



Elevation of transgene expression level by flanking matrix attachment regions (MAR) is promoter dependent: a study of the interactions of six promoters with the RB7 3' MAR

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Abstract

We have analyzed effects of a matrix attachment region (MAR) from the tobacco RB7 gene on transgene expression from six different promoters in stably transformed tobacco cell cultures. The presence of MARs flanking the transgene increased expression of constructs based on the constitutive CaMV 35S, NOS, and OCS promoters. Expression from an induced heat shock promoter was also increased and MARs did not cause expression in the absence of heat shock. There was also no effect of MARs on the pea ferredoxin promoter, which is not normally expressed in this cell line. Importantly, most transgenes flanked by RB7 MAR elements showed a large reduction in the number of low expressing GUS transformants relative to control constructs without MARs.

Abbreviations: 35S – cauliflower mosaic virus 35S promoter; AtAhas – *Arabidopsis thaliana* acetohydroxyacid synthase promoter; bp – base pairs; CaMV – cauliflower mosaic virus; cDNA – complementary DNA; GmHspL – *Glycine max* (soybean) heat shock protein 17.6L promoter; *gusA* – β -glucuronidase cDNA; GUS – β -glucuronidase protein; kb – kilobase pairs; LIS – lithium diiodosalicylate; MAR(s) – matrix attachment region(s); NOS – nopaline synthase 5' region; nosT – nopaline synthase 3' region; *nptII* – neomycin phosphotransferase II cDNA; OCS – octopine synthase 5' region; ocsT – octopine synthase 3' region; PCR – polymerase chain reaction; PEV – position effect variegation; PsFed1 – *Pisum sativum* (pea) ferredoxin I promoter; SEM – standard error of the mean.

Introduction

DNA sequences necessary for the proper formation, maintenance, or regulation of chromatin structure, including those that define domain boundaries, can be considered 'chromatin elements'. Such elements most likely function by interacting with specific architectural proteins (reviewed by Sun & Elgin, 1999). We have been studying the effects on transgene expression of matrix attachment regions (MARs), a class of chromatin elements operationally defined by the ability to

bind specifically to isolated nuclear matrices. MARs have been isolated and studied from a diverse range of eukaryotes including mammals, birds, insects, and plants (Mirkovitch et al., 1984; Cockerill & Garrard, 1986; Mielke et al., 1990; Breyne et al., 1994; Spiker & Thompson, 1996; Allen et al., 2000). The primary MAR used in our studies is a 1.2 kb fragment isolated from the 3' flanking region of the RB7 root-specific gene of tobacco (Conkling et al., 1990; Hall et al., 1991). The RB7 MAR is AT-rich (73.2%), like most MARs, and has a high affinity for isolated tobacco nuclear matrix (Michalowski et al., 1999). The RB7 MAR has been used in numerous studies of plant transformation including tobacco cells (Allen et al.,

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1996), tobacco plants (Ülker et al., 1999), rice plants (Vain et al., 1999), poplar explants (Han et al., 1997), sorghum cells (Able et al., 1999), and pine callus (Levéé et al., 1999). However, these and other experiments with plant systems have focused almost entirely on test constructs involving the cauliflower mosaic virus (CaMV) 35S promoter (Allen et al., 1996; Han et al., 1997; Ülker et al., 1999; Vain et al., 1999). We are aware of only one report presenting a side-by-side comparison of MAR effects on two different promoters (Mlynárová et al., 1996). The intent of the report by Mlynárová et al. (1996) was to determine the lower limit of variation in transgenic populations, and the analysis therefore focused on a small number of primary transformant lines that had been pre-selected for low epigenetic instability. Since our goal is to analyze the effects of MARs on epigenetic phenomena, we have chosen to analyze MAR effects in random populations of independent primary transformants.

In this study, we have used a modified, rapid assay procedure to analyze effects of the RB7 MAR on six different promoters to determine the extent of their interactions. The promoters tested were derived from *Agrobacterium* T-DNA, a plant DNA virus (cauliflower mosaic virus; CaMV), and three different plant genomes. They confer various levels of constitutive or inducible expression. We tested the matrix binding ability of these promoters in an *in vitro* assay, and found only weak activity that did not correlate with expression levels or with the magnitude of the MAR effect. Highly active promoters exhibited significant increases in β -glucuronidase (GUS) activity in MAR constructs as compared to controls, but the presence of MARs did not significantly increase GUS activity from weak promoters. Importantly, most transgenes flanked by MARs showed a large reduction in the number of low expressing GUS transformants (i.e., kanamycin-resistant calli that fail to express GUS above background level), suggesting that MARs can reduce the frequency of gene silencing in primary transformants.

Materials and methods

Plasmid constructs

Standard cloning techniques (Sambrook et al., 1989) were used to create the plasmid constructs described in Figure 1 from the starting materials listed below. Detailed sequence-based maps are available upon request.

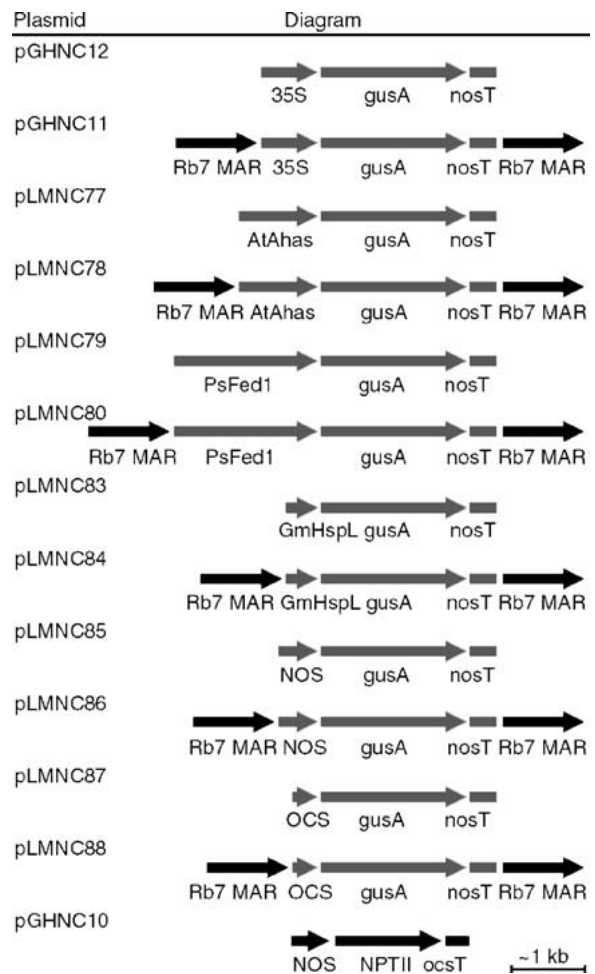


Figure 1. Schematic diagram of the promoter plasmid constructs used in this study. Diagrams for reporter and selection constructs are drawn approximately to scale. Each construct is named (e.g., pGHNC12, pGHNC11), and the *nptII* selectable marker plasmid is also diagrammed. In pBluescriptII SK+ (Stratagene), the rest of the plasmid is not shown. 35S, cauliflower mosaic virus 35S promoter; AtAhas, *Arabidopsis thaliana* acetohydroxyacid synthase promoter; CaMV, cauliflower mosaic virus; GmHspL, *Glycine max* (soybean) heat shock protein 17.61 promoter; gusA, cDNA for β -glucuronidase; NOS, nopaline synthase promoter region; nosT, nopaline synthase 3' region; *nptII*, neomycin phosphotransferase II cDNA; OCS, octopine synthase promoter region; ocsT, octopine synthase 3' region; PsFed1, *Pisum sativum* (pea) ferredoxin I promoter; Rb7 MAR, 3' MAR of the *Nicotiana tabacum* Rb7 gene.

The *gusA* reporter plasmid, pGHNC12 (Allen et al., 1996), is a derivative of pBluescriptII (Stratagene, La Jolla) and the 3.0 kb *EcoR* I-*Hind* III fragment of pBI221 (Jefferson et al., 1987). This fragment contains a chimeric β -glucuronidase (*gusA*) gene under transcriptional control of a cauliflower mosaic virus (CaMV) 35S promoter and terminated by a polyadenylation signal from the nopaline synthase gene

(nosT; Depicker et al., 1982). The plasmid pGHNC11 (Allen et al., 1996), which contains two directly repeating RB7 MARs as 1.2 kb *Cla* I-*Sca* I fragments from pRB7-6 (Hall et al., 1991) and 3.0 kb *Eco*R I-*Hind* III fragment of pBI221 (Jefferson et al., 1987), was used to generate all the other 5 MAR containing plasmids.

Five promoters were subcloned into the *Bam*H I-*Hind* III sites pBI221 (Jefferson et al., 1987) to produce new chimeric promoter β -glucuronidase (*gusA*) gene fusions. A 1177 bp promoter fragment of the *Arabidopsis thaliana* acetohydroxyacid synthase gene (G Hall, personal communication) was sub-cloned via PCR (primers: 5' ATC AGA AGC TTC GAA AGT AGC 3' and 5' AGG CGG ATC CGT TGT TCA GGA GAA 3') to produce pLMNC71 (AtAhas). A ca. 2 kb *Bam*H I-*Hind* III restriction fragment of the ferredoxin I gene from *Pisum sativum* was sub-cloned from pRE1091 (Elliott et al., 1989) to generate pLMNC72 (PsFed1). The promoter of the *Glycine max* heat shock protein 17.61 gene (Kilby et al., 1995) was also sub-cloned via PCR (primers: 5' GCT AGA AGC TTC TGA AAT TGG GTC 3' and 5' TCT TTA TGT TTT TGG CGT CTT CCA 3') into pBI221 to create pLMNC74 (GmHspL). A *Bgl* II-*Hind* III restriction fragment containing the promoter region of the nopaline synthase gene was sub-cloned from pGPTV-kan (Becker et al., 1992) into pBI221 to generate pLMNC75 (NOS). Finally, a *Bgl* II-*Hind* III restriction fragment containing the octopine synthase promoter region of pLkB07 (Mankin et al., 1997) was sub-cloned via PCR from pLMNC03 (primers: 5' AAC AGC TAT GAC CAT GAT TAC GCC 3' and 5' CGG GAG ATC TCC TTG AGG CCA CAC 3') into pLMNC76 (OCS). All promoter fragments subjected to PCR during subcloning were sequenced in each direction to ensure that there were no PCR-induced mutations. All sequences were as predicted. The *Eco*R I-*Hind* III *gusA* reporter gene fragments from pLMNC71-76 were sub-cloned into pGHNC11 and pGHNC12 to create pLMNC77-88 (Figure 1).

In co-transformation experiments, pGHNC10 (Allen et al., 1996) was used as the selection plasmid. This plasmid contains the neomycin phosphotransferase II (*nptII*) gene under the transcriptional control of a NOS promoter and is terminated by an octopine synthase 3' polyadenylation signal (ocsT).

Tobacco nuclear matrix binding assay

Nuclear matrices were isolated from tobacco NT-1 cells, and exogenous nuclear matrix binding assays

were performed as described by Hall et al. (1991), (Hall & Spiker, 1994). In summary, isolated nuclei are lysed, and stripped of histones by extraction with lithium diiodosalicylate (LIS). Then the genomic DNA is digested with restriction endonuclease. The genomic DNA fragments are retained to act as competitors during the binding assay. Plasmids containing DNA fragments to be tested for matrix binding are cut with restriction enzymes to generate 5' overhangs on each fragment and end labeled with α^{32} P using a Klenow fill in reaction. In our experiments, each binding assay contained approximately $3 \bullet (10^5)$ nuclear matrices (determined by counting in a hemocytometer) and 5 ng (ca. 50,000 cpm) of probe. For each assay, nuclear matrices and probe were mixed together and incubated at 37°C for 3 h with resuspension every 20 min. Following incubation, matrix bound probe was separated from unbound probe by centrifugation. DNA was purified from the pellet and supernatant, and equal fractions of the total input probe (T), pellet (P), and supernatant (S) DNA were electrophoretically separated in an agarose gel. The gel was dried, and the labeled DNA was detected by autoradiography.

Plasmids containing DNA fragments to be tested for matrix binding were cut with restriction enzymes to generate 5' overhangs on each fragment; pLMNC71-76 and pGHNC12 were cut with *Hind* III and *Xma* I to release the promoter fragments, and pRB7-6 (Hall et al., 1991) was cut with *Spe* I and *Xho* I to release the RB7 MAR fragment. The plasmid vector fragment serves as a convenient negative control in each assay. Fragments were end labeled with 32 P using a Klenow fill in reaction. The nuclear matrices were prepared by digestion of nuclear halos with *Hind* III and *Xba* I; these restriction enzymes are not denatured or removed, but they do not cut within most of the test fragments. Residual nuclease activity in the matrix preparations did digest some promoter fragments and reduced the total signals as seen in Figure 2.

Transformation by microprojectile bombardment

The *Nicotiana tabacum* cell line NT-1 was originally obtained from G. An (Washington State University, Pullman). Suspension cultures were grown at 27°C on NT-1 medium (1 \times MS salts, 180 mg/l KH_2PO_4 , 100 mg/l *myo*-inositol, 1 mg/l thiamine HCl, 200 μ g/l 2,4-dichlorophenoxyacetic acid, 3% sucrose, pH adjusted to 5.7 with KOH) as described by Allen et al. (1996). Early log phase cells, 3–4 days after transfer,

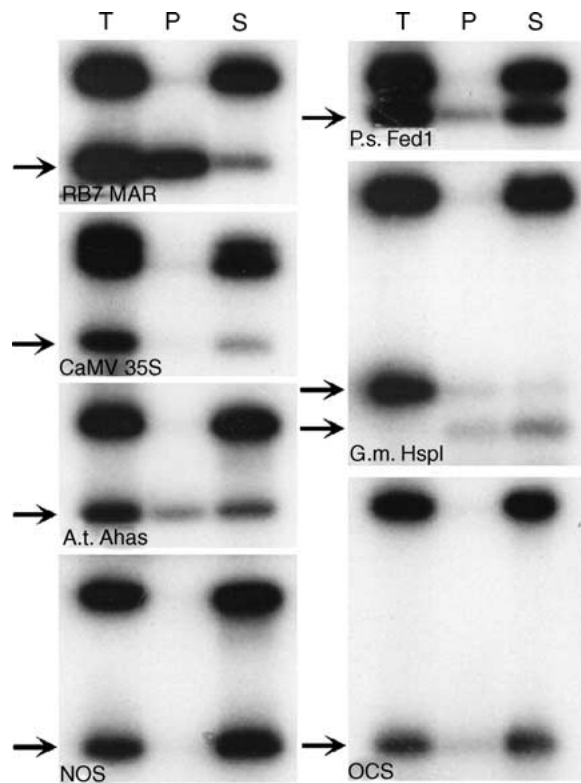


Figure 2. Nuclear matrix binding assays indicate that the RB7 MAR, AtAhas, GmHspL, OCS, and PsFed1 promoters all bind to tobacco matrices. Plasmid DNAs containing promoters or RB7 MAR sequences were digested with restriction endonucleases to separate test DNA from vector DNA, and end-labeled with ^{32}P . The labeled DNA fragments were then incubated with isolated tobacco nuclear matrices as described in Materials and methods. Matrices were pelleted by centrifugation, and DNA was purified as described by Hall et al. (1991). Equal fractions of total (T), matrix pelleted (P), and supernatant (S) DNA were analyzed by gel electrophoresis. Arrows indicate the test fragment (e.g., RB7 MAR, OCS) in each panel. No binding could be detected with the 35S and NOS promoters. The GmHspL promoter was partially digested by residual nuclease activity within the nuclear matrix preparations, but binding is clearly observed. Low levels of binding are observed for the PsFed1, AtAhas, and OCS promoters, which can be compared to the strong binding observed for the RB7 MAR.

were transformed by microprojectile bombardment using a co-transformation procedure modified from Allen et al. (1996). As discussed in Results, the main modifications involved use of a higher cell density during bombardment and an abbreviated selection process prior to the analysis of GUS expression. Cell suspensions (50 ml) were centrifuged and resuspended in 0.5 ml fresh culture medium per gram wet weight of cells. Aliquots (0.5 ml) of the concentrated suspension were spread onto sterile lens paper that had been placed on NT-1 medium solidified with 0.8% phytagar.

Plated cells were returned to 27°C for ca. 3 h prior to bombardment. Microprojectile bombardments were performed with a particle accelerator (PDS-1000/He, BioRad) using 1100 psi rupture disks, a 9 cm sample distance, 1.0 μm gold beads, and 28 mm Hg partial vacuum.

Each batch of cells was co-transformed with a mixture of expression plasmid DNA (52 pg per base pair of plasmid size per bombardment) and selection plasmid DNA (13 pg per base pair). Plasmids were prepared and precipitated onto the gold beads as described by Allen et al. (1996). Petri plates were sealed with gas-permeable tape after bombardment and the cells allowed to recover at 27°C for ca. 48 h. The cells from each plate were then resuspended in 2 ml of fresh NT-1 medium and 0.5 ml aliquots spread onto plates containing NT-1 medium supplemented with 50 mg/l kanamycin. After 2 weeks, kanamycin resistant calli were transferred to fresh NT-1 medium supplemented with 50 mg/l kanamycin for an additional 14 days of secondary selection. Calli that survived and grew on the secondary selection plate were used in their entirety for GUS assays, except for those containing constructs driven by the soybean heat shock promoter (GmHspL). We obtained an average of 148 transformants per plate (SEM = 10) in all cases except those involving test constructs driven by the NOS promoter, where the average was 65 ± 10 transformants per plate (see Results). We often obtained a slightly larger number of transformants when using MARs in the test constructs, but this difference was never large enough to be statistically significant.

To compare induced and non-induced activities of the GmHspL heat shock promoter, each transformed callus was split in half after secondary selection, and the two halves transferred separately to fresh selective medium. After 6 days, half of the split calli were subjected to heat shock (2 h at 40°C). Induced and uninduced calli were harvested 7 days after transfer, which was 24 h after the heat shock stimulus.

GUS assays

Harvested calli were ground in 200 μl GUS-Light plant lysis buffer (Tropix) with a motor-driven Kontes 'pellet pestle' for 20 s in an ice-water bath. Cell extracts were cleared by centrifugation (13,500 rpm, 5 min, 4°C), and stored at -70°C until assayed. GUS activity was measured using an automatic microtiter plate reading luminometer (LumiStar, BMG

Table 1. Summary statistics

Promoter	Control			MAR-Flanked			Fold increase ^d
	Mean ^a	Non-Exp. ^b	Number ^c	Mean ^a	Non-Exp. ^b	Number ^c	
35S	16.5 ± 3.61	11	46	87.9 ± 29.1	0	47	5.3 (4.2)
AtAhas	7.2 ± 2.3	23	45	11.6 ± 2.3	5	46	1.6 (0.8)
GmHspL(-) ^c	1.1 ± 1.1	46	47	1.3 ± 1.1	44	46	1.1 (0.6)
GmHspL(+) ^c	98.1 ± 42.5	15	46	306 ± 110	13	45	3.1 (2.7)
NOS	114 ± 35.4	17	41	551 ± 117	4	45	4.9 (3.0)
OCS	26.0 ± 8.51	23	47	397 ± 97.4	6	48	15.3 (8.3)
Psfed1	4.5 ± 1.2	24	42	4.8 ± 1.1	25	44	1.0 (1.0)

^aMean GUS activity ($\mu\text{U}/\text{mg}$) \pm standard error.

^bNumber of transformants with GUS activities of $\leq 1 \mu\text{U}/\text{mg}$.

^cTotal number of transformants which survived secondary selection (out of 48 that survived primary selection).

^dMean GUS activity for MAR transformants divided by mean GUS activity for control transformants. Parenthesis indicate the ratio obtained when low expressing GUS transformants are not excluded from the calculation.

^eFor constructs containing the heat shock promoter (GmHspL) data from the uninduced (-) and induced (+) populations are shown separately.

Technologies) and the GUS-Light assay system (Tropix). For each measurement, 20 μl of cleared cell extract was mixed with 70 μl of GUS Reaction Buffer (Tropix) and incubated at room temperature for 30 min before being mixed with 100 μl of Light Emission Accelerator (Tropix). Five seconds after mixing, light emission was measured for 5 s. A standard curve for GUS activity was generated using recombinant β -glucuronidase protein (Sigma) resuspended in extraction buffer. GUS enzymatic units (μU) are as defined by the manufacturer (one unit will liberate 1.0 μg of phenolphthalein from phenolphthalein glucuronide per hour at 37°C). Protein concentrations were determined in the cleared cell extracts using a Lowry-type protein assay (DC Protein Assay Kit, Bio-Rad; Lowry et al., 1951).

Results

Matrix binding assay

We initially asked whether any of the promoter sequences chosen for this study contained an internal MAR, reasoning that the activity of such constructs might be less affected by the addition of flanking MARs. All three of our plant derived promoters, *A. thaliana* acetohydroxyacid synthase promoter (AtAhas; G Hall personal communication), *Glycine max* heat shock protein 17.61 promoter (GmHspL; Kilby et al., 1995) and *P. sativum* ferredoxin I promoter (Psfed1; Elliott et al., 1989), showed detectable, but

weak, binding to isolated tobacco matrices (Figure 2). The octopine synthase promoter region (OCS; Mankin et al., 1997) also bound very weakly; however, neither CaMV 35S (Depicker et al., 1982) nor nopaline synthase (NOS; Becker et al., 1992) promoters showed detectable binding activity in our standard assay (Hall et al., 1991, Michalowski et al., 1999).

Expression analysis

Under our new experimental design (Materials and methods), we assayed calli grown on solid media, rather than suspension cultures, and assays were carried out 4 weeks after transformation instead of the 10–12 weeks used previously. The 4 week selection period is sufficient to kill wild type cells and extends well beyond the 2–3 day transient expression peak for bombarded NT1 cells (Daniell et al., 1990; Southgate et al., 1995). The MAR effects we observe with this protocol are not as large as those we previously reported with the suspension culture protocol (Allen et al., 1993, 1996). The reason for this difference is not known, although some possibilities will be considered in the Discussion. However, it is clear that the presence of MARs does have a large impact on reducing the number of transformants that fail to express GUS.

Overall, MARs consistently increased average GUS expression when used with an active promoter, but had little impact on expression when used in combination with promoters that are weak in NT1 cells (Table 1 and Figure 3). The PsFed1 promoter is most

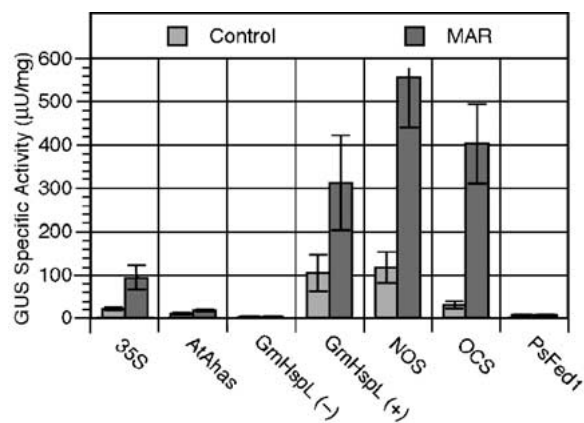


Figure 3. Promoters active in NT-1 cells are expressed at increased levels in MAR-flanked transgenes. Mean GUS expression ($\mu\text{U}/\text{mg}$) plotted on a linear scale for transformants with control (gray bars) and MAR-flanked (black bars) transgenes. Both uninduced (-) and induced (+) expression levels are graphed for the heat shock promoter (GmHspL). Error bars represent one standard error. Descriptive statistics are given in Table 1.

active in green tissue (Elliott et al., 1989) and thus has little activity in NT-1 cells (which lack developed plastids and are incapable of photosynthesis). As expected, we observed only weak GUS expression from PsFed1 transformants, ranging from 0–30 $\mu\text{U}/\text{mg}$. GmHspL transformants prior to heat shock had GUS activities ranging from 0 to 52 $\mu\text{U}/\text{mg}$, with only three transformants (1 control, 2 MAR) expressing detectable amounts. There was no MAR effect with either PsFed1 or GmHspL constructs. The AtAhas promoter produced slightly higher GUS activities (0–81 $\mu\text{U}/\text{mg}$, average 7.2 $\mu\text{U}/\text{mg}$), but the 1.7-fold MAR effect on these constructs is not statistically significant.

When the GmHspL promoter was induced by a heat shock treatment 24 h prior to harvest, the average expression of control constructs increased by a factor of 89, and GUS activity averaged 3-fold higher for MAR flanked transgenes than for the same genes without MARs. In addition, all three of the strong constitutive promoter constructs we tested showed significant increases in gene expression when flanked by MARs. Constructs driven by the 35S, NOS, and OCS promoters showed 5.3, 4.9, and 15.3-fold MAR effects, respectively.

Interestingly, the NOS promoter, which is normally rather weak (Scofield et al., 1992), exhibited a high average expression level (mean = 114 $\mu\text{U}/\text{mg}$) in these experiments (Table 1). We speculate that this result may be explained by the fact that the NOS promoter was also driving the selectable marker.

The presence of two NOS promoters would both increase the probability of gene silencing mediated by promoter homology and create an indirect selective pressure against promoter-mediated silencing of the GUS reporter. Consistent with the prediction of increased silencing, we observed on average only 65 transformants per plate (SEM = 10) for NOS promoter constructs, while we obtained an average of 148 ± 10 transformants per plate for the other five promoters we tested.

The most striking characteristic of these populations is that the MAR-flanked reporter genes have significantly fewer low expressing GUS transformants (Figure 4). We employed a blocked experimental design in order to obtain insight into the variation among bombarded plates (Figure 4(a)). After 2 weeks of selection (50 mg/l kanamycin), 12 randomly chosen primary transformants per bombarded plate were transferred to a secondary selection plate (50 mg/l kanamycin). Analysis reveals a significant reduction in low expressing GUS ($\leq 1 \mu\text{U}/\text{mg}$) lines in populations of transformants with MAR-flanked reporter genes (Figures 4 and 5). Only the GmHspL and PsFed1 promoters failed to show a significant reduction in low expressing GUS calli. PsFed1 and non-induced GmHspL promoters are essentially inactive in NT-1 cells, while variability in expression of induced GmHspL constructs may have been increased by the short assay period. Excluding these data, we calculate an approximately 5-fold decrease in the number of low expressing transformants obtained with the other four promoters.

Discussion

The Rb7 MAR effects (ratio of expression for MAR v.s. control constructs) we report are smaller than those we originally reported with a suspension culture assay (Allen et al., 1996). Experiments that reproduce the conditions of the original experiments have consistently given large MAR effects, even when conducted with different constructs and a different microprojectile bombardment device. However, large variations in the reported magnitude of MAR effects have been reported in the literature. For example, Cheng et al. (2001) recently reported effects of the Rb7 MAR in rice ranging from 3 to 650-fold for different reporter genes. In our laboratories, we have also seen large variations with different assay procedures. As a working hypothesis, we propose that differences in the magnitude of MAR effects arise

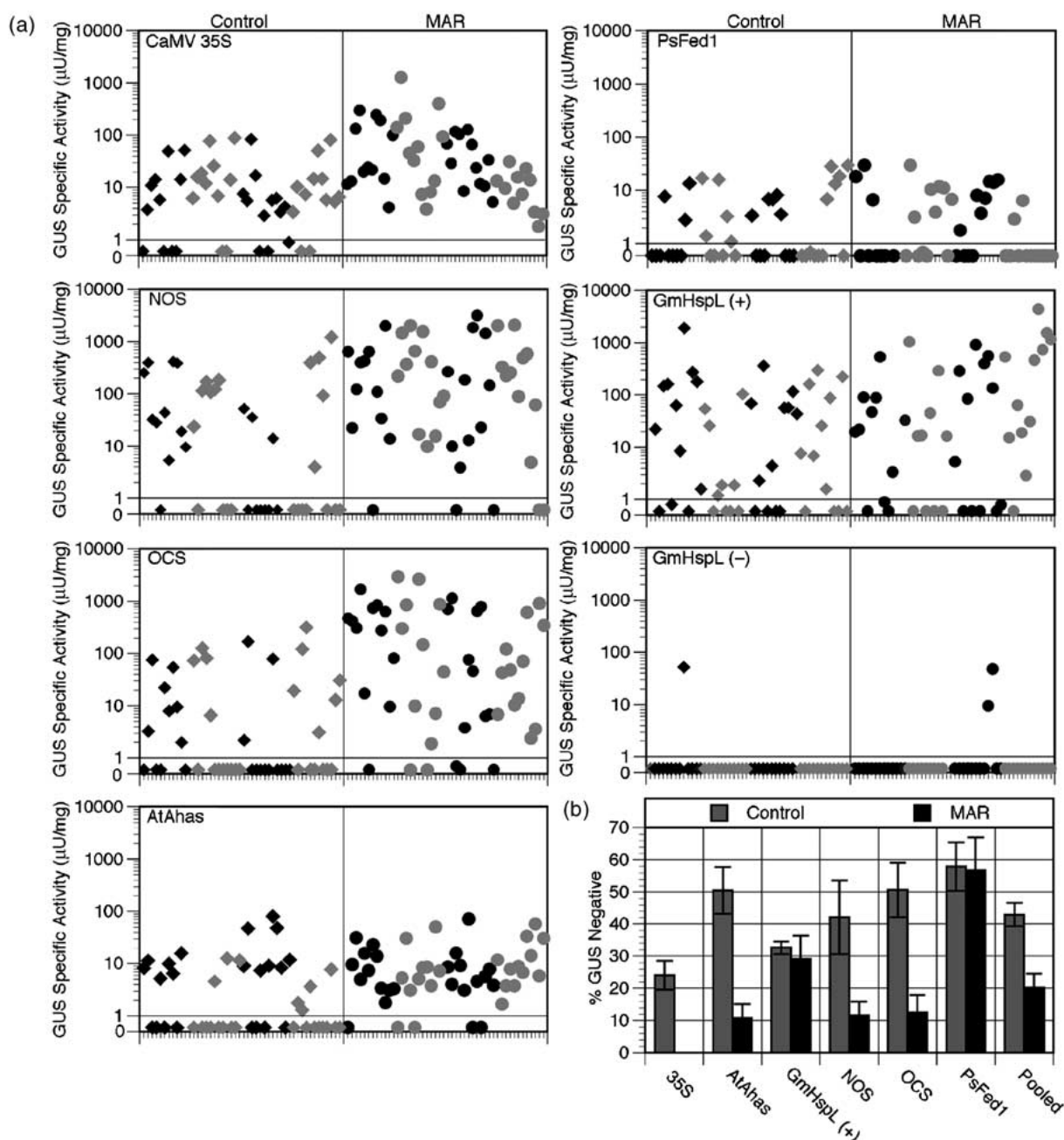


Figure 4. GUS expression analysis by replicate transformation plate. (a) GUS activities of individual transformants are plotted on a logarithmic scale and separated into groups corresponding to replicate transformation plates to allow visualization of both transformant-to-transformant variation and bombardment-to-bombardment variation. A linear scale has been added to display GUS activities between 0 and 1 $\mu\text{U}/\text{mg}$; these values are not significantly above background due to the sensitivity of the assay. Transformants with control (\blacklozenge , \blacklozenge) and MAR flanked (\bullet , \bullet) transgenes are graphed for each promoter. Each transformation plate is shown by alternating black (\blacklozenge , \bullet) and gray (\blacklozenge , \bullet) symbols and represents 12 randomly picked microcalli from a single primary selection plate. Each point on the x -axis represents an individual transformant. GmHspL promoter data points from induced (+) and uninduced (-) calli represent clonal transformant lines. Calli which failed to grow during the second selection period are not included (see Materials and methods for details). (b) For each promoter, the mean percent of surviving, low expressing GUS ($\leq 1 \mu\text{U}/\text{mg}$) transformants per replicate transformation plate is graphed (see Figure 4(a) and Materials and methods) for control and MAR-flanked reporter genes. Each of the four transformation plates are represented by 12 randomly picked calli. For the 35S promoter, there were no low expressing GUS calli among transformants with MAR-flanked reporter genes. Uninduced GmHspL promoter data is not included because only three transformants were expressing above background. Pooled data represent a combined mean for all the promoters graphed here. Error bars represent one standard error. Descriptive statistics are given in Table 1.

mainly from variations in the degree of silencing experienced by control constructs. For example, using an assay involving 10–12 weeks of selection in liquid culture we have previously observed that about 50% of KmR transformants carrying the 35S-GUS construct (pGHNC12) exhibit very low or undetectable levels of GUS activity (Allen et al., 1996). The corresponding figure for the present assay protocol is 24%. One important component of this variation is likely to be the time (or the number of cell divisions) between transformation and assay. It is well established in animal systems that the probability of transgene silencing increases with the number of cell generations (Ronai et al., 1999; Wiersma et al., 1999; Zentilin et al., 2000), and it is known that MARs can reduce this type of progressive silencing (Wiersma et al., 1999). In the experiments we report here, transformants were cultured only 4 weeks post-transformation (5 weeks for GmHspL transformants), as compared to the 12–16 weeks we used previously. In addition, our transformants were maintained as solid calli instead of as suspension cultures. In calli, fewer cells are actively dividing and much of the total GUS activity is expected to come from cells that have undergone only a few divisions subsequent to the transformation event (e.g., see, Constabel, 1984; Constabel & Shyluk, 1994). Considering these factors, we expect control transformants to exhibit less extensive gene silencing in the present protocol than in our previous experiments with suspension cultures which is consistent with the observed results. If the principal effect of MARs is to reduce gene silencing, one would also expect a lower MAR effect when there is less silencing in control populations.

The hypothesis that MARs reduce gene silencing is consistent with our observation that the number of low-expressing GUS transformants was greatly reduced when MAR-flanked constructs were used in the transformation, both in these experiments and in our previous work. In the present experiments, we cannot rule out the possibility that low expression may sometimes be associated with the absence of a functional GUS gene, but previous results indicate that MARs can reduce the incidence of silencing in complex loci. For example, Ülker et al. (1999) found that MARs greatly reduced silencing in backcross progeny of tobacco plants that expressed the transgene as primary transformants. Similar results were obtained for rice by Vain et al. (1999).

Even when the low expressing GUS transformants are removed from the calculations, there are still

significant MAR effects for the constitutive promoters 35S, NOS, and OCS (Table 1). One interpretation of this result might be that MARs affect expression levels of active genes by a mechanism different from that by which they affect the frequency of silencing – for example, by acting as enhancer facilitators (van der Geest & Hall, 1997) or by relieving torsional strain as base unpairing regions (Kohwi-Shigematsu & Kohwi, 1990; Bode et al., 1992; deBelle et al., 1998). However, it is equally possible that this seemingly quantitative effect actually reflects the summation of qualitative effects on portions of complex transgene loci, or an effect on the frequency of silencing in various somatic cell lineages. Additional experiments will be required to resolve this question.

Exceptions to the rule that MARs reduce the number of low-expressing GUS transformants can be found in our data for the PsFed1 and GmHspL promoters. For PsFed1 and the uninduced GmHspL populations, this result may be a simple consequence of the very low inherent activities of these promoters. However, the fact that MARs do not reduce the number of low expressing transformants in induced GmHspL populations seems significant, and may reflect the fact that these calli were maintained in a non-induced, transcriptionally inactive, state until 24 h prior to harvest. However, further investigation will be required to determine the basis for this difference.

MARs offer many benefits in transgenic applications. We have shown here that the RB7 MAR can increase recovery of active transformants and raise average expression levels for a variety of promoters. These benefits were obtained without altering the specificity of environmentally or developmentally regulated promoters. MARs seem to be most beneficial when the corresponding control constructs are poorly expressed or frequently silenced, so they may prove especially useful when stable transgene expression is otherwise difficult to achieve.

Acknowledgements

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