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Suppression of Transgene Silencing by Matrix Attachment Regions in Maize: A Dual Role for the Maize 5′ *ADH1* Matrix Attachment Region

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Matrix attachment regions (MARs) are DNA sequences that bind an internal nuclear network of nonhistone proteins called the nuclear matrix. Thus, they may define discrete gene-containing chromatin loops in vivo. We have studied the effects of flanking transgenes with MARs on transgene expression levels in maize callus and in transformed maize plants. Three MAR elements, two from maize (*Adh1* 5′ MAR and *Mha1* 5′ MAR) and one from yeast (ARS1), had very different effects on transgene expression that bore no relation to their affinity for the nuclear matrix in vitro. In callus, two of the MAR elements (*Adh1* 5′ MAR and ARS1) reduced transgene silencing but had no effect on the variability of expression. In transgenic plants, *Adh1* 5′ MAR had the effect of localizing β-glucuronidase expression to lateral root initiation sites. A possible model accounting for the function of *Adh1* 5′ MAR is discussed.

INTRODUCTION

Gene expression is influenced not only by the presence or absence of nearby transcription factors but also by the structure of surrounding chromatin. When chromatin competes with the transcriptional machinery, stochastic expression or epigenetic effects may result. To understand fully the regulation of gene expression inside the nucleus, it is necessary to consider the different levels of structural organization of DNA in chromatin and the factors involved in establishing, maintaining, and modifying these structures.

Three levels of chromatin compaction (the 11-, 30-, and 300-nm fibers, respectively) are thought to be required for packaging the genome inside the nucleus. The third level of compaction results from the folding of the 30-nm fiber into loops of various sizes attached to the nuclear matrix. In addition, dense masses of heterochromatin can be distinguished cytologically from euchromatin in interphase nuclei. Variable repression of transcription often is associated with heterochromatin and frequently manifests itself as position effect variegation (reviewed by Wakimoto, 1998; Wallrath, 1998). In plants, variable expression may be observed among independent transformation events expressing the same transgene from different positions within the genome.

Despite the fact that little is known about both the structure and the protein composition of heterochromatin in plants, gene-silencing phenomena often are attributed to heterochromatin effects (Avramova, 2002). The genomes of many commercially important crop plants contain large amounts of repetitive DNA. In maize, ~80% of the genome is composed of highly repetitive DNA that is organized either as morphologically visible knobs (Peacock et al., 1981; Ananiev et al., 1998; Fransz et al., 2000) or as large, densely methylated blocks in the intergenic space (SanMiguel et al., 1996).

In maize, genes may be separated from one another by highly repetitive DNA stretches of up to 80 kb (Avramova et al., 1996; Tikhonov et al., 1999). In closely related grass genomes, orthologous genes with high sequence homology are separated by intergenic regions that differ in sequence from those found in maize (Chen et al., 1997; Tikhonov et al., 1999). Given the importance of the genomic context for the function of a gene, these findings suggest an apparent paradox: orthologous genes in related species function within very different chromosomal settings.

One model accounting for this apparent paradox proposes that genes and blocks of repetitive DNAs might exist in different, structurally separated nuclear compartments (Lamond and Earnshaw, 1998; Cockell and Gasser, 1999) and that mislocation alters expression. Therefore, genes

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may have "anchors" that position them in the spatial architecture of the nucleus (Flavell, 1994). This anchoring function may be mediated by matrix-attachment regions (MARs). MARs are defined operationally as DNA sequences that bind preferentially to the proteins of the nuclear matrix. In eukaryotic genomes, MARs typically are localized at the borders of gene domains, implicating them in the formation of individual loops of higher order chromatin structure. In plants, these putative gene loops are relatively small (3 to 10 kb) (Breyne et al., 1992; van der Geest et al., 1994; Chinn et al., 1996; van Drunen et al., 1997; Avramova et al., 1998).

Previously, we reported that the intergenic stretches of repetitive DNA in the maize *Adh1* region are segregated into topologically sequestered units and that the microcolinearity of gene composition between grass species is mirrored by the similar placement of orthologous genes between MARs (Avramova et al., 1995, 1998). Thus, the positioning of orthologous genes within their respective structural domains appears to be conserved in evolution (Avramova et al., 1998; Tikhonov et al., 2000). This is consistent with the ideas that endogenous genes within loops are in structural domains that separate them from neighboring nongenic or genic sequences and that transgene position may influence the availability of specific factors that affect transgene expression. Flanking transgenes with MARs could decrease the probability of silencing, reduce the variability of expression, or both.

Plant MARs have been implicated in a variety of gene expression phenomena (Breyne et al., 1992; Allen et al., 1993; Schöffl et al., 1993; Mlynarova et al., 1994, 1996; van der Geest et al., 1994; Iglesias et al., 1997; Matzke and Matzke, 1998; Ülker et al., 1999; Vain et al., 1999), suggesting that MARs are *cis*-regulatory elements. In other transgenic studies, MARs have failed to play a role as gene effectors (L. Sidorenko, W. Bruce, S. Maddock, and T. Peterson, unpublished data). This indicates that the role of MARs may be more elusive and complicated than initially expected. In particular, the effects of flanking a transgene with MARs appear to be highly variable in plants. One reason could be that in many cases, the samples studied have been too small to describe in full the expression level distributions among independent transformants. We suspected that a systematic study with larger samples might help elucidate the underlying basis of this reported variability.

In this study, we used maize cell cultures that could be transformed at high frequency to generate sufficient numbers of independent transformants to allow us to examine the underlying gene expression distributions without bias. Other factors that have complicated studies involving MARs are variability in transgene copy number and homology-dependent silencing effects observed in transformants with high transgene copy numbers. Here, transformations were performed under conditions in which predominantly low-copy inserts were introduced, minimizing the impact of these factors.

The effects on transgene expression of a heterologous MAR from yeast and of two endogenous maize MARs were studied. When enough transformants were analyzed, the distributions of transgene expression levels were found to be bimodal in every case, which complicated statistical analysis. We found that two of the MARs reduced the probability of transgene silencing, without affecting the variability, whereas the third had no effect on transgene expression. There was no relationship between the strength of binding to the nuclear matrix in vitro and the effects on transgene expression in vivo. Finally, the effect of the maize *Adh1* 5′ MAR on transgene expression was examined in regenerated maize plants. The results suggested that this MAR may display both activating and repressing roles, functionally relating it to other recognized dual-function elements such as two immunoglobulin MARs (κ and μ) and the polycomb response elements described in animal studies (reviewed by Lyko and Paro, 1999).

RESULTS

Affinity of MARs for the Maize Nuclear Matrix in Vitro

Three MARs were used in this study: the yeast autonomously replicating sequence *ARS1*, which binds to the yeast nuclear matrix (Amati and Gasser, 1988) and affects transgene expression in tobacco (Allen et al., 1993), and two MARs associated with the 5′ regions of two maize genes, *Adh1* and *Mha1*, which encode alcohol dehydrogenase and a H^+-ATPase, respectively.

The relative affinity of each MAR for the maize nuclear matrix was tested by in vitro binding assay. As shown in Figure 1, the MAR located at the 5′ end of *Mha1* displayed the highest affinity under the assay conditions, and the yeast ARS1 sequence bound less efficiently than either of the maize MARs (Figures 1A and 1B). In the presence of a large excess of *Escherichia coli* competitor DNA (150 μg/mL), ~10 times more *Mha1* 5′ MAR remained bound compared with *Adh1* 5′ MAR (Figure 1B).

Inhibition of *Adh1* 5′ MAR binding by the addition of cold *Mha1* 5′ MAR and vice versa (Figure 1C) confirmed the relative matrix affinity for each MAR: 50 ng/mL cold *Mha1* 5′ MAR completely abolished *Adh1* 5′ MAR binding, whereas 50 ng/mL cold *Adh1* 5′ MAR left ~9% of labeled *Mha1* 5′ MAR bound to the matrices (Figure 1C). This finding indicates that the highest affinity for the matrix is displayed by *Mha1* 5′ MAR. Because *Adh1* and *Mha1* 5′ MARs can compete with each other, some of the components required for the matrix binding of *Adh1* 5′ MAR also must be involved in binding to *Mha1* 5′ MAR.

MAR Elements Do Not Affect Expression before Integration in Black Mexican Sweet Maize Cells

Before investigating the effects of MARs on transgene expression, we assayed the transient expression of a lu-

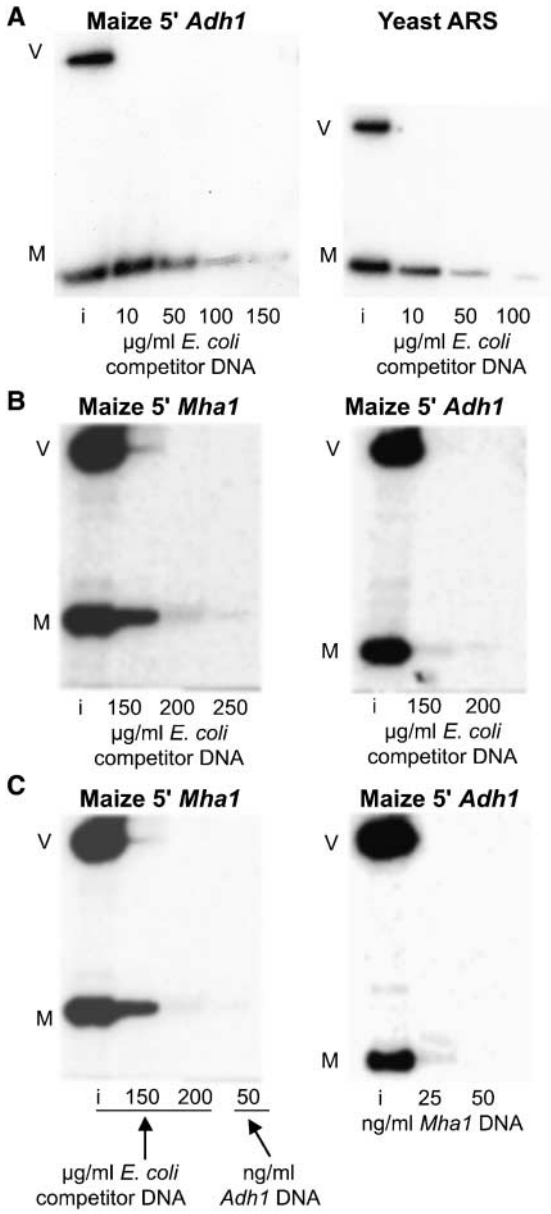

Figure 1. Binding Assay Results for the Three MARs Used in this Study.

Vector and MAR fragments are labeled v and M, respectively. Labeled input DNA, without nuclear matrices, is shown in lane i. Adjacent lanes show the DNA fragments recovered from the matrix fraction in the presence of various concentrations of competitor DNA.

(A) Yeast ARS1 sequences (right) bound maize matrices with a lower affinity than the maize *Adh1* 5′ MAR (left). In the presence of a 1000-fold molar excess of competitor DNA (100 μg/mL), 3% of yeast ARS1 and 10% of *Adh1* 5′ MAR remained matrix bound.

(B) *Mha1* 5′ MAR (left) displayed the highest affinity for binding to the matrix. In the presence of a 1500-fold molar excess of competitor (150 μg/mL), 30% of *Mha1* 5′ MAR remained bound, whereas under the same conditions, only 3.5% of *Adh1* 5′ MAR (right) remained bound.

ciferase (*LUC*) reporter gene in Black Mexican Sweet (BMS) maize suspension cells. Because MARs often colocalize with gene regulatory elements (Gasser and Laemmli, 1987; Bonifer et al., 1994; Phi-Van and Strätling, 1996), it was important to separate effects caused by enhancers/silencers (eventually contained in the transformation constructs) from effects resulting from MAR activity. Effects on transgene expression displayed only after its stable integration into the genome might reflect a MAR role in affecting the chromatin structure at the integration site.

The plasmid constructs used in the study are shown in Figure 2A. Each *LUC* construct was introduced into BMS cells in combination with a fixed level of a 35S::β-glucuronidase (*GUS*) plasmid (PHP264), and *LUC* expression was normalized to *GUS* expression. The expression levels of constructs in which 35S::*LUC* was flanked by each of the three MARs against a control vector lacking MARs (PHP1528) were compared.

None of the three MAR elements tested had any significant effect on the expression level of *LUC* compared with the control vector without MARs (Figure 2B). Thus, none of the tested MAR-containing fragments appeared to carry significant enhancer/silencer activity when expressed transiently.

Expression Levels of Stably Integrated Transgenes in BMS

To assay the effects of each MAR on the expression of stably integrated transgenes, we used the *BAR* gene as a selectable marker to obtain a population of independently transformed BMS calli. 35S::*BAR* and 35S::*LUC* genes were introduced in *trans* on separate constructs. The transformation procedure generating independent transgenic lines has been used routinely (Grotewold et al., 1998; Bruce et al., 2000). In the present study, transformation under these conditions generated stably transformed lines representing individual insertion events. DNA gel blot hybridization analysis of a random sample of stably transformed lines displayed unique integration patterns for each event with more than one inserted copy (Figures 3A and 3B).

Regulating the DNA dose, low-copy-number (lanes 1 to 6) or multiple-copy-number (lanes 7 to 12) transgene insertions

(C) Binding of *Mha1* 5′ MAR in the presence of different concentrations of *E. coli* DNA or 50 ng/mL unlabeled *Adh1* 5′ MAR (left). Nine percent of *Mha1* 5′ MAR remained bound to the matrix in the presence of 50 ng/mL unlabeled *Adh1* 5′ MAR. When the binding of labeled *Adh1* 5′ MAR was challenged with different concentrations of unlabeled *Mha1* 5′ MAR (right), 25 ng/mL of the specific competitor left only 3% of *Adh1* 5′ MAR bound to the matrix, and 50 ng/mL unlabeled *Mha1* 5′ MAR completely abolished the matrix binding of *Adh1* 5′ MAR.

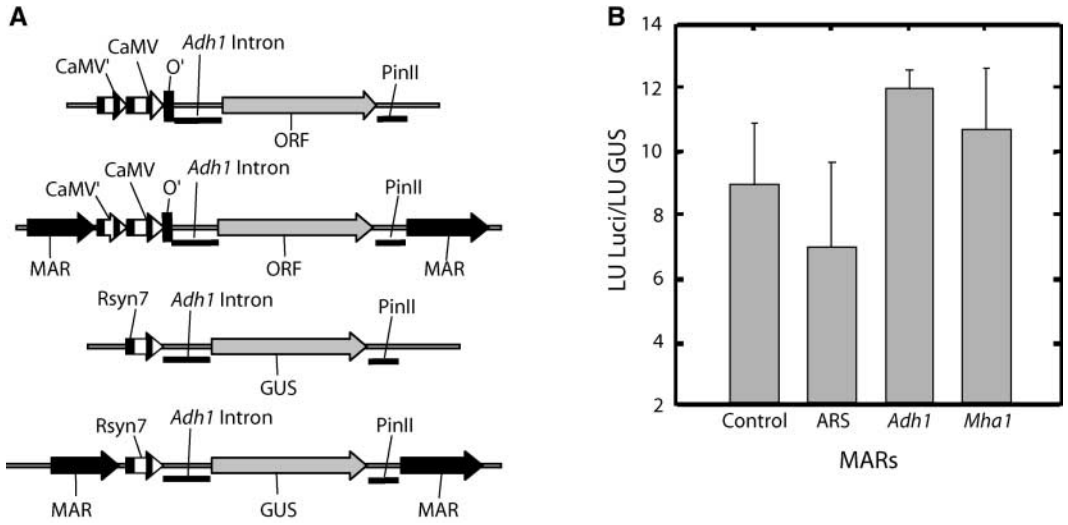

Figure 2. Vectors Used in the Transformation and Transient Assay Studies.

(A) The vectors used for BMS transformations. In the first construct, for PHP264, the open reading frame (ORF) equals *GUS*; for PHP1528, ORF equals *LUC*; and for PHP3528, ORF equals BAR. In the second construct, for PHP5438, MAR equals ARS1 and ORF equals *LUC*; for PHP5456, MAR equals ARS1 and ORF equals BAR; for PHP6248, MAR equals *Adh1* 5′ and ORF equals *LUC*; for PHP6344, MAR equals *Adh1* 5′ and ORF equals BAR; for PHP6486, MAR equals *Mha1* 5′ and ORF equals *LUC*; and for PHP6487, MAR equals *Mha1* 5′ and ORF equals BAR. The third construct is PHP6086 containing the *Rsyn7* promoter. The fourth construct is PHP7917 containing the *Rsyn7* promoter and the *Adh1* 5′ MAR elements.

(B) Effects of MARs on the transient expression of 35S::*LUC*. Five replicas for each treatment were assayed for *LUC* and *GUS* expression. The 35S::*LUC* vectors PHP1528, PHP5483 (ARS1), PHP6248 (*Adh1*), and PHP6486 (*Mha1*) were mixed 5:1 with the 35S::*GUS* vector PHP264. Relative levels of gene expression were calculated by normalizing *LUC* to the level of *GUS* expression. An *F* test indicated no significant difference between means ($P = 0.21$). LU, light units.

could be achieved (Figure 3B). These experiments were performed under previously established conditions known to generate independently transformed lines with low-copy-number insertions (see Methods). In the control treatments, neither the reporter nor the selectable marker contained MARs, whereas in the test treatments, both the reporter and the selectable marker were flanked with one of the three MARs. *LUC* expression levels in BMS calli transformed with each of the four vector combinations are displayed in Figure 4A. In each case, >50 *LUC*-expressing events were analyzed per treatment. Reproducibly, the transgene expression levels were distributed bimodally. With smaller sample sizes (20 to 30), the expression level distributions were not significantly different from log normal (see Discussion).

Adh1 5′ MAR and ARS1 Reduce Transgene Silencing in BMS

Because *LUC* expression levels were not distributed normally among transformants, even after log transformation, the data could not be analyzed by analysis of variance. Instead, we used a nonparametric approach that compares cumulative distributions.

Although differences in the *LUC* expression level distributions between the various MAR elements and the control can be seen by visual inspection, the histograms shown in Figure 4A include data only from *LUC*-expressing events. By contrast, the cumulative distribution function (see Methods) effectively summarizes the data from both expressing and nonexpressing events. This allows quick determination of the percentile expressing at a certain level. However, instead of plotting against expression level on the *x* axis, we plotted against the quantile so that we could make comparisons across treatments. The cumulative distribution function graphs (Figure 4B) reveal that flanking *LUC* with either *Adh1* 5′ MAR or ARS1 increased the proportion of events that express the *LUC* transgene, whereas flanking *LUC* with *Mha1* 5′ MAR had no effect on the expression level distribution.

To examine these effects further, the *LUC*-expressing events were subdivided into high and low expressors by selecting a threshold between the two peaks of each bimodal distribution shown in Figure 4A. A single expression value [4 ln(light units/μg protein)] was chosen arbitrarily and used as the cutoff between high- and low-expressing events. The same cutoff value was applied consistently for all treatments and for all data sets. The graph in Figure 5 clearly indicates

that flanking the reporter with ARS1 or *Adh1* 5′ MAR increased the frequency of higher expressing cells. By contrast, flanking the reporter with *Mha1* 5′ MAR had no detectable effect.

ARS1 increased the number of high-expressing events primarily at the expense of nonexpressing events, whereas *Adh1* 5′ MAR nearly tripled the number of high-expressing events at the expense of both nonexpressing and low-expressing events (Figure 5). Both ARS1 and *Adh1* 5′ MAR decreased the number of nonexpressors approximately two-fold. This finding indicates that these two MARs increased average expression by converting a population of silent cells into cells expressing the reporter gene (i.e., they reduced transgene silencing).

MARs May Increase Transgene Expression Levels but Do Not Influence the Variability of Expression

The net result of reducing transgene silencing is an increase in the average expression of *LUC* (Table 1). When the *LUC* transgene was flanked with ARS1, *Adh1* 5′ MAR, or *Mha1* 5′ MAR, the average level of *LUC* expression was increased 5.8-, 26.8-, or 1.5-fold, respectively. Although *Mha1* 5′ MAR had a greater affinity for the nuclear matrix than *Adh1* 5′ MAR, its effect on transgene expression was significantly lower than that of *Adh1* 5′ MAR. In addition, yeast ARS1, the MAR with the lowest binding affinity for the maize nuclear matrix proteins in vitro, increased the average *LUC* expression levels more than *Mha1* 5′ MAR. Thus, the affinity for the matrix per se cannot serve as a reliable indicator for predicting the effects of MARs on transgene expression in vivo.

The increase in average *LUC* expression associated with ARS1 and *Adh1* 5′ MAR resulted from a significant increase in the number of high expressors (Table 1, Figure 5). However, neither MAR affected significantly the range of expression seen among the transformants (Table 1), indicating that the presence of MARs flanking the transgene did not decrease the variability of expression. Thus, neither MAR influenced the level of variation of transgene expression to any measurable extent.

Effects of Maize *Adh1* 5′ MAR on *GUS* Expression in Transgenic Maize Plants

The effect of *Adh1* 5′ MAR was explored further in regenerated transgenic maize plants using the *GUS* gene as a reporter. The reporter gene was cloned in a vector under the control of a synthetic promoter, *Rsyn7* (see Methods), flanked by *Adh1* 5′ MAR (Figure 2) and introduced with *Ubi*::*PAT*, also flanked by *Adh1* 5′ MAR. The control constructs did not have MARs flanking *Rsyn7*::*GUS* or *Ubi*::*PAT*. Mature T1 plants grown in the greenhouse were analyzed for *GUS* expression enzymatically and by histochemical staining.

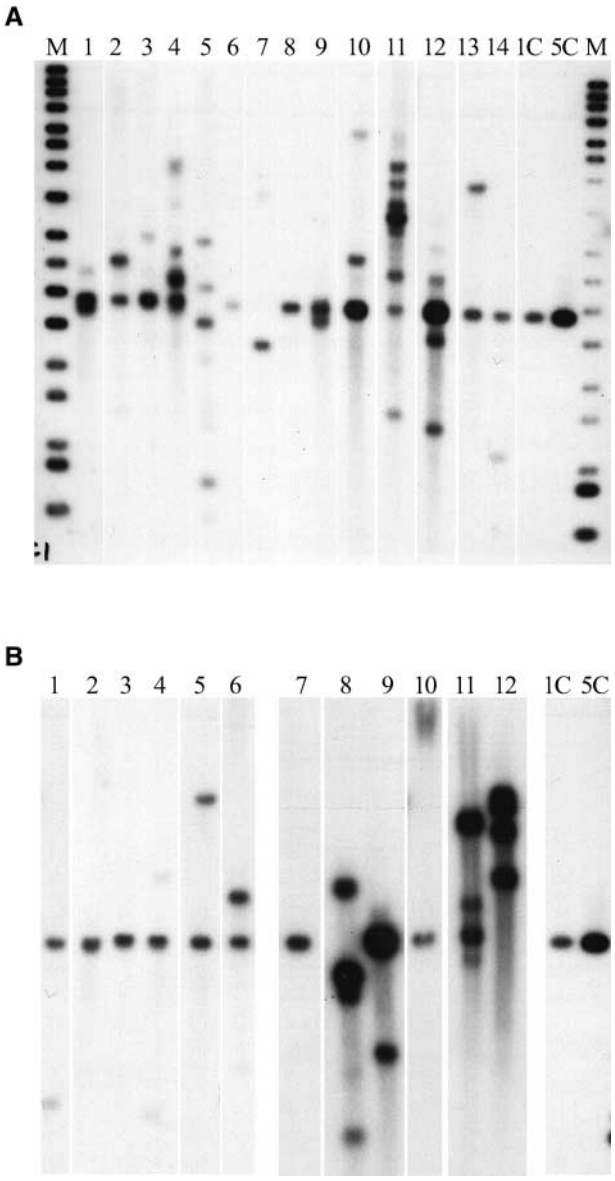

Figure 3. DNA Gel Blot Analysis and Copy-Number Frequency Distribution for BMS Transgenic Lines.

(A) A random sample of stably transformed lines representing independent transformation events. The lines shown were from two filters from the same bombardment experiment. Lanes marked M contained molecular mass marker fragments. Lanes 1C and 5C show hybridization signals from one and five transgene copies, respectively.

(B) Copy numbers of random samples of BASTA-resistant BMS calli transformed with low-dose DNA. Lines with predominantly one or two copy numbers per genome are shown in lanes 1 to 6. High-dose DNA resulted in multiple insertions, which are shown in lanes 7 to 12. Lanes 1C and 5C are as described in **(A)**.

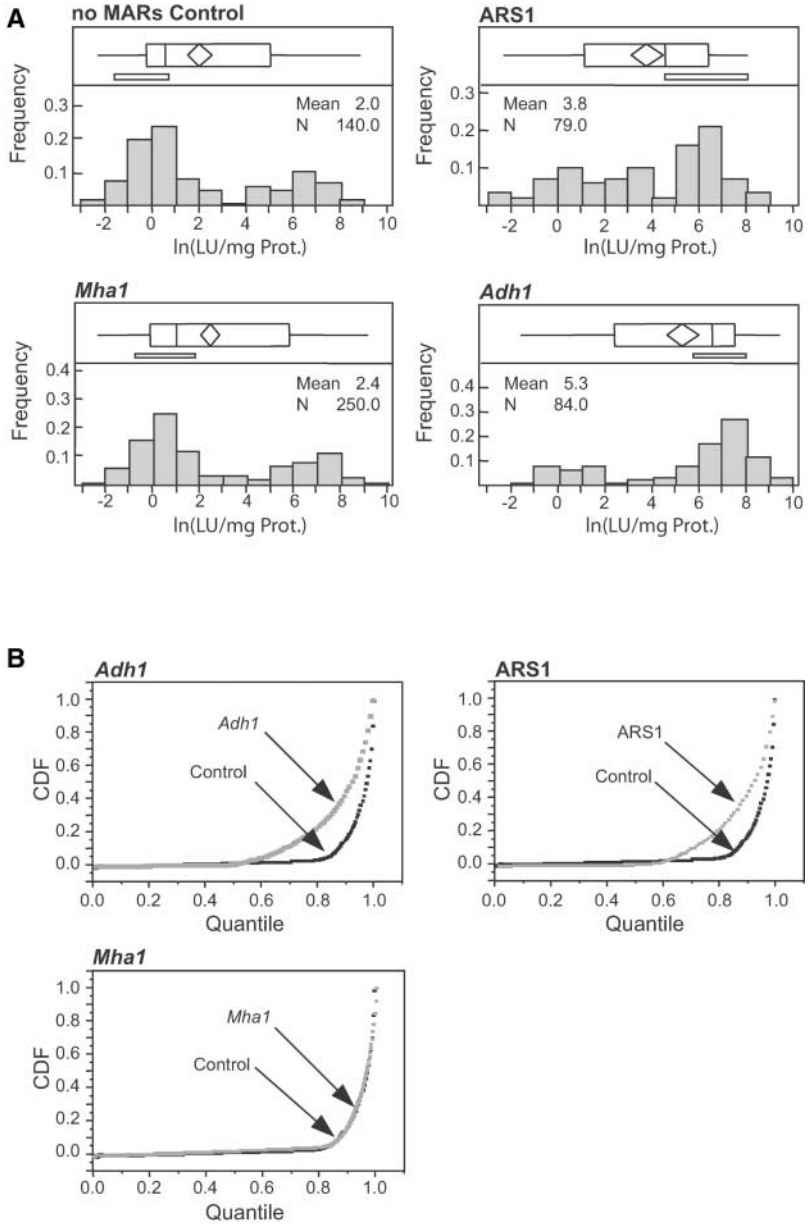

Figure 4. *LUC* Expression in BMS Transformants.

(A) Transgene expression level distributions are shown in the histograms. The *y* axis corresponds to *LUC* expression on a logarithmic scale [ln(LU/μg protein)], where LU indicates light units. Above each histogram is an outlier box plot. The box represents the interquartile range, or the difference between the 25th and 75th percentiles. The "whiskers" (the horizontal lines outside of the box) represent the range (computed as 150% of the interquartile range). The vertical line inside the box represents the median, and the diamond represents the mean. The bracket underneath the box identifies the most dense 50% of all observations. All distributions were bimodal, and the shape of the distributions is similar for no-MARs and *Mha1*, indicating that *Mha1* had no effect on expression. ARS1 and *Adh1* both increased the expression level and shifted the majority of expressing events to the higher expressing peak of the bimodal distribution.

(B) Cumulative distribution function (CDF) graphs. *Adh1* 5′ MAR and ARS1 both have distributions significantly different from that of the no-MAR control. *Mha1* 5′ MAR has the same distribution as the no-MAR control.

Eight *Adh1* 5′ MAR transformants and eight non-MAR control transformants were analyzed. In seven of eight test plants transformed independently with *Rsyn7*::*GUS* flanked by *Adh1* 5′ MAR, *GUS* expression in all tissues was lower than that in control plants transformed with *Rsyn7*::*GUS* without MARs. In root tissue of constructs without MAR, the relative activity (*GUS* assay units normalized to total soluble protein) ranged from 1223 to 2,086,225, with an average of 329,561 units. For *Adh1 5′* MAR transformants, the respective units were 339 to 7237, with an average of 3789. The probability for the means of the non-MAR controls and *Adh1* 5′ MAR transformants by *t* test was $P > 0.05$. This finding indicates that *GUS* activity in the two types of transformants was significantly different, being much lower in plants expressing *GUS* from the construct flanked by the MARs.

Histochemical analysis of transformed roots provided an even more striking picture. *Rsyn7* functions as a constitutive promoter (B.A. Bowen, W.B. Bruce, G. Lu, L.E. Sims, and L.A. Tagliani [2000], U.S. patent 6,072,050). Accordingly, most of the *GUS* staining in control plants was observed throughout the plant, including all cell types in the roots (Figure 6A). By contrast, plants transformed with *Rsyn7*::*GUS* flanked by *Adh1* 5′ MAR exhibited *GUS* expression only in patches of epidermal and cortical cells localized at lateral root-emerging sites (Figures 6B and 6C). The restriction of *GUS* expression to small regions of root cells would explain the lower level of overall *GUS* expression activity established above using the biochemical approach.

The timing of expression coincided with the earliest stages of emergence of the lateral roots and was restricted exclusively to cells in the parent root. No significant expression was observed in the meristems of growing lateral roots. Low levels of *GUS* expression also were seen in ear tissue, confined primarily to the inner and outer glumes (data not shown).

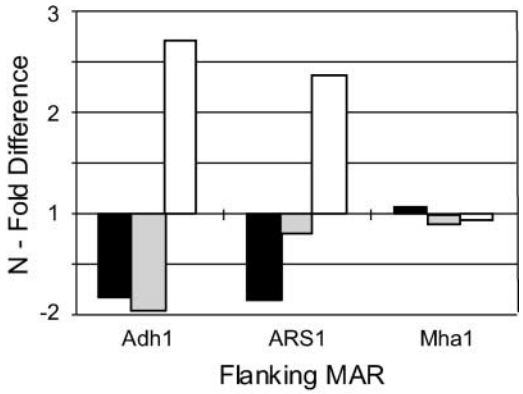

Figure 5. N-fold difference graph of *LUC* expression in BMS.

BASTA-resistant transformants were classified as nonexpressors (black bars), low expressors (gray bars), or high expressors (white bars), as described in Results. A single expression value [4 ln(LU/μg protein)] was used as the cutoff for all data sets. Values indicate the *n*-fold difference from the control without MARs in numbers of transformants in each category. The main effect of ARS1 and *Adh1* MARs was to increase the number of high-expressing events at the expense of nonexpressors and low expressors. *Mha1* had no effect on expression levels.

DISCUSSION

DNA Elements Involved in Chromatin-Dependent Gene Regulation

The complex and inconsistent behavior of MARs on transgene expression suggests that our understanding of the nature of MAR effects must be incomplete. In addition to enhancers and promoters, various other classes of *cis*-DNA elements may affect a nearby gene's expression by influencing how a given chromosomal environment is established, maintained, and modified. Such DNA sequences may direct nuclear sublocalization, may nucleate or propagate specific forms of higher order chromatin structures, and may define the boundaries of "open" and "closed" domains. Presumably, a single segment of DNA containing all of these elements would be unaffected by chromosomal position, shield a promoter from nearby regulatory elements, and promote copy number–dependent gene expression.

Some reports have suggested elements that could mediate any or all of these effects in any context. These include special chromatin structures (*scs* and *scs′*), insulators, locus control regions, polycomb response elements, and MARs (reviewed by West et al., 2002). Other studies have indicated that the functions of these elements are elusive, more complicated than initially expected, and sometimes even antagonistic (Cai and Levine, 1995; Avramova and Tikhonov, 1999).

Because the majority of transgenic events involve insertions into uncharacterized chromosomal locations, the role of the transgenic DNA must be evaluated carefully. The modular structure and frequently overlapping *cis*-elements of promoter regions suggest that the effects observed (or not observed) at their native locations are a result of their combined influences (Nabirochkin et al., 1998). Evidently, MARs may exhibit interactions with neighboring sequences. MARs collaborate with enhancers for the transcriptional activation of immunoglobulin and β-globin genes to generate an extended domain of accessible chromatin (Forrester et al., 1994; Jenuwein et al., 1997). Likewise, the 5′ and 3′ MARs from the tomato *Heat Shock Cognate 80* (*HSC80*) gene are essential for regulated expression, but only when combined with transgenes that harbor introns of *HSC80*. The absence of either MAR or any of the introns reduces or eliminates *HSC80* expression (Chinn and Comai, 1996; Chinn et al., 1996). Finally, the only study to explore the relationship

Table 1. Summary of Results in BMS Callus

MAR	Relative Binding Strength	Mean Expression Level[a]	*N*-Fold Increase over Control	*N*-Fold Increase in High Expressors	Range[b]
No MAR	N/A[c]	8.0	1.0	N/A	11.24
ARS1	1	20.7	5.8	2.4	10.44
Adh1 5′	3	214.9	26.8	2.7	11.17
Mha1 5′	30	11.8	1.5	1.0	11.52

[a] Light units/μg protein.
[b] 1.5 × interquartile range (same as whiskers in Figure 4) expressed as ln(maximum) − ln(minimum) in ln(light units/μg protein).
[c] N/A, not applicable.

between the stability of promoter-driven transgene expression and its integration site in plants established that some stably expressing lines were correlated with integrations at natural matrix binding sites (Iglesias et al., 1997).

These facts provide a context for interpreting the differences observed with the *Adh1* and *Mha1* MARs in this work. All constructs used in the study contained the first intron of maize *Adh1*, which is known to enhance maize transgene expression (Callis et al., 1987). Therefore, cooperation between *Adh1* 5′ MAR and the first intron of *Adh1* could be important for the *Adh1* 5′ MAR effect in vivo and less so for the *Mha1* 5′ MAR effect. At any rate, binding by proteins that compete for these two MARs in vitro (Figure 1) must be insufficient to mediate transgene silencing in vivo.

The nature of the *Adh1* 5′ MAR DNA fragment used to flank the reporter gene constructs in these studies has been reported (Avramova and Bennetzen, 1993; Tikhonov et al., 2000). The entire fragment displayed matrix binding activity, and splitting the region significantly diminished its affinity for the matrix. The adjacent downstream region that did not display any matrix binding capacity defined the 3′ boundary of the region that we called MAR.

The non-MAR region, which contains recognized *Adh1* gene regulatory elements (Walker et al., 1987; Paul and Ferl, 1991), was not included in the fragment used to flank the constructs in the experiments described here. However, the presence of cryptic, *cis*-regulatory elements within the MAR region are not excluded; in fact, they could explain the effects observed (as discussed above and below). The fact that MAR fragments affected *LUC* expression in BSM cells only after stable integration in the genome suggests that MARs function as regulatory elements only after they bind to the nuclear matrix.

Does a Higher Affinity for the Matrix in Vitro Predict a Higher Effect in Vivo?

If MARs exert their effect through binding to the nuclear matrix, then MARs with a higher matrix affinity might be expected to have more pronounced effects on transgene expression. Allen et al. (1996) found that a tobacco MAR displaying greater affinity for tobacco matrices than the yeast ARS1 MAR increased reporter gene expression 140-fold compared with a 24-fold increase with ARS1. They concluded that there is a correlation between MAR binding strength and the effects of MARs on transgene expression.

In this study, we found no correlation between MAR binding strength and MAR effects on transgene expression in vivo: the maize *Adh1* 5′ MAR and the yeast ARS1 decreased transgene silencing, whereas the maize *Mha1* 5′ MAR had no apparent effect on average expression levels, despite binding maize nuclear matrices more strongly than *Adh1* 5′ MAR. This result suggests that binding strength in vitro does not necessarily correlate with effects on transgene expression in vivo. One possible explanation for the discrepancy between the two studies could be that, in the former case, the conclusion was made after comparing a homologous and a heterologous MAR. We would have drawn a similar conclusion had we compared the homologous MAR from maize (*Adh1* 5′ MAR) with the heterologous MAR from yeast (ARS1).

If the basis of the interactions between a MAR and the matrix is competition, it is possible that a higher affinity of *Mha*1 5′ MAR for the matrix makes it less available for the binding of transcriptional regulators that, under similar circumstances, interact with other available MARs (e.g., *Adh1* 5′ MAR). In other words, *Mha1* 5′ MAR might be involved more in structural than in regulatory function. Defining MARs as “strong” or “weak” on the basis of in vitro binding affinity may reflect a different nature of the DNA-protein interactions (Tikhonov et al., 2000).

Adh1 5′ MAR as a Positive/Negative Effector of Gene Expression: The Binary Model

An unexpected result of this study was the observation that *Adh1* 5′ MAR may have a dual function, both activating (in callus) and repressing (in transgenic plants) transcription. The postulated mechanism behind a dual-function capacity is the ability of a DNA element to assemble both activating and repressive complexes. These may result from changes in their chromatin structure or in protein–protein interactions that affect DNA binding activity (Diamond et al., 1990). A bi-

nary model, termed binary because of its on/off nature, had been suggested to explain a positive and a negative regulation from a single DNA element (Walters et al., 1995, 1996). An important factor in the development of this model was the distinction made between expressing and nonexpressing cell populations (Walters et al., 1995).

Several aspects of this model agree well with our results. MAR-flanked, stably integrated constructs increased the number of expressors at the expense of nonexpressors (Figure 5) but were not able to reduce expression variability (Figure 4A). Therefore, MARs appear to protect transgenes from repression by counteracting the silencing effect. Thus, a function of a MAR might be to stabilize transcription, not modulate its level. The shift toward expressing cells observed here, without an increase in transcription levels within the population, suggests that MARs increased the chance of creating active templates. The frequency of such occurrences would depend on the availability of factors involved in the formation of regulatory complexes.

Bimodal Distribution Pattern

Large-sample analysis of transformed cells revealed a bimodal distribution pattern for the expression level data (Figure 4A). Some transformation studies have reported expression level data that are distributed normally after log transformation (Mlynarova et al., 1994; Allen et al., 1996), whereas others have reported a non-log-normal distribution (Breyne et al., 1992). In our analysis, graphing of the findings from 20 to 30 events did not result in a bimodal profile. Analysis of 30 to 50 events sometimes resulted in a bimodal distribution, but most of the time the pattern was not clear. However, samples of $>$50 events always resulted in a bimodal distribution curve. One possible explanation for this effect could be the wide range in transgene expression levels. When small sample numbers are analyzed, the events might be scattered across the range, obscuring any pattern.

A bimodal expression pattern was related to gene silencing caused by high copy numbers of integrated transgenes (Hobbs et al., 1990). Because the transformants in our case contain predominantly low copy numbers, we favor the possibility that the bimodal distribution resulted from a combination of silenced and expressing events. Probably, MARs increase the likelihood that an integrated transgene will be expressed, not the level of its expression. If MARs make a construct more efficient by creating an individual region, they would tend to increase the number of sites at which activity could occur after integration, and therefore the number of expressing cells. The probability of establishing expression would depend on the interplay between the chromatin at the integration site and the control elements of the construct. The action of the MARs is consistent with the view that they counteract the silencing.

How Do MARs Affect Expression in Vivo?

One possibility is that MARs act as structural elements that may create autonomously regulated chromatin loops. In addition to such a structural role, MARs may function as regulatory elements responsible for the state of chromatin, either through their inherent unwinding capability (Bode et al., 1992; Benham et al., 1997) or by binding regulatory proteins. SATB1 and Bright are two proteins that bind specifically the μ-immunoglobulin MAR (Dickinson et al., 1997; Dillon et al., 1997; Kohwi et al., 1997; Liu et al., 1997), and they illustrate how the same MAR may act as a positive regulator in B-cells and as a negative regulator in the non-B lineage (Scheuermann and Chen, 1989; Cunningham et al., 1994).

We suggest that the effects of the maize *Adh1* 5′ MAR in the roots might be explained by a similar mechanism. Because in control (without MAR) plants *GUS* is expressed in all cell types, the loss of *GUS* expression in the majority of the root cells in constructs with MAR indicated a silencing potential for the MAR fragment. The specificity of this

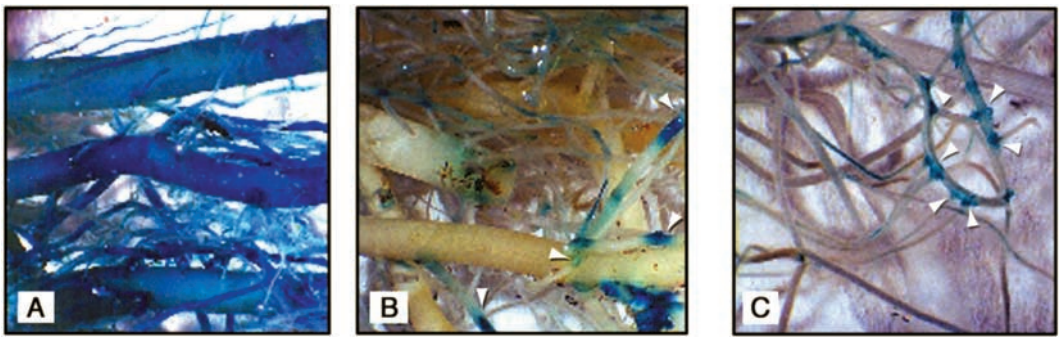

Figure 6. Plants Stably Transformed with the *Rsyn7*-Driven *GUS* Gene.

Histochemical analysis of *GUS* expression in roots of transgenic maize plants transformed with *Rsyn7*::*GUS* unflanked **(A)** or flanked (**[B]** and **[C]**) with *Adh1* 5′ MAR. The expression patterns in **(B)** and **(C)** show GUS activity primarily at the sites of lateral root emergence (arrowheads).

silencing function (except in nonrandom cell clusters where a transcriptionally active *GUS* state was preserved) suggested that root-specific protein factors might be involved.

Therefore, the different effects displayed by the same MAR in BMS cells and in whole plants could be attributable to different *trans*-acting factors present in BMS cells versus plants, to different interactions between the MAR and the two promoters, to different interactions between the MAR and the two transgene coding sequences, or to any combination of these causes.

Conclusion

The establishment and maintenance of appropriate expression patterns requires a balance between positive and negative regulatory mechanisms. The decision to establish an activating or a repressive complex may be the result of a stochastic binding of transcription factors, of the site of integration of transfected DNA into transcriptionally active or inactive regions of the nucleus, of the cell cycle state, of the age of the transformed cell, or of the cell's epigenetic status. The decision may be influenced by modulating the concentrations of the participating proteins or by modulating the protein binding capacity of DNA. The differentiation between a silenced and an expressing state may occur at the time of its establishment or during the process of its maintenance. The prevention of silencing by MARs could operate at either level.

Two of the MARs suppressed silencing of a *LUC* transgene in BMS callus, but none of the MARs used in this study displayed any effect on the variability of transgene expression among transformants. Contrary to a general belief that a MAR with the highest affinity for the nuclear matrix in vitro would have higher effects in vivo, we found that the two characteristics did not define each other. Thus, MAR binding affinity alone is insufficient to predict MAR effects in vivo.

Sample size, nature of MAR, proximity to promoter, and whether cultured cells or whole plants were analyzed may account, at least partly, for the variance in the behavior of these elements, explaining some reported controversies. Finally, the effect of *Adh1* 5′ MAR on *LUC* expression in BMS callus was markedly different from the effect on *GUS* expression in transgenic maize plants, revealing a dual nature of *Adh1* 5′ MAR. These results support the idea that MARs are not only structural elements but may be involved actively in maintaining a state that controls promoter activity (C. Brouwer and B. Bowen, unpublished data).

METHODS

Nuclear Matrix Binding Assays

Nuclear matrix binding assays (MARs) were performed essentially as described by Avramova and Bennetzen (1993). Matrices were isolated from 0.5 A_{260} units of leaf nuclei. A total of 50 ng of MAR containing plasmid (pUC19 containing ARS1, *Adh1* 5′ MAR, or *Mha1* 5′ MAR) was cut with HindIII-EcoRI, HindIII-NotI, or HindIII, respectively, to separate each MAR insert from the plasmid backbone. The DNA was end-labeled with γ-^{32}P-ATP and incubated with the isolated nuclear matrices corresponding to the residual protein obtained after extraction of 0.3 to 0.5 A_{260} units of starting nuclei.

Varying concentrations of *Escherichia coli* DNA (100- to 2500-fold molar excess) were used as competitors to prevent nonspecific binding (the A-T content of *E. coli* DNA is ~50%) and to compare the affinity of different DNA fragments for proteins in the matrix preparation. After the incubation, the 100-μL reaction was placed in a centrifuge, and the nuclear matrices were pelleted. The supernatant was discarded, and DNA that remained bound to the pelleted nuclear matrices was run on a gel, transferred to a nylon membrane, and exposed to film.

Vector Construction

Plasmid maps for vectors used in this study are shown in Figure 2A. PHP264 consists of an enhanced 35S promoter of *Cauliflower mosaic virus* (bases −421 to −90 and −421 to +2; Gardner et al., 1981), a 79-bp fragment from the 5′ leader sequence of *Tobacco mosaic virus* (Gallie et al., 1987), the first intron of the maize (*Zea mays*) *Adh1-S* gene (Dennis et al., 1984), the coding sequence of the β-glucuronidase (*GUS*) gene (Jefferson et al., 1987), and the potato proteinase II gene (bases 2 to 310; An et al., 1989). PHP264 and all other plasmids in this study have pUC-derived backbones. The vectors PHP1528 and PHP3528 are similar to PHP264 except for the coding sequence. PHP1528 contains the firefly luciferase (*LUC*) gene (de Wet et al., 1987), and PHP3528 contains the *BAR* gene from *Streptomyces hygroscopicus* (Thompson et al., 1987).

An 839-bp EcoRI-HindIII fragment region of ARS1 (Struhl et al., 1979) was modified by site-specific mutagenesis (Sambrook et al., 1989) so that it contained a BamHI site on the 3′ end and a NotI site on the 5′ end. This modified ARS1 fragment was inserted into PHP1528 at the 5′ and 3′ ends of the 35S::*LUC* cassette, to create PHP5438 (Figure 2). PHP5456 (Figure 2) was made by replacing the *LUC* coding sequence with the *BAR* coding sequence, using unique sites in the promoter and the terminator.

The 948-bp BamHI-PstI fragment of the maize *Adh1* gene (Dennis et al., 1984) containing *Adh1* 5′ MAR (Avramova and Bennetzen, 1993) was cloned into pBluescript SK+ (Stratagene). *Mha1* 5′ MAR (−2562 to −1442 of GenBank entry U09989; Jin and Bennetzen, 1994) also was subcloned into pUC19. Vectors containing these MARs flanking *LUC* or *BAR* (PHP6248, PHP6344, PHP6486, and PHP6487) (Figure 2A) were constructed by inserting the MARs into sites at the 5′ or 3′ ends of PHP1528 or PHP3528, as described for PHP5438 and PHP5456.

PHP6086, PHP6608, PHP7819, and PHP7917 were constructed in a similar manner. The enhanced 35S promoter of *Cauliflower mosaic virus* was replaced with the *Rsyn7* promoter, and *GUS* was used as the reporter. *Rsyn7* is a root-specific, constitutively expressed promoter consisting of a synthetic sequence of ~140 bp containing three TGACG core motifs and a TATA box. This synthetic promoter has been described in detail elsewhere (B.A. Bowen, W.B. Bruce, G. Lu, L.E. Sims, and L.A. Tagliani [2000], U.S. patent 6,072,050; Lu and Bruce, 2000).

Transformation Methods

The Black Mexican Sweet cell line (kindly provided by Dave Somers, University of Minnesota, Minneapolis-St. Paul) was subcultured

twice per week using 586 medium (modified MS2-D medium; Bittel et al., 1996). Two days before transformation, cells were subcultured and prepared for bombardment essentially as described previously (Bruce et al., 2000). One day before bombardment, cells were placed in osmoticum (586 medium plus 3% polyethylene glycol 8000) at a density of 200 mg/mL. Ten nanograms of reporter plasmid (PHP1528, PHP5438, PHP6248, or PHP6486) or 10 ng of selectable marker plasmid (PHP3528, PHP5456, PHP6344, or PHP6487) was precipitated onto 750 μg of 1.8-μm tungsten beads (General Electric, Fairfield, CT), and each preparation was divided into six aliquots for bombardment. Twenty nanograms of total DNA was used in all experiments.

Under the established transformation conditions, the DNA dose used yielded a majority of events that contain two or fewer copies of unselected transgenes. This fact has been determined and confirmed by numerous quantitative DNA gel blot analyses. An example of the copy-number distribution under the standard protocol conditions is shown for the control lines used in this study (Figure 3B). Based on this distribution and on massive previous experience, we assume that the MAR-flanked lines would have similar copy-number insertions. In the absence of the respective blots, however, the possibility that these particular MAR transformants might carry a different copy-number distribution cannot be excluded.

A total of 0.5 mL of cells was pipetted in a 2-cm circle onto sterile filter sets (consisting of a grade 391 filter on top and a grade 363 filter [both Whatman] beneath) premoistened with 750 μL of the same medium used for osmoticum. Cells were bombarded with a PDS1000 helium gun (Bio-Rad) using a 1100-p.s.i. rupture disk. Immediately after bombardment, cells were removed by placing the top filter on solid 586 medium containing 3% Gel-Rite (Merck & Co., Rahway, NJ) without selection. Three days after bombardment, the cells were scraped off the filter, suspended in 4 mL of 586 liquid medium, and divided into aliquots (1 mL/plate) on four plates of 586 solid medium containing the herbicide BASTA (AgrEvo, Wilmington, DE) at 3 mg/L. Stable transformants were recovered at 4 to 8 weeks after bombardment and were subcultured twice to confirm BASTA resistance. Events were assayed for *LUC* expression at 7 days after the second subculture. Expression levels were normalized to total protein levels (Bradford, 1976) and reagents from Bio-Rad.

For transient gene expression assays, Black Mexican Sweet cell lines were subcultured at 24 h before bombardment and placed in osmoticum at 4 h before shooting. A total of 100 mg of cells was plated onto filters. Ten micrograms of the test *LUC* constructs was mixed with 2 μg of PHP264 and precipitated onto 1.0-μm tungsten beads (General Electric or Bio-Rad). Bombardments were performed using a 600-p.s.i. rupture disc. At 20 h after bombardment, cells were harvested and assayed for *LUC* and *GUS* expression.

Stably transformed plants were produced from embryogenic callus induced from immature embryos of Hi-Type II maize (Armstrong et al., 1991). Particle bombardments of DNA constructs and subsequent tissue culture steps were conducted essentially as described (Klein et al., 1989; Bowen, 1992). PCR and DNA gel blot analyses were conducted on resistant calli and subsequently on selected transformed plants to confirm transgene incorporation.

Regenerated transgenic plants (T0) were grown to maturity in a greenhouse and crossed to a proprietary Pioneer inbred line to produce T1 plants. These were grown in a greenhouse until flowering (5 days after silking). Tissues, including leaf, tassel, ear, stem, and whole roots, were collected at or near flowering and incubated in a solution of 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-glucuronic acid cyclohexylamine (Rose Scientific, Edmonton, Alberta, Canada) at 37°C for 20 h essentially as described by Capellades et al. (1996) and then cleared in 70% ethanol.

Quantitative *GUS* assays were conducted by homogenizing fresh root tissue in Eppendorf tubes using a Kontes pestle (Vineland, NJ) in a solution of 50 mM sodium phosphate, pH 7.0, 10 mM EDTA, and 1 mM DTT. The tubes were centrifuged for 5 min at 4°C, and the supernatant was recovered and subjected to Gus Light assay from Tropix, Inc. (Bedford, MA), according to manufacturer's protocol. Concentrations of total soluble protein were determined using the method of Bradford (1976).

Gene Expression Assays and Data Analysis

Callus tissue (100 mg) was ground in 300 μL of 0.1 M phosphate buffer, pH 7.8, and 1 mM DTT, and 10% of the cleared extract was diluted with 200 μL of buffer. A total of 100 μL of 1 mM luciferin was added, and light units (LU) were measured using a 10-s integration time on a single-well luminometer (model 2010; Analytical Luminescence Laboratories, San Diego, CA). For stable transformants, LU were normalized to total soluble protein. Transient gene expression levels were normalized by dividing *LUC* expression measurements (in LU/μL) by *GUS* expression levels (in LU/μL) measured in an equivalent volume of extract using the Gus Light kit from Tropix.

Statistical Analysis

Graphic and statistical analyses of log-transformed *LUC* expression level data were performed using JMP version 3.1.5 for Macintosh (SAS Institute, Cary, NC). To test for normality, we used the Shapiro-Wilk test. The cumulative distribution function was calculated with the formula

$$CDF(\text{point } k) = \frac{\sum_{i=1}^{k} (LU/\mu g)}{\sum_{i=1}^{n} (LU/\mu g)},$$

where n is the total number of transformation events and $k = 1, 2, 3, \ldots n$.

Upon request, all materials described in this article and owned by us will be made available in a timely manner for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this article that would limit their use for noncommercial research purposes.

Accession Number

The GenBank accession number for *Mha1* 5′ MAR is U09989.

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