistent with activation in all plants homozygous for the mutants (Table 1). The dark pigment phenotype conferred by the reactivated transgene was equivalent in all three mutants. When the backcross progeny were Pl’ and –r, the anthocyanin color in the anthers (where the transgenes are not expressed) could be used to assess whether the plant was heterozygous or homozygous for a particular mutant (Figure 3D). Each of the homozygous mutants upregulate expression of Pl’, resulting in darkly pigmented anthers (DORWEILER et al. 2000; HOLLICK and CHANDLER 2001). Independent of whether anther color could be used to assess whether the mutation was heterozygous or homozygous, every plant was crossed with appropriate mutant testers to determine or confirm the mutant genotype. Not all crosses were successful, but in all cases when a plant was confirmed homozygous for the mutations, the transgene was activated and no activation was seen in plants heterozygous for the mutations.

Reactivation of the 35SBTG transgene occurs at the transcriptional level: Given that all three mutants increase transcription of the B’ and Pl’ alleles (DORWEILER et al. 2000; HOLLICK and CHANDLER 2001), a reasonable hypothesis was that the mutations also increase transcription from the transgene. Because of the stronger pigment phenotype, the experiments examining RNA levels and transcription were performed with the 35SBTG lines. RNA was isolated from sheath tissue of transgenic plants heterozygous or homozygous for each mutation and examined on Northern blots. The darkly pigmented plants (homozygous for either mopl-1, rmr1-1, or rmr2-1) had dramatic increases in transgene encoded b1 RNA (Figure 4A). No b1
RNA was detectable in the plants heterozygous for the mutations. RNA from the \textit{bar} gene, originally used to select for transformants, was also increased in plants homozygous for the mutants (data not shown).

To investigate whether the increased \textit{b1} RNA levels were caused by increases in transcription or increases in RNA stability, nuclear run-on assays were performed (Figure 4B). Transcription of the 35SBTG transgene is dramatically increased in plants homozygous for the mutants. The transgene activation results in an active B protein, as two genes that are controlled by B, \textit{c2} and \textit{a1} (Coe et al. 1988) are also transcribed. There was also an increase in transcription of the \textit{bar} gene, and the vector backbone (+SK). This transgene locus contains several rearranged copies of the introduced constructs (data not shown), which may have fortuitously placed the vector sequences adjacent to a promoter.

Expression of promoter sequences can trigger RNA directed DNA methylation and subsequent transcriptional silencing (Mette et al. 2000). Thus, a probe for the CaMV 35S promoter was included in the nuclear run-on experiments to determine if transcription from the CaMV 35S promoter could be detected, but no promoter transcripts were detected (Figure 4B). Northern blot analysis was also performed on small RNA enriched samples to determine if siRNAs homologous to the transgene were present. Small RNAs were not detected with probes specific for any part of the 35SBTG construct (data not shown), although this protocol has been successfully used in the laboratory to detect small RNAs for other experiments (data not shown).

**Heritability of the transgene reactivation:** Plants containing the activated 35SBTG transgene were used to test whether the transgene is efficiently resilenced or if it remained active in progeny plants after outcrosses to wild type individuals. Multiple individual plants with the active 35SBTG transgene and homozygous for either \textit{mop1-1}, \textit{rmr1-1} or \textit{rmr2-1} were outcrossed...
with \( b r-g \) \( Pl \) stocks, which contained dominant functional \( Mop1, Rmr1 \) and \( Rmr2 \) alleles (Figure 5A). Purple seeds were planted and the resulting plants were scored for pigment level (Table 2). With the \( rmr1-1 \) experiment, all of the resulting plants were green, indicating that the transgene was efficiently resiledenced in the presence of a functional \( Rmr1 \) allele. For \( mop1-1 \) and \( rmr2-1 \), the transgene was resiledenced in many of the progeny, but there were multiple families in which the transgene remained active in some of the progeny (Table 2, Figure 6A), all of which had functional alleles of \( Mop1 \) and \( Rmr2 \). Southern blot analysis indicated no obvious rearrangements of the transgene associated with resiencing or continued activation (data not shown).

A second outcross was done with the Tg-a, \( mop1-1 \) or \( rmr2-1 \) heterozygotes (Figure 5B). In these experiments, \( \frac{1}{2} \) the progeny would be expected to be wild type and \( \frac{1}{2} \) would be heterozygous for each mutation. For both sets of outcrosses, almost all of the plants gave rise to large numbers of dark progeny (Table 2), and this continued for a third generation of outcrosses (data not shown). In the second generation of outcrossing after reactivation, the number of plants expressing the transgene in each family was usually much higher than in the previous generation (Figure 6B), with the most dramatic results observed in the \( mop1 \)-derived families.

**DNA methylation correlates with silencing of the transgene:** Mutants that show reduction in DNA methylation can exhibit loss of gene silencing (BENDER 2004; MITTELSTEN SCHEID and PASZKOWSKI 2000). To test whether DNA methylation changes within the transgene correlated with its activation, methylation patterns of the transgene in plants heterozygous and homozygous for each mutation were examined.

Methylation of DNA within the 35S promoter and the adjacent \( adh1 \) intron sequence was assayed using Southern blots. When possible, the \( adh1 \) intron sequence was used as a probe so
that the 35S promoter driving the $b1$ transgene could be distinguished from the 35S promoter driving the $bar$ transgene, but in some cases a 35S promoter probe was used. Multiple restriction sites with different methylation sensitivities are distributed across the promoter/intron region (Figure 7A). Restriction sites were chosen such that both symmetrical (CNG) and asymmetrical (CNN) cytosine methylation could be evaluated. Symmetrical CG methylation was not evaluated as in this region there were no CG methylation sites within the recognition sites for methylation sensitive enzymes that could be distinguished with the probes utilized for this study.

Methylation was compared in the heterozygous and homozygous mutants in the first generation that reactivation was observed (BC$_1$). Differential transgene methylation was observed, with a decrease in CNG methylation correlating with reactivation (Figure 7B and D). One exception was that CNN methylation persisted at one site upon reactivation, but only in the transgene upregulated in homozygous $rmr1-1$ plants (Figure 7C). Transgenes reactivated in $mop1-1$ and $rmr2-1$ homozygous plants were hypomethylated at this site (Figure 7C).

DISCUSSION

Mutations in three different genes that are required for paramutation ($mop1$, $rmr1$ and $rmr2$) have been shown to reactivate two silenced transgenes. Nuclear run-on experiments demonstrated that the transgenes were silenced at the transcriptional level and transcription increases in the presence of the mutants. The fact that the mutants were able to prevent paramutation and reactivate transcriptionally silent transgenes suggests that there are shared mechanisms between paramutation and transcriptional silencing of transgenes. Importantly, with
two of the mutations, the transgene can remain active after the wild type protein is reintroduced, suggesting that the absence of the wild type proteins establishes an epigenetic state that can be immune to silencing in the presence of the wild type proteins. Decreases in DNA methylation correlated with increased transgene expression. In the \textit{rmr1-1} mutant that retained asymmetrical methylation of one site in the transgene, the transgene was efficiently resiled upon introduction of wild type proteins by outcrossing. In contrast, in the \textit{mop1-1} and \textit{rhr2-1} lineages that lost all tested DNA methylation, the transgene could escape resilingencing.

All of the mutants were originally isolated based on their ability to prevent paramutation. The transgenes that they reactivated contain the \textit{b1} gene, which is subject to paramutation. It could be argued that the transgenes were reactivated simply because they contained \textit{b1} sequences; this is unlikely because none of the transgenes contain the sequences required for \textit{b1} paramutation, which are located approximately 100 kb upstream of the \textit{b1} gene (STAM \textit{et al.} 2002). Additional experiments with other transcriptionally silenced transgenes will reveal the generality of transgene reactivation by the mutants.

In a subset of individuals, the transgenes reactivated by \textit{mop1-1} and \textit{rhr2-1} were able to escape resilingencing when outcrossed with wild type plants, suggesting that the absence of the MOP1 and RMR2 proteins can induce a heritable chromatin state immune to silencing upon reintroduction of the wild type proteins. The number of individuals with active transgenes increased over multiple generations of outcrossing with wild type plants, demonstrating that the immunity to silencing was highly heritable, and actually increased over multiple generations of outcrossing. In Arabidopsis, failure to resilience has been described as delayed resilingencing for several mutants shown to impact TGS (MITTELSTEN SCHEID \textit{et al.} 1998). However, it is difficult to directly compare our results with those previously reported, because heritability was not a
focus of the previous studies nor was the activity of the transgene followed over multiple
generations. It has been speculated (Brzeski 2004; Mittelesten Scheid et al. 1998) that delayed
resilencing could be due to an epigenetic condition that takes multiple generations to re-establish
in a wild type or heterozygous plant. The data reported here are not consistent with that idea, as
the number of plants maintaining an active state increases rather than decreases over multiple
generations.

The ability of an expressed locus to remain active even when the mutation causing
activation is segregated away is reminiscent of the ddm1 mutant in Arabidopsis. Plants carrying
the ddm1 mutation accumulate developmental abnormalities that are associated with activation
of previously silent loci (Kakutani 1997; Kakutani et al. 1996). The unlinked loci can remain
active in subsequent outcrosses to wild type (Stokes and Richards 2002).

In contrast to the results observed for rmr2-1 and mop1-1 derived lines, transgenes
reactivated by rmr1-1 are effectively resilenced upon outcrossing with wild type plants. This is
similar to sil1 [hda6 (Probst et al. 2004)] and drd1 mutants, which are capable of reactivating
silent loci, but not in a manner that is heritable when the mutation is segregated away (Furner et
al. 1998; Kanno et al. 2004).

Another interesting interpretation of the heritability data involves acquired characters and
genetic assimilation, first described by C.H. Waddington in the 1950s. Alternative phenotypes,
or polyphenisms, in Drosophila melanogaster were induced by subjecting individuals to multiple
types of environmental stressors (Waddington 1953; Waddington 1956; Waddington 1959).
After selectively breeding the individuals that exhibited polyphenisms under the influence of the
environmental stressor, the acquired trait was eventually assimilated, such that the trait would be
displayed in some progeny without requiring exposure to the inducing stressor. Waddington
demonstrated that the assimilated traits were heritable and polygenic, and speculated that the exposure of the organism to environmental stressors uncovered hidden genetic variability that could be incorporated via selective breeding into the heritable information passed onto the progeny of the flies exhibiting the phenotypes.

With genetic assimilation, the frequency of the phenotype increases over multiple generations of selective breeding, just as the number of Tg-a containing plants increased over multiple generations of outcrossing in this study. Since Waddington’s pioneering work, there have been many interesting reports of polyphenisms, and it is clear that epigenetic modifications can alter gene expression and cause divergent, heritable phenotypes. One intriguing speculation is that the underlying mechanism of genetic assimilation is alterations in chromatin structure or other epigenetic marks resulting in a concomitant shift in gene expression.

Methylation of cytosine in a symmetrical CG context is a highly conserved DNA modification found across the plant, animal, and fungal kingdoms (ANTEQUERA and BIRD 1993; FINNEGAN et al. 1996). Plants contain additional types of DNA methylation such as methylation of symmetrical CNG sequences and of asymmetrical CNN sequences (reviewed in CHAN et al. 2005). Decreases in symmetrical DNA methylation in the promoter correlated with 35SBTG transgene activation by the mutations. However, these mutations do not globally demethylate the genome as centromeric and rDNA repeats remain methylated in the mutants (DORWEILER et al. 2000; Lin and Chandler, unpublished data). Many of the mutants in other organisms that have been able to reactivate silent transgenes also affect DNA methylation. Initially, TGS was highly correlated with cytosine methylation at silenced loci, leading to the speculation that methylation was a major contributor to TGS (KILBY et al. 1992). However, further investigations revealed that maintenance of DNA methylation is not required for TGS at all loci (AMEDEO et al. 2000;
Extensive genetic studies in Arabidopsis have indicated that control of DNA methylation and transcriptional silencing may be locus and sequence specific (CAO and JACOBSEN 2002; LIPPMAN et al. 2003), suggesting that a number of different mechanisms, both methylation dependent and independent, could potentially affect a given locus.

When 35SBTG was reactivated by rmr1-1, hypomethylation was observed at CNG sequences within the 35S CaMV promoter. However, asymmetrical (CNN) cytosine methylation persisted upon reactivation by rmr1-1 in at least one site within the promoter/enhancer region of the transgene. In contrast, transgenes reactivated by mop1-1 and rmr2-1 showed reduced methylation at all symmetric and asymmetric sequences tested within the promoter/enhancer region. This distinction is intriguing because the persistence of asymmetrical methylation upon reactivation coincides with an immediate resilencing of the transgene upon introduction of the wild type protein by outcrossing. Possibly, the persistence of asymmetric methylation in the transgene activated by the absence of RMR1 facilitates resilencing in the next generation. Conversely, the loss of CNN methylation in the promoter region of the reactivated transgenes in the mop1-1 and rmr2-1 lineages may reflect a heritable chromatin state that hinders resilencing by wild type proteins.

Although the underlying mechanism of silencing of the 35SBTG is not known, it seems likely that this silencing phenomenon involves RNA directed chromatin changes. Recently, mop1 was cloned and shown to be the maize ortholog of RDR2 (ALLEMAN et al. 2006), an Arabidopsis protein involved in an RNAi-mediated transcriptional silencing pathway correlating with DNA methylation of the silenced locus (CHAN et al. 2004). The proposed function of MOP1 in paramutation is to amplify tandem repeat specific RNAs to a level sufficient to trigger
chromatin changes (ALLEMAN et al. 2006). This suggests that an RNA signal (“small” or otherwise) is necessary for mediating both paramutation at $bl$ and transcriptional silencing of the 35SBTG. Perhaps the RNA signal for transcriptional silencing of the transgene used in this study was not observed because it exists below the sensitivity range of the techniques used. Consistent with an involvement in RNA-mediated silencing, $mop1$ does reactivate silenced Mutator transposons (LISCH et al. 2002), whose regulation correlates with siRNAs (SLOTKIN et al. 2005).

The correlation of asymmetric methylation with transcriptional silencing has been previously demonstrated (DIEGUEZ et al. 1998). It has been proposed that asymmetric methylation is established and maintained in response to small RNA signals that direct methylation at loci sharing sequence identity with small RNAs (CHAN et al. 2005). Symmetric methylation can be established and maintained by this mechanism, but can also be maintained by an RNA independent mechanism (AUFSATZ et al. 2002; JONES et al. 2001).

The results herein, together with extensive studies from Arabidopsis, suggest a model for transcriptional silencing at this locus where an RNA signal is generated and amplified by maize DCL3, RDR2 (XIE et al. 2004), AGO4 (ZILBERMAN et al. 2003), and RNA polymerase IV (HERR et al. 2005) orthologs, and interpreted by other proteins in this pathway, including the de novo methyltransferase DRM1/2, the maintenance methyltransferases CMT3 (CAO et al. 2003) and MET1 (AUFSATZ et al. 2004), and the putative SNF2 chromatin remodeling protein DRD1 (KANNO et al. 2004). It is possible that similar to $mop1$ (which encodes the maize RDR2 ortholog), $rmr1$ and $rmr2$ encode other proteins involved in establishing or interpreting RNA directed chromatin formation.
To summarize, maize proteins encoded by \textit{mop1}, \textit{rmr1} and \textit{rmr2} are required for transcriptional silencing of some transgenes, in addition to their previously reported roles in paramutation and transposon silencing. Intriguingly, the loss of the MOP1 and RMR2 proteins results in a chromatin structure that enables the transgene to be maintained in an active state after reintroducing the wild type protein through crosses. In the case of \textit{mop1}, this means that restoration of RDRP activity does not always result in resilencing, suggesting that amplified RNAs (siRNA or others) are not sufficient for silencing. It is likely that further investigation into the unique properties of paramutation, transposon silencing, and transgene silencing will continue to yield novel insights.
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Table 1. Activation of two silent transgenes.

|                  | 35SBTG |          | BBBS |          |
|------------------|--------|----------|------|----------|
|                  | No.    | No.      | No.  | No.      |
|                  | Active | Silent   | $\chi^2$ | Active | Silent   | $\chi^2$ |
| mop1-1 (1:1)     | 213    | 192      | 1.1  | 27       | 26       | 0.02     |
| rmr1-1 (1:1)     | 55     | 52       | 0.09 | 15       | 13       | 0.14     |
| rmr2-1 (1:3)     | 26     | 95       | 0.79 | 28       | 96       | 0.39     |

Each of the three mutants was introduced into the silent transgene lines as indicated in Figure 2. The resulting BC$_1$ progeny segregated active and silent transgenes. The observed ratios were consistent with the plants carrying active transgenes being homozygous for each mutant; the expected ratio for this hypothesis is summarized for each mutant in Figure 2.

*Chi square tests ($\chi^2$) were performed to estimate the degree of confidence for each hypothesis. The hypothesis tested in each case is indicated in parentheses next to the indicated mutation.

$^1$Forty-four of these individuals had ACS scores between 1-4, consistent with a genotype of $Rmr1/rmr1-1$. In contrast, eight individuals had ACS scores of 6-7. These eight individuals could be due to reversion of $Pl'$ to $Pl$-Rh (HOLLICK et al. 2000), with these individuals being $Rmr1/rmr1-1$ and not activating the transgene. Alternatively, the plants with ACS6-7 could have been $rmr1-1$ homozygotes, but the transgene was not activated. Unfortunately testcrosses of these 8 individuals were not obtained so the $rmr1$ genotype could not be assessed.
Table 2. Immunity of activated transgenes to silencing in subsequent generations.

| Inducing mutation | First generation outcross |  | Second generation outcross |  |  |
|-------------------|---------------------------|--|---------------------------|--|--|
|                   | Homozygotes               | Tg-a progeny/ | total | Heterozygotes | Tg-a progeny/ | total |
|                   | yielding                  | progeny       | scored | yielding      | progeny       | scored |
|                   | total outcrossed          | total outcrossed | scored | total outcrossed | scored |
| mop1-1            | 7/10                      | 87/198        | 9/9    | 1491/1566     |
| rmr2-1            | 5/8                       | 21/127        | 11/12  | 370/620       |
| rmr1-1            | 0/44                      | 0/877         | NA¹    | NA¹           |

Homozygous mutants were outcrossed with wild type as described in Figure 5A. Some individuals produced progeny that maintained an active transgene (Tg-a) in the presence of wild type proteins (First generation outcross). The Tg-a, heterozygous mutant plants were outcrossed again to wild type as described in Figure 5B and the results are summarized in the columns under Second generation outcross.

¹NA: Not applicable
FIGURE LEGENDS

Figure 1. The 35SBTG (A) and BBBS (B) constructs contain the same \( b1 \) genomic sequences but expression is driven by different promoters, which control expression in different tissues. Each construct was cobombarded with a 35S CaMV promoter: \( bar \) construct as a selectable marker. For unknown reasons, both transgenes were expressed in the aleurone even in individuals displaying a silenced phenotype in vegetative tissue. The ears shown in the pictures were a result of crossing an individual hemizygous for the transgene with a wild type, non-transgenic individual, producing the 1:1 segregation of purple:colorless kernels.

Figure 2. Crossing strategy to test the ability of three paramutation mutants to reactivate the transgenes. Plants with silent transgenes (Tg-s) were crossed individually with plants homozygous for each of the three mutants. \( F_1 \) progeny were crossed again with plants homozygous for the appropriate mutant to generate a backcross population segregating homozygous and heterozygous mutations, and hemizygous for or lacking the transgene. The resulting genotypes and the phenotypes expected for activation of the transgene by each homozygous mutant are listed for each experiment; data are in Table 1. Both \( mop1 \) and \( rmr2 \) are linked to \( b1 \), \( rmr1 \) segregates independently. The original transgene lines were \( Pl \), which becomes paramutated to \( Pl' \) when crossed with the \( Pl' \) carried in the \( rmr1-1 \) and \( rmr2-1 \) stocks. Thus, because \( Pl' \) is in all plants, it is not shown.

Figure 3. Reactivation of the silent transgenes resulting in dark purple pigmentation throughout all the vegetative tissue of the plant, while the transgene remained silent in plants heterozygous for the mutations. (A) The 35SBTG transgene was reactivated in plants homozygous for each of
the mutations. These photographs illustrate \textit{rml1-1/rml1-1} plants and are representative of the phenotype observed with all three mutants. (B-C) The BBBS transgene was also reactivated in the plants homozygous for the mutations. Plants heterozygous for the mutations have a silent transgene and are green (B), while plants homozygous for the mutations have a reactivated transgene with an intermediate level of pigmentation in the leaf and culm (C). In plants homozygous for each mutation \textit{Pl} is upregulated producing darkly pigmented anthers (D); neither transgene is expressed in the anthers.

Figure 4. Steady state transcript abundance and the transcription rate of several elements of the 35SBTG transgene were increased in the homozygous mutant plants relative to heterozygous siblings. (A) Northern blot of total RNA from transgenic siblings segregating homozygous and heterozygous mutants hybridized with the \textit{b1} probe. The ethidium bromide stained gel shows the bands corresponding to rRNA, a loading control. (B) Nuclear run-on analysis reveals that the transgene is transcriptionally silenced in wild type and that transcription increases in the presence of each homozygous mutant. Slot blots containing the indicated gene sequences were hybridized with RNA isolated from transcription assays with nuclei of the indicated genotypes. The ubiquitin gene (\textit{ubi}) is the positive control. The \textit{b1}, \textit{sk+}, 35S and \textit{bar} sequences were all in the 35SBTG locus. The \textit{c2} and \textit{a1} genes are transcriptionally activated by B (Coe et al. 1988). Additional biological replicates revealed comparable results to those shown.

Figure 5. To test for resilencing upon introduction of wild type proteins, plants with an active transgene (Tg-a) and homozygous for each mutation were crossed with non-transgenic, wild type plants. Phenotypic data for the resulting progeny are reported in the First generation outcross
columns of Table 2 and in Figure 6A. A subsequent outcross to wild type non-transgenic plants was done with Tg-a plants heterozygous for the mutations. Phenotypic data for the resulting progeny data are reported in the Second generation outcross columns of Table 2 and in Figure 6B.

Figure 6. Activation of the previously silent transgene can be maintained after outcrossing to a wild type individual. After the initial reactivation in the homozygous mutants and subsequent outcrossing to wild type, some progeny retained an active transgene in mop1-1 and rmr2-1 heterozygotes. Seed from each ear resulting from an outcross becomes one family. For each family, the percentage of plants with active transgenes (Tg-a) is indicated. (A) Data from First Generation Outcross diagrammed in Figure 5A. (B) Data from Second Generation Outcross diagrammed in Figure 5B.

Figure 7. DNA methylation is different in the promoter/enhancer regions of silent (Tg-s) and active (Tg-a) transgenes. (A) Restriction sites for enzymes with differing methylation sensitivities are present within the promoter (CaMV 35S) and enhancer (adh1 intron) sequences of 35SBTG. Restriction sites’ locations and methylation sensitivities are indicated and the predicted band sizes for digestions used in Figure 7B and 7C are described below the promoter/enhancer map. (B) DNA samples from leaf tissue of individuals with the indicated genotype were digested with HindIII (methylation insensitive) and PstI (methylation sensitive), Southern blots were prepared and hybridized with the CaMV probe indicated in Figure 7A. Each lane contains DNA from a different plant. The product corresponding to complete digestion is 418 bp, and the 1164 bp fragment corresponds to no digestion of the PstI site within the 35S
CaMV promoter. Representative data is shown for mop1-1 mutants, similar results were observed for all mutants. (C) DNA was extracted from leaves of individual plants and subjected to electrophoresis and Southern blotting. The resulting blot was hybridized with the adh1 intron probe illustrated in Figure 7A. The 921 bp fragment results when the site is methylated and consequently not cut; when the site is hypomethylated and fully digested, a 737 bp fragment is observed. The two lower bands indicated with dashed arrows are the endogenous adh1 gene; two distinct alleles are segregating. (D) Summary of methylation across the promoter/enhancer region of the 35SBTG.
Figure 1.
A.

Expressed Silenced

35S pro /adhl intron  b1 gene  35S pro bar

B.

Expressed

B-bolivia promoter  b1 gene  35S pro bar

Silenced
Figure 3. Reactivation of silent transgenes by mutants.
Figure 4.

A. b1

rRNA

B. ubi

b1
c2
a1
sk+
35S
bar
A. First outcross: \( \text{Tg-a} \) mutant \( \text{-} \) mutant \( \times \) wild type (+) wild type (+)

Progeny of first outcross: (Table 2: First generation outcross)

B. Second outcross: + Mutant \( \text{Tg-a} \) - wild type (+) wild type (+)

Progeny of second outcross: (Table 2: Second generation outcross)
Figure 6.

A.

B.
Figure 7.

A. 

```
| Enzyme | Site 1 | Site 2 |
|--------|--------|--------|
| BstNI  | Dde I  | Pst I  |
| HindIII| Dde I  |        |
| Dde I  |        | Hinf I |
| Pst I  |        |        |
```

```
CaMV 35S promoter  PROBE
```

```
adh1 intron  PROBE
```

```
P-H 718 bp
```

```
B-D 186 bp
```

```
D-D 737 bp
```

```
insensitive  CNG  CNN
```

B. 

```
mop1-1/+, Tg-s
```

```
mop1-1 homo, Tg-a
```

```
H-H 718 + 446 bp (1164 bp)
```

```
H-P 450 bp
```

C. 

```
rmr1-1  rmr2-1  mop1-1
```

```
| Tg-a | Tg-s | Tg-a | Tg-s | Tg-a | Tg-s |
|------|------|------|------|------|------|
| D-D 737 + D-B 184 (921 bp) |
```

```
D-D 737 bp
```

```
Endogenous adh1
```

D. 

```
Ddel  Hinfl  PstI  Ddel
```

```
CaMV 35S promoter  adh1 intron
```

```
Hypermethylated when TG-s
```

```
Hypomethylated when TG-a by mop1-1 and rmr2-1
```

```
Hypomethylated when TG-a by rmr1-1
```
Figure 2.

A. **Initial cross:**

\[
\begin{align*}
\text{Mop1} b & \quad \text{TG-s} \quad \times \quad \text{mop1-1} B' \\
\text{Mop1} b & \quad - \\
\hline
\text{Mop1} b & \quad \text{TG-s} \\
\text{mop1-1} B' & \quad \times \quad \text{mop1-1} B'
\end{align*}
\]

**F1:**

\[
\begin{align*}
\text{Mop1} b & \quad \text{TG-s} \\
\text{mop1-1} B' & \quad - \\
\hline
\text{backcross} & \\
\hline
\text{mop1-1} B' & \quad \times \quad \text{mop1-1} B'
\end{align*}
\]

**BC1:**

\[
\begin{align*}
\text{Genotype:} & \quad \text{Frequency:} & \quad \text{Phenotype:} \\
\text{mop1-1} B' & \quad \frac{1}{2} & \quad \text{Dark purple plant} \\
\text{mop1-1} B' & \quad - & \\
\text{Mop1} b & \quad \frac{1}{2} & \quad \text{Lightly pigmented plant from B'} \\
\text{mop1-1} B' & \quad -
\end{align*}
\]

B. **Expected frequency in purple seeds:**

\[
\begin{align*}
\text{Genotype:} & \quad \text{Frequency:} & \quad \text{Phenotype:} \\
\text{rmr1-1} b \quad r-r & \quad \text{TG-s} & \frac{1}{2} & \quad \text{Dark purple plant} \\
\text{rmr1-1} b & \quad \text{r-r or r-g} & \quad - & \quad \text{Lightly pigmented plant from B'}
\end{align*}
\]

C. **Expected frequency in purple seeds:**

\[
\begin{align*}
\text{Genotype:} & \quad \text{Frequency:} & \quad \text{Phenotype:} \\
b \quad \text{rmr2-1} & \quad \frac{1}{2} & \quad \text{Dark purple plant; purple anthers} \\
b \quad \text{rmr2-1} & \quad \text{R-r or R-r or r-r or r-g} & \quad - \\
\text{TG-s} & \quad 4/12 & \quad \text{Green plant speckled anthers}
\end{align*}
\]

\[
\begin{align*}
\text{Genotype:} & \quad \text{Frequency:} & \quad \text{Phenotype:} \\
b \quad \text{rmr2-1} & \quad \frac{1}{2} & \quad \text{Dark purple plant; purple anthers} \\
b \quad \text{rmr2-1} & \quad \text{R-r or R-r or r-r or r-g} & \quad - \\
\text{TG-s} & \quad 4/12 & \quad \text{Green plant speckled anthers}
\end{align*}
\]

\[
\begin{align*}
\text{Genotype:} & \quad \text{Frequency:} & \quad \text{Phenotype:} \\
b \quad \text{rmr2-1} & \quad \frac{1}{2} & \quad \text{Green plant; purple anthers No transgene} \\
b \quad \text{rmr2-1} & \quad \text{R-r or R-r} & \quad - \\
\text{TG-s} & \quad 2/12 & \quad \text{Green plant; purple anthers No transgene}
\end{align*}
\]