Identification of Epigenetic Regulators of a Transcriptionally Silenced Transgene in Maize

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ABSTRACT

Transcriptional gene silencing is a gene regulatory mechanism essential to all organisms. Many transcriptional regulatory mechanisms are associated with epigenetic modifications such as changes in chromatin structure, acetylation and methylation of core histone proteins, and DNA methylation within regulatory regions of endogenous genes and transgenes. Although several maize mutants have been identified from prior forward genetic screens for epigenetic transcriptional silencing, these screens have been far from saturated. Herein, the transcriptionally silent b1 genomic transgene (BTG-silent), a stable, epigenetically silenced transgene in Zea mays (maize), is demonstrated to be an effective phenotype for a forward genetic screen. When the transgene is reactivated, a dark purple plant phenotype is evident because the B1 transcription factor activates anthocyanin biosynthesis, making loss of silencing mutants easy to identify. Using BTG-silent, ten new putative mutants were identified and named transgene reactivated1 through 11 (tgr1-6 and tgr8-11). Three of these mutants have been examined in more detail, and molecular and genetic assays demonstrated that these mutants have both distinct and overlapping phenotypes with previously identified maize mutants that relieve epigenetic transcriptional silencing. Linkage analysis suggests that tgr2 and tgr3 do not correspond to a mutation at previously identified maize loci resulting from other forward genetic screens, while tgr1 shows linkage to a characterized gene. These results suggest that the mutants are a valuable resource for future studies because some of the mutants are likely to reveal genes that encode products required for epigenetic gene regulation in maize but are not currently represented by sequenced mutations.

KEYWORDS
epigenetics
transgene
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Transcriptional regulation of gene expression is essential for the normal growth and development of organisms. Transcriptional regulation is accomplished via the association of transcription factors with genetic regulatory elements nearby or adjacent to the regulated gene and transcription factor accessibility to those sequences, which is dependent on the chromatin structure. In eukaryotic species, the establishment and maintenance of a particular conformation of chromatin involves an interdependent association of differentially methylated DNA, modified histones, and variations in nucleosome distribution and compaction (reviewed by Goldberg et al. 2007). A particular chromatin structure and its associated gene expression state is sometimes heritable across cell divisions, contributing to cellular differentiation and development (Jarillo et al. 2009).

The methylation of cytosines in a symmetric CG context is highly conserved in plants and mammals and is generally considered to be associated with the regulation of gene expression, although the exact nature of the relationship between gene expression and DNA methylation is not completely understood (reviewed by Lee et al. 2010). In plants, cytosine methylation within gene promoters, repetitive sequences and transposons can be associated with transcriptional gene silencing (TGS) via the RNA-dependent DNA methylation (RdDM) pathway and can involve asymmetric cytosine residues (CHH, where H is any residue other than G) in addition to symmetric cytosine residues (CG, CHG). This pathway is reliant upon the activity of a set of proteins that produce small interfering RNAs (siRNAs) homologous to target loci and mediate epigenetic features on the chromosome at target loci (reviewed by Matzke et al. 2009; Simon...
and Meyers 2010). Although RdDM has been characterized most extensively in Arabidopsis, genetic screens in maize have identified several components of the pathway and the maize orthologs have been implicated in the regulation of both endogenous and transgenic loci, including those that participate in paramutation (reviewed by Arteaga-Vazquez and Chandler 2010).

Paramutation occurs when two alleles interact in trans to heritably change the expression level of one allele (reviewed by Chandler 2010). Paramutation in maize has been described at several genes involved in the flavonoid biosynthetic pathway and include r1, b1, pl1, and pl1 (Brink 1956; Coe 1959; Hollick et al. 1995; Sidorenko and Peterson 2001) (reviewed by Chandler et al. 2000). Each of these genes encodes a transcription factor that regulates the expression of the enzymes required for pigment biosynthesis, allowing transcriptional activity of these genes to be readily detected by the visual observation of pigment in plant tissues. At the b1 gene, paramutation requires the presence of tandem hepta-repeat sequences located 100 kb upstream of the b1 transcription start site (reviewed by Chandler 2010). This well-characterized example of paramutation involves transcriptional silencing of one allele, associated with changes in DNA methylation and chromatin structure within the tandem repeats (Haring et al. 2010; Stam et al. 2002a; Stam et al. 2002b).

The b1 and pl1 systems have been used in genetic screens to identify mutants required for paramutation. Using either active Mutator (Mu) transposable elements or EMS mutagenesis, genetic screens based on these paramutation systems have identified several mutants. The b1 paramutation alleles were used to identify the mediator of paramutation (mop) mutants (Dorweiler et al. 2000; Sidorenko et al. 2009). Map-based cloning revealed that the first locus characterized, mop1, encodes a putative RNA-dependent RNA-polymerase similar to Arabidopsis RDR2 (Alleman et al. 2006). More recently, a dominant mutation designated Mop2-1 was cloned and shown to encode a protein similar to the second largest subunits of Pol IV and Pol V in Arabidopsis (Sidorenko et al. 2009). The pl1 paramutation system was used to identify several mutants designated required to maintain expression (Hale et al. 2007; Hollick and Chandler 2001; Stonaker et al. 2009). rrm1 encodes a putative SNF2-like ATPase chromatin remodeler (Hale et al. 2007) and rrm6 encodes a protein similar to Arabidopsis NRPD1, which is the large subunit of the plant specific DNA-dependent RNA polymerase (Pol IV) (Erhard et al. 2009). rrm7 encodes an allele of mop2 (Stonaker et al. 2009). Identification and cloning of these genes provides strong evidence that RNA-directed transcriptional gene silencing is one mechanism underlying paramutation. While the endogenous b1 and pl1 systems have been useful in the discovery of genes required for both paramutation and TGS, many of the maize orthologs for Arabidopsis RdDM mutants have yet to be identified (reviewed by Arteaga-Vazquez and Chandler 2010). Approaching gene discovery through multiple screens will enhance the likelihood of characterizing as many components of epigenetic gene regulation as possible in this important model organism.

The mop1-1, rrm1-1, and rrm2-1 mutants were identified in genetic screens for paramutation, and Mop1, Rrm1, and Rrm2 are also required for epigenetic silencing of two transgenes (McGinnis et al. 2006), including the b1 genomic transgene (BTG). This transgene includes the maize b1 genomic sequence (transcribed region including introns and exons) driven by the heterologous, highly expressed 35SS cauliflower mosaic virus promoter (Figure 1A); plants actively expressing this transgene are purple. A stably silent line was identified (BTG-silent) in which the plant tissues were green due to transcriptional silencing of the transgene, even when the transgene remained active in the kernel (McGinnis et al. 2006). Because none of the sequences required for b1 paramutation are included in the transgene (reviewed by Chandler 2010), and all of the described experiments used maize stocks that did not carry b1 alleles that participate in paramutation, the transcriptional silencing of this transgene is not directly related to b1 paramutation. BTG-silent provides a powerful marker for investigating epigenetic gene silencing and a system in which heritable changes in gene expression can be correlated with specific epigenetic marks and mechanisms (McGinnis et al. 2006).

To identify other genes involved in epigenetic gene regulation, a forward genetic screen based on reactivation of BTG-silent was conducted using EMS mutagenized maize. Reactivation of the transgene is easily scored by visually monitoring accumulation of anthocyanin pigmentation. Herein, we report that the BTG-silent transgene and EMS mutagenesis were effectively used to identify multiple genes required for transcriptional silencing of transgenes in maize; these genes are referred to as transgene-reactivated (tgr). Initial genetic and molecular characterizations of these mutants are reported.

**MATERIALS AND METHODS**

**Genetic stocks and plant material**

The b1 genomic transgenic line has been described previously (McGinnis et al. 2006). Briefly, the line is transgenic for the 35SBTG construct, which is composed of the 35S Cauliflower Mosaic Virus promoter (35S CaMV), the first intron of maize alcohol dehydrogenase 1 (included as an enhancer of expression), and the genomic...
sequence spanning the complete coding region and both the 5’ and 3’ UTRs of the B-I allele of the maize bt1 gene (McGinnis et al. 2006) (Figure 1A). B-I encodes a transcription factor which activates anthocyanin biosynthetic genes, leading to red or purple plant pigmentation in tissues where it is expressed (Selinger et al. 1998). Following transformation of the maize inbred line CG00526 with the 35SBTG construct, the transgenic line has been crossed for many generations with stocks that were recessive for bt1 and the functionally redundant r1 locus, wild-type for the other transcriptional regulators and wild-type for all the biosynthetic enzymes required for pigment production. In this line, BTG is stably, heritably, and transcriptionally silenced (McGinnis et al. 2006). The chromosomal location of BTG is unknown, but the same transgenic event was used for all experiments described herein. Details on the genotype of this stock are available upon request.

Mutagenesis and forward genetic screen

Mutagenesis was conducted by treatment of nontransgenic pollen with ethyl methanesulfonate (EMS) (Neuffer et al. 1997). EMS was diluted in paraffin oil and applied to pollen collected from nontransgenic b r-g stocks. After an incubation period, treated pollen was applied to ears of plants hemizygous or homozygous for BTG-silent. Ears were covered and allowed to mature for the remainder of the field growing season. Resulting seeds (the M1 generation) were planted and self-pollinated to make any introduced mutations homozygous. The M1 generation was monitored for increased pigment in plant tissues, which could be indicative of dominant or semidominant alleles that reactivated the transgene—none were observed. After self-pollination, 60 transgenic M2 seeds were planted (McGinnis et al. 2006). Approximately 500 M2 families were screened, resulting in 10 putative recessive mutants identified. One M2 family was originally designated as tgr7, but it only segregated a very small number of dark plants, and no dark plants segregated upon replanting. This line was dropped from analysis; consequently, there is no putative mutant corresponding to tgr7.

Nomenclature

Mutant families from this screen were designated tgr for transgene reactivated. For each individual, two independent loci are relevant for the experiments, one is the putative mutated locus (tgr1 through tgr11), and the other is the transgene (BTG-silent/active). Transgenic lines are designated by their tgr family number and the activation status of BTG. For example, a given family, which results from planting seed from a single ear, will be segregating wild-type and mutant tgr alleles and segregating the transgene, which will be silent or active. Genotyping is not possible as the mutated loci are as yet uncloned, but we refer to the various combinations of loci using the hypothesis that plants bearing silent transgenes are heterozygous for the recessive mutant or homozygous wild-type, while plants with active transgenes are homozygous for the mutation.

Bisulfite conversion and DNA methylation analysis

Genomic DNA was isolated from adult leaf tissue using the DNeasy Plant Mini Kit (Qiagen; www.qiagen.com) according to the manufacturer’s instructions. Three BTG-silent and three BTG-active individual plants were used from segregating populations for each mutant. For bisulfite treatment, 200-500 ng of genomic DNA was converted using the MethyEasy Xceed Rapid DNA Bisulphite Modification Kit (Human Genetic Signatures Pty Ltd; North Ryde, Australia; www.geneticsignatures.com) according to the manufacturer’s instructions. The Kismeth plant bisulfite sequencing primer design program (Gruntman et al. 2008) (http://katahdin.mssm.edu/kismeth) was used to design degenerate primers that were used to amplify converted DNA from the transgene. Primer locations were selected to selectively amplify the 35SBTG construct promoter and transcriptional enhancer regions. The selected primers designed to amplify portions of the 35S CaMV promoter + adh1 intron are as follows (R refers to pyrimidine):

KM64: 5’-AAAGGAYAGTAGAAAGGAGGTTGGA-3’;
KM69: 5’-CAAACCTTTCRCRCTTCTAAACAC-3’;
KM73: 5’-CCTCTTTCRCAARTTCACATCT-3’;
KM78: 5’-ATYATTGATAAGGGAAAGG-3’.

Nested PCR was used to amplify desired products. For the first round of PCR, KM64 and KM69 primers were used. PCR conditions were as follows: 94°C for 3 min (1×); 94°C for 30 sec, 52°C for 1 min, 72°C for 1.5 min (30×); 72°C for 10 min (1×). 2 μl of first-round PCR product were used as template for the nested PCR reaction using KM73 and KM78 primers. PCR conditions were as follows: 94°C for 3 min (1×); 94°C for 30 sec, 44°C for 1 min, 72°C for 1.5 min (30×); 72°C for 10 min (1×). PCR products were gel purified using the Wizard SV Gel and PCR Clean-up System (Promega; www.promega.com), cloned into the pCR4 TOPO TA cloning vector (Invitrogen; www.invitrogen.com) and transformed to TOP10 Chemically competent cells (Invitrogen). Plasmid DNA was isolated using the QIAGEN Plasmid Mini Kit (Qiagen) according to the manufacturers’ instructions. Cloned inserts were verified by PCR using insert specific primers and by restriction digestion using the EcoRI enzyme with restriction sites within the pCR4 vector. M13R primers were used for sequencing. The analysis included three individual plants for each of the seven different types of plant (tgr1 BTG-s, tgr1 BTG-a, tgr2 BTG-s, tgr2 BTG-sec, tgr2 BTG-a, tgr3 BTG-s, and tgr3 BTG-a). For each individual plant, 10-12 clones were sequenced and subjected to analysis. The Kismeth plant bisulfite sequencing analyzer (Gruntman et al. 2008) (http://katahdin.mssm.edu/kismeth) was used for methylation analysis. Clones that appeared to be duplicate representation of the same molecule were identified and removed from analysis (Henderson et al. 2010) as these might artificially overrepresent the methylation pattern and bias the data. Because a nonproofreading enzyme was used for PCR amplification, any sequences with more than 0.8% non C/T mismatches in the analyzed region were also removed them analysis. Removing these potentially duplicate or error-containing clones resulted in the evaluation of between 10 and 31 clones for each mutant genotype and BTG expression level that was analyzed. An endogenous unmethylated sequence, Probe A/Ptr1 (Stam et al. 2002a), was used as a control to confirm that the retention of cytosines in the sequences did not result from incomplete conversion reactions. The PCR conditions are the same as those described for analysis of methylation in the 35S CaMV promoter of BTG and primers used to amplify this control sequence are:

KM447: 5’-TTGGAAGATYAGATGGGATGTTGA-3’;
KM 448: 5’-GGATGGATGAAATATAATAYAGTAG-3’;
KM449: 5’-CAATCGATCCCTCTCTCTCTC-3’;
KM450: 5’-TCTAACATCTACATCTAACAC-3’.

Linkage relative to putative maize RdDM components with characterized mutants in maize

The BTG-silent lines had been crossed for multiple generations with a stock that is polymorphic relative to the inbred line B73, enabling B73 to be used as the outcross parent for constructing mapping.
SSR genotyping

Polymerase chain reaction (PCR) conditions and cycling profile are based on the original protocol established for maize SSR mapping (Sharopova et al. 2002). Briefly, PCR reactions for genotyping were as follows: 1X Bioline PCR Buffer (www.bioline.com; as per manufacturer’s instructions); 2.5 mM MgCl2; 0.4 mM each dNTP (dATP, dCTP, dGTP, dTTP); 50 ng each forward and reverse SSR primer; 0.3 units Bioline TAQ Polymerase; and 50 ng of genomic DNA template. PCR reactions were brought up to a final volume of 15 μl with sterile H2O.

All PCR reactions were performed in a 96-well thin-walled microtiter style plate in a Perkin Elmer thermocycler using the following program: 95°C for 1 min, 65°C for 1 min, 72°C for 1 min for one cycle and then decreased 1°C per cycle, until the annealing temperature is 55°C. The regime is then 95°C for 1 min, 55°C for 1 min, 72°C for 1.5 min, repeated for a total of 30 cycles. After the addition of 5× loading dye (3 μl), 4% Super Fine Resolution agarose (Amresco) gels were loaded with 7.5 μl of each PCR reaction and run with constant voltage of 90-100 V for ~120 min, which achieved the best resolution. Most maize SSR polymorphism band sizes range between 90 and 150 bp and are well resolved using these conditions that were polymorphic in the parental lines for the mapping populations. Chromosomal location, names of the linked markers, and primer sequences for each marker are provided in the supporting information (Table S1).

RESULTS AND DISCUSSION

The silent b1 transgene is an effective phenotype for the identification of mutants defective in transgene silencing

The transgene is stably silent in wild-type backgrounds, resulting in plants without observable anthocyanin pigmentation in most tissues (Figure 1B). Based on nuclear run on assays, DNA methylation, and genetic analysis, the transgene is transcriptionally and epigenetically silenced in wild-type plants (McGinnis et al. 2006). Although prior work had shown that three genetic factors identified as required for paramutation were also required for maintaining transgene silencing (McGinnis et al. 2006), reactivation of BTG-silent had not been used for forward genetic screens. As it is likely that transcriptional silencing is dependent upon many proteins, a forward genetic screen for transgene reactivation was conducted to identify additional proteins required for silencing this locus.

For this screen, nontransgenic pollen was mutagenized with EMS and applied to ears of plants that were homozygous or hemizygous for BTG-silent. Plants representing the M1 and M2 generations were visually screened for evidence of loss of transgene silencing associated with dominant or recessive mutations, respectively (Figure 1C). In the M1 generation, ~3000 plants were screened, and all demonstrated a silent transgene phenotype, revealing no dominant nor semidominant mutations.

Within the ~500 M2 families, a total of 10 were observed to segregate plants with dark pigment, indicative of reactivated transgenes, and these were designated tgr1 through tgr6 and tgr8 through tgr11. In many M2 families, other morphological or chlorophyll pigment phenotypes were observed, consistent with a successful mutation by EMS (Neuffer et al. 1997). In most of the putative tgr mutants, the plants were very dark, consistent with extensive upregulation of the transgene (Figure 1C). The tgr3 mutant was an exception, as it exhibited a unique phenotype consistent with only a modest reactivation of the transgene (Figure 1D).

Chi square (χ²) analysis was used to test the fit of the observed values to the expected values for the segregation of recessive mutations. Nine of the ten families demonstrated segregation consistent with the presence of a single, recessive, mutated allele (Table 1). The tgr9 family segregated significantly fewer individuals than expected for a recessive mutation over multiple generations (Table 2), indicating that this may not be a fully penetrant allele, that there may be reduced transmission of tgr9, or reduced viability of tgr9 homozygotes.

Previous work demonstrated that mutations in two genes that encode maize orthologs of the Arabidopsis RdDM pathway resulted in reactivation of BTG-silent (McGinnis et al. 2006); mop1 that encodes an RNA-dependent RNA polymerase (Alleman et al. 2006), and rnr1 that encodes a protein related to chromatin remodeling proteins (Hale et al. 2007). Maize mutants for the orthologs of every known component of this pathway in Arabidopsis have not yet been identified, and more than one silencing pathway could function at a given locus. Thus, the putative mutants from this screen might represent mutations in uncharacterized genes, new mutations in the maize RNA-directed transcriptional gene silencing pathway, or mutations in genes encoding proteins previously implicated in other gene silencing pathways.

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**Table 1 Segregation of BTG-active plants in M₂ families**

| Family | Hypothesis | No. of BTG-active Plants | No. of BTG-silent Plants | χ² |
|--------|------------|--------------------------|-------------------------|----|
| tgr1   | (1:3)      | 4                        | 8                       | 0.44 |
| tgr2   | (1:3)      | 4                        | 25                      | 1.94 |
| tgr3   | (1:3)      | 6                        | 18                      | 0.00 |
| tgr4   | (1:3)      | 2                        | 8                       | 0.13 |
| tgr5   | (1:3)      | 2                        | 8                       | 0.13 |
| tgr6   | (1:3)      | 5                        | 16                      | 0.02 |
| tgr8   | (1:3)      | 4                        | 7                       | 0.76 |
| tgr9   | (1:3)      | 3                        | 28                      | 3.88* |
| tgr10  | (1:3)      | 3                        | 7                       | 0.43 |
| tgr11  | (1:3)      | 11                       | 19                      | 2.17 |

* The null hypothesis reflects a ratio consistent with the segregation of a recessive mutation in a population that resulted from self-pollination of a heterozygous individual.

* Chi square tests (χ²) were used to estimate the degree of confidence for the hypothesis (P = 0.05) for each mutant family.

* Significant difference.
Two mutants exhibit unique phenotypes and segregation patterns in the M3 generation

Segregating M2 families included BTG-silent and BTG-active plants, identified as green and purple plants, respectively (Figure 1). The BTG-silent individuals were potentially homozygous for the mutation or homozygous for the wild-type allele, while the BTG-active individuals were potentially homozygous for a recessive allele. To test these hypotheses and to analyze the genetic behavior of the newly discovered putative mutants in further generations, BTG-silent, M2 individuals were self-pollinated, and the activity of the transgene was observed in the M3 progeny. These tests were completed for seven of the mutants because tgr4, tgr5, and tgr8 proved difficult to propagate due to reduced seed set and low germination frequencies. With two exceptions discussed below, the segregation ratios observed in the M3 generation were similar to those observed in the M2 generation (Table 2).

For tgr2, a reduced number of green plants and the appearance of a new phenotypic class with sectors (Figure 2) did not fit the simple hypothesis for segregation of a single recessive mutation (Table 2). This sectored phenotype (BTG-sec) is consistent with the transgene only being reactivated in some cell lineages, or with the transgene becoming resilienced in a subset of cells in the presence of an activating mutation. While the presence of the third phenotypic class might suggest tgr2 is semidominant, with heterozygous individuals exhibiting the sectored phenotype and homozygous mutant individuals exhibiting the confluent, darkly pigmented phenotype, the segregation ratio did not match that expectation based on $\chi^2$ analysis, nor the hypothesis that tgr2 is a dominant mutation. Furthermore, the M1 generation that produced tgr2 did not exhibit a BTG-active phenotype, which is inconsistent with the hypothesis that tgr2 is a dominant or semidominant allele. The tgr2 data also did not match the expectation for two independent, recessive mutations (Table 2). This suggests that the tgr2 mutation has unexplained but complex genetic characteristics. Simple genetic analysis of these traits is further complicated by the persistence of the BTG-active phenotype after the inducing mutation has been segregated away. This characteristic of tgr2 is described in more detail below.

In tgr3 families, fewer green plants than expected for a recessive mutation are observed. Similar to tgr2, in tgr3 families, the segregation ratios in the M1 generation do not match a 1:3, 3:1, or 7:9 ratio (Table 2), which would be consistent with recessive, dominant, or two independent mutations, respectively. As discussed for tgr2, this result may be indicative of complex genetic behavior of the tgr3-1 allele.

Based upon their diverse genetic behaviors and ease of propagation, tgr1, tgr2, and tgr3 were selected for further investigation and subjected to more extensive molecular and genetic analysis.

Transgene reactivation is heritable in tgr2 lines

It was previously demonstrated that transgene activity persisted after segregating away the mop1 and rmr2 mutants (McGinnis et al. 2006). This was referred to as heritability of activation, and this activation became increasingly stable after subsequent generations of being maintained in wild-type backgrounds. Heritability of activation was tested in tgr1-, tgr2-, and tgr3- derived lines (Figure 3). For this assay, BTG-active plants in segregating families were crossed with nontransgenic, nonmutant genetic stocks, and transgenic plants were observed for pigmentation. Transgenic progeny from tgr2 parents were all darkly pigmented, indicating that the transcriptionally active state persisted through meiosis and was heritable, and that the transgene was not resilienced in the presence of the wild-type Tgr2 allele. This is the same phenotype previously observed for the mop1 and rmr2 mutants.
In *tgr1*-derived lines, the progeny of outcrossed plants were all green, indicating that silencing was efficiently restored upon introduction of the wild-type allele. The *tgr1* phenotype is similar to that reported for a *rmr1* mutant in previous studies (McGinnis et al. 2006). For lines derived by outcrossing *tgr3* individuals with active transgenes, heritability was observed at a relatively low level compared with *tgr2*-derived lines. Thus, the *tgr* mutants collectively present a full spectrum of epigenetically heritable and nonheritable phenotypes for in depth studies on transmission of epigenetic information from one generation to the next.

**DNA methylation in *tgr1* and *tgr2* mutants correlates with transgene reactivation**

Consistent with their involvement in the RdDM pathway, loss of Mop1, Rmr1, and Rmr2 resulted in reactivation of the BTG-silent correlated with a reduction in cytosine methylation of the 35S CaMV

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**Figure 3** Crossing strategy used for testing the heritability of transgene expression in *tgr1*, *tgr2*, and *tgr3*. Plants with active transgenes were outcrossed for one generation with nontransgenic, nonmutant plants to observe if the transcriptional activity would persist through meiosis and the reintroduction of wild-type proteins in the next generation. In the crosses the female parent is listed first and the male parent listed second. Reciprocal crosses varying whether the transgene was transmitted through male or female were done to compare heritability through both parents.

**Figure 4** Methylation of the 35S CaMV promoter in three *tgr* mutants. Bisulfite sequencing was used to determine whether there were differences in CG, CHG, and CHH methylation of the 35S promoter in *tgr* BTG-active (tgr BTG-a) plants for *tgr1*, *tgr2*, and *tgr3* relative to control siblings carrying BTG-silent (BTG-s). The *tgr2* mutation includes an additional sectored phenotype (Figure 2) denoted as *tgr2* BTG-sec. The level of methylation is reported as the percentage of total cytosines in the 35S CaMV promoter exhibiting methylation. The number of clones for each genotype is indicated in parentheses, the analyzed region represents a total of 60 cytosines.
promoter within the transgene (McGinnis et al. 2006). To determine whether transgene reactivation in the newly discovered mutants correlated with changes in DNA methylation, cytosine methylation of the 35S CaMV promoter in the transgene was determined using sequence analysis of PCR products from bisulfite converted genomic DNA.

The adh1 intron was used to specifically amplify the 35S CaMV promoter directly driving bt expression (Figure 1A) and not the 35S promoter associated with the bar transgene that was used for selection when it was cotransformed (McGinnis et al. 2006). In nonmutant BTG-silent plants (six clones), 76.39% (CG, 88.33%; CHG, 80.55%; CHH, 73.10%) of the cytosines were methylated within the analyzed portion of the 35S CaMV promoter, which included 220 base pairs and a total of 60 cytosine residues. Approximately 410 base pairs of the immediately adjacent adh1 intron of the transgene was also analyzed; this region was consistently unmethylated in converted DNA extracted from BTG-active and BTG-silent plants (data not shown).

Methylation within the 35S CaMV promoter region in BTG-active plants from segregating tgr1, tgr2, and tgr3 families were compared with BTG-silent plants within the same families. In combination, these three mutants allowed for analysis of plants demonstrating a range of pigmentation, phenotypic heritability, and inheritance patterns.

In tgr1 and tgr2 mutants, the BTG-active plants were hypomethylated when compared with their BTG-silent siblings in all methylation contexts (Figure 4), which suggests that Tgr1 and Tgr2 may function in a gene silencing pathway that is associated with cytosine methylation in the promoters of regulated genes. This is consistent with observations of mop1, rmr1, and rmr2 mutants (McGinnis et al. 2006). Extensive hypomethylation was also observed in tgr2 BTG-active individuals, although some methylation (<10%) is apparent in these plants. In contrast, promoter methylation levels in BTG-silent and BTG-active appear to be similar in tgr3 families. In tgr3 mutants, the low level of BTG activation could mean that there is a slight up-regulation of transcripational activity in the presence of cytosine methylation, but it is also possible there is a very modest change in methylation that is below the sensitivity level for the detection technique. Further investigation into this relationship may yield additional insight into the correlation between DNA methylation and transcripational activity.

### Table 3 Tgr1 is linked to Rmr6

| Linked Gene | SSR Locus Name | Number of Individuals | Number of Individuals | Number of Individuals |
|-------------|----------------|-----------------------|-----------------------|-----------------------|
|             |                | Homozygous for Tgr    | Heterozygous          | Homozygous for B73    |
|             |                | Parental Allele       | Individuals           | Parental Allele       |
| Mop1 (chromosome 2) | UMC1465 | 4 | 10 | 20 |
| Mop2 (chromosome 2) | UMC2403 | 6 | 19 | 8 |
| Rmr1 (chromosome 6) | UMC2320 | 7 | 18 | 8 |
| Rmr6 (chromosome 1) | BNLG1025 | 26 | 12 | 0 |

### tgr1 is linked to rmr6

The loss of epigenetic gene silencing characteristic of the mutants identified in this screen is similar to the phenotypes used to identify mop1, mop2, rmr1, and rmr6 mutants (reviewed by Arteaga-Vazquez and Chandler 2010). This phenotypic similarity may be an indication that the tgr mutants represent alleles of these cloned genes. Similar to the phenotypes previously reported for rmr1 mutants (McGinnis et al. 2006), tgr1 exhibited DNA hypomethylation, but a lack of meiotic heritability of transcriptional reactivation of BTG. The persistence of transgene activity into the next generation after outcrossing as demonstrated by tgr2-1, rmr2-1, and mop1-1 confounds allelism analysis by complementation analysis for some mutants, so a molecular approach was used to examine linkage of the tgr mutants with genes with previously sequenced mutations, including Mop1, Mop2, Rmr1, and Rmr6. Cloning and sequencing of the rmr2-1 mutation has not been published.

To test for linkage of tgr1 with sequenced mutations, a total of 36 tgr1 BTG-active individuals were analyzed with SSR markers tightly linked to cloned genes associated with epigenetic gene regulation in maize, mop1, mop2, rmr1, and rmr6. For three genes no linkage was identified (Table 3). For an Rmr6-linked marker, the tgr1 parental allele was overrepresented, suggesting that the gene bearing the tgr1 mutation lies on chromosome 1, and is linked to rmr6. The presence of heterozygous individuals in this small population suggests that tgr1 is not an allele of rmr6. Several developmental abnormalities have been described for rmr6-1 and rmr6-2 alleles, including abnormal leaf polarity and male inflorescence development (Parkinson et al. 2007), none of which have been observed in segregating tgr1 families. Further mapping and complementation tests will be required to determine the molecular identity of tgr1.

There is also some evidence of an overrepresentation of the B73 allele of a chromosome 2 localized marker in this population. Segregation distortion has been reported in other maize mapping populations, and the effect loci seem to vary in a population-dependent manner (Sharopova et al. 2002). The presence of a higher number of individuals with the B73 allele than the tgr parent allele for this marker may either be caused by observation of segregation in a relatively small population or reflect an example of segregation distortion in the population.

### Table 4 Tgr2 is not linked to previously cloned components of the maize RdDM pathway

| Linked Gene | SSR Locus Name | Number of Individuals | Number of Individuals | Number of Individuals |
|-------------|----------------|-----------------------|-----------------------|-----------------------|
|             |                | Homozygous for Tgr    | Heterozygous          | Homozygous for B73    |
|             |                | Parental Allele       | Individuals           | Parental Allele       |
| Mop1 (chromosome 2) | UMC1465 | 6 | 13 | 18 |
| Mop2 (chromosome 2) | UMC2403 | 6 | 13 | 20 |
| Rmr1 (chromosome 6) | UMC2320 | 9 | 27 | 4 |
| Rmr6 (chromosome 1) | UMC1035 | 7 | 17 | 15 |
tgr2 is not linked to cloned mutations with similar phenotypes

In addition to the characteristic loss of epigenetic silencing phenotype, tgr2 exhibited DNA hypomethylation and heritability of transgene reactivation, which are phenotypes that have been previously reported for mop1 and rmr2 mutants (McGinnis et al. 2006). Tgr2 linkage was analyzed in a similar manner to that described for tgr1 to determine if these common phenotypes were an indication of allelism between tgr2 and previously sequenced mutations. To test linkage of the tgr2 BTG-active phenotype with the candidate genes, 38 individuals were analyzed. For each analyzed gene, populations of purple plants exhibiting the tgr2-related BTG-active phenotype were not genotypically biased toward the tgr2 parent allele for that locus (Table 4), suggesting a lack of linkage between the phenotype and the genetic locus being tested. This suggests that although tgr2 shares many phenotypes with previously characterized mutations in these genes, it is unlikely to represent an allele of one of these genes. For some markers, the B73 allele was detected in more individuals than the tgr2 parental allele, indicating an unusual segregation pattern and potential segregation distortion.

tgr3 is not linked to mop1, mop2, rmr1, or rmr6

While more subtle than that observed for other mutants (Figure 1D), the tgr3 phenotype is indicative of a reduction in the epigenetic silencing at BTG. While the BTG-a phenotype is notably distinct from that observed for other mutants, loss of silencing is consistent with the phenotype of mop1, rmr1, and rmr2 mutants. Thus, linkage was tested for tgr3 as described for tgr1 and tgr2 (Table 5). In tgr3 BTG-active plants, the parental mutant alleles were not overrepresented and the B73 parental alleles were not underrepresented, demonstrating that tgr3 is not an allele of mop1, mop2, rmr1, or rmr6. The B73 allele appeared to be overrepresented for a marker on chromosome 1; segregation distortion toward the B73 allele was reported for several markers in this chromosomal region in an intermated B73 / Mo17 population (Sharopova et al. 2002).

CONCLUSIONS

These results demonstrate that reactivation of the silent 35SSBTG transgene in maize is an effective epigenetic phenotype for use in a forward genetic screen. The application of this screen led to the identification of multiple mutants, which exhibit some distinct phenotypes relative to one another and to previously identified maize mutants that can reactivate the silent transgene. Mapping studies suggest that tgr2 and tgr3 do not represent alleles of previously cloned genes with similar phenotypes in maize, while tgr1 resides on chromosome 1 and is linked to rmr6. Further, tgr2- and tgr3-derived lines exhibited persistent transgene reactivation after the reactivating mutation had been segregated away, meaning that BTG-silent provides a useful platform for studying heritable changes in gene expression in plants. Additional study of these mutants should yield further insight into epigenetic gene regulation.

Table 5 Tgr3 is not linked to previously cloned components of the maize RdDM pathway

| Linked Gene       | SSR Locus Name | Number of Individuals Homozygous for Tgr Parental Allele | Number of Heterozygous Individuals | Number of Individuals Homozygous for B73 Parental Allele |
|-------------------|----------------|----------------------------------------------------------|-----------------------------------|----------------------------------------------------------|
| Mop1 (chromosome 2) | UMC1465        | 7                                                        | 14                                | 18                                                        |
| Mop2 (chromosome 2) | UMC1823        | 6                                                        | 21                                | 12                                                        |
| Rmr1 (chromosome 6) | UMC2320        | 10                                                      | 14                                | 8                                                         |
| Rmr6 (chromosome 1) | UMC2560        | 3                                                        | 13                                | 20                                                        |

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