Diverse Gene-Silencing Mechanisms with Distinct Requirements for RNA Polymerase Subunits in *Zea mays*

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**ABSTRACT** In *Zea mays*, transcriptional regulation of the *b1* (booster1) gene requires a distal enhancer and MEDIATOR OF PARAMUTATION1 (MOP1), MOP2, and MOP3 proteins orthologous to *Arabidopsis* components of the RNA-dependent DNA methylation pathway. We compared the genetic requirements for MOP1, MOP2, and MOP3 for endogenous gene silencing by two hairpin transgenes with inverted repeats of the *a1* (anthocyaninless1) gene promoter (*a1pIR*) and the *b1* gene enhancer (*b1IR*), respectively. The *a1pIR* transgene induced silencing of endogenous *A1* in *mop1-1* and *mop3-1*, but not in *Mop2-1* homozygous plants. This finding suggests that transgene-derived small interfering RNAs (siRNAs) circumvented the requirement for MOP1, a predicted RNA-dependent RNA polymerase, and MOP3, the predicted largest subunit of RNA polymerase IV (Pol IV). Because the *Arabidopsis* protein orthologous to MOP2 is the second largest subunit of Pol IV and V, our results may indicate that hairpin-induced siRNAs cannot bypass the requirement for the predicted scaffolding activity of Pol V. In contrast to *a1pIR*, the *b1IR* transgene silenced endogenous *B1* in all three homozygous mutant genotypes—*mop1-1*, *Mop2-1*, and *mop3-1*—suggesting that transgene mediated *b1* silencing did not involve MOP2-containing Pol V complexes. Based on the combined results for *a1*, *b1*, and three previously described loci, we propose a speculative hypothesis of locus-specific deployment of Pol II, MOP2-containing Pol V, or alternative versions of Pol V with second largest subunits other than MOP2 to explain the mechanistic differences in silencing at specific loci, including one example associated with paramutation.

**ANTHOCYANINS** are plant pigments that confer red pigmentation to plant tissues. Because the phenotype for anthocyanin production is easily scored by visual observation, it is a favored model system for studies of transcriptional gene regulation. Anthocyanin biosynthesis is mediated through biosynthetic enzymes, including the products of the *anthocyaninless1* (*a1*), *a2*, *colorless2* (*c2*), and *bronze* (*bs*) genes in maize (Neuffer et al. 1997). These biosynthetic genes are coordinately regulated through the activity of a MYB-like transcription factor, encoded by *colored aleurone1* (*c1*) or *purple plant1* (*pl1*) as a heterodimer with a helix-loop-helix transcription factor encoded by *booster1* (*b1*) or *red1* (*r1*) (Goff et al. 1992; Cone et al. 1993). The *b1*-*r1*-encoded and the *c1*-*pl1*-encoded transcription factors are functionally duplicate with different alleles at each gene expressed in different tissues (Chandler et al. 1989; Radicella et al. 1992; Cone et al. 1993). The combination of allelic tissue-specific patterns of expression at each gene produces the wide range of seed and plant pigmentation patterns (Styles 1967; Hollick et al. 1995; Selinger and Chandler 1999, 2001).

Because the regulatory genes also have epialleles (reviewed by Arteaga-Vazquez and Chandler 2010), regulation of anthocyanin biosynthesis in maize also provides a model for the study of epigenetic regulation. For example, the *B* and *B-I* alleles of the *b1* gene have identical DNA sequences (Stam et al. 2002a,b) and produce functional B1 protein, but *B*’ is expressed at a much lower level than *B-I* (Patterson et al. 1993). These two alleles participate in paramutation (Coe et al. 1966), which is an interaction that leads to the epigenetic conversion of the highly expressed, paramutable *B-I* into a lowly expressed, paramutagenic *B*’ allele (Arteaga-Vazquez and Chandler 2010; Chandler 2010).
The paramutation of B-I by B requires tandem repeats located ~100 kb upstream of the b1-coding sequence (Stam et al. 2002a,b). While both B’ and B-I contain seven copies of the tandem repeats, alleles of b1 that do not participate in paramutation (referred to as neutral) have only one copy of the sequence repeated in B-I and a1pIR silences A1 in mop1-1/+.

Paramutation at b1 and pl1 has been the subject of extensive genetic analysis, which has led to the isolation of multiple trans-acting regulators required for b1 paramutation. Molecular cloning of these trans-acting factors, including the products of the mediator of paramutation (mop) and required for maintenance of repression (rmr) genes, indicates that paramutation requires the activity of proteins homologous with Arabidopsis thaliana proteins involved in small RNA-induced DNA methylation and heterochromatin formation (referred to as RNA-directed DNA methylation, or RdDM) (Alleman et al. 2006; Hale et al. 2007; Erhard et al. 2009; Sidorenko et al. 2009; Stonaker et al. 2009).

mop1 encodes a protein that is most similar to Arabidopsis RNA-dependent RNA polymerase RDR2 (Dorweiler et al. 2000; Alleman et al. 2006), which physically interacts with Pol IV (Ream et al. 2009). Mop2 is allelic with mr7 (Stonaker et al. 2009) and encodes a conserved protein homologous to the A. thaliana second largest subunit of RNA polymerase IV.
and V (Sidorenko et al. 2009). mop3 (Materials and Methods) is allelic to rmr6 (Erhard et al. 2009), which is predicted to encode the largest subunit of Pol IV. Mop2-1 is a dominant mutation with respect to preventing paramutation, but is recessive in the release of silencing at the b1 enhancer (Sidorenko et al. 2009). Both mop3-1 and mop1-1 are recessive alleles compared to their wild-type counterparts for both phenotypes (Dorweiler et al. 2000; this report). According to the current model for RdDM in Arabidopsis, Pol IV and RDR2 are involved in the generation of double-stranded RNAs (dsRNAs) that are processed by other pathway components into small interfering RNAs (siRNAs), while Pol V is transcriptionally active at a regulated locus, generating transcripts that act as scaffolds for the proteins that initiate DNA and chromatin modifications (Haag et al. 2012, reviewed by Axtell 2013) associated with transcriptional gene silencing (reviewed by Zhang and Zhu 2011).

A model for the roles of MOP1, MOP2, and MOP3 in the regulation of b1 enhancer repeats is based upon sequence homology to Arabidopsis RdDM components and their predicted protein functions (Mop1, Mop2, and Mop3) with other required proteins to generate siRNAs that interact with the MOP2-containing Pol V holoenzyme to initiate epigenetic modifications and transcriptional silencing of b1. This model accounts for the loss of silencing of b1 in plants deficient for MOP1, MOP2, and MOP3 activities and is consistent with the predicted function of all three predicted proteins (Alleman et al. 2006; Sidorenko et al. 2009; Stonaker et al. 2009). However, prior findings indicate that the b1 enhancer repeats are predominantly transcribed by Pol II (Sidorenko et al. 2009; Arteaga-Vazquez et al. 2010), and although siRNA levels homologous with the b1 enhancer repeats are reduced in mop1-1 homozygous plants, in wild-type plants these siRNA levels are similar in transcriptionally silenced B', transcriptionally active B-I, and neutral b1 alleles (Arteaga-Vazquez et al. 2010).

Transcriptional silencing and paramutation are conceptually, and mechanistically, separable, although they share some genetic requirements (Hollick et al. 1995; Sidorenko and Peterson 2001; McGinnis et al. 2006; Jia et al. 2009; Sidorenko et al. 2009). For example, although an inverted repeat transgene with homology to a promoter region for the endogenous a1 gene (a1pIR) is sufficient to induce transcriptional silencing after one generation of exposure, silencing does not persist in nontransgenic progeny of outcrossed individuals and therefore is not an example of paramutation (Arteaga-Vazquez et al. 2010). However, an inverted repeat transgene that contains copies of the b1 enhancer tandem repeat (b1IR) is sufficient to induce paramutation of the paramutagenic B-I allele; i.e., the transgene-induced B' state persists in nontransgenic progeny of outcrossed individuals (Arteaga-Vazquez et al. 2010). Due to the self-annealing potential of the inverted repeat transcripts, both inverted repeat transgenes are expected to bypass the need for the proteins producing dsRNAs (Pol-IV and RNA-dependent RNA polymerases) and provide a useful tool for identifying potentially distinct genetic requirements for silencing paramutated loci and other loci targeted by a RdDM-like mechanism.

We examined the effects of transgenes with inverted repeats of the a1 promoter and the b1 enhancer repeat units on the expression of the corresponding endogenous genes in the mop1-1, Mop2-1, and mop3-1 mutant backgrounds. We found that the genetic requirements for b1 enhancer-mediated silencing and a1 promoter-mediated silencing differ, suggesting the activity of diverse silencing mechanisms involving distinct combinations of RNA polymerases in plants.

### Materials and Methods

#### Plant materials

Hemizygous transgenic plants, resistant to Ignite (Bayer CropScience), were crossed to plants homozygous for the various mop mutations using standard genetic stocks for each mutant. For mop1-1 and Mop2-1, the mutations have been described previously (Dorweiler et al. 2000; Alleman et al. 2006; Sidorenko et al. 2009). Cloning of the a1pIR and b1IR transgenes are described in Supporting Information, File S1, Materials and Methods. Isolation of the mop3-1 mutation is described below. The resulting F1 families were screened for herbicide resistance, and resistant plants were either self-pollinated or crossed to plants homozygous for the mop mutation. Segregating F2 families from these crosses were screened for the presence of a transgene and a mop mutation using herbicide resistance and molecular genotyping.

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**Table 1 Pigment phenotypes in F2 families segregating for a1pIR transgene and mop1-1**

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<thead>
<tr>
<th>Transgene</th>
<th>mop1-1 genotype</th>
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<th>Family 2</th>
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*a Total number of individuals in this category.
*b Individuals with this phenotype and genotype were not detected.
*c Number in parentheses indicates number of plants with sectored phenotype. This is a subset of the total number of plants in that category.
and were scored for pigment production at flowering. F2 families were analyzed by chi square to identify families with the expected segregation of each mutation and transgene, and families with chi square values that failed to reject the null hypothesis at $P = 0.01$ were included in the final results.

**Plant growth conditions**

Plants were grown in Arizona, Florida, or Juana Diaz, Puerto Rico, outside in the field under ambient conditions. F2 families segregating the b1pIR transgene and mop3-1 were grown in a greenhouse (Department of Biological Science, Florida State University, Tallahassee, FL) under controlled conditions and under ambient conditions in the field in Juana Diaz, Puerto Rico.

**Isolation and characterization of the mop3-1 mutation**

The mop3-1 mutation was isolated from an EMS-mutagenized population similar to that described for Mop2-1 (Sidorenko et al. 2009). To determine the identity of the mop3-1 mutation, a large mapping population was developed by crossing B73 with homozygous mop3-1 and by repeated backcrossing to the homozygous mop3-1 stock. In the backcross populations, plants with a dark pigment phenotype (402 individuals) were used for mapping using polymorphic markers. Molecular marker genotyping mapped mop3-1 to chromosome 1L in the vicinity of (~4 Mb) the previously characterized rmr6 gene, encoding the largest subunit of plant-specific RNA polymerase Pol IV (Erhard et al. 2009). The rmr6 gene was a strong candidate gene for mop3-1, and therefore the coding sequence and intron/exon boundaries of the rmr6 gene was PCR-amplified and sequenced. Results of sequencing revealed a G-to-A mutation consistent with EMS mutagenesis within the acceptor site of intron 13. Sequencing of the cDNA fragment that spanned the mutation site in mop3-1 revealed that the mutation leads to the use of an alternative splicing site 13 bp downstream, leading to a frameshift and a premature stop codon (Figure S3).

**Genotyping for the transgene and mutations**

DNA was extracted from leaves using a modified CTAB protocol involving <100 mg of tissue placed into deep 96-well plates with one 3-mm bead each. Tissue was disrupted by shaking plates in a Mixer Mill (Retsch) for 1 min at 30 Hz. DNA was extracted in CTAB/1% β-mercaptoethanol, followed by chloroform:octanol extraction. DNA was extracted from the resulting aqueous phase with isopropanol and washed twice with 70% ethanol. Pellets were dried overnight before resuspending in 100 μL of nuclease-free water. Presence of the transgene was initially determined by resistance to the herbicide Ignite (Bayer CropScience) and then confirmed by PCR with bar-gene-specific primers KM 93 (5’ GAAGTCCAGCTGC CAGAAAC) and KM 94 (5’ AGTCGACCGTGTACGTCTCC). Mop1 and Mop2 genotyping assays were conducted previously described (Sidorenko et al. 2009; Labonne et al. 2013) while KM 553 (5’ TCCACTGAACATTTGCTTGC) and KM 554 (5’ GGTITTCCCTACAGCCCTTTC) were used to amplify Mop3 and sequence the resulting PCR product.

**Figure 2** Pigment phenotypes of representative plants for a1pIR experiments. Phenotypes observed in segregating families of individuals hemizygous or lacking the a1pIR transgene and heterozygous or homozygous for mop1-1 (A–D), mop3-1 (E–H), or Mop2-1 (I–L) mutant alleles. G and H include insets of a higher magnification view.
Table 2 Pigment phenotypes in F₂ families segregating for a1pIR transgene and mop3-1

<table>
<thead>
<tr>
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<th>Family 6</th>
<th>Family 7</th>
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</table>

² Total number of individuals in this category.
³ Individuals with this phenotype and genotype were not detected.
⁴ Number in parentheses indicates number of plants with sectored phenotype. This is a subset of the total number of plants in that category.

Analysis of cytosine methylation

Genomic DNA was extracted as described for genotyping, treated with RNaseA, and purified using the Zymo Genomic Clean and Concentrator. For each genotype, 100 ng of genomic DNA was digested with PstI (Promega) for 4 hr in the manufacturer-recommended buffer and subjected to a PCR program of 28 cycles using primers KM1289 (5'-TTTCCAGAGCATGATACG) and KM1290 (5'-CGCACACTGTGCTTGCCT). KM1290 is homologous to genomic sequence outside of the tandem repeat array to ensure that the endogenous b1 enhancer repeat region and not the transgene was amplified by this primer combination. This assay measures methylation of DNA in the first repeat of the 7 tandem repeats in the endogenous locus, which is the repeat most distal to b1. Four biological replicates of each genotype were analyzed.

Results

MOP1 is not required for a1pIR transgene-induced silencing of endogenous A1

To test our hypothesis that production of dsRNA from the a1pIR hairpin will induce silencing of the functional allele (A1) of the endogenous a1 gene, B-I/B-I or B-I/b plants hemizygous for the a1pIR transgene (a1pIR/-) (Figure 1A) were crossed with a B' mop1-1 mutant stock (Figure 1B). The B-I/B-I; a1pIR/- or B-I/b; a1pIR/- plants used in these crosses exhibited the speckled brown-green to green plant pigmentation consistent with reduced anthocyanin content (Neuffer et al. 1997) due to a1pIR-induced silencing of the endogenous A1 gene. In the mop1-1/+ F₁ plants that were nontransgenic, the color specified by the B-I allele was reduced to a light, speckled pigmentation because of B-I paramutation by B', and pigmentation of transgenic mop1-1/+ F₁ plants was also reduced due to A1 inactivation by a1pIR. The B'/B mop1-1/+; a1pIR/- or B'/b mop1-1/+; a1pIR/- F₁ plants were backcrossed to the B' mop1-1 stock (Figure 1B), and progeny were molecularly genotyped for the mop1-1 mutation and the transgene. Plants were also visually scored for intensity of pigmentation. Because paramutation of B-I to B' is irreversible in wild-type backgrounds (Patterson et al. 1993), it is expected that nontransgenic mop1-1/+ heterozygotes will have light pigmentation because of continued maintenance of B' silencing. The nontransgenic mop1-1/mop1-1 homozygotes were expected to have dark pigmentation due to disruption of B' silencing (Dorweiler et al. 2000).

Indeed, examination of nontransgenic plants in segregating families revealed that all 56 mop1-1/+ plants had the expected light pigment phenotype (Table 1 and Figure 2A). Analysis of nontransgenic mop1-1 homozygous plants revealed that 26 plants had the expected dark phenotype (Figure 2B) and that 13 plants were lighter than expected. Presence of the light plants among nontransgenic mop1-1 homozygotes was unexpected and could have resulted from the presence of an unknown anthocyanin modifier in these crosses or from incomplete mop1-1 penetrance, although the latter was not previously reported (Dorweiler et al. 2000).

The expectation for the transgenic a1pIR plants was that mop1-1/+ should have a light phenotype due to maintenance of B' silencing and silencing of the endogenous A1 gene by a1pIR. Examination of this progeny class revealed that all 69 plants were lightly pigmented (Table 1 and Figure 2C). The prediction for the transgenic mop1-1 homozgyotes was that a dark plant color would be observed if maintenance of both B' silencing and A1 silencing by a1pIR was disrupted by the mop1-1 mutation. In this progeny class, all 62 transgenic homozygous mop1-1 plants had a light B' phenotype (Table 1 and Figure 2D). Because B' is upregulated in the majority of nontransgenic plants homozygous for mop1-1, but all a1pIR transgenic mop1-1 homozygous plants are light, this result indicates that the transgene continues to silence endogenous A1. The silencing response of the a1pIR transgene was consistent across all F₂ families (Table 1) and indicates that MOP1 is not required for a1pIR silencing of A1.

MOP3 is not required for a1pIR transgene-induced silencing of endogenous A1

MOP3 is predicted to be the largest subunit of the Pol IV holoenzyme (Materials and Methods) and, based on similarity
to the RdDM model from *Arabidopsis*, is expected to function with MOP1 upstream of siRNA biogenesis (Haag and Pikaard 2011). To assay whether the a1pIR transgene-induced A1 promoter silencing requires Pol IV activity, plants transgenic for the a1pIR were crossed with mop3-1/+ heterozygous plants (Figure S1). The resulting F1 were light B’ due to b1 paramutation and A1 inactivation by a1pIR. Transgenic F1 plants were crossed again with B’ mop3-1 individuals, and F2 progeny segregating for the presence or absence of the transgene and for the wild-type and mutant mop3-1 alleles were analyzed for pigmentation (Table 2).

Analysis of nontransgenic progeny revealed, as expected, that most of the wild-type (46 of 48) and mop3-1/+ (62 of 63) plants were lightly pigmented (Table 2 and Figure 2E) due to the maintenance of B’ silencing in these individuals and that 24 of 25 homozygous mop3-1 individuals had dark pigmentation (Table 2 and Figure 2F), indicating that B’ silencing was released. One homozygous mop3-1 plant was lightly pigmented, which was not expected for homozygous mop3-1 mutants. As described for the mop1-1 segregating families, this could result from either incomplete penetrance or an unknown modifier of anthocyanin expression segregating in these families. The prediction for the transgenic mop3-1 homozygotes was that dark plant color would be observed if both maintenance of B’ silencing and A1 silencing by a1pIR were disrupted by the mop3-1 mutation. Analysis of this progeny class revealed that all 16 transgenic mop3-1 homozygous plants had decreased anthocyanin pigmentation (Table 2 and Figure 2H). Because B’ is upregulated in 24 of 25 nontransgenic plants homozygous for mop3-1 (Figure 2G), but all a1pIR transgenic mop3-1 homozygous plants have reduced pigmentation (Figure 2H), this result indicates that the transgene continues to silence endogenous A1 and that MOP3 is not required for a1pIR silencing of A1.

**Somatic sectors of anthocyanin pigmentation suggest sporadic silencing of a1pIR**

Close examination of transgenic wild-type and mop3-1/+ plants revealed atypical pigmentation patterns that manifested as sectors of light speckled B’ tissue and green non-anthocyanin-pigmented tissue (Figure 2G) in a subset (40 of 72) of these plants (Table 2). Sectoring was also observed in seven transgenic mop3-1 homozygotes, consisting of alternating darkly pigmented and green sectors (Figure 2H). Upon replanting a subset of families segregating mop1-1 and a1pIR, some sectored plants were also observed, suggesting that this is not limited to mop3-1-containing lineages.

Sectored pigmentation was observed very infrequently in the mop1-1 experiments, and much more frequently in the mop3-1 experiments, but in both cases was observed only in crosses with a1pIR, suggesting that this sectoring is due to changes in the a1pIR transgene expression. In addition to sectoring described here with b1-specified plant body pigmentation, similar sectoring with distinct, sharp boundaries was also observed with the a1pIR transgene in experiments where anther pigmentation specified by alleles of r1 and pl1 genes was used as a reporter (L. Sidorenko, unpublished data). In all instances, the lightly pigmented sectors were interpreted as somatic tissues wherein A1 was silenced by a1pIR, the anthocyanin-expressing sectors as tissues where a1pIR-mediated silencing of A1 was not occurring, and the intensity of pigmentation in these darker sectors was as specified by the regulatory genes active in that plant/tissue. This interpretation of sectoring is also consistent with differential release of silencing in sectors observed in experiments involving transgenes that confer anthocyanin pigmentation in maize (McGinnis et al. 2006; Madzima et al. 2011). Therefore, while the exact cause of the sectored pigmentation remains unknown, the most likely possibility is that in the darker sectors the a1pIR transgene is expressed at levels that are insufficient for A1 silencing.

**MOP2 is required for endogenous A1 silencing by a1pIR**

Similar to the case in *Arabidopsis*, a MOP2-encoded protein could be the second largest subunit of both Pol IV and Pol V RNA polymerases, although there are two other expressed maize genes with a high degree of similarity to MOP2 (Sidorenko et al. 2009). To determine whether MOP2 is required for a1pIR-induced silencing of the A1 gene, a1pIR transgenic plants were crossed with B’ Mop2-1 (Figure 1A). Crosses between a1pIR/+ and B’ Mop2-1 and the expectations for segregating progeny were similar to those diagrammed in Figure 1B for mop1-1. Mop2-1 is a recessive allele with regard to the maintenance of B’ silencing, and as expected, the resulting F1 plants were lightly pigmented due to a combination of B’I paramutation by B’ and A1

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### Table 3 Pigment phenotypes in F2 families segregating for a1pIR transgene and Mop2-1

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<th>Mop2-1 genotype</th>
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<sup>a</sup> Total number of individuals in this category.

<sup>b</sup> Individuals with this phenotype and genotype were not detected.
inactivation by a1pIR. Transgenic F1 plants were crossed with 
B9 Mop2-1 heterozygous or homozygous individuals, and pig-
mentation was assessed in individual plants of segregating F2 
families (Table 3). A loss of B9 silencing phenotype was ob-
served across all families in all 22 nontransgenic homozygous 
Mop2-1 individuals (Figure 2I), which were darker than their 
19 nontransgenic heterozygous siblings (Figure 2J), which is 
consistent with upregulation of B9. In transgenic plants from 
the same F2 families, Mop2-1 heterozygotes had light pigment 
(33 plants) as expected, and all 7 homozygous 
Mop2-1 individ-
uals were darker than their heterozygous 
Mop2-1 trans-
genic siblings (Table 3; Figure 2, K and L). The fact that all 
homozygous 
Mop2-1 plants with and without a1pIR were 
dark indicates that hairpin transgene-induced silencing of en-
dogenous AI is prevented in Mop2-1 homozygous mutants 
and that MOP2-containing complexes are required for a1pIR 
promoter-induced silencing.

b1IR-induced silencing of b1 does not require MOP1, 
MOP2, or MOP3

Similar to the a1pIR transgene, expression of the b1IR trans-
gene is expected to generate transcripts that can self-anneal to 
form dsRNAs (Figure 3A). The b1IR transgene contains an 
inverted repeat of a single 853-bp repeat from a tandem hepta 
repeat that serves as an enhancer of the b1 gene (Belele et al. 
2013). Prior studies have shown that the b1IR transgene, 
which produces dsRNA of the 853-bp repeat, is suf-
ficient to 
induce transcriptional silencing and paramutation of an endog-
igenous B-I allele (Arteaga-Vazquez et al. 2010). To determine if 
the b1IR transgene requires MOP1, MOP2, or MOP3 for silenc-
ing, the b1IR transgene was crossed with 
mop1-1, 
Mop2-1, a n d 
mop3-1 mutants. Crosses with 
Mop2-1 were similar to those 
with mop1-1 (Figure 3B), and crosses with mop3-1 are dia-
grammed in Figure S2. In the b1IR tests with 
mop1-1, 
Mop2-1, and 
mop3-1, all F1 progeny were lightly pigmented, 
consistent with B’ silencing in heterozygous mutants exhibit-
ing maintenance of paramutation (data not shown). F1 prog-
eny were crossed with B’ individuals either heterozygous or 
homozygous for the appropriate mutant allele. The progeny 
plants were molecularly genotyped and scored for plant 
pigmentation.

In families segregating for the presence or absence of the 
b1IR transgene and the mop1-1 mutant or wild-type allele, all 
nontransgenic homozygous 
mop1-1 individuals (15) exhibited
the expected dark pigmentation phenotype consistent with a release of \( B' \) silencing (Table 4 and Figure 4B). All b1IR transgenic homozygous mop1-1 individuals (29) were lightly pigmented, indicative that b1IR-induced \( b1 \) silencing persisted in the absence of MOP1 (Table 4 and Figure 4D).

In families segregating for the presence or absence of the b1IR transgene and heterozygous or homozygous for the Mop2-1 allele, 13 of the 16 homozygous Mop2-1 individuals that lacked the transgene exhibited the expected dark pigment phenotype consistent with release of \( B' \) silencing (Table 5; Figure 4, E and F). The observation that not all Mop2-1 homozygous plants had the dark phenotype is similar to previous findings that suggested incomplete penetrance for disruption of silencing maintenance phenotypes (Sidorenko et al. 2009). The 18 homozygous Mop2-1 plants that were transgenic for b1IR were all lightly pigmented, indicating that the transgene silenced \( B' \) in the absence of MOP2 (Table 5 and Figure 4H).

In families segregating for b1IR and the mop3-1 mutation, 13 of the 18 nontransgenic homozygous mop3-1 individuals

<table>
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<td>ND(^b)</td>
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<td></td>
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<td>Light</td>
<td>ND(^b)</td>
<td>ND(^b)</td>
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</tr>
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\(^a\) Total number of individuals in this category.
\(^b\) Individuals with this phenotype and genotype were not detected.

![Figure 4](https://example.com/f4.png)

**Figure 4** Genotype and corresponding pigment phenotypes of representative plants for b1IR experiments. Plant pigment phenotypes observed in segregating families of transgenic b1IR/– and nontransgenic individuals heterozygous or homozygous for mop1-1 (A–D), Mop2-1 (E–H), or mop3-1 (I–L) mutant alleles.
exhibited dark pigmentation consistent with release of B’ silencing (Table 6; Figure 4, I and J). Among b1IR transgenic individuals, all 18 plants heterozygous for mop3-1 were lightly pigmented, as were 13 of the 14 plants homozygous for mop3-1 (Table 6; Figure 4, K and L). This result indicates that the b1IR transgene silenced B’ in the absence of MOP3 activity.

**DNA methylation of endogenous b1 hepta repeat associated with B1 silencing is reduced in MOP1, but not in MOP2 and MOP3 mutants**

Specific regions of the b1 tandem enhancer repeats are methylated in B’/B’ but not B-1/B-1 plants (Haring et al. 2010). To determine if B1 upregulation in response to loss of MOP1, MOP2, or MOP3 and B1 silencing by the b1IR transgene were also associated with changes in the endogenous b1 gene tandem repeat methylation, we analyzed a differentially methylated region of the tandem enhancer repeats using methylation-sensitive enzyme digestion of genomic DNA followed by polymerase chain reaction. This region is assayed with digestion by PstI at a site in the first repeat of the tandem hepta repeats. Consistent with previous reports (Haring et al. 2010), we found that this region of the tandem enhancer repeats was methylated in nontransgenic B’/B’ individuals that were heterozygous for any mutation (Figure 5). When B’ silencing was alleviated in nontransgenic mop1-1 mutant individuals, we observed hypomethylation of this region of the enhancer tandem repeat, as evidenced by reduced amplification due to digestion with a methylation-sensitive enzyme (Figure 5). In nontransgenic Mop2-1 and mop3-1 individuals, methylation persisted in darkly pigmented plants in which B1 was apparently upregulated (Figure 5). In plants transgenic for the b1IR transgene, methylation of the repeats and pigmentation indicating expression of B1 is similar to that of B’/B’ plants (Figure 5). These data demonstrate that methylation is reduced in mop1-1/mop1-1 mutants with upregulated B1 expression, but persists in Mop2-1/Mop2-1 and mop3-1/mop3-1 plants with upregulated B1 expression.

**Discussion**

We investigated the ability of transgene-derived dsRNAs to initiate silencing of endogenous genes in maize mutants deficient in three proteins known to be required for epigenetic transcriptional silencing and found that two different transgenes had distinct requirements. In plants transgenic for a1pIR, which contains an inverted repeat of the endogenous a1 gene promoter, mop1-1 and mop3-1 homozygous individuals exhibited silencing equivalent to nonmutant individuals. This indicates that neither MOP1 nor MOP3 are required for RNA-induced promoter-mediated silencing when dsRNAs are provided from a transgenic source. Based on the current model for the Arabidopsis RdDM pathway (Haag and Pikaard 2011), both MOP1 and MOP3 are expected to function upstream of dsRNA biogenesis in a Pol IV-mediated gene silencing pathway (Alleman et al. 2006; Erhardt et al. 2009). In contrast to MOP1 and MOP3, MOP2 was required for a1pIR-induced silencing of A1. MOP2 is orthologous with an Arabidopsis NRPD2/E2 protein that is incorporated in both Pol IV and Pol V complexes (Ream et al. 2009). Our observation that MOP2, but not MOP3, is required for a1pIR-induced silencing suggests that MOP2 functions in Pol V to silence A1 in response to the a1pIR transgene. Taken together, these results demonstrate that a1pIR-mediated silencing of A1 is regulated in accordance with the RdDM pathway model, as described for Arabidopsis (Axtell 2013).

Intriguingly, the b1IR silencing results do not meet the expectations of silencing in response to the Arabidopsis model for the RdDM pathway. The b1IR was competent to mediate silencing of b1 in plants homozygous for any of the three tested mutations, suggesting that the b1IR transgene bypassed the need for the siRNA biogenesis function normally provided by MOP1, MOP2, and MOP3, as the a1pIR transgene did in

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**Table 5 Pigment phenotypes in F2 families segregating for b1IR transgene and Mop2-1**

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Mop2-1 genotype</th>
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<tr>
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<sup>a</sup>Total number of individuals in this category.

<sup>b</sup>Individuals with this phenotype and genotype were not detected.

---

**Table 6 Pigment phenotypes in F2 families segregating for b1IR transgene and mop3-1**

<table>
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<th>Transgene</th>
<th>mop3-1 genotype</th>
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</table>

<sup>a</sup>Total number of individuals in this category.

<sup>b</sup>Individuals with this phenotype and genotype were not detected.
the absence of MOP1 and MOP3 function. The fact that hairpin-mediated silencing at the b1 locus can occur in the absence of MOP2 could be an indication that b1 enhancer silencing does not require MOP2-containing Pol V function. Because transcription within the endogenous b1 enhancer is sensitive to α-amanitin, a strong inhibitor of Pol II, but not Pol IV and Pol V polymerase activity (Haag et al. 2012), it was hypothesized that the b1 enhancer may be transcribed predominantly by Pol II (Arteaga-Vazquez et al. 2010). Although b1 silencing associated with paramutation is disrupted in both mutants, transcription within the endogenous b1 enhancer is not altered in either mop1-1 (Arteaga-Vazquez et al. 2010).

Figure 5 Methylation of the b1 enhancer repeats in transgenic and nontransgenic plants homozygous or heterozygous for mop1-1, Mop2-1, or mop3-1. Genomic DNA from individual plants in families segregating for the b1IR transgene and mop1-1 (top), Mop2-1 (middle), or mop3-1 (bottom) was digested with PstI (cut) and subjected to PCR to amplify the first repeat of the endogenous tandem repeat array that is differentially methylated in B9 and B-1 (Haring et al. 2010). Undigested DNA from each plant was also subjected to the same PCR conditions (uncut) as a control.

Figure 6 Hypothetical model for silencing of endogenous genes with inverted-repeat-containing transgenes. In this model, the requirement for MOP1 and MOP3 is bypassed by abundant double stranded RNA (dsRNA) production from a hairpin. The b1 enhancer-hairpin-induced silencing of the endogenous b1 gene occurs in Mop2-1 homozygous plants because an alternative RNA polymerase provides a scaffolding transcript. This alternative RNA polymerase could be Pol II or another, yet uncharacterized, polymerase (Pol-N), possibly a Pol V variant containing one of the two other MOP2-like proteins in maize. At the A1 promoter dsRNA silencing occurs through mechanisms that are similar to that of RdDM in Arabidopsis with Pol V transcription generating scaffolding transcripts.
predict whether the divergent mechanisms in our hypothesis are driven by differences in sequence of the target loci or by the tissue-specific differences in expression of the two other MOP2-like proteins in maize. Determining the genetic requirements for silencing of additional maize loci, including those with paramutation, through the use of promoter/enhancer hairpins and determining the genome-wide occupancy of Pol II and IV and the distinct variants of Pol V will refine the proposed hypothesis and provide insights into the extent to which this model may apply to other loci.

Acknowledgments

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Diverse Gene-Silencing Mechanisms with Distinct Requirements for RNA Polymerase Subunits in *Zea mays*

Amy E. Sloan, Lyudmila Sidorenko, and Karen M. McGinnis
Figure S1  Diagram of crosses testing effects of the mop3-1 mutation on a1pIR hairpin-induced A1 silencing. In the year when initial crosses were done, a hot and dry summer combined with the reduced fitness of mop3-1 homozygotes, necessitated using heterozygous mop3-1/+ plants for the initial crosses. The F1 plants carrying mop3-1/+ and the a1pIR transgene were self-pollinated and the resulting F2 families were examined.
Figure S2  Diagram of crosses testing effects of the mop3-1 mutation on b1IR hairpin transgene mediated silencing of b1. The rationale for using mop3-1/+ plants is similar to that described in Figure S1. Plants in BC₁ families segregating dark non-transgenic (mop3-1 homozygotes) were genotyped and the results are presented in Table 6.
Figure S3  Schematic drawing of the mop3-1 allele. cDNA was amplified from a mop3-1 mutant and sequenced. Sequence traces were aligned using LaserGene Seqman (DNASTAR, Madison, WI). Analysis of cDNA sequence indicated that an alternative splice site (purple letters) is used instead of the intron 13 splice acceptor site (green letters) which is mutated in mop3-1. Use of this alternative splicing site leads to a frame shift and a premature stop codon (red letters). Genomic to cDNA alignments and protein translations (segments shown here) were done using Spidey at (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/).
Supporting Information Materials and Methods

Construction of a1pIR and b1IR transgenes

The a1pIR transgene was generated by cloning PCR amplification of ~150 bp of the maize a1 gene promoter using pMZ2011 plasmid (Klein et al., 1989) as a template. Two primers were used for amplification of both repeats: vc1447a (5’GACTAGTGGCGGCAGAGATATGCGCAGTAG3’ containing SpeI and Ascl restriction sites) and vc1447b (5’GTTGGTTGCGATCGCCCTAGGAGACTGCTGCTCCAGTTCC3’ containing AvrII and restriction SgfII sites). The resulting a1 promoter fragments were cloned in an inverted orientation in the pMCG161 vector (McGinnis et al. 2005) using restriction sites within multiple cloning sites flanking a loop-forming intron as described in (McGinnis et al. 2005) producing the pVC4300 plasmid. Next, HindIII and Swal restriction sites were used to release the entire hairpin and the 3’ terminator from the pVC4300 and the released fragment was inserted in the HindIII and Swal digested pMCG7942 vector, producing pVC4318 that was used for Agrobacterium mediated maize transformation to produce a1pIR transgenes. pMCG7942 is a pBIN19 based vector with a 4x 35S:Omega Leader:Bar plant selectable marker, and the maize ubiquitin1 gene promoter and 5’UTR driving expression of the inverted repeat cassette.

To produce the b1IR hairpin construct, the 856 bp repeat was amplified from the pMS14 plasmid, a subclone of the pBΔ described in (Belele et al. 2013) that includes the b1 enhancer repeat. The first copy of b1 repeat was amplified using VC977A (GGTTGGTTGCGATCGCCCTAGGCGGATGCTGCATCCTTG with AvrII site) and VC977B (GGACTAGTGGCGGCAGAGATATTCGGTATAAAAGTTGT with Ascl site) primers. Resulting PCR product was digested with AvrII and Ascl and inserted in pMCG7942 digested within AvrII and Ascl. The second repeat amplified using VC1130F (5’GGATCAGTGCTGCATCCTTG with SpeI site) and VC1130R (5’GGATCAGTGCTGCATCCTTG with SpeI site) primers. The resulting construct was named pVSIR2 and used to produce b1IR transgenic plants.