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* (*bz*) 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*- and *r1*-encoded and the *c1*- and *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 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).

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Figure 1 Outline of experiment testing effects of the *mop1-1* mutation on a1pIR silencing of endogenous *A1* gene. (A) Diagram of the hairpin construct and overall experiment outline. (B) Crossing scheme used to test effects of the *mop1-1* mutation on the a1pIR silencing of *A1*. Because the location of the a1pIR transgene is not known, it is assumed to be unlinked with the *a1* and *b1* loci. While *mop3-1* is unlinked to *a1* and *b1*, *mop1* and *b1* are 20 cM apart on chromosome 2 (Alleman *et al.* 2006) and expected to segregate together in ~80% of plants. *Mop2-1* is 27 cM from *b1* (Sidorenko *et al.* 2009) and expected to segregate with *b1* in ~73% of plants. Expected phenotypes are indicated below each genotype.

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 *B'*. These repeats are necessary and sufficient to participate in paramutation from ectopic locations (Belele *et al.* 2013), are required for transcriptional enhancement of *b1* (Louwers *et al.* 2009), and enhance transcription of a reporter gene (Belele *et al.* 2013). The current model for the transcriptional regulation involves changes in chromatin conformation and chromatin looping events that bring the enhancer repeats in proximity with the *b1* promoter (Louwers *et al.* 2009). This is consistent with observed changes in chromatin structure, nucleosome occupancy, post-translational histone modifications, and DNA methylation at the *b1* enhancer repeats associated with the differential transcriptional regulation of *B'* and *B-I* (Haring *et al.* 2010).

Paramutation at *b1* and *p11* 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 *rmr7* (Stonaker *et al.* 2009) and encodes a conserved protein homologous to the *A. thaliana* second largest subunit of RNA polymerase IV

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

Transgene	<i>mop1-1</i> genotype	Plant color	Family 1	Family 2	Family 3	Family 4
<i>a1pIR</i>	Heterozygous (69) ^a	Dark	ND ^b	ND ^b	ND ^b	ND ^b
		Light (69) ^a	23 (1) ^c	26 (1) ^c	5	15
<i>a1pIR</i>	Homozygous (62) ^a	Dark	ND ^b	ND ^b	ND ^b	ND ^b
		Light (62) ^a	16 (7) ^c	20 (9) ^c	ND ^b	26 (5) ^c
—	Heterozygous (56) ^a	Dark	ND ^b	ND ^b	ND ^b	ND ^b
		Light (56) ^a	21	17	4	14
—	Homozygous (39) ^a	Dark (26) ^a	11	6	1	8
		Light (13) ^a	1	5	ND ^b	7

^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 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* based upon sequence homology to *Arabidopsis* RdDM components and their mutant phenotypes has MOP1, MOP2, and MOP3 functioning 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 an 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

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.

and were scored for pigment production at flowering. F_2 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. F_2 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 μ l 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' AGTCGACCGTGACGTCTCC). *Mop1* and *Mop2* genotyping assays were conducted as previously described (Sidorenko *et al.* 2009; Labonne *et al.* 2013) while KM 553 (5' TCCACTGAACATTGCTTTGTC) and KM 554 (5' GGTTCCTCCCTACAGCCTTTC) were used to amplify *Mop3* and sequence the resulting PCR product.

Table 2 Pigment phenotypes in F₂ families segregating for *a1pIR* transgene and *mop3-1*

Transgene	<i>mop3-1</i> genotype	Plant color	Family 5	Family 6	Family 7	Family 8
<i>a1pIR</i>	Wild type (24) ^a	Dark	ND ^b	ND ^b	ND ^b	ND ^b
		Light (24) ^a	8 (4) ^c	13 (8) ^c	3 (1) ^c	ND ^b
<i>a1pIR</i>	Heterozygous (48) ^a	Dark	ND ^b	ND ^b	ND ^b	ND ^b
		Light (48) ^a	15 (7) ^c	21 (13) ^c	6 (4) ^c	6 (3) ^c
<i>a1pIR</i>	Homozygous (16) ^a	Dark	ND ^b	ND ^b	ND ^b	ND ^b
		Light (16) ^a	5 (5) ^c	6 (6) ^c	2 (1) ^c	3 (3) ^c
—	Wild type (48) ^a	Dark (2) ^a	ND ^b	2	ND ^b	ND ^b
		Light (46) ^a	17	16	7	6
—	Heterozygous (63) ^a	Dark (1) ^a	ND ^b	1	ND ^b	ND ^b
		Light (62) ^a	25	18 (1) ^c	12 (2) ^c	8
—	Homozygous (25) ^a	Dark (24) ^a	5	5	7	7
		Light (1) ^a	ND ^b	ND ^b	ND ^b	1

^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 sectorized 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 *Pst*I (Promega) for 4 hr in the manufacturer-recommended buffer and subjected to a PCR program of 28 cycles using primers KM1289 (5' TTCGACGAGCATA GATACG) and KM1290 (5' CGCAGCACTGTTGTTGTTCT). 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* homozygotes was that a dark plant color would be observed if maintenance of both *B'* silencing and *A1* silencing by *a1pIR* were 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

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

Transgene	<i>Mop2-1</i> genotype	Plant color	Family 9	Family 10	Family 11
<i>a1pIR</i>	Heterozygous (33) ^a	Dark	ND ^b	ND ^b	ND ^b
		Light (33) ^a	7	18	8
<i>a1pIR</i>	Homozygous (7) ^a	Dark (7) ^a	1	4	2
		Light	ND ^b	ND ^b	ND ^b
—	Heterozygous (19) ^a	Dark	ND ^b	ND ^b	ND ^b
		Light (19) ^a	ND ^b	17	2
—	Homozygous (22) ^a	Dark (22) ^a	6	14	2
		Light	ND ^b	ND ^b	ND ^b

^a Total number of individuals in this category.

^b Individuals with this phenotype and genotype were not detected.

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*

Figure 3 Outline of experiment testing effects of the *mop1-1* mutation on b1IR silencing of the endogenous *b1* gene. (A) Diagram of the transgene b1IR hairpin construct and outline of experiment that tests the MOP1, MOP2, and MOP3 requirement for b1IR silencing of the endogenous *b1* gene. (B) Crossing scheme used to test effects of the *mop1-1* mutation on b1IR-mediated silencing of *b1*. Location of the b1IR transgene is not known and was assumed to be unlinked to the *b1* gene. Assumptions regarding linkage between *b1* and *mop* mutants are similar to those in Figure 1. Expected plant phenotypes are indicated below each genotype.

inactivation by a1pIR. Transgenic F₁ plants were crossed with *B' Mop2-1* heterozygous or homozygous individuals, and pigmentation was assessed in individual plants of segregating F₂ families (Table 3). A loss of *B'* silencing phenotype was observed 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 *B'*. In transgenic plants from the same F₂ families, *Mop2-1* heterozygotes had light pigment (33 plants) as expected, and all 7 homozygous *Mop2-1* individuals were darker than their heterozygous *Mop2-1* transgenic 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 endogenous *A1* 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 transgene 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 sufficient to induce transcriptional silencing and paramutation of an endogenous *B-I* allele (Arteaga-Vazquez *et al.* 2010). To determine if the b1IR transgene requires MOP1, MOP2, or MOP3 for silencing, the b1IR transgene was crossed with *mop1-1*, *Mop2-1*, and *mop3-1* mutants. Crosses with *Mop2-1* were similar to those with *mop1-1* (Figure 3B), and crosses with *mop3-1* are diagrammed 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 exhibiting maintenance of paramutation (data not shown). F₁ progeny 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

Table 4 Pigment phenotypes in F₂ families segregating for *b1IR* transgene and *mop1-1*

Transgene	<i>mop1-1</i> genotype	Plant color	Family 12	Family 13	Family 14	Family 15
<i>b1IR</i>	Wild type (21) ^a	Dark	ND ^b	ND ^b	ND ^b	ND ^b
		Light (21) ^a	3	7	6	5
<i>b1IR</i>	Heterozygous (57) ^a	Dark (1) ^a	ND ^b	ND ^b	ND ^b	1
		Light (56) ^a	15	17	14	10
<i>b1IR</i>	Homozygous (29) ^a	Dark	ND ^b	ND ^b	ND ^b	ND ^b
		Light (29) ^a	7	3	7	12
—	Wild type (31) ^a	Dark (1) ^a	ND ^b	ND	1	ND ^b
		Light (30) ^a	10	4	5	11
—	Heterozygous (42) ^a	Dark (1) ^a	ND	1	ND ^b	ND ^b
		Light (41) ^a	7	15	7	12
—	Homozygous (15) ^a	Dark (15) ^a	8	3	3	1
		Light	ND ^b	ND ^b	ND ^b	ND ^b

^a Total number of individuals in this category.

^b Individuals with this phenotype and genotype were not detected.

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

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.

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

Transgene	<i>Mop2-1</i> genotype	Plant color	Family 16	Family 17	Family 18
<i>b1IR</i>	Heterozygous (31) ^a	Dark	ND ^b	ND ^b	ND ^b
		Light (31) ^a	17	10	4
<i>b1IR</i>	Homozygous (18) ^a	Dark	ND ^b	ND ^b	ND ^b
		Light (18) ^a	6	10	2
—	Heterozygous (32) ^a	Dark (2) ^a	1	1	ND ^b
		Light (30) ^a	15	13	2
—	Homozygous (16) ^a	Dark (13) ^a	5	6	2
		Light (3) ^a	1	1	1

^a Total number of individuals in this category.

^b Individuals with this phenotype and genotype were not detected.

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-I/B-I* 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; Erhard *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

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

Transgene	<i>mop3-1</i> genotype	Plant color	Family 19
<i>b1IR</i>	Heterozygous (18) ^a	Dark	ND ^b
		Light	18
<i>b1IR</i>	Homozygous (14) ^a	Dark	1
		Light	13
—	Heterozygous (19) ^a	Dark	ND ^b
		Light	19
—	Homozygous (18) ^a	Dark	13
		Light	5

^a Total number of individuals in this category.

^b Individuals with this phenotype and genotype were not detected.

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 *Pst*I (cut) and subjected to PCR to amplify the first repeat of the endogenous tandem repeat array that is differentially methylated in *B'* and *B-I* (Haring *et al.* 2010). Undigested DNA from each plant was also subjected to the same PCR conditions (uncut) as a control.

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

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.

et al. 2010) or *Mop2-1* mutants (Sidorenko *et al.* 2009). Collectively, these observations lead to a hypothesis that *b1* silencing (Figure 6) requires a scaffolding RNA polymerase(s) that does not contain MOP2 as a second largest subunit. This RNA polymerase might be Pol II or an alternatively assembled Pol-V-like complex containing one of the two other MOP2-like proteins encoded in the maize genome (Sidorenko *et al.* 2009), although relatively little is known about the function of these proteins and there are no currently known mutant alleles for these genes.

In plants where silencing of *B1* was apparent, including genotypically *B'/B'* plants heterozygous for any mutation and plants exhibiting b1IR transgene-induced silencing of *B1*, we observed methylation of a previously identified differentially methylated region of the enhancer repeats (Haring *et al.* 2010). This suggests that, in addition to inducing heritable silencing of *B1* as previously reported (Arteaga-Vazquez *et al.* 2010), the b1IR transgene induces cytosine methylation of the enhancer repeats, as does exposure to *B'* in wild-type plants. In *mop1-1/mop1-1* plants, hypomethylation occurred when silencing was alleviated, but methylation persisted in *Mop2-1/Mop2-1* and *mop3-1/mop3-1* plants exhibiting upregulation of *B1*. This suggests that silencing and methylation of the tandem enhancer repeats can occur separately from one another, although methylation of this region and level of *B1* expression are associated in wild-type *B'* and *B-I* plants (Haring *et al.* 2010). This difference could indicate that other RNA polymerases act in concert with MOP1 to maintain methylation in the absence of MOP2 and MOP3 or that MOP2 and MOP3 function in a parallel pathway to pathways that induce methylation of regulated loci.

In support of a model for Pol IV/Pol II-induced silencing, long noncoding RNAs are proposed to act as scaffolding molecules similar to that described in humans and other organisms that lack RNA polymerases IV and V (Tsai *et al.* 2010; Geisler and Collier 2013). There is also evidence in *Arabidopsis* that Pol IV and Pol V activity can be uncoupled (Wierzbicki *et al.* 2008, 2012; Pontes *et al.* 2009; Lopez *et al.* 2011) and that Pol II- and Pol V-occupied sites are frequently found in close proximity to one another (Wierzbicki *et al.* 2012). Therefore, it is feasible that, in the absence of Pol V, Pol II might be positioned to compensate for this activity.

The proposed model is also supported by results from a previous study that reported on maize promoter hairpin transgenes targeting three endogenous loci for silencing: *ms45*, *5126*, and *pg47* (Sidorenko *et al.* 2009). MOP2 was shown to be required for hairpin transgene-induced silencing of *ms45* and *5126*, but not *pg47*, which appears to be predominantly expressed in tissues with relatively low *Mop2* expression. To generalize our hypothesis to include the five different loci silenced by hairpin transgenes, three loci use MOP2-containing Pol V complexes for downstream siRNA targeting, and the other two engage either Pol II RNA polymerase or variants of Pol V (Figure 6). Based upon the limited number of loci that have been tested so far, it is not possible to

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.

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GENETICS

Supporting Information

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

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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 *F*₁ plants carrying *mop3-1/+* and the *a1pIR* transgene were self-pollinated and the resulting *F*₂ 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/>).

File S1

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'GACTAGTGGCGCGCCGCGCAGAGATATGCCGGTAG3' containing *SpeI* and *Ascl* restriction sites) and vc1447b (5'GTTGGTTGCGATCGCCCTAGGAGACTGCTGCTCCAGTTCC3' containing *AvrII* and restriction *SgfI* 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 *SwaI* 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 *SwaI* 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 (GGTTGGTTGCGATCGCCCTAGGCCATGGGTTTGCTGCATCCTTG with *AvrII* site) and VC977B (GACTAGTGGCGCGCCCCAAGTATTCGGTATAAAAGTTGT 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'GGATCACTAGTCCATGGGTTTGCTGCATCCTTG3' with *SpeI* site) and VC1130R (5'GGATCACTAGTCCAAGTATTCGGTATAAAAGTTG3' with *SpeI* site) primers. The resulting construct was named pVSIR2 and used to produce b1IR transgenic plants.