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A tobacco matrix attachment region reduces the loss of transgene expression in the progeny of transgenic tobacco plants

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Summary

The RB7 matrix attachment region (MAR), when flanking a *uidA* (*GUS*) reporter gene, has been previously shown to increase *uidA* gene expression by 60-fold in stably transformed tobacco suspension cell lines. We have now used the same co-transformation procedure to determine the effect of flanking MARs on *uidA* gene expression in tobacco plants. The neomycin phosphotransferase selection gene and *uidA* reporter gene on separate plasmids were co-transformed into seedlings by microprojectile bombardment. In primary transgenic plants, the average *uidA* expression in plants with MARs was twofold greater than in control plants without MARs, but there was no effect on variation of expression. GUS activity was not proportional to the number of integrated *uidA* transgenes over the entire range of copy numbers. However, in the lower part of the copy number range, MAR lines show a tendency for expression to increase with copy number. Transgene expression in backcross progenies of the MAR-containing lines averaged threefold higher than in control progenies. MARs also reduced the loss of transgene expression in the BC_1 generation. Sixty-three per cent of the 21 MAR-containing primary transformants, but only 20% of the 14 control primary transformants, produced backcross progenies in which no loss of transgene expression was observed. These observations are discussed in the context of homology-dependent gene silencing.

Introduction

Matrix Attachment Regions (MARs), also known as Scaffold Attachment Regions (SARs), are DNA sequences that bind specifically to the nuclear matrix and have been proposed to organize chromatin into loop domains (reviewed by Bode *et al*., 1996). Early studies in animal systems claimed that flanking transgenes with MARs increased overall levels of transgene expression and reduced the transformant-to-transformant variation in levels of expression (Bonifer *et al*., 1990; Grosveld *et al*., 1987; Klehr *et al*., 1991; Phi-Van *et al*., 1990; Stief *et al*., 1989). In contrast, more recent studies with animal systems indicate that MARs increase the overall levels of transgene expression but do not normalize expression to make it proportional to transgene copy number (Kalos and Fornier, 1995; McKnight *et al*., 1992; Poljak *et al*., 1994).

We have shown previously (Allen *et al*., 1993; Allen *et al*., 1996) that MARs from either yeast or tobacco increase the overall levels of expression of a reporter gene, *uidA*, in tobacco suspension cells. Although the mean expression levels were increased by up to 60-fold, we saw little decrease in variation. Other reports of work in plant systems using a variety of MARs indicate that an augmenting effect on transgene expression is a common phenomenon, but the magnitude of these effects was substantially less than that previously reported on suspension cultures (van der Geest *et al*., 1994; Han *et al*., 1997; Liu and Tabe, 1998; Mlynarova *et al*., 1994; Mlynarova *et al*., 1995; Schoffl *et al*., 1993). There are several possible explanations for the reported differences in the magnitude of the MAR-mediated augmentation of transgene expression, which include target tissue, DNA delivery method, vector design and MAR sequence used.

Work presented here allows a direct comparison between tobacco plants and suspension culture cells transformed with the same tobacco RB7 MAR constructs and co-transformation methods. We found that MAR-induced augmentation of transgene expression in plants is lower than in suspension cells, and in the same range as demonstrated by others using a variety of MARs with plants transformed by *Agrobacterium*. A possible explanation of these results is that MAR effects on transgene expression are greater in rapidly dividing cells than in non-dividing, differentiated cells.

The use of plants also allowed us to examine the effect of MARs on stability of transgene expression in the progeny of the primary transformants. Transgene expression is frequently lost after one or a few sexual generations (Brandle *et al*., 1995; Finnegan and McElroy, 1994; Flavell, 1994; Hart *et al*., 1992; Kumpatla *et al*., 1997; Lowe *et al*., 1995; Lynch *et al*., 1995; Matzke *et al*., 1994). Such losses have been a major problem in transgenic plants produced by direct DNA transformation (Pawlowski *et al*., 1998). Although there are many reports

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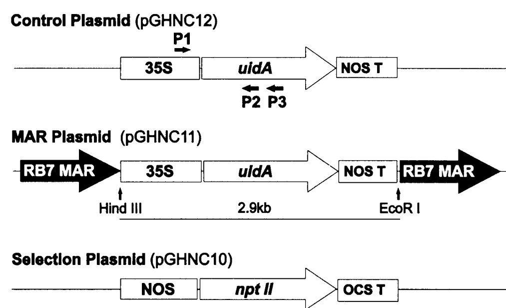

Figure 1. Schematic diagrams of plasmid constructions used in transformation experiments.
Details of plasmid construction have been published previously (Allen *et al.*, 1996). The arrows P1 and P2 indicate the locations of PCR primers used to identify transgenic plants carrying the *uidA* gene. Arrows P1 and P3 indicate the locations of primers used in estimating the copy number by quantitative PCR analysis. In plasmid pGHNC11, the chimeric *uidA* gene was flanked with two copies of the RB7 MAR (1167 bp) arranged as direct repeats. The selection plasmid contained the *nptII* gene from Tn5 of *E. coli*, driven by a NOS promoter and terminated by an OCS 3′ sequence.

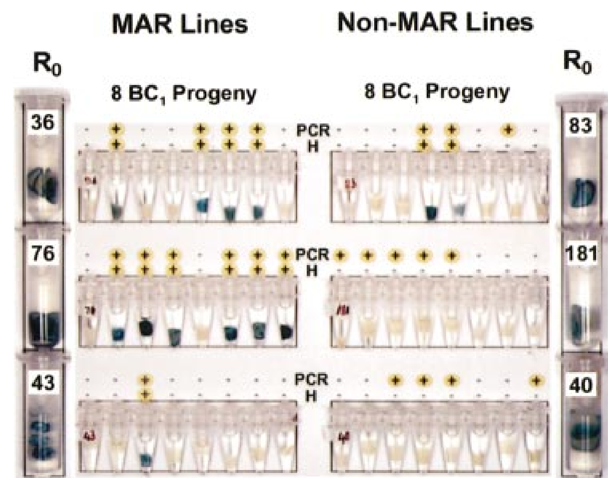

Figure 2. Examples of histochemical staining and PCR analysis for selected BC_1 lines.
R_0 histochemical staining results are compared with those for corresponding BC_1 progenies. H stands for histochemical assay and PCR for PCR analysis for the *uidA* gene. PCR results are based on the primers P1 and P2. A circled plus sign indicates positive results for particular assay and an uncircled minus sign indicates negative results. Details of histochemical and PCR procedures are described in Experimental procedures.

of MAR effects on primary transformants, the only information presently available on MAR effects in progeny is from *Agrobacterium*-derived transformants (Mlynarova *et al.*, 1996).

Our study shows that tobacco RB7 MARs flanking the reporter gene have a dramatic effect on the genetic stability of reporter gene expression. Loss of reporter gene expression occurred frequently in progenies of control transformants lacking flanking MARs. Only 20% of the 14 primary control transformants had backcross progenies in which no gene silencing was observed. In contrast, 63% of the MAR-containing primary transformants produced backcross progenies with stable transgene expression.

Results

Production of R_0 and BC_1 plants

Eleven-day-old seedlings were co-transformed by microprojectile bombardment using a 1:4 ratio of selection plasmid (pGHNC10) carrying the *nptII* gene and a *uidA* reporter plasmid as previously described (Allen *et al.*, 1993). The reporter plasmid carried a CaMV 35S promoter driving the *uidA* gene and either lacked flanking MARs (pGHNC12) or contained flanking MARs (pGHNC11) (Figure 1). We obtained 37 kanamycin-resistant lines from co-transformations with pGHNC10 and pGHNC11 (MAR lines), and 25 kanamycin-resistant lines from co-transformations with pGHNC10 and pGHNC12 (non-MAR lines).

Kanamycin-resistant R_0 plants were screened by PCR for the presence of the *uidA* reporter gene using primers P1 and P2 (Figure 1). The 532 bp amplification product included a 3′ portion of the 35S promoter and part of the 5′ end of the *uidA* coding sequence. Plants carrying the 532 bp fragment [designated PCR (+)] were used for further analysis. These included 21 R_0 MAR and 16 R_0 non-MAR co-transformed lines.

R_0 lines were crossed onto wild-type tobacco to produce backcross progeny (BC_1). Eight randomly selected BC_1 progeny from each R_0 line were analyzed. BC_1 progeny were tested by qualitative PCR to identify plants carrying the *uidA* gene. Two non-MAR R_0 lines, out of the 30 that were tested, did not pass the PCR amplifiable sequence to BC_1 progeny.

Histochemical assay results

The histochemical procedure of Jefferson (1987) was used to identify transformants that had β-glucuronidase (GUS) activity, and to determine whether MARs affected the pattern of expression within individual plants. Punches from three adjacent leaves from each R_0 plant were assayed. Twelve of 16 non-MAR and 16 of 21 MAR lines produced blue staining leaf punches and were therefore designated GUS (+). No overt difference in intensity or pattern of staining was associated with the presence of MARs (results not shown). Furthermore, the fraction of non-expressing transgenic lines, as determined by this assay, was approximately equal between MAR and non-MAR arrays (5/21 and 4/16, respectively).

Because the RB7 MAR was originally isolated from a clone containing a root specific gene (Hall *et al.*, 1991), we also tested roots for expression. Root and leaf samples from kanamycin resistant R_0 plants were analyzed by the

Table 1. Summary of histochemical staining assay results for BC_1 progeny

	Non-MAR lines		MAR lines	
	Total	%	Total	%
R_0 generation				
R_0 lines tested	14		21	
R_0 lines expressing *uidA*	10	71	16	76
BC_1 generation				
R_0 lines with non-silenced progeny	2/10	20	10/16	63
R_0 lines with completely silenced progeny	6/10	60	1/16	6
R_0 lines with partially silenced progeny	2/10	20	5/16	31
Total PCR (+) BC_1 individuals	46/107	43	82/165	50
PCR (+) BC_1 individuals expressing *uidA*	8/46	17	47/82	57

R_0 lines with non-silenced progeny are those in which the presence of the PCR amplified *uidA* fragment (PCR +) was 100% correlated with GUS activity (GUS +) as evidenced by histochemical staining assay. R_0 lines with completely silenced progeny are those in which all of the progeny carrying the PCR amplified fragment failed to express *uidA* gene. R_0 lines with partially silenced progeny are those in which the presence of the PCR amplified *uidA* fragment were not always correlated with GUS activity. None of the lines that had a PCR amplified fragment but failed to express the *uidA* gene in the R_0 generation produced *uidA* expressing BC_1 progeny.

histochemical GUS assay. When *uidA* was expressed in leaves, expression was also typically observed in roots (data not shown). Although some plants expressed *uidA* only in leaves, none of the plants tested expressed the gene only in roots. Similar results were obtained with both MAR and non-MAR lines.

BC_1 progeny from R_0 plants that expressed *uidA* were also analyzed by the GUS histochemical assay and by PCR using the same primers used to detect the *uidA* transgene in R_0 plants. Eight progeny from each R_0 line tested were assayed (Figure 2). Ten of 16 (63%) GUS (+) R_0 MAR lines produced BC_1 progeny in which GUS activity was detected in all progeny that carried the *uidA* gene (Table 1). However, only 2 of 10 (20%) non-MAR lines produced BC_1 progeny in which the presence of the *uidA* gene was correlated with expression. A Chi-square test showed that there was a statistically significant difference between MAR and non-MAR lines.

MAR and non-MAR R_0 transformants produced similar numbers of PCR (+) BC_1 progeny. Approximately 43% (46 of 107) non-MAR BC_1 and about 50% (82/165) of MAR BC_1 progeny were PCR (+). However, the proportion of PCR (+) progeny that expressed the gene differed greatly between MAR and non-MAR progeny arrays. Table 1 shows that GUS activity was detected in approximately 57% (47 of 82) of PCR (+) BC_1 plants with MARs, but in only about 17% (8 of 46) of PCR (+) non-MAR plants.

Some GUS (+) R_0 lines yielded BC_1 progeny in which the transgene expression was completely lost. Comparison of staining results with PCR data revealed that 6 of 10 (60%) of the non-MAR GUS (+) R_0 lines produced BC_1 progeny in which the transgene was inactive in all PCR (+) progeny tested. In contrast, only 1 of 16 (6%) GUS (+) R_0 MAR lines produced progeny in which the transgene was completely inactive (see lines 181 and 40 in Figure 2 as examples).

Other GUS (+) R_0 lines produced BC_1 progeny in which the transgene was inactive in some progeny, but not in others, despite PCR (+) results in all cases (e.g. line 83 in Figure 2). Five of 16 (31%) GUS (+) R_0 MAR lines and 2 of 10 (20%) GUS (+) R_0 non-MAR lines were in this category.

PCR results were corroborated by Southern blot analysis of a random sample of PCR (+) plants. Results for 24 R_0 plants (12 MAR and 12 non-MAR) and 24 BC_1 progeny (12 MAR and 12 non-MAR) that were PCR (+) are shown in Figure 3.

Determination of transgene copy number by quantitative PCR

Transgene copy number was determined by a quantitative PCR assay. In the R_0 generation, non-MAR lines carried an average of 21 integrated copies of the transgene per nucleus, while MAR lines had an average of eight copies. This observation was consistent with results reported previously (Allen *et al.*, 1993; Allen *et al.*, 1996). Copy number in non-MAR lines ranged from 2 to 75, while the number of copies in MAR lines varied from 1 to 60.

The distribution of *uidA* copy numbers in the primary transformants is shown in Figure 4(a). Although the difference in average number of transgene copies between MAR and non-MAR populations is statistically significant, a substantial proportion of this difference is attributable to two non-MAR lines (102 and 123), each of which carried more than 70 copies. Copy number variance in non-MAR lines was significantly higher than in MAR lines. If these two high copy lines (102 and 123) were excluded from the analysis, copy number was not significantly different between MAR and non-MAR populations.

The presence of MARs did not affect average transgene copy number in progeny. BC_1 progeny of both MAR and non-MAR lines carried an average of eight copies. The apparent reduction in average copy number in the non-MAR group is caused by under-representation of some high copy R_0 lines in the BC_1. No progeny carrying the *uidA* sequence were recovered among BC_1 arrays from two non-MAR R_0 lines, 112 and 123, which carried 10 and 74 copies, respectively. Additionally, R_0 lines with large numbers of transgene copies produced very few PCR (+)

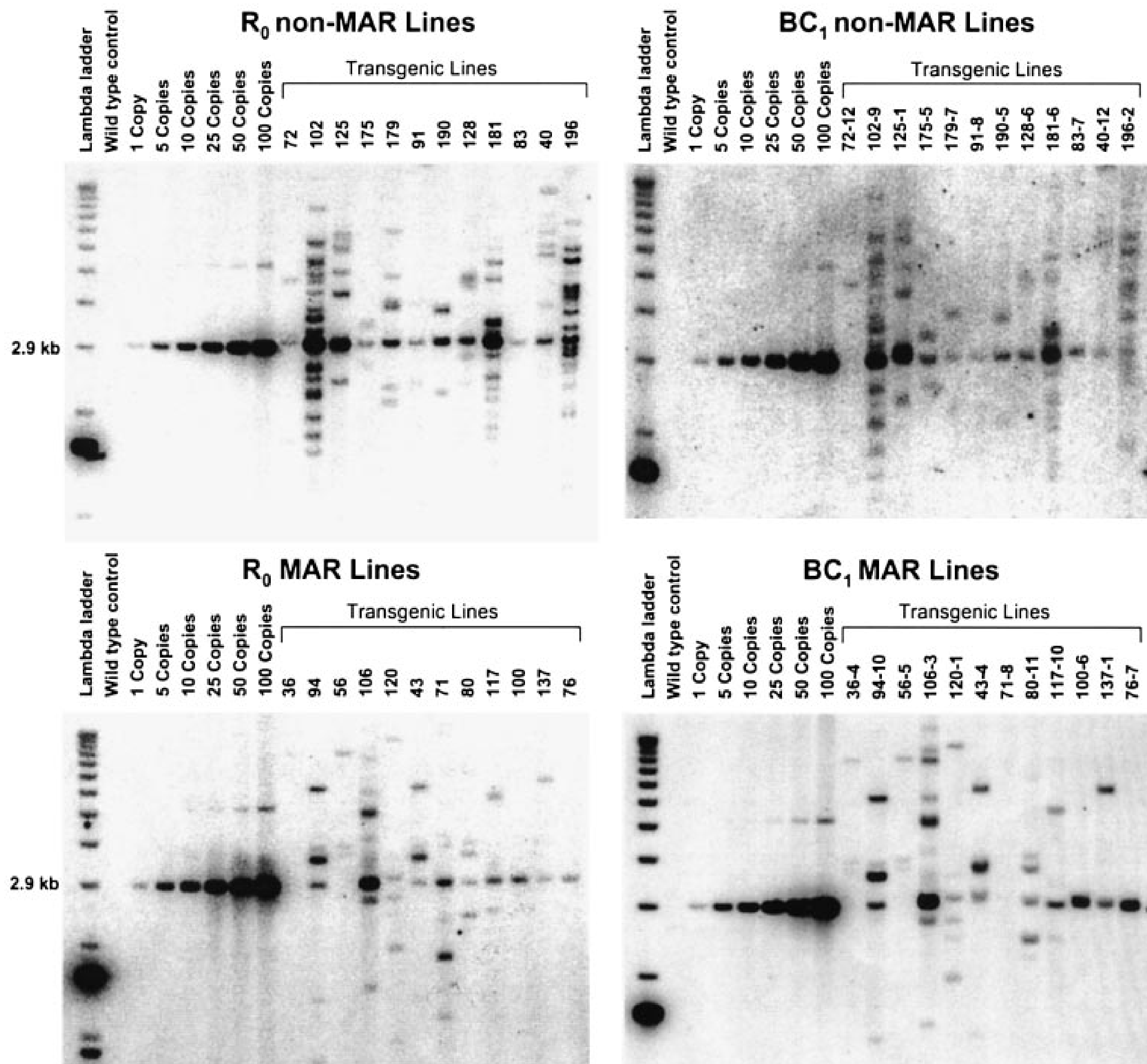

Figure 3. Integration patterns of the *uidA* gene determined by Southern blot analysis.
Twelve randomly chosen control and 12 MAR lines were analyzed in the R_0 and BC_1 generations. One randomly selected BC_1 progeny plant from each R_0 line was assayed. Ten μg genomic DNA was digested with *Hin*dIII and *Eco*RI and fractionated in a 0.8% agarose gel. The DNA was blotted onto a nylon membrane and probed with a ^{32}P-labeled 2.9 kb fragment isolated from MAR plasmid, as described in Experimental procedures. Copy number reconstruction lanes contain 10 μg of non-transformed (control) genomic DNA spiked with 0, 1, 5, 10, 25, 50 and 100 copies of the 8116 bp MAR plasmid per nucleus based on the assumption that tobacco genome size is 6.6 pg/2C (Bennett and Smith, 1991).

progeny. For example, non-MAR line 125 produced only one PCR (+) individual out of eight progeny tested. The PCR (+) progeny had the same copy number (52) as the R_0 parent. The distribution of *uidA* copy number in the BC_1 generation is shown in Figure 4b.

DNA blot analysis

Southern blot analyses were carried out on R_0 and BC_1 plants to examine patterns of transgene integration. Genomic DNA from 12 randomly chosen MAR lines and 12 non-MAR lines was digested with *Hin*dIII and *Eco*RI. Digestion with these enzymes was expected to release a fragment of 2.9 kb, including the promoter, *uidA* coding sequence and the terminator, if no rearrangement had occurred (Figure 1). However, as can be seen in Figure 3, digestion of genomic DNA produced complex fragment patterns. Such patterns are typical of transgenics produced by direct DNA transfer (Allen *et al.*, 1996; Gordon-Kamm *et al.*, 1990; Kumpatla *et al.*, 1997; Peng *et al.*, 1995; Scheid *et al.*, 1991; Tomes *et al.*, 1990; Wan and Lemaux, 1994). Similarly complex patterns were seen in both MAR and non-MAR lines.

Although PCR analysis indicated the presence of the

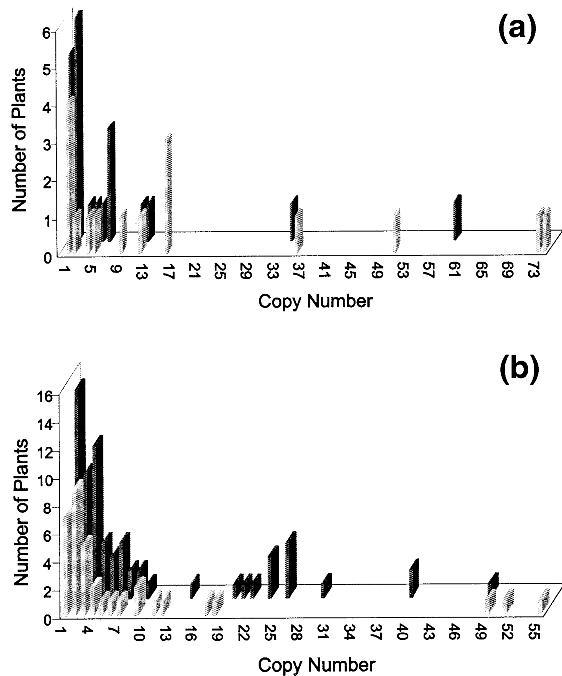

Figure 4. Copy number distribution in the R_0 and BC_1 generations.
The copy number distribution in the R_0 generation (a) and the BC_1 generation (b) are shown. Lighter bars in the front row represent the number of control plants in each copy number category and darker bars in the back row represent the number of MAR plants in each copy number category.

uidA gene in R_0 lines 82, 60 and 44, no GUS activity was detected in these lines by either histochemical or luminometric GUS assays. Some hybridizing bands larger or smaller than 2.9 kb were observed, but Southern blot analysis failed to yield the expected 2.9 kb band in these lines, suggesting that rearrangement had occurred (data not shown).

Southern analysis was also performed on the BC_1 progeny representing the same lines analyzed in the R_0 generation. A single, randomly chosen PCR (+) BC_1 plant was chosen to represent each R_0 line used for the analysis (Figure 3). Fragment profiles are the same for R_0 and BC_1 generations in both MAR and non-MAR lines, suggesting that the transgene inserts are linked and are not segregated in the backcross progeny. This result is consistent with previous reports indicating that transgenes are frequently integrated into a single chromosomal locus and do not segregate from each other in crosses (Cooley *et al.*, 1995). Although the identity of fragment size and intensity in a few BC_1 progenies are not clear for some of the lines (e.g. lines 72 and 40 in Figure 3), additional Southern analyses (including analyses of doubled haploid lines generated from the same R_0 plants) confirm that both the fragment sizes and relative intensities are identical to the corresponding R_0 plants (data not shown). We conclude, therefore, that the loss of transgene expression is due to gene silencing rather than to segregation of active and inactive loci or to deletion or rearrangements in the progeny.

Quantitation of GUS activity

A luminometric assay (Bronstein *et al.*, 1994) was used to quantify GUS activity in R_0 and BC_1 plants. Leaf punches (1 cm) from the third fully opened leaf (from top) from plants that had just initiated flowering were assayed. The mean expression in R_0 plants was 48.7 fg GUS μg^{-1} of total soluble protein in non-MAR lines, and 99.1 fg μg^{-1} in MAR lines. GUS levels ranged from 2.9 to 269 fg μg^{-1} in non-MAR lines, and from less than 1–686 fg μg^{-1} in MAR lines. Median values were 9.4 fg μg^{-1} in non-MAR lines and 39.1 fg μg^{-1} in MAR lines.

GUS activity data from R_0 plants were skewed toward low values, so that a logarithmic transformation was required prior to statistical analysis (Nap *et al.*, 1993). Logarithmic transformation of the non-MAR and MAR R_0 GUS activity data resulted in a normal distribution (*W*-test for normality, Shapiro and Wilk, 1965). Although R_0 MAR lines exhibited a mean GUS activity level twice that observed in non-MAR lines, this difference was not statistically significant in either untransformed or transformed data. Similarly, variance and coefficient of variation (CV%) among MAR lines were also not statistically different from that of non-MAR lines.

Eight BC_1 progeny from each R_0 line were tested. Progeny of MAR and non-MAR lines were grown together to minimize environmental variation. The eighth leaf (counted from the base of the plant, including cotyledons) from PCR (+) progeny was harvested when it reached a width of 3 cm and a length of 5 cm. Average GUS concentration in extracts from non-MAR plants was 235 fg GUS μg^{-1} total protein, while MAR plants averaged 712 fg μg^{-1}. Expression ranged from 0 to 1925 fg μg^{-1} in the non-MAR group and between 0 and 11971 fg μg^{-1} in the MAR group. The median was 44 fg μg^{-1} for non-MAR lines and 121 fg μg^{-1} for the MAR group.

GUS activity data from BC_1 plants were also skewed toward low values and, in this case, logarithmic transformation failed to normalize the data. Therefore, statistical analysis was carried out using procedures that are less sensitive to the effects of skewed data [Wilcoxon Mann–Whitney two-sample *Z*-test (Steel and Torrie, 1980) and O'Brien's W50 test (O'Brien, 1981) which is a modification of Levene's test of homogeneity of variances (Snedecor and Cochran, 1980)]. Analysis of untransformed data with these tests indicated that mean GUS activity in MAR lines was significantly higher (threefold) than in non-MAR lines. The presence of MARs did not affect either the variance or the coefficient of variation. Details of statistical analyses can be obtained from the corresponding author.

Relationship between expression level and transgene copy number

Figure 5 shows the distribution of GUS activity as a function of *uidA* copy number. Analysis of both R_0 (Figure 5a) and

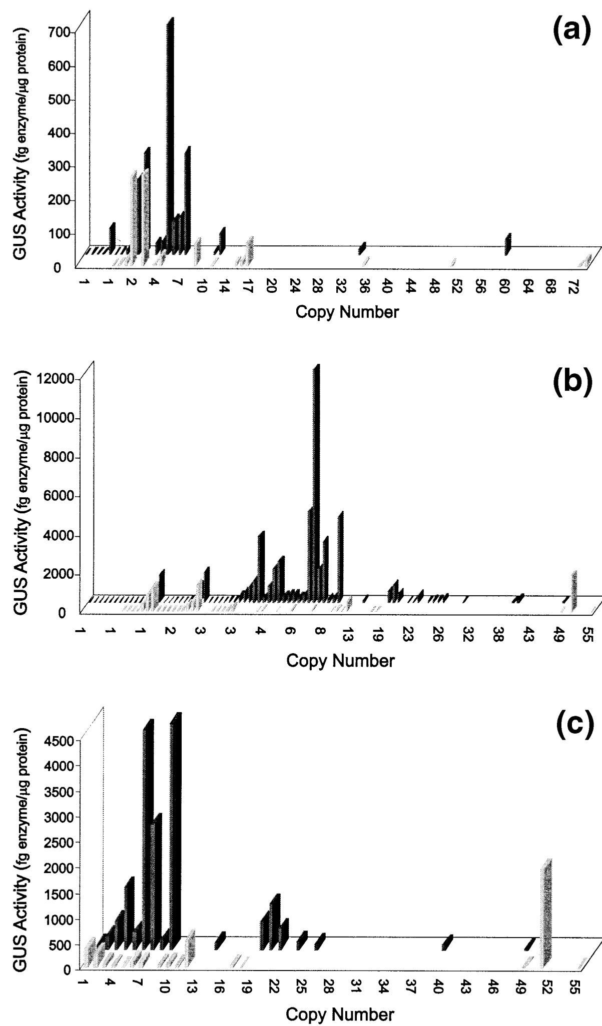

Figure 5. *uidA* expression as a function of gene copy number in stably transformed lines.
GUS specific activity versus copy number is plotted for R_0 plants (a) and BC_1 progenies (b). Each bar represents an independent R_0 transformant or a backcross progeny plant. Darker bars (back row) represent individual MAR transformants and lighter bars (front row) represent individual non-MAR transformants. In (c) mean GUS specific activity for all BC1 plants having the same copy number was plotted versus the copy number. R_0 plants and BC_1 progenies were analyzed for *uidA* gene copy number by a quantitative PCR assay and GUS activity was measured by a luminometric assay, as described in Experimental procedures.

BC_1 (Figure 5b,c) indicated that neither non-MAR nor MAR lines showed a correlation between copy number and expression over the full range of copy numbers. MAR and non-MAR lines, however, showed dramatic differences in the shape of these distributions. In non-MAR lines the expression was reduced as the copy number increased, while in MAR lines expression showed a strong tendency to increase with copy number up to about nine copies. This tendency is most clearly seen in Figure 5c which shows average GUS activity for each copy number class rather than for each individual plant.

Regression analysis of R_0 data up to seven copies revealed a significant correlation between copy number and expression level in MAR lines, but not in non-MAR lines. The same analysis on the BC_1 data also indicated a significant correlation between copy number and expression in MAR lines up to nine copies, but not in non-MAR lines.

Discussion

This work allowed us to compare the effects of flanking tobacco RB7 MAR sequences on transgene expression in tobacco plants and suspension cultures transformed by direct DNA transfer. Importantly, the use of plants allowed us to investigate the effects of MARs on transgene expression in a subsequent generation.

On average, R_0 plants transformed with the MAR-flanked transgene produced approximately twice as much GUS enzyme activity as plants transformed with the non-MAR construct. In arrays of BC_1 progeny from these plants, MAR lines had an average of threefold higher GUS activity than non-MAR lines. The increased MAR effect in the second generation seems to be attributable to a higher silencing frequency in the control lines, which was not seen in MAR lines. Experiments currently in progress to determine whether the reduction in gene silencing will continue into further generations.

The magnitude of these MAR effects is similar to that for several comparable experiments with 35S::*uidA* constructs in tobacco plants transformed with *Agrobacterium* vectors. Thus, van der Geest *et al.* (1994) observed an approximately threefold increase with a phaseolin MAR and Mlynarova *et al.* (1994, 1995) observed two- to fourfold increases with the 'A element' MAR from the chicken lysozyme gene. Two other groups have reported larger MAR effects on 35S::*uidA* constructs. Liu and Tabe (1998) have recently reported that a 2.0 kb *Arabidopsis* MAR increases median expression in a population of transgenic tobacco plants by five- to 10-fold. It is difficult to compare these median values to mean values, and it may be significant that these authors assayed GUS activity in leaves from plants maintained in sterile culture rather in a greenhouse or growth chamber. However, as Liu and Tabe note, it is important to be aware that different MARs may have different effects. Another large MAR effect was reported by Han *et al.* (1997) in poplar explants. These authors used a binary vector containing the tobacco RB7 MAR and observed a five- to 10-fold increase in production of kanamycin-resistant callus and shoots as well as a similar increase in total GUS activity in stem explants during the first month after co-cultivation. However, these experiments did not separate effects on transformation efficiency from effects on gene expression. Therefore, the MAR effect, although impressive, is not directly comparable

to measurements in the leaves of fully developed transgenic plants.

One of the larger MAR effects observed so far in plant leaf tissue was reported by Schoffl *et al.* (1993), who obtained five- to ninefold increases in the expression of a *uidA* gene driven by a heat shock promoter in transgenic tobacco plants. Perhaps importantly, the expression assay in these experiments involved measuring GUS accumulation only during a 2 h period after activation of the previously inactive promoter, a procedure that should provide a more direct measure of transcriptional potential than obtained by analyzing steady state levels of enzyme activity.

It is well-documented that direct DNA transfer often leads to complex arrangements and multicopy insertions, which can increase the frequency of gene silencing in both plants (Cooley *et al.*, 1995; Kumpatla *et al.*, 1997; Kumpatla *et al.*, 1998) and animals (reviewed in Henikoff, 1998; Wolffe, 1997). If MARs act to reduce gene silencing, one might therefore expect MAR effects to be larger in plants transformed by microprojectile bombardment than in plants transformed with *Agrobacterium* vectors. However, our results with plants indicate that MAR effects in our system are similar to those in comparable experiments with *Agrobacterium*-mediated transformation. Thus, most of the differences between our initial results and those of other groups are likely to reflect differences between suspension cultures and plant leaf tissue, rather than differences in transformation procedures or integration patterns.

A model for MAR effects

Laemmli and co-workers (Käs *et al.*, 1993; Poljak *et al.*, 1994; Zhao *et al.*, 1993) have proposed a 'chromatin-opening' model in which HMG I/Y proteins are intimately involved in MAR function. Both HMG I/Y (Reeves and Nissen, 1990) proteins and H1 histone (Izaurralde *et al.*, 1989) proteins have been shown to bind to the AT-tracts found in MARs. The chromatin-opening model suggests that HMG I/Y type proteins interact with MARs to displace H1 histones, and thus to disrupt the co-operative interactions that lead to a condensed chromatin structure. Histone H1 displacement by HMG I/Y proteins could 'open' chromatin and permit access to DNA by RNA polymerase and other factors required for transcription.

A study of MAR effects in transgenic mice has shown that MARs have a great effect on transgene expression in rapidly growing embryonic tissues in contrast to developed mouse tissues (Thompson *et al.*, 1994). Further work demonstrated that HMG I/Y protein levels are elevated in the undifferentiated and rapidly dividing embryonic cells, in contrast to differentiated cells. These data led to the proposal that HMGI/Y levels may be important for mediating the MAR effect (Thompson *et al.*, 1995). We speculate that the differences we observed in RB7 MAR effects might reflect a lower abundance of high mobility group (HMG) proteins in differentiated leaf cells as opposed to rapidly dividing, undifferentiated suspension culture cells.

Generational stability

Methods of stabilizing transgene expression are of interest in biotechnology because transgene expression is often lost over the course of one or more generations following the initial transformation (Assaad *et al.*, 1993; Brandle *et al.*, 1995; Finnegan and McElroy, 1994; Flavell, 1994; Hart *et al.*, 1992; Hobbs *et al.*, 1990; Kilby *et al.*, 1992; Linn *et al.*, 1990; Lowe *et al.*, 1995; Lynch *et al.*, 1995; Meyer and Heidman, 1994; Meyer *et al.*, 1992; Kumpatla *et al.*, 1997; Pawlowski *et al.*, 1998). In order to assess the generational stability of transgene expression in our system, we compared the frequency of GUS-positive individuals in populations of primary transformants and their backcross progeny.

In the R_0 generation, histochemical staining revealed no substantial difference between the frequencies of expression in the MAR and non-MAR populations. Very different results were obtained in the BC_1 generation, however. Histochemical analysis revealed that transgene expression was completely lost between the R_0 and BC_1 generations in 60% of the non-MAR transformed lines, but in only 6% of the MAR transformed lines. Perhaps more importantly, 63% of R_0 plants containing MAR constructs gave rise to BC_1 progeny in which the presence of *uidA* DNA was always associated with GUS enzyme activity, while only 20% of non-MAR plants met this criterion. Comparisons of Southern profiles obtained for R_0 and BC_1 plants indicate that loss of expression between generations is due to silencing rather than segregation, deletion or rearrangements, as noted above (Figure 3). We suggest, therefore, that flanking MARs reduce the frequency of transgene silencing from one generation to the next. Further experiments will be designed to determine if this MAR effect persists in subsequent generations.

Copy number effect

MAR effects are seen almost exclusively in low copy number transformants. Transgene expression shows a tendency to increase with copy number up to 9–10 copies, but is sharply reduced even in MAR lines when the copy number exceeds this value. We have previously suggested that one explanation for this observation could be that MARs reduce DNA pairing interactions that would otherwise lead to homology-dependent transcriptional gene silencing, but that this effect is lost at higher copy numbers (Allen *et al.*, 1996). However, the sharpness of the 'threshold' for loss of expression (see Figure 5c), together with recent evidence supporting a threshold model for post-transcriptional gene silencing (Jorgensen *et al.*, 1998;

Lindbo *et al.*, 1993; Que *et al.*, 1997; Smith *et al.*, 1994; Tanzer *et al.*, 1997), suggests another interpretation. According to this model MARs would, indeed, reduce transcriptional gene silencing, but a decline in expression at higher copy numbers would occur when the increased transcriptional activity pushes GUS mRNA above a threshold value to trigger a post-transcriptional degradation process such as that described by Tanzer *et al.* (1997). Consistent with this hypothesis, we originally observed a higher copy number threshold in experiments with the yeast ARS-1 MAR (Allen *et al.*, 1993), which had a smaller overall effect on gene expression. If the model is correct, we would also predict that positive MAR effects would be observed at higher copy numbers with weaker promoters, or if only initial transcription rates rather than RNA or protein accumulation were measured.

In summary, this study provides insight into several critical issues. First, the effects we see with the RB7 MAR in plants transformed with microprojectile bombardment are, in general, quite comparable to those seen for other MARs in plants transformed with T-DNA vectors. Thus the very large MAR effect we observe in suspension culture cells transformed by microprojectile bombardment (Allen *et al.*, 1993; Allen *et al.*, 1996) is most likely attributable to our use of rapidly dividing, undifferentiated cells rather than to some unique property of the RB7 MAR or to the complexity of the insertion events we obtain with direct DNA delivery. Second, although MARs increase expression, they do not significantly reduce variation among individuals in our R_0 or BC_1 transformant populations. We postulate that much of the remaining variation derives from post-transcriptional silencing events and that the 9–10 copy 'threshold' above which MAR effects decline is related to an mRNA expression threshold above which homology-dependent RNA degradation is initiated. Most importantly, we have shown that MARs reduce the loss of expression that would otherwise occur between the R_0 and BC_1 generations. Additional experiments are needed to determine whether such MAR effects can continue for many generations, but our data provide an indication of how 'chromatin elements' such as MARs may be useful in a practical context.

Experimental procedures

Transformation vectors, explant preparation and transformation

Plasmids used for microprojectile bombardment are shown in Figure 1, and details of their construction have been published previously (Allen *et al.*, 1996). *Nicotiana tabacum* cv Petite Havana SR1 seeds were sterilized with three successive washes of 10% Clorox and 1% Tween 20 for 3 min and rinsed twice with sterile, distilled water. Rinsed seeds were spaced approximately 5 mm apart on MS basal medium (4.4 g l^{-1} MS salts (Murashige and Skoog, 1962) (Sigma), 1 ml l^{-1} 1000X B-5 vitamins (Gamborg *et al.*, 1968) (Sigma), 30 g l^{-1} sucrose, 8 g l^{-1} agar, pH 5.6) in 100 mm × 20 mm Petri plates. Plates were sealed with Parafilm® and seeds were germinated in a growth chamber with 18 h light/6 h dark, at 25–27°C. The seedlings were bombarded in the PDS-1000/He (BioRad) after 11–14 days. Eleven-day-old seedlings had two cotyledons and two young leaves. Bombardment parameters were as follows: rupture disk pressure of 650 psi, 1.6 μm gold particles, seedlings placed on the second slot from the microcarrier launch assembly and 700 mmHg of vacuum. MAR plasmid transformation mixtures contained 400 ng of pGHNC11 and 100 ng pGHNC10, whereas the control transformation mixtures contained 400 ng of pGHNC12 and 100 ng pGHNC10. Each plate was bombarded twice. Following bombardment the plates were resealed and returned to the growth chamber for 2 days. Cotyledons and young leaves from bombarded seedlings were excised and transferred onto callus induction medium which consists of MS basal medium plus (per litre) 0.5 mg 6-benzylaminopurine and 2 mg naphthalene acetic acid. The final pH was adjusted to 5.6. Leaves were put onto media with adaxial surfaces in contact with the media. The explants were transferred after 3–5 days onto shoot induction medium which consists of MS basal medium plus (per litre) 1.25 mg 6-benzylaminopurine and 100 mg kanamycin at a pH of 5.7. Explants were transferred to fresh shoot induction medium at 2 week intervals until shoots were obtained which could be excised for rooting. Shoots were transferred onto rooting medium, which consists of MS basal medium plus (per litre) 0.5 mg indoleacetic acid and 100 mg kanamycin at a pH of 5.6. Shoots that rooted on kanamycin containing medium were transferred to pots and placed in the greenhouse.

Backcrossing transgenic R_0 plants

BC_1 families were produced by crossing wild-type SR1 pollen donor plants onto R_0 transgenic females. BC_1 seed resulting from the backcrosses were then germinated to produce progeny arrays (families) for analysis.

Histochemical staining assay for β-glucuronidase (GUS)

Leaf disks (1 cm each) of R_0 plants were taken from three adjacent fully expanded leaves using a cork borer and put into microfuge tubes containing 2 ml of a 0.5 mg ml^{-1} solution of 5-bromo-3-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) (Jefferson, 1987) supplied from Clontech for histochemical staining. After overnight incubation at 37°C, leaf disks were cleared in 70% ethanol.

For BC_1 plant histochemical analysis, one leaf disk (6 mm) was taken from the seventh leaf from the bottom counting cotyledons of 30–40-day-old plants grown in growth chambers.

DNA isolation

DNA was isolated from frozen tissue collected from transgenic tobacco plants using a modified CTAB extraction procedure of Murray and Thompson (1980) as described by Johnson *et al.* (1995).

Qualitative PCR analysis

The presence of the *uidA* gene in putatively transformed plants was confirmed with qualitative PCR. Amplification was carried out using a sense primer (P1) (5′-ATGACGCACAATCCCACTAT-3′) and

an antisense primer (P2) (5′-GCCAGTTCAGTTCGTTGTTC-3′) which yielded a 532-bp product if the gene was present (see Figure 1). PCR was performed for 28–30 cycles. Amplification conditions were: 5 min at 94°C, followed by 28 cycles of 30 sec each at 94°C, 30 sec at 63°C, 30 sec at 72°C, and 7 min at 72°C. Amplification was carried out in a 25 μl volume consisting of final concentration of 50 mM KCl, 10 mM Tris–HCl (pH 9.0 at 25°C), 0.1% Triton® X-100, 1.5 mM $MgCl_2$, 20 μM dideoxynucleotide mixture (Promega), 500 nM sense primer, antisense primer, 6.25 units Taq polymerase (Boehringer Mannheim), and 75 ng template DNA. Samples were separated by electrophoresis in a 1.2% agarose gel in TBE buffer (Sambrook *et al*., 1989).

Quantitative PCR analysis

Transgene copy number was determined by quantitative PCR assay using primers P1 and P3 (Figure 1). Amplification was carried out using the sense primer (P1, 5′-ATGACGCACAATCCCACTAT-3′) and an antisense primer (P3, 5′-TGGTGTAGAGCATTACGCTG-3′). The total size of the PCR product was 646 bp. Amplification was carried out under the following conditions: 5 min at 94°C; 18 cycles of 1 min at 94°C, 1 min at 64°C and 1 min at 72°C; and 7 min at 72°C. Amplification reactions were carried out in a microtiter plate. Each well contained 50 μl of reaction mixture described above supplemented with 50 μg bovine serum albumin. One hundred ng of template DNA was included in each reaction. Samples were separated by electrophoresis in a 0.8% agarose gel in TBE buffer. Gels were blotted and the blots hybridized with labeled probe as described below. Signal intensities for standards and unknown samples were determined with a Molecular Dynamics phosphorimager and the associated software.

DNA blot analysis

Ten μg of plant genomic DNA was digested with *Eco*RI and *Hin*dIII (Boehringer Mannheim) in the same reaction buffer for 5 h in a reaction volume of 50 μl, containing 100 units of *Eco*RI and 100 units of *Hin*dIII according to the instructions of the manufacturer. Copy number reconstructions contained 10 μg of non-transformed (control) genomic DNA spiked with 0, 1, 5, 10, 25, 50 and 100 copy number equivalents of the 8116 bp MAR plasmid (pGHNC11) per nucleus. The fragments were then separated by electrophoresis for 16 h in a 0.8% agarose gel in TBE and blotted onto MagnaGraph nylon transfer membranes (Micron Separations) by overnight capillary transfer according to Sambrook *et al*. (1989). Membranes were pre-hybridized at 65°C for 3 h and hybridized overnight at 65°C using a 2.9 kb gel-isolated *Hin*dIII and *Eco*RI fragment from pGHNC11 (Figure 1). The probe was ^{32}P-labelled (NEN-DuPont) by random-primed synthesis (Boehringer Mannheim) according to the manufacturer's instructions. Hybridized membranes were then placed on BioMax MS intensifying screens (Kodak) with BioMax film (Kodak) and exposed for 2 days at –80°C.

Quantitative GUS assays

Primary transformants. The putative kanamycin resistant transformants that rooted were transferred to the greenhouse. Since the speed of growth differed from transformant to transformant, we used flowering times to determine sampling times. Samplings for the luminometric assay were carried out just prior to flowering. At this time the flower buds had just appeared on the plants. Five leaf punches for each transformant were collected from the third fully open leaf from the top using a cork borer (1 cm) and snap frozen in liquid nitrogen. The GUS activity was analyzed using the 'Plant GUS-Light' kit (Tropix, Bedford, MA, USA). Leaf disks were ground in liquid nitrogen using a Kontes pestle. Three hundred μl of Plant Lysis Buffer (Tropix plant GUS-Light kit) which contains 50 mM sodium phosphate pH 7, 10 mM EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100) was then added to each sample and the mixture was incubated for 5 min at room temperature. The sample was centrifuged and 10 μl supernatant was incubated with the chemiluminescent substrate for 30 min at room temperature. The amounts of reagents added were reduced threefold to accommodate the use of an automated microtiter-plate luminometer (Lumistar, BMG Industries). Reactions were stopped by the addition of 100 μl light emission accelerator and signal intensities were measured. Purified β-glucuronidase (Clontech) was used for standardizing the assay. Buffer controls and extracts from untransformed control plants were also included in the experiments. The value obtained with extracts of untransformed plant was subtracted from the values obtained for other samples.

Backcross progenies. The youngest leaf (eighth leaf counting the cotyledons, approximately 3 cm wide and 5 cm long) from 6-week-old plants grown at 28°C and an 18 h light/6 h dark cycle were collected as a whole into microfuge tubes and stored in –80°C until assayed. The samples were ground in liquid nitrogen and 500 μl Plant Lysis Buffer were added to each sample. Samples were assayed by the luminometric GUS assay described above.

Protein content of the extracts was estimated in a microtiter plate reader (Bio-Tek Instruments) by the Bradford assay (Bradford, 1976) (Bradford Protein Assay Dye, Bio-Rad, Hercules, CA, USA). Standards were prepared from bovine gamma globulin diluted in Plant Lysis Buffer. GUS activities that were determined from the same samples were normalized using the total protein data.

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