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The Influences of Two Plant Nuclear Matrix Attachment Regions (MARs) on Gene Expression in Transgenic Plants

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Nuclear matrix attachment regions (MARs) are thought to influence gene expression by anchoring active chromatin to the proteinaceous nuclear matrix. In this study, two plant DNA fragments with strong MAR activity were selected and tested for their effects on expression of a linked reporter gene in transgenic tobacco. One MAR was isolated from the 5′ flanking region of a pea vicilin gene previously reported to be expressed in a copy number-dependent manner in transgenic tobacco. A second MAR was isolated from the genome of *Arabidopsis thaliana* by preselection for autonomously replicating sequence (ARS) activity in yeast. Flanking copies of the *A. thaliana* MAR stimulated median reporter gene expression in transgenic plants by five to ten fold. Neither MAR significantly reduced the variation in transgene expression between individual transformants, or conferred copy number-dependence in gene expression.

Key words: Autonomously replicating sequence — Copy number-dependence — Genomic position effect — Scaffold attachment region — Tobacco — Transgene expression.

It has long been observed that a gene transferred to plants is expressed at levels that vary widely between individual transformed plants containing the gene at different genomic locations (Peach and Velten 1991). This occurs despite the initial selection of transgenic individuals expressing a linked selectable marker gene at a level sufficient to overcome the toxicity of a selection agent such as a herbicide or antibiotic. There has been considerable interest in dampening or eliminating these so-called position effects using nuclear matrix attachment regions.

Abbreviations: A, adenosine; ARS, autonomously replicating sequence; *At, Arabidopsis thaliana*; *bar*, bialaphos resistance gene from *Streptomyces hygroscopicus*; *ftz, fushi tarazu* gene from *Drosophila melanogaster*; GUS, β-glucuronidase; ln, natural log; MAR, matrix attachment region; *ocs*, gene encoding octapine synthase from *Agrobacterium tumefaciens*; SAR, scaffold attachment region; T, thymidine; T-DNA, transferred DNA (by *A. tumefaciens*); *uidA*, gene encoding β-glucuronidase from *Eschericia coli*; *ura3*, gene encoding orotidine-5′-phosphate carboxy-lyase from *Saccharomyces cerevisiae*; *vic, vicilin* gene from *Pisum sativum*; 35S promoter, promoter for the 35S transcript of the cauliflower mosaic virus.

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Nuclear scaffold or matrix attachment regions (SARs or MARs) are fragments of genomic DNA defined by their ability to bind to a proteinaceous nuclear matrix preparation in vitro (Mirkovitch et al. 1984, Cockerill and Garrad 1986). It has been suggested that in the context of plant research, the term MAR be used in preference to SAR in order to avoid confusion with the same abbreviation used for systemic acquired resistance (van der Geest et al. 1994). The MARs that have been characterised from plants are very similar to MARs from animal origins in that they have a high content of A/T and contain multiple copies of several short consensus sequences (Slatter et al. 1991, Breyne et al. 1992b, Schoffl et al. 1993, van der Geest et al. 1994). MARs are thought to represent the boundaries of functional chromatin domains (Phi-Van and Stratling 1988) and the anchor points of chromatin loops visualised by microscopy of metaphase chromosomes (Gasser and Laemmli 1987). The affinity of MARs for the nuclear matrix is conserved between widely differing groups of organisms. A MAR from one organism will bind to nuclear matrices from many different sources, including yeasts, animals and plants (Mielke et al. 1990, Breyne et al. 1992b, Stief et al. 1989).

MARs have been proposed to standardise the expression of each integrated transgene in a transformed cell or organism such that overall expression is proportional to integrated gene copy number (Phi-Van et al. 1990). It is thought that the MARs anchor the introduced DNA to the nuclear matrix, after integration into the host genome, thereby isolating the new gene in a chromatin loop which acts as an independent regulatory domain (Spiker and Thompson 1996). In contrast, randomly-inserted transgenes, particularly those present as multiple copies, are often expressed at low or undetectable levels in a transgenic host. Suggested mechanisms for this so-called genomic "position effect" include the spreading of an inhibitory chromatin structure from the surrounding genomic DNA into the inserted DNA, and methylation of cytosine residues in the inserted DNA sequences (Peach and Velten 1991).

A range of effects of MARs on mean expression level and variation in expression of transgenes in plants has been reported (Breyne et al. 1992b, Allen et al. 1993, 1996, Mlynarova et al. 1994, 1995, van der Geest et al. 1994). The different effects reported in these and other studies may arise from differences between individual MARs. With the aim of isolating MARs which were capable of protecting

plant transgenes against genomic position effects, we selected two plant DNA regions with strong affinity for the nuclear matrix. Furthermore, one MAR was isolated from the upstream region of a gene previously reported to be expressed in a copy number-dependent manner in transgenic tobacco (Higgins et al. 1988). We tested the hypothesis that the copy number-dependent expression seen with the whole vicilin gene could be conferred on a heterologous reporter gene by addition of the vicilin MAR.

The second MAR was selected from a library of *A. thaliana* genomic DNA that was first screened for clones able to act as autonomously replicating sequences (ARSs) in the yeast, *Saccharomyces cerevisiae*. Some animal MARs have been reported to act as ARS in yeasts (Brun et al. 1990, Amati and Gasser 1990). A yeast ARS has been reported to act as a MAR in yeast (Amati and Gasser 1988) and plants (Allen et al. 1993). It has been reported that strength of matrix binding affinity of MARs correlates with the ability to act as ARS in yeast (Brun et al. 1990), so we employed a preliminary ARS screen with the aim of facilitating isolation of strong MARs from the *A. thaliana* genome.

Our approaches resulted in the isolation of two strong MARs which were tested for the ability to buffer transgene expression against deleterious position effects in transgenic tobacco. Neither MAR significantly reduced variation in transgene expression between individual transformants, or conferred copy number-dependence in gene expression. However, median reporter gene expression was stimulated by the presence of flanking copies of the *A. thaliana* MAR. These results suggest that MARs are heterogeneous with respect to their effects on transgene activity.

Materials and Methods

ARS assay—1. Pre-selection of A.thaliana genomic DNA fragments with ARS activity. The shuttle vector, pJW3, was constructed by removing the yeast ARS from pFL38 (Bonneaud et al. 1991) and replacing it with a multi-cloning site. The pJW3 plasmid contained the *ura3* selectable marker gene for yeast, but no ARS. Restriction fragments of *A. thaliana* genomic DNA were then inserted into pJW3. The ligated DNA was transformed into *E. coli*, then a mixed population of plasmid DNA was purified from a plate containing several hundred ampicillin-resistant *E. coli* colonies. This mixture of plasmids was transformed into *S. cerevisiae*, strain M2915-8A (Chen et al. 1993) using the lithium acetate procedure of Gietz et al. (1992). Total DNA was isolated (Ward 1990) individually from several of the resulting yeast colonies which grew after incubation for 3 to 5 days at 30°C on synthetic complete medium lacking uracil (Sherman 1991). These "yeast-selected" plasmids were recovered by back-transformation into *E. coli* (Ward 1990). Plasmids were also isolated individually from several randomly-selected, that is "unselected", *E. coli* colonies from the original transformation.

2. Measurement of ARS activity. The ARS activities of several of the "yeast-selected" and "unselected" plasmids described above were quantified by measuring their transformation efficiencies in yeast. In addition, a pea vicilin gene fragment (Higgins et al. 1988) was inserted into the pJW3 vector and tested. A DNA fragment with known MAR and ARS activity from the *fushi tarazu* (*ftz*) gene of *Drosophila melanogaster* (Amati and Gasser 1990) was also inserted into pJW3 and used as a positive control in the assay. The plasmid DNAs were purified using a boiling lysis method and quantified by estimating ethidium bromide fluorescence after electrophoresis on a 0.8% agarose gel (Sambrook et al. 1989). The efficiency of transformation of yeast by each of the plasmids of interest was then determined by transformation of the purified, quantified plasmid DNA into *S. cerevisiae*, by the lithium acetate method (Gietz et al. 1992).

MAR assay—A. thaliana nuclei were isolated by the methods described by Hamilton et al. (1972), with minor modifications. *A. thaliana* callus was maintained axenically on B5 medium from Sigma, plus 0.5 μg ml^{-1} 2,4 D and 0.05 μg ml^{-1} kinetin. Callus was homogenised in 2 to 4 volumes of NB 1 buffer (20 mM HEPES pH 7.6, 20 mM KCl, 10 mM EDTA pH 7.6, 0.05 mM spermine, 0.125 mM spermidine, 0.5 mM PMSF, 5 mM β-mercapto-ethanol). The homogenate was filtered through 25 μm nylon mesh and nuclei were banded on a 35%/60% Percoll step gradient by centrifugation at 4,000 × *g* for 10 minutes in a swinging bucket rotor at 4°C.

Nuclear matrices were prepared by treating isolated nuclei with 5 mM lithium-3,5-diidosalicylate (LIS) to remove histones, using a method similar to that of Slatter et al. (1991). Briefly, nuclei corresponding to 10 to 20 A_{260} units were washed in 1 ml of NB 2 buffer (20 mM HEPES pH 7.6, 20 mM KCl, 0.05 mM spermine, 0.125 mM spermidine, 0.5 mM PMSF, 10 μg ml^{-1} leupeptin). The nuclear matrix was stabilised by incubation at 42°C for 20 min in 0.5 ml of NB 2 buffer containing 0.5 mM $CuSO_4$ (Mirkovitch et al. 1984, Slatter et al. 1991, Breyne et al. 1992b). Histones were removed by adding 5 ml of NB 3 buffer (20 mM HEPES pH 7.6, 2 mM EDTA, 0.1 M lithium acetate, 0.1% digitonin, 5 mM lithium diiodosalicylate) and incubating the nuclei at room temperature for 15 min. Histone-depleted matrices were collected by centrifugation at 5,000 × *g* for 10 min in a swinging bucket rotor. Matrices were washed 3 to 5 times in NB 4 buffer (20 mM HEPES pH 7.6, 70 mM NaCl, 20 mM KCl, 10 mM $MgCl_2$, 0.05 mM spermine, 0.125 mM spermidine, 0.1% digitonin, 0.5 mM PMSF, 10 μg ml^{-1} leupeptin). The matrices were suspended in 0.5 ml NB 4 buffer plus 500 units restriction enzymes and incubated at 37°C for 2 to 3 hours. This treatment released approximately ninety-five percent of the endogenous, nuclear DNA into solution.

Binding assays were performed by adding end-labelled plasmid DNA fragments to aliquots of matrices without further purification. The DNA fragments were generated by digestion of each test plasmid with the appropriate restriction enzymes, followed by end-filling with the Klenow (large) fragment of *E. coli* DNA polymerase I (PolIk) in the presence of ^{32}P-dATP. For the binding assay, 2 to 3 A_{260} units of the nuclear matrices were incubated, at 37°C for 2 h, with 50 ng of end-labelled plasmid DNA fragments. The plant genomic DNA acted as competitor in the binding assay. After the incubation, the mixture was centrifuged at 13,000 × *g* in an eppendorf bench centrifuge for 5 min. One-tenth of each pellet (P) and supernatant (S) fraction was electrophoresed on a 0.8% agarose gel. For comparison, 5 ng of the original input (I) labelled plasmid DNA mixture was also electrophoresed. The gel was dried onto Whatmann 3MM paper and exposed to X-ray film overnight.

Gene constructs and tobacco transformation—All DNA manipulations were performed using standard procedures (Sambrook et al. 1989). The *35S-uidA* reporter gene was created by first-

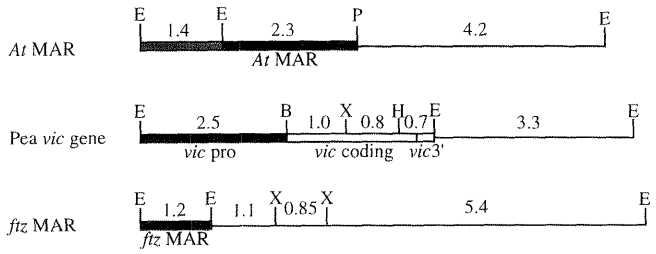

Fig. 1a

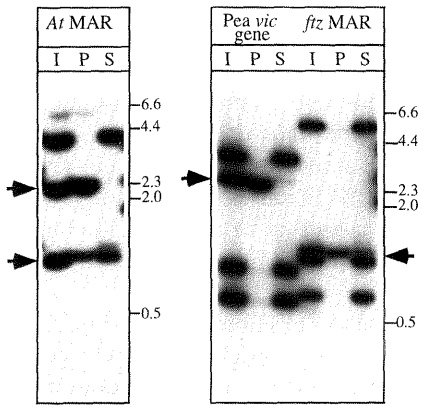

Fig. 1b

Fig. 1 Binding of pea and *A. thaliana* MARs to the nuclear matrix of *A. thaliana*. (a) Restriction maps of the tested plasmids showing the sites that were digested to generate the mixture of DNA fragments that was incubated with the nuclear matrix preparation. E, *Eco*RI; P, *Pst*I; B, *Bam*HI; X, *Xba*I; H, *Hin*dIII. DNA fragments that demonstrated matrix affinity are shaded. Sizes of fragments (kb) are indicated on the maps. The *vicilin* gene promoter extends from the left hand *Eco*RI site to 100 bp upstream of the *Bam*HI site. The *vicilin* protein coding region extends from 60 bp upstream of the *Bam*HI site to 380 bp downstream of the *Hin*dIII site (position marked by a vertical line in the diagram). The *vicilin* gene 3′ non-coding region extends from 380 bp downstream of the *Hin*dIII site to the right hand *Eco*RI site. (b) Binding of DNA fragments to the nuclear matrix. I, input DNA=total mixture of labelled DNA fragments added to assay; P, pellet containing nuclear matrix-bound DNA fragments; S, supernatant containing unbound DNA fragments. Arrowheads indicate DNA fragments bound to the matrix. Sizes (kb) of molecular weight markers are shown at the right of each panel.

ly excising the *35S-uidA-ocs* gene from pKiwi101 (Janssen and Gardner 1989) with *Xba*I and *Xho*I. The fragment was ligated into the *Xba*I and *Xho*I sites of pBluescript KS+ (Stratagene) to create pCC1. The *35S-uidA* region was excised from pCC1 as a *Not*I-*Eco*RI fragment. The 5′ over-hanging ends of the fragment were blunted with PolIk, and the fragment was ligated with a 3.2 kb, end-filled *Xba*I-*Xho*I fragment from pCW66 (Wandelt et al. 1991), containing the 3′ region of the pea vicilin gene, to create pLiu2. A 2.4 kb *Nco*I-*Xba*I fragment containing the vicilin coding region was excised from pCW66 and inserted upstream of the *35S-uidA-vic* gene in pLiu2 to create the progenitor of the spacer control construct. The vicilin coding region showed no MAR activity in binding assays (see Fig. 1a, b).

Reporter genes with upstream MARs were constructed as follows. A 2.0 kb *Nsp*I fragment incorporating the 5′ MAR of the pea vicilin gene from −0.3 to −2.3 kb from the start of transcription was excised from pCW66, end-filled with PolIk and inserted into pLiu2 upstream of the *35S-uidA-vic* gene in the same orientation with respect to the chimeric reporter gene as it had to the original vicilin gene. The 2.3 kb *A. thaliana* MAR shown in Fig. 1 was excised as a *Xba*I-*Hin*dIII fragment, end-filled and inserted upstream of the *35S-uidA-vic* gene in pLiu2.

The binary vector, pTAB10 (Tabe et al. 1995), containing a *35S-bar* selectable marker gene, was modified by inversion of the *35S-bar-ocs* gene. The resulting plasmid, pJW9, contained a unique *Eco*RI site adjacent to the right T-DNA border. The *35S-uidA-vic* gene fragment, either alone or with an upstream spacer control region, *vic* MAR or *At* MAR were excised from the respective pUC-based plasmids as *Eco*RI fragments and inserted into the *Eco*RI site of pJW9. The direction of transcription of the *35S-uidA-vic* gene was towards the *35S-bar-ocs* gene. This placed the test sequences between the *35S* promoter of the *uidA* gene and the adjacent host genomic DNA after integration of the T-DNA into the tobacco genome. The two constructs with a 3′ MAR were created by inserting an end-filled, *Xba*I-*Hin*dIII fragment containing the 2.3 kb *At* MAR into an end-filled *Pst*I site downstream of the *35S-bar-ocs* gene to produce the T-DNAs shown in Fig. 2. The 3′ MAR fragments were in the same orientation as the 5′ *At* MAR. The gene constructs were transferred to *Agrobacterium tumefaciens*, by tri-parental mating (Ditta et al. 1980). The *A. tumefaciens* transformants were then used to infect leaf explants of tobacco cv. Wisconsin 38, as described by Higgins et al. (1988), with the following modifications. The strain of *A. tumefaciens* used was AGL1 (Lazo et al. 1991) and transgenic shoots were selected on medium containing 10 $\mu g\ ml^{-1}$ phosphinothricin and 150 $\mu g\ ml^{-1}$ timentin. Total protein was extracted from leaves of the transgenic plants in sterile culture and GUS activity was assayed fluorimetrically as described by Jefferson (1987).

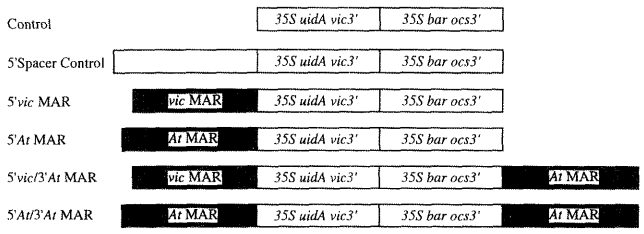

Fig. 2 The structure of *uidA* gene constructs with and without MARs that were transferred to transgenic tobacco by *A. tumefaciens*. DNA fragments with MAR activity are shaded.

Results

Isolation and characterisation of two plant MARs—Selection in *S. cerevisiae* resulted in the isolation of eight *A. thaliana* genomic clones which had ARS activity comparable to that of the bona fide yeast ARS from the pFL38

vector (Table 1). Eight randomly selected *A. thaliana* genomic clones ("unselected", Table 1) were also tested for ARS activity. These clones had not been selected by prior transformation into *S. cerevisiae*. The results indicated that relatively few *A. thaliana* genomic DNA fragments had detectable ARS activity, and that the average transformation efficiency of these unselected clones in *S. cerevisiae* was much lower than that of the selected clones. The plasmid containing the pea vicilin gene promoter showed ARS activity (Table 1).

After ARS selection and screening, plasmids were mapped with restriction enzymes and tested for binding to *A. thaliana* nuclear matrix preparations. The plasmids tested were 8 yeast-selected *A. thaliana* genomic clones, 8 unselected *A. thaliana* genomic clones, the pea vicilin gene and the *ftz* MAR fragment cloned in the pJW3 vector. Results for the strongest MARs are shown in Fig. 1. In these cases, the fragments with MAR activity were recovered almost exclusively in the matrix-bound (pellet) fraction. Three out of the eight ARS-selected *A. thaliana* genomic DNA fragments showed strong binding to the *A. thaliana* nuclear matrix. In contrast, only one out of the eight randomly chosen clones showed weak affinity for the nuclear matrix, as well as weak ARS activity (Table 1). The majority of DNA fragments in the digests showed no affinity for the matrix preparation, demonstrating the specificity of the assay (see Fig. 1b). We were therefore able to facilitate isolation of MARs from the *A. thaliana* genome by a prior selection for ARS in yeast.

The strongest *A. thaliana* MAR isolated in this screen was contained in a 2.3 kb genomic DNA fragment with matrix binding affinity equivalent to 100% of the positive control. The 2.3 kb fragment was adjacent to a 1.4 kb genomic fragment with weaker matrix binding affinity (Fig. 1). The region from −2.5 kb to the transcription start of the pea *vicilin* gene also had both strong ARS and MAR activities (Table 1, Fig. 1). In subsequent constructs, this region was truncated to remove the transcrip-

Table 1 ARS and MAR activities of *A. thaliana* genomic DNA fragments and a pea *vicilin* gene

Plasmid DNA[a]		ARS activity: yeast transformation efficiency (%)[b]	MAR activity: matrix binding affinity (%)[c]
A. thaliana genomic clones: Unselected	PR1	0	0
	PR2	0	0
	PR3	5	0
	PR4	2	12
	PR5	0	0
	PR6	0	0
	PR7	0	0
	PR8	3	0
: Yeast-selected	JW11	29	0
	JW12	29	50
	JW13	24	0
	JW31	42	0
	JW32	55	100
	JW51	20	0
	JW52	60	0
	JW53	37	50
Pea *vicilin* gene		32	136
ftz MAR[d]		30	100
pFL38[e]		100	n.d.

[a] Plasmid DNA tested in each assay. The *A. thaliana* genomic DNA fragments and the *D. melanogaster ftz* MAR fragment were cloned in vector pJW3. The pea *vicilin* gene was cloned in pUC118 for the MAR assay. A 2.5 kb *Eco*RI fragment containing the vicilin gene promoter was cloned into pJW3 for the ARS assay.

[b] Transformation efficiency of the test DNA in *S. cerevisiae* as a percentage of the transformation efficiency of the yeast ARS control (pFL38 vector, Bonneaud et al. 1991) in the same experiment.

[c] Proportion of the MAR DNA fragment bound to the nuclear matrix as a percentage of the bound proportion of the *D. melanogaster ftz* MAR positive control.

[d] Positive control for MAR assay. The percentage of the *ftz* MAR fragment which was recovered in the pellet fraction, $[P/(S+P)] \times 100$, varied between 58% and 67% in different assays. P=pellet, S=supernatant.

[e] Positive control for ARS assay.

tion start site of the *vicilin* gene and the proximal promoter elements responsible for the normal organ-specific pattern of expression of the *vicilin* gene in the developing seed (Morton et al. 1995). The truncated, 2.0 kb fragment from −0.3 kb to −2.3 kb relative to the start of transcription of the *vicilin* gene was shown to contain all of the matrix binding affinity of the full-length, 2.5 kb region (results not shown).

MAR test constructs—The effects of the 2.3 kb *A. thaliana* MAR and the 2.0 kb pea *vicilin* MAR on expression of a chimeric *uidA* reporter gene in stably-transformed tobacco were examined. Gene constructs contained the test sequences either upstream of the reporter gene or flanking the two transferred genes (Fig. 2). The chimeric *uidA* gene was driven by a 0.3 kb cauliflower mosaic virus 35S promoter which directs readily detectable, essentially constitutive gene expression in transgenic plants (Ow et al. 1987, Benfey et al. 1989). The selectable marker gene, *bar*, which confers resistance to the herbicide, phosphinothricin, was also controlled by a 35S promoter. In each construct, the upstream MAR DNA fragment was positioned between the right T-DNA border and the 5′ end of the *35S-uidA* gene. The MAR would therefore lie between the reporter gene and the flanking tobacco genomic DNA after integration into the host genome. The 3′ region of the chimeric *uidA* gene was from the *vicilin* gene. Although it did not contain MAR activity (Fig. 1b), it was identical to the 3′ region of the pea *vicilin* gene previously reported to be expressed in a copy number-dependent manner in developing seeds of transgenic tobacco (Higgins et al. 1988). Where present, the downstream MAR DNA fragment was positioned 3′ to the *35S-bar* gene.

Control constructs either lacked an inserted DNA fragment between the T-DNA border and the 35S promoter of the *uidA* gene, or included a non-MAR DNA fragment comprised of the coding region of the *vicilin* gene, which was approximately the same length as the MAR fragments tested. This latter "spacer control" controlled for effects of placing the reporter gene further into the T-DNA. Position in the T-DNA has previously been reported to influence gene expression (Breyne et al. 1992a). The gene constructs were transferred to *Nicotiana tabacum*, cv. Wisconsin 38, using *A. tumefaciens* and transgenic plants were selected on medium containing phosphinothricin. Expression of the *uidA* reporter gene was estimated by measuring the activity of the protein product, β-glucuronidase (GUS), in leaf extracts from a number of individual transformants containing each construct.

Effects of MARs on transgene expression in tobacco—A wide range of GUS activities were recorded in each population of transformed plants (Fig. 3). Because such data do not follow a normal distribution, it is invalid to apply statistical tests dependent on a normal distribution (Nap et al. 1993). For this reason, we have used distribution-free statistical tests. Firstly, we compared the median rather than the mean GUS activities of each population (Table 2 and

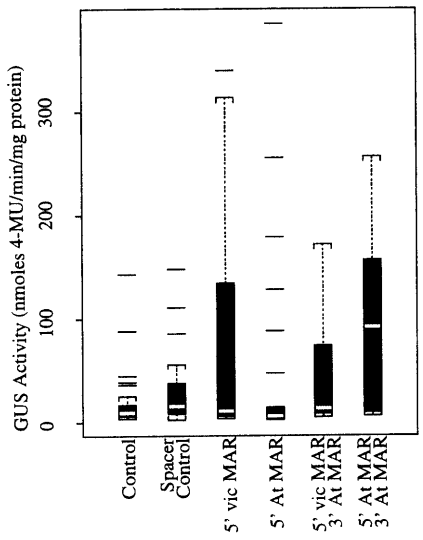

Fig. 3a

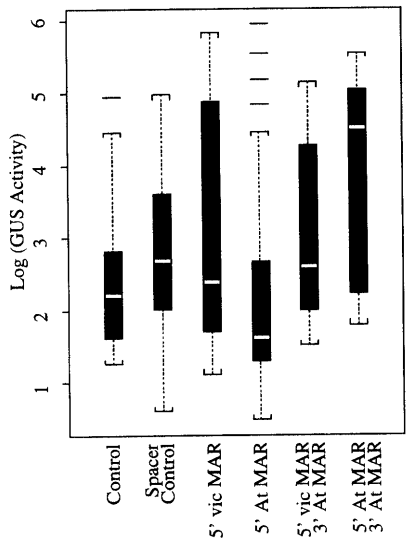

Fig. 3b

Fig. 3 The GUS activities of individual transformed plants in each experimental group represented by a box plot (Cleveland 1993) which illustrates the scatter of GUS expression levels in each group: (a) raw data, (b) log-transformed data. The horizontal white bar shows the median of the group. It is exceeded by 50% of the values. The ends of the shaded box are the quartiles, which either exceed 25% of the group or are exceeded by 25% of the group. The distance between the quartiles is the interquartile range, and the dashed appendages enclose all the values with 1.5 times the interquartile range of the nearest quartile. The values outside these categories are indicated by separate horizontal lines. The data for the control and spacer control groups are from experiment 1 in both cases (Table 2). 4-MU; 4-methyl umbelliferone.

Table 2 Median reporter gene activity in transgenic tobacco containing constructs with and without MARs

Construct[a]	Number of plants[b]	Median GUS activity[c]
Control, experiment 1	46	9.0
Control, experiment 2	23	22.7
Spacer Control, expt. 1	26	14.5
Spacer Control, expt. 2	21	9.9
5′*vic* MAR/*uidA-bar*	41	10
5′*At* MAR/*uidA-bar*	27	5.0
5′*vic* MAR/*uidA-bar*/3′*At* MAR	18	13.3
5′*At* MAR/*uidA-bar*/3′*At* MAR	28	91

[a] Gene constructs are described in Fig. 2.
[b] Number of transgenic tobacco plants with detectable GUS activity. Plants with no detectable GUS activity were discarded. GUS-negative putative transformants constituted a similar proportion of each population (data not shown).
[c] nmol 4-methyl-umbelliferone (mg protein)$^{-1}$ min^{-1}. Data from two separate experiments with each of the control constructs are included to demonstrate the extent of variation between experiments. The data from experiment 1 is plotted in Fig. 3, for both control constructs.

Fig. 3). Data from two separate experiments with each of the control constructs are included in Table 2 to demonstrate the degree of variation between repeat experiments involving the same construct.

The most striking effect of the MARs was seen in the case of the construct flanked by copies of the *A. thaliana* MAR (5′ *At* MAR/3′ *At* MAR). This group of transgenic plants showed a median GUS activity five to ten-fold higher than the medians of all the other populations (Table 2). This increase in median GUS activity was observed only when the *At* MAR was present at both the 5′ and 3′ ends of the T-DNA. The fact that populations containing constructs with only one copy of the *At* MAR did not show increased median GUS expression argues that the *At* MAR was not acting simply as an enhancer.

The transformation efficiency for the 5′ *At* MAR/3′ *At* MAR construct was no greater than the efficiencies observed for the other constructs (data not shown). If the flanking MARs had stimulated the activity of the *bar* gene in the same way that they had for the *uidA* gene, we might have expected to recover a higher percentage of transformed plantlets for the 5′ *At* MAR/3′ *At* MAR construct. The fact that we did not, suggests that there was no such stimulation of the expression of the *bar* gene in this population, although this point was not investigated in detail. The observation that flanking MARs can differentially affect two adjacent genes has been reported by others (Mlynarova et al. 1995).

In order to compare the variation in GUS activities between individual transformants within groups of plants containing different constructs, a distribution-free test, Median Absolute Deviation, (MAD, Sprent 1989) was applied. The test was performed on the raw data and on the data after log transformation. Group MAD values were compared using a permutation test and no differences that were significant for both transformed and untransformed data were found between groups. This indicated that there was no significant effect of any of the MAR combinations on the degree of variation of transgene activity. The medians and degree of variation of the experimental groups are most clearly compared graphically, using a box plot (Fig. 3).

We investigated the relationship between reporter gene copy number and expression level in selected individual transformants bearing constructs with or without the flanking regions of the *vicilin* gene. The chimeric *uidA* gene in all the gene constructs contained the 3′ region of the *vicilin* gene. This region did not contain MAR activity (see Fig. 1b), but was identical to the 3′ region of the pea *vicilin* gene previously expressed in transgenic tobacco. Thus, the flanking regions of the *35S-uidA* gene in the 5′ *vic* MAR and 5′ *vic* MAR/3′ *At* MAR constructs were identical to those of the *vicilin* gene previously reported to be expressed in a copy number-dependent manner in developing seeds of transgenic tobacco (Higgins et al. 1988). Furthermore, in the 5′ *vic* MAR/3′ *At* MAR construct, a 3′ MAR was also present downstream of the *35S-bar* gene. Copy number of the *uidA* gene was estimated by Southern blotting analysis for transformants representing the observed range of GUS activities in the Spacer Control population and in the 5′ *vic* MAR/3′ *At* MAR population. We found no correlation between gene copy number and GUS activity in extracts from leaves of plants from either population (Table 3). Although the seed-specific transcriptional control elements of the *vicilin* gene were not present in the *vicilin* MAR, we also tested for any seed-specific activity of the *vicilin* MAR. Nei-

Table 3 Relationship between reporter gene activity and gene copy number

Construct[a]	Plant	*uidA* gene copy number[b]	GUS activity[c]
Spacer Control	11-3	1	124
	11-2	3	10
	11-12	3	5.7
	11-11	4	44
	11-6	5	3.5
5′*vic* MAR/*uidA-bar*/3′*At* MAR	14-1	1	100
	14-10	1	54
	14-11	1	14
	14-12	2	7.8
	14-17	2	75
	14-27	3	54

[a] Constructs are described in Fig. 2.
[b] Determined by Southern blotting (results not shown).
[c] nmol 4-methyl-umbelliferone (mg protein)$^{-1}$ min^{-1}.

ther a general increase in activity, nor a correlation between gene copy number and activity of the *uidA* gene in maturing seeds of the plants bearing the 5′ *vic* MAR/3′ *At* MAR construct was found (data not shown).

Discussion

The diversity of the reported effects of MARs on mean expression level and variation in expression of transgenes in plants (Breyne et al. 1992b, Allen et al. 1993, 1996, Mlynarova et al. 1994, 1995, van der Geest et al. 1994) may be due, at least in part, to diversity in MARs themselves. The nuclear matrix preparations used in binding assays are complex mixtures of many different proteins, including abundant, relatively non-sequence-specific MAR binding proteins (von Kreis et al. 1991) as well as components of replication and transcription complexes (Jackson 1991). It is therefore possible that individual DNA fragments that demonstrate binding to matrices may interact with overlapping or distinct subsets of nuclear proteins. In this study, we assayed the effects on transgene expression of two MARs. In addition to having MAR activity, the DNA fragments had ARS activity and one fragment was part of a gene that was expressed in a dose-dependent manner in a transgenic host.

The results of our experiments with both MARs are in agreement with the results of others who have reported lack of copy number-dependent expression of chimeric genes linked to heterologous MARs in transgenic plants (Allen et al. 1993, Mlynarova et al. 1995). Similarly, it has now been reported that MAR activity alone is not sufficient to confer copy number-dependent transgene expression in animal systems (Bonifer et al. 1994, Huber et al. 1994, Polijak et al. 1994). In contrast with some findings (Breyne et al. 1992b, Schoffl et al. 1993, van der Geest et al. 1994, Mlynarova et al. 1994, 1995), we did not see a reduction in the variation of expression of MAR-linked transgenes in transgenic tobacco.

It has been reported that a MAR-containing promoter from a gene encoding the seed storage protein, phaseolin, from bean (*Phaseolus vulgaris*), was able to confer copy number-dependent, seed-specific expression on a chimeric *uidA* gene in transgenic tobacco (van der Geest et al. 1994). Our results show that an analogous pea gene encoding the seed storage protein, vicilin, also has a 5′ MAR. The *vicilin* MAR DNA sequence (Higgins et al. 1988) shares the common distinguishing features of other MARs; it is A/T rich (79%A/T) and contains numerous copies of a minimal MAR consensus sequence (ATATTT, Schoffl et al. 1993). It is possible that this MAR may contribute to regulation of the seed-specific expression of the *vicilin* gene in situ. However, we have demonstrated that the *vicilin* MAR, when placed in an appropriate context with a chimeric gene driven by a heterologous promoter, and flanked by the 3′ region of the *vicilin* gene, does not have the capacity to confer dose-dependent expression of the gene in a transgenic host (Table 3).

We found that selection for DNA fragments with ARS activity in yeast facilitated the isolation of strong MARs from the genome of *A. thaliana*. A 2.3 kb *A. thaliana* MAR was found to stimulate expression of a linked reporter gene in transgenic tobacco when it was present at both the 5′ and 3′ flanks of the construct. Our results agree with those of others who have found that MARs can often stimulate transgene expression, but are not sufficient to completely over-ride genomic position effects in plants (Allen et al. 1993, 1996), as in animal systems (Bonifer et al. 1994, Polijak et al. 1994). These reports contrast,

to some extent, with those that demonstrate a reduction in variability of transgene expression associated with MARs (Breyne et al. 1992b, Schoffl et al. 1993, van der Geest et al. 1994, Mlynarova et al. 1995). Thus, in combination with the results of others, our data indicate that the MARs identified by in vitro binding assays can have differing effects in the various assay systems that have been used to probe their functions in transgenic plants. In our study, we have seen different effects of two MARs tested under the same experimental conditions. The *5′ At* MAR stimulated median reporter gene expression when in combination with a *3′ At* MAR, whereas the *vic* MAR did not. This suggests that the heterogeneous results reported may reflect heterogeneity in the nature of the MARs themselves. Furthermore, the precise spacing between MARs and adjacent genes may be important in determining the effects of MARs on gene expression. This point has not yet been investigated. Some MARs demonstrate promise as positive regulators of plant transgene activity, but further advances in our understanding of their mechanisms of action will depend on more refined characterisation of the MARs and the proteins with which they interact.

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References

Allen, G.C., Hall, G.E., Jr., Childs, L.C., Weissinger, A.K., Spiker, S. and Thompson, W.F. (1993) Scaffold attachment regions increase reporter gene expression in stably transformed plant cells. *Plant Cell* 5: 603–613.

Allen, G.C., Hall G.E., Jr., Michalowski, S., Newman, W., Spiker, S., Weissinger, A.K. and Thompson, W. (1996) High level transgene expression in plant cells: Effects of a strong scaffold attachment region from tobacco. *Plant Cell* 8: 899–913.

Amati, B.B. and Gasser, S.M. (1988) Chromosomal ARS and CEN elements bind specifically to the yeast nuclear scaffold. *Cell* 54: 967–978.

Amati, B. and Gasser, S.M. (1990) Drosophila scaffold-attached regions bind nuclear scaffolds and can function as ARS elements in both budding and fission yeasts. *Mol. Cell. Biol.* 10: 5442–5454.

Benfey, P.N., Ren, L. and Chua, N.-H. (1989) The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. *EMBO J.* 8: 2195–2202.

Bonifer, C., Yannoutsos, N., Kruger, G., Grosveld, F. and Sippel, A.E. (1994) Dissection of the locus control function located on the chicken lysozyme gene domain in transgenic mice. *Nucl. Acids Res.* 22: 4202–4210.

Bonneaud, N., Ozier-Kalogeropoulos, O., Li, G., Labouesse, M., Minvielle-Sebastia, L. and Lacroute, F. (1991) A family of low and high copy replicative, integrative and single-stranded *S. cerevisiae/E. coli* shuttle vectors. *Yeast* 7: 609–615.

Breyne, P., Gheysen, G., Van Montagu, M. and Depicker, A. (1992a) Effect of T-DNA configuration on transgene expression. *Mol. Gen. Genet.* 235: 389–396.

Breyne, P., Van Montagu, M., Depicker, A. and Gheysen, G. (1992b) Characterisation of a plant scaffold attachment region in a DNA fragment that normalises transgene expression in tobacco. *Plant Cell* 4: 463–471.

Brun, C., Dang, Q. and Miassod, R. (1990) Studies of an 800-kilobase DNA stretch of the *Drosophila* X-chromosome: co-mapping of a subclass of scaffold-attached regions with sequences able to replicate autonomously in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 10: 5455–5463.

Chen, X.-J., Guan, M.-X. and Clarke-Walker, G.D. (1993) MGM101, a nuclear gene involved in maintenance of the mitochondrial genome in *Saccharomyces cerevisiae*. *Nucl. Acids Res.* 21: 3473–3477.

Cleveland, W.S. (1993) *Visualising Data.* Hobart Press, Summit, New Jersey.

Cockerill, P.N. and Garrad, W.T. (1986) Chromosomal loop anchorage of the *kappa* immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites. *Cell* 44: 273–282.

Ditta, G., Stanfield, S., Corbin, D. and Helsinki, D.R. (1980) Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proc. Natl. Acad. Sci. USA* 77: 7347.

Gasser, S.M. and Laemmli, U.K. (1987) A glimpse at chromosomal order. *Trends Genet.* 3: 16–22.

Gietz, D., Jean, A.S., Woods, R.A. and Schiestl, R.H. (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucl. Acids Res.* 20: 1425.

Hamilton, R.H., Kunsch, U. and Temperli, A. (1972) Simple, rapid procedures for isolation of tobacco leaf nuclei. *Anal. Biochem.* 49: 48–57.

Higgins, T.J.V., Newbigin, E.J., Spencer, D., Llewellyn, D.J. and Craig, S. (1988) The sequence of a pea vicilin gene and its expression in transgenic tobacco plants. *Plant Mol. Biol.* 11: 683–695.

Huber, M.C., Bosch, F.X., Sippel, A.E. and Bonifer, C. (1994) Chromosomal position effects in chicken lysozyme gene transgenic mice are correlated with supression of DNase I hypersensitive site formation. *Nucl. Acids Res.* 22: 4195–4201.

Jackson, D.A. (1991) Structure-function relationships in eukaryotic nuclei. *Bioessays* 13: 1–10.

Janssen, B.-J. and Gardner, R.C. (1989) Localised transient expression of GUS in leaf discs following co-cultivation with *Agrobacterium*. *Plant Mol. Biol.* 14: 61–72.

Jefferson, R.A. (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol. Biol. Rep.* 5: 387–405.

Lazo, G.R., Stein, P.A. and Ludwig, R.A. (1991) A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. *Biotechnol.* 9: 963–967.

Mielke, C., Kohwi, Y., Kohwi-Shigematsu, T. and Bode, J. (1990) Hierarchical binding of DNA fragments derived from scaffold-attached regions: correaltion of properties in vitro and function in vivo. *Biochemistry* 29: 7475–7485.

Mirkovitch, J., Mirault, M.-E. and Laemmli, U.K. (1984) Organisation of the higher-order chromatin loop: specific DNA attachment sites on the nuclear scaffold. *Cell* 39: 223–232.

Mlynarova, L., Jansen, R.C., Conner, A.J., Stiekema, W.J. and Nap, J.-P. (1995) The MAR-mediated reduction in position effect can be uncoupled from copy number-dependent expression in transgenic plants. *Plant Cell* 7: 599–609.

Mlynarova, L., Loonen, A., Heldens, J., Keizer, P., Stiekema, W.J. and Nap, J.-P. (1994) Reduced position effect in mature transgenic plants conferred by the chicken lysozyme matrix-associated region. *Plant Cell* 6: 417–426.

Morton, R., Quiggin, D. and Higgins, T.J.V. (1995) Regulation of seed storage protein gene expression. *In* Seed development and germination. Edited by Kigel, J. and Galili, G. pp. 103–138. First ed. Marcel Dekker, Inc., New York.

Nap, J.-P., Kiezer, P. and Jansen, R. (1993) First-generation transgenic plants and statistics. *Plant Mol. Biol. Rep.* 11: 156–164.

Ow, D., Jacobs, J.D. and Howell, S.H. (1987) Functional regions of the cauliflower mosaic virus *35S* RNA promoter determined by use of the firefly luciferase gene as a reporter of promoter activity. *Proc. Natl. Acad. Sci. USA* 84: 4870–4874.

Peach, C. and Velten, J. (1991) Transgene expression variability (position effect) of CAT and GUS reporter genes driven by linked divergent T-DNA promoters. *Plant Mol. Biol.* 17: 49–60.

Phi-Van, L. and Stratling, W.H. (1988) The matrix attachment regions of the chicken lysozyme gene co-map with the boundaries of the chromatin domain. *EMBO J.* 7: 655–664.

Phi-Van, L., von Kries, J.P., Ostertag, W. and Stratling, W.H. (1990) The chicken lysozyme 5′ matrix attachment region increases transcription from a heterologous promoter in heterologous cells and dampens position effects on the expression of transfected genes. *Mol. Cell. Biol.* 10: 2302–2307.

Polijak, L., Seum, C., Mattioni, T. and Laemmli, U.K. (1994) SARs stimulate but do not confer position independent gene expression. *Nucl. Acids Res.* 22: 4386–4394.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual.* (2nd Ed.). Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York.

Schoffl, F., Schroder, G., Kliem, M. and Rieping, M. (1993) An SAR sequence containing 395 bp DNA fragment mediates enhanced, gene-dosage-correlated expression of a chimeric heat shock gene in transgenic tobacco plants. *Trans. Res.* 2: 93–100.

Sherman, F. (1991) Getting started with yeast. *Methods Enzymol.* 194: 3–21.

Slatter, R.E., Dupree, P. and Gray, J.C. (1991) A scaffold-associated DNA region is located downstream of the pea plastocyanin gene. *Plant Cell* 3: 1239–1250.

Spiker, S. and Thompson, W.F. (1996) Nuclear matrix attachment regions and transgene expression in plants. *Plant Physiol.* 110: 15–21.

Sprent, P. (1989) *Applied Nonparametric Statistical Methods.* Chapman and Hall, London.

Stief, A., Winter, D.M., Stratling, W.H. and Sippel, A.E. (1989) A nuclear DNA attachment element mediates elevated and position-independent gene activity. *Nature* 341: 343–345.

Tabe, L.M., Wardley-Richardson, T., Ceriotti, A., Aryan, A., McNabb, W., Moore, A. and Higgins, T.J.V. (1995) A biotechnological approach to improving the nutritive value of alfalfa. *J. Anim. Sci.* 73: 2752–2759.

van der Geest, A.H.M., Hall, G.E., Jr., Spiker, S. and Hall, T.C. (1994) The phaseolin gene is flanked by matrix attachment regions. *Plant J.* 6: 413–423.

von Kries, J.P., Buhrmester, H. and Stratling, W.H. (1991) A matrix-/scaffold atttachment region binding protein: identification, purification and mode of binding. *Cell* 64: 123–135.

Wandelt, C., Knibb, W., Schroeder, H.E., Khan, M.R.I., Spencer, D., Craig, S. and Higgins, T.J.V. (1991) The expression of an ovalbumin and a seed protein gene in the leaves of transgenic plants. *In* Plant Molecular Biology 2. Edited by Herrman, R.G. and Larkins, B. pp. 471–478. Plenum Press, New York.

Ward, A.C. (1990) Single-step purification of shuttle vectors from yeast for high frequency back-transformation into *E. coli. Nucl. Acids Res.* 18: 5319.

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