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Reduced Position Effect in Mature Transgenic Plants Conferred by the Chicken Lysozyme Matrix-Associated Region

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Matrix-associated regions may be useful for studying the role of chromatin architecture in transgene activity of transformed plants. The chicken lysozyme A element was shown to have specific affinity for tobacco nuclear matrices, and its influence on the variability of transgene expression in tobacco plants was studied. T-DNA constructs in which this element flanked either the β-glucuronidase (GUS) reporter gene or both reporter and selection gene were introduced in tobacco. The variation in GUS gene activity was reduced significantly among mature first-generation transgenic plants carrying the A element. Average GUS activity became somewhat higher, but the maximum attainable level of gene expression was similar for all three constructs. Transient gene expression assays showed that the A element did not contain general enhancer functions; therefore, its presence seemed to prevent the lower levels of transgene expression. The strongest reduction in variability was found in plants transformed with the construct carrying the A elements at the borders of the T-DNA. In this population, expression levels became copy number dependent. The presence of two A elements in the T-DNA did not interfere with meiosis.

INTRODUCTION

The introduction of transgenes and their subsequent expression in plants has become an important tool for the analysis of a wide range of fundamental plant developmental processes (Verma, 1993). However, in general, a seemingly random and large variation in the levels of expression of a newly introduced transgene is found among transgenic plants that each carry essentially the same transgene (Dean et al., 1988; Blundy et al., 1991; Peach and Velten, 1991). This variation hampers analysis and reflects the limited knowledge of the regulation of transgene integration and expression in a plant genome.

In addition, a variety of epistatic interactions has been described for (partially) homologous transgene loci in the plant genome (Jorgensen, 1992; Kooter and Mol, 1993; Matzke and Matzke, 1993). Higher order genome organization together with the particular site of integration of the transgene in that genome is supposed to be at least in part responsible for the observed phenomena (Slatter and Gray, 1991; Laemmli et al., 1992; Matzke and Matzke, 1993). During interphase, chromatin architecture is thought to consist of topologically constrained DNA loops that at their bases are associated with a proteinaceous nuclear structure called the "scaffold" or "matrix" (Wolffe, 1992). By virtue of their binding to matrix preparations, DNA elements containing nuclear matrix-associated (MAR) or scaffold-associated (SAR) regions have been isolated from various organisms (Garrard, 1990), including plants (Izaurralde et al., 1988; Mielke et al., 1990; Hall et al., 1991; Slatter et al., 1991; Breyne et al., 1992). To prevent potential confusion with the acronym "SAR" for systemic acquired resistance (Ward et al., 1991; Enyedi et al., 1992), we will use the terms matrix and MAR and thus ignore the convention of naming these structures based on the method of isolation.

MAR elements are thought to be the boundaries of DNA loops that insulate the genes encoded on that loop, thus demarcating a regulatory domain of gene expression (Laemmli et al., 1992). MARs are AT rich and share DNA topoisomerase II and other rather loosely defined consensus sequences. They appear to be evolutionarily conserved but do not cross-hybridize (Garrard, 1990; Laemmli et al., 1992), so currently it is not possible to predict a MAR from a given sequence.

One of the best characterized MAR elements is the chicken A element, which is localized far upstream of the chicken lysozyme gene (Phi-Van and Strätling, 1988). In transformed animal cell lines, this MAR has been shown to increase the overall level of transgene expression and to decrease its position-

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dependent variability when placed around a reporter gene (Stief et al., 1989). This effect extends to heterologous promoters and cells (Phi-Van et al., 1990) as well as to the tissue specificity of transgene expression (McKnight et al., 1992). In addition, an A element binding protein has been characterized (von Kries et al., 1991), with characteristics that support the DNA loop hypothesis. Other MAR elements have also been reported to decrease positional variation (Grosveld et al., 1987; Reitman et al., 1990; Klehr et al., 1991).

In plants, MAR elements from soybean, humans (Breyne et al., 1992), and yeast (Allen et al., 1993) have been functionally tested in tobacco calli. The soybean MAR has been shown to "normalize" reporter gene expression (Breyne et al., 1992), whereas the human β-globin MAR has little influence on the variability of reporter gene expression (Breyne et al., 1992). The yeast element enhanced gene expression, as did another MAR element from soybean (Schöffl et al., 1993). In both cases, no reduction in variability of gene expression was observed.

In this study, we showed that the chicken A element is able to bind to the tobacco nuclear matrix, and we demonstrated that the addition of the A element at the borders of the T-DNA considerably decreases the variability of reporter gene expression in full-grown primary transformants of tobacco. The effect was less pronounced when the A element flanks only the reporter gene. An animal MAR element therefore is able to contribute to improved predictability of transgene expression in transformed plants.

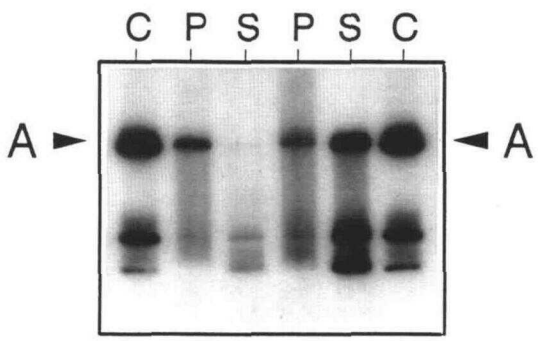

Figure 1. Affinity of Chicken Lysozyme A Element for Tobacco Matrices.

The binding of fragments from the A element–containing plasmid pUC-B-1-X1 in the presence or absence of *E. coli* competitor DNA is shown. The DNA fragment that contains the A element is indicated by an arrowhead on both sides of the figure. The two other fragments represent pUC vector DNAs of 1.4 kb (XbaI-AvaII fragment) and 1 kb (AvaII-BamHI fragment), respectively. The 200-bp AvaII fragment is not shown. In the rightmost P and S lanes, sheared *E. coli* DNA was added as competitor in a 300-fold excess over the amount of probe added. C, labeled input DNA; P, pellet fraction; S, supernatant fraction. Without competitor DNA, ~15% of the total label was found in the pellet fraction; in the presence of competitor, this amount decreased to about 2.5%.

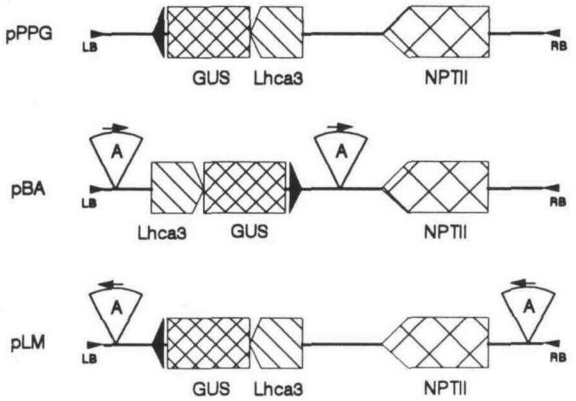

Figure 2. Schematic Overview of the T-DNA Region of Plasmids.

For pPPG, pBA, and pLM, the relative positions of the GUS gene, the nopaline synthase terminator (black triangles), and the NPTII gene are shown with respect to the left border (LB) and right border (RB) of the T-DNA. *Lhca3* is used to indicate the promoter of the gene encoding apoprotein 2 of the light harvesting complex of photosystem I of potato. The chicken lysozyme A element is indicated as an open triangle labeled A. The arrows on top of the open triangles show the orientation of the A element. In both pBA and pLM, the orientation of the A element with respect to the *Lhca3* promoter is the same as the orientation of this element in the chicken lysozyme gene from which the element was originally isolated. The T-DNA regions are not drawn to scale. Detailed cloning steps are described in Methods.

RESULTS

Chicken A Element Interacts with the Tobacco Matrix

The chicken A element was analyzed for MAR activity with respect to tobacco matrices. Nuclear matrices isolated from protoplasts were incubated with the end-labeled A element and pUC vector fragments, the latter serving as internal controls. As shown in Figure 1, the vector fragments were confined to the supernatant, while the fragment containing the A element was retained in the matrix-containing pellet. Sheared *Escherichia coli* competitor DNA, when added in a 300-fold excess with respect to the amount of probe added, was not able to inhibit completely the association with the matrix-associated element. Because the aspecific competitor DNA was not able to abolish the association, the A element had specific affinity for the tobacco matrix. A more precise mapping using restriction sites within the A element indicated the presence of multiple sites binding with different affinities to the tobacco matrix (data not shown), as had previously been demonstrated for oviduct matrices (von Kries et al., 1990). Because this heterologous MAR element had an affinity for plant nuclear matrices, this feature of plant chromatin structure was apparently conserved. The matrix binding assay tests the potential for binding of an element to the matrix, but need not

imply functionality in vivo (Garrard, 1990). Because the different binding sites are likely to act cooperatively (von Kries et al., 1990, 1991), we reasoned that a functional analysis of the influence of the A element on gene expression in plants would be best performed with the full element first.

Reduced Variation of β-Glucuronidase Gene Expression

We constructed two A element–containing Agrobacterium T-DNA vectors starting from plasmid pPPG, as shown in Figure 2. pPPG is a derivative of the binary vector pBin19 (Bevan, 1984). It carries the neomycin phosphotransferase II (NPTII) gene (*aphA2*) for kanamycin resistance selection and the β-glucuronidase (GUS) reporter gene (*uidA*; Jefferson et al., 1987) for easy and convenient monitoring of plant gene expression. The GUS gene was placed under control of the promoter from the potato gene designated *Lhca3.St.1*, encoding apoprotein 2 of the light-harvesting complex of photosystem I (Nap et al., 1993b). The A element was cloned in the orientation as present in the original chicken lysozyme gene to surround either the GUS reporter gene, yielding pBA, or both the GUS reporter and NPTII selection genes, yielding pLM. For each of these three constructs, more than 40 independent transformants were obtained by standard leaf disc transformation (Horsch et al., 1985).

To minimize the influence of environmental conditions and to ensure physiological equivalence as much as possible, all plants were grown in a fully controlled growth room. No alterations in photosynthetic efficiencies were observed in any of the three populations (data not shown), which indicated that no gross alterations in overall physiology had occurred as a result of the transformation. From all plants, leaf discs were assayed for GUS activity. Figure 3 shows the frequency distribution of GUS activity in the populations of transgenic plants. The transformation vector pBin19 has the selectable marker at the right border sequence. Therefore, kanamycin-resistant plants need not contain the GUS gene. Using DNA gel blot

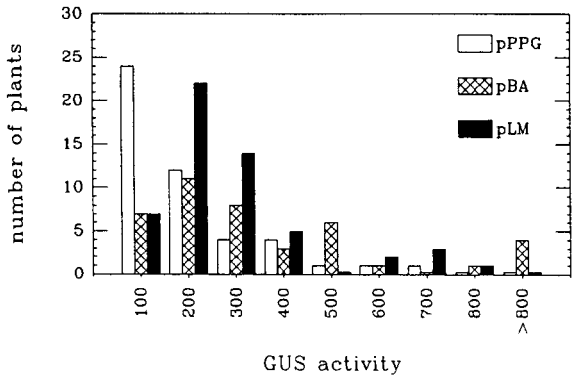

Figure 3. Frequency Distribution of GUS Activity.

The number of plants is plotted against the GUS activity. Plants are grouped into classes, the highest activity of which is given on the x-axis. The GUS activities in the class labeled 200 thus contain all plants with an activity ranging from 101 to 200 pmol of methylumbelliferone per minute per microgram of protein. The GUS activity taken for classification is the mean of all measurements performed for each individual plant. The series are named according to the T-DNA construct used for plant transformation.

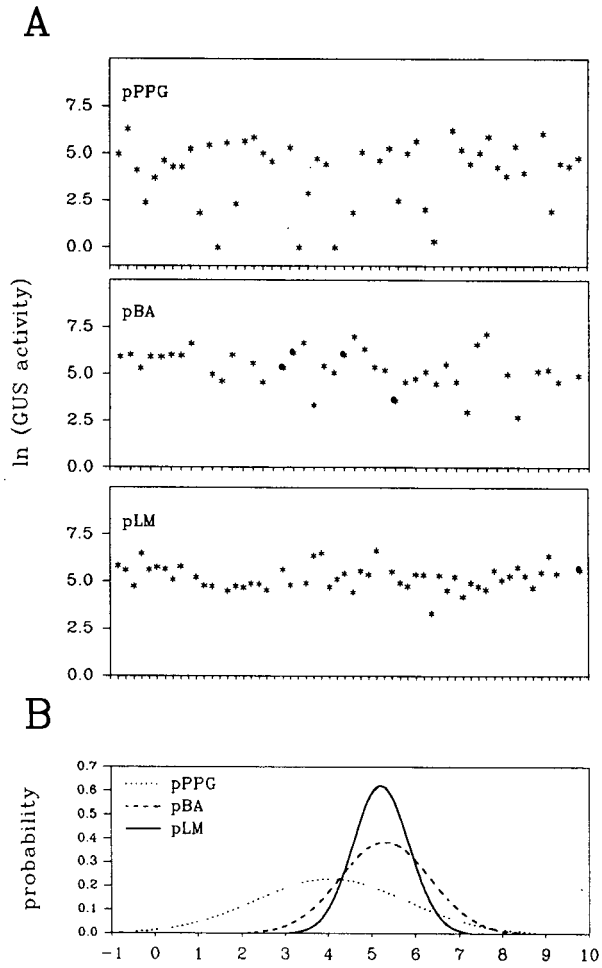

Figure 4. Decreased Variability of GUS Activity.

(A) The natural logarithm (ln) of the GUS activity of each individual transformant known to contain the GUS gene is plotted for each of the populations of plants, which are named according to the T-DNA construct they contain. The value plotted is the mean of the natural logarithms of the activities of all measurements performed. Each bar on the x-axis represents a transformant. The statistics describing the three populations are given in Table 1.

(B) Probability density curves summarizing the three populations of plants. The mean and variance used to calculate these curves are given in Table 1.

analysis, most plants without detectable GUS activity were shown not to contain the GUS gene. These plants were omitted from the populations.

The distribution of transgene activity in a population of first-generation transgenic plants is markedly skewed (Figure 3; see also Peach and Velten, 1991; Nap et al., 1993a), and proper statistical analysis requires a logarithmic transformation (Nap et al., 1993a). In Figure 4A, the means of the (natural) logarithms of the GUS activities were plotted for each individual plant. These plots and the accompanying statistics, as shown in Table 1, demonstrate a marked reduction in the range of GUS gene expression in the pBA and pLM populations compared to the control pPPG population. The contribution of sampling and experimental error to the total variation was negligible (Table 1; var versus cvar). The reduction in variability was most pronounced in the pLM population. The derived probability densities (Figure 4B) show the markedly reduced variation of GUS gene expression in the presence of the A element.

Enhancer Activity of the A Element

In addition to a reduction in variability, the overall level of GUS activity was higher in the pBA and pLM populations. The presence of the A element may thus increase transcription as has been observed in animal cell lines (Stief et al., 1989). To distinguish between true enhancer activity and a putative role in chromatin architecture, the three constructs were evaluated in transient expression assays using PEG-mediated transformation of tobacco leaf protoplasts. Chloramphenicol acetyltransferase (CAT) gene activity was used as an internal standard. Table 2 shows the GUS and CAT activities per equivalent amount of GUS gene. No marked differences were observed in the GUS-to-CAT ratio. As an alternative transient expression assay, particle bombardment of thin cell layer explants of tobacco stems was performed. The number of blue spots obtained with pLM and pBA was not higher than the number of blue spots obtained with pPPG (T. Creemers-Molenaar, personal communication). Both approaches showed that the A element carried no general enhancer elements.

MAR-Mediated Copy Number-Dependent GUS Gene Expression

Generally, no clear-cut correlation between the number of transgene inserts and level of transgene expression is found in transformed plants (Hobbs et al., 1993). The presence of MAR elements may result in such a correlation, as was demonstrated in animal cell lines (e.g., Stief et al., 1989). To study the influence of copy number on transgene expression levels, the number of inserts was estimated in all transformants by determining the number of fragments on DNA gel blots that represented junctions between plant DNA and T-DNA. The analysis was

Table 1. Statistics of Data

			Natural Logarithmic Scale								
Plants	DNA	No.	Mean	SE	P[a]	var[b]	cvar[b]	SE[c]	P[d]	Fold[e]	RT Mean[f]
All	pPPG	47	4.04	0.25	na	3.01	2.87	0.63	na	na	56.8
	pBA	40	5.30	0.16	***	1.05	0.96	0.24	***	3	200.3
	pLM	53	5.21	0.09	***	0.41	0.34	0.08	***	8	183.1
1 Copy	pPPG	13	3.43	0.50	na	3.19	3.03	1.30	na	na	30.9
	pBA	8	5.23	0.36	**	1.05	1.02	0.56	0.10	na	186.8
	pLM	22	4.78	0.10	**	0.21	0.16	0.06	***	19	119.1
2 Copies	pPPG	12	3.94	0.60	na	4.26	4.19	1.82	na	na	51.4
	pBA	8	5.32	0.22	*	0.39	0.35	0.21	**	12	204.4
	pLM	16	5.54	0.11	*	0.21	0.16	0.07	***	26	254.7
>2 Copies	pPPG	16	4.49	0.38	na	2.28	2.09	0.83	na	na	89.1
	pBA	20	5.35	0.28	*	1.53	1.38	0.50	0.20	na	209.8
	pLM	12	5.50	0.21	*	0.55	0.48	0.24	*	4	244.2

[a] Probability according to the *t*-test for location in case of unequal variances (Sokal and Rohlf, 1981) with respect to the corresponding control population pPPG.

[b] Variance (var) corrected for the variation caused by sampling and experimental error (cvar), using restricted maximum likelihood (REML) analysis (Payne et al., 1990).

[c] Standard error of the corrected variance as estimated by REML analysis.

[d] Probability according to the *F*-test for homogeneity of variances (Sokal and Rohlf, 1981) with respect to the corresponding control population pPPG.

[e] Reduction of variability with respect to the corresponding control population pPPG.

[f] Mean GUS activity after retransformation (RT) to the scale of measurement (in picomoles per minute per microgram of protein).

No., number of plants; SE, standard error of mean; var, variance; na, not applicable; ***, significant at $P < 0.001$; **, significant at $P < 0.01$; *, significant at $P < 0.05$.

Table 2. Transient Expression Assays of Tobacco Protoplasts

Plasmid[a]	GUS/CAT Ratio[b]
pPPG	0.95
pBA	0.90
pLM	0.90

[a] In all cases, 10 μg of plasmid was used in combination with 1 μg of pRT103cat.

[b] To correct for the size of the MAR-containing plasmids, GUS activity was calculated per equivalent GUS gene by dividing the pPPG GUS activity by a factor of 1.5. The GUS-to-CAT ratio is given in picomoles of methylumbelliferone per minute per picogram of CAT enzyme. Data shown are the means of two independent experiments.

performed for both the left (GUS gene inserts) and the right (NPTII gene inserts) border fragments, as shown in Table 3. Left border truncations were found in all three populations. This agrees with current knowledge of Agrobacterium-mediated gene transfer to plants, with the right border being the most conserved (Zambryski, 1992). Because all three populations had left border truncations, the A element had no (pseudo)border activity, and the presence of two A elements did not interfere with T-DNA transfer. No direct repeats of the T-DNA were found, and inverted repeats were only observed in two plants of the PPG population. Because tandem or inverted repeats were not a common form of T-DNA arrangement in the plants studied, most integration events were probably independent.

The distribution of copy numbers among the three populations was markedly different; the pLM population had the largest number of plants carrying precisely one complete copy of the T-DNA (Table 3). In Figure 5, the natural logarithm of the GUS activity is plotted versus the GUS gene copy number. Figure 5 and the accompanying statistics (Table 1) show that a large variability in GUS gene activity was still observed in pPPG plants having equal copy numbers. For pBA plants, there was some reduction in variation. However, in pLM plants, the variation in levels of GUS gene expression between plants carrying the same copy number was reduced highly significantly. Twenty-two pLM plants with one GUS gene copy had virtually the same level of gene expression. The 16 pLM plants with two copies also exhibited little variability and showed twice the GUS activity of the one-copy plants (Table 1; RT mean). In the few pLM plants with higher T-DNA copy numbers, GUS activity appeared to reach an averaged level of activity that was roughly equivalent to the activity of plants having two copies (Figure 5).

Meiotic Stability

Whereas MAR sequences are supposed to influence chromatin architecture in the interphase nucleus, little is known about the behavior of these elements during cell division and meiosis. Our analysis of primary transformants suggested that proper mitosis occurred. Selected plants from the pPPG and pLM populations carrying one copy were selfed, and their offspring were tested for both kanamycin resistance and GUS activity. The 3:1 segregation ratio for both traits, shown in Table 4, confirmed the DNA gel blot estimation of the single copy and showed inheritance in a Mendelian fashion. Therefore, the presence of the A element as a repeat in the T-DNA did not interfere with meiosis.

DISCUSSION

The chicken lysozyme A element had specific affinity for the tobacco nuclear matrix. Probably as a result, a marked decrease in variability of transgene expression was found in mature transgenic plants when this A element was surrounding the transgene. Because the presence of the A element was also meiotically stable, this heterologous MAR was, thus, functional in transgenic plants and their offspring. The relationship of chromatin structure, MAR action, and gene expression, as

Table 3. Distribution of Border and GUS Gene Fragments

	Number of Plants													
		Border Fragments[a]				GUS Gene Copy Number[b]								
Plasmid[c]	Total[d]	ND[e]	LB = RB	LB<RB	LB>RB	0	1	2	3	4	5	6	7	>7
pPPG	52	8	15	25	4	3	13	12	8	3	2	1	2	0
pBA	46	5	13	23	5	5	8	8	9	3	4	1	0	3
pLM	60	4	29	20	7	6	22	16	4	3	2	1	0	2

[a] The number of border fragments determined by DNA gel blot hybridization. LB, number of left border fragments; RB, number of right border fragments.

[b] The number of left border fragments was taken as the number of GUS gene copies.

[c] Plant populations are named according to the T-DNA construct they contain.

[d] The total number of kanamycin-resistant plants obtained after transformation.

[e] ND, the number of plants from which not both left and right border fragments were determined.

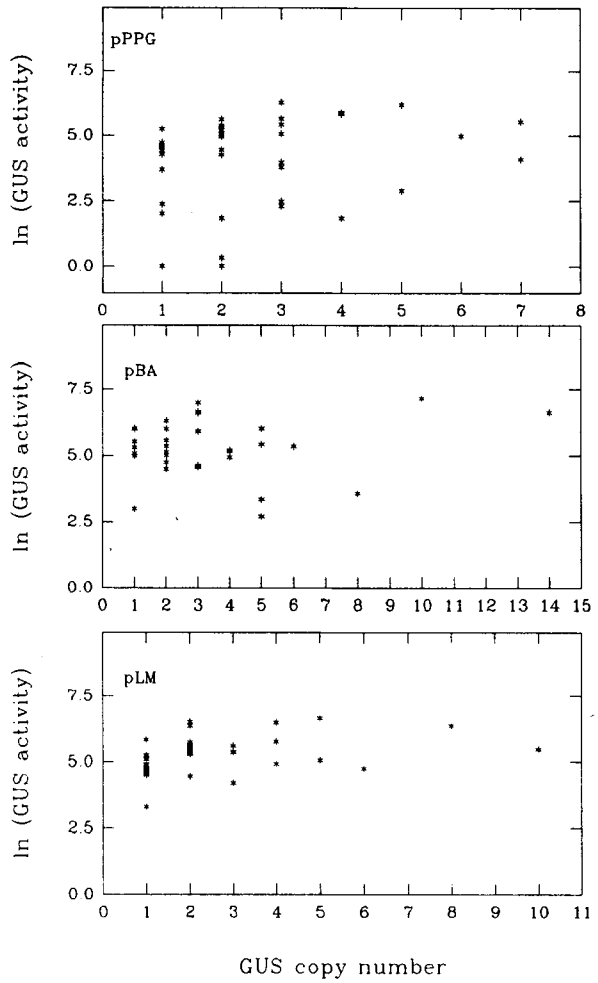

Figure 5. GUS Activity as a Function of GUS Gene Copy Number.

For each of the three populations of plants, the number of GUS gene copies, which was determined by the number of left border fragments as shown by DNA gel blot analysis, is plotted against the natural logarithm (ln) of GUS activity. Due to the way these data are plotted, not all individual plants (see Tables 1 and 3) are discernible as such. The statistics describing the subpopulations of plants are given in Table 1.

predicted by the DNA loop model (Laemmli et al., 1992), can therefore be extended to plants. This is in agreement with the apparently conserved characteristics of MAR sequences (Garrard, 1990). It has been demonstrated that the human β-globin MAR sequence has hardly any affinity for plant matrices and has little effect on transgene variability (Breyne et al., 1992), suggesting a functional relation between affinity and insulating capacity. The conclusion that the globin MAR has no effect is, however, based on the assumption of a normal distribution of gene expression, which should be reevaluated (Nap et al., 1993a).

Part of the A element used in our study has been recently shown to have the least affinity for animal matrices from a series of three MAR elements (Luderus et al., 1992). The relation between affinity for the nuclear matrix and the ability to insulate from the influence of surrounding chromatin is therefore not yet clear.

MAR versus Enhancer Activity

The observed increase in the average level of gene expression in the pBA and pLM populations is not due to the presence of an enhancer in the A element. Generally, MARs and enhancers occur on the same DNA fragments (Gasser and Laemmli, 1987), but are thought to reflect different aspects of gene regulation. MAR sequences behave differently in functional enhancer assays with plants. The soybean P1 and human β-globin MARs contain no enhancer activity (Breyne et al., 1992), whereas the *Drosophila fushi tarazu* MAR (Breyne et al., 1992) and yeast ARS-1 MAR (Allen et al., 1993) do, although the latter MAR increases gene expression only twofold. These elements also act differently in stable transformation; the P1 MAR appears to silence transcriptional activity, and the ARS-1 MAR increases it dramatically. The soybean heat shock gene MAR also promotes gene expression (Schöffl et al., 1993). These data suggest that different MAR elements differ considerably in their properties.

In animal cell lines, the A element enhances transgene activity ~10-fold (Phi-Van et al., 1990), but no such increase is

Table 4. Segregation Ratios of Kanamycin Resistance and GUS Activity

	Offspring					
	NPTII			GUS		
Parent	R[a]	S[b]	P[c]	+[d]	−[e]	P[c]
PPG-10	84	25	0.70	39	21	0.10
PPG-11	97	30	0.80	47	13	0.65
PPG-15	111	30	0.36	41	19	0.30
PPG-41	81	23	0.57	46	14	0.88
LM-9	88	23	0.35	20	6	1.0
LM-13	48	15	0.94	51	9	0.10
LM-18	47	15	1.0	20	4	0.48
LM-38	97	22	0.12	45	15	0.88
LM-43	100	30	0.69	32	13	0.68
LM-61	53	15	0.67	13	7	0.44

[a] Resistant.
[b] Sensitive.
[c] Probability according to the chi-square test for goodness of fit corrected for continuity. $P > 0.05$ indicates that the data follow a 3:1 Mendelian segregation.
[d] (+), presence of GUS activity.
[e] (−), no GUS activity.

observed in transgenic mice (McKnight et al., 1992). Our results showed that the maximum attainable level of gene expression did not depend on the presence of the A elements in the T-DNA. This suggested that stable integration in the presence of the A elements was not enhancing transcription but was able to prevent the occurrence of low levels of GUS gene expression. The presumed shielding by the A elements, as predicted by the DNA loop hypothesis, would thus seem to be more concerned with silencers in the plant genome.

Selection versus Loop Size

The reduction of variability in reporter gene expression was observed in both the pBA and the pLM populations, but was most pronounced for pLM where the A elements were next to the borders of the T-DNA (Table 1). This may suggest that the size of the presumed loop generated by the A elements was important. Loop sizes observed in animal systems range from 5 to 200 kb (Jackson et al., 1990), with smaller loops carrying more highly expressed genes than the large loops (Gasser and Laemmli, 1987). The potential loop size in the pLM construct, ~8 kb, was within this range, whereas the potential loop in the pBA construct was only ~4 kb. The latter size may be too small to ensure optimal MAR activity. At this time, it cannot be excluded that the precise orientation of elements also played a role in the differences in the reduction of variability. Whereas the orientation of the A elements with respect to the promoter GUS gene cassette is identical in both pBA and pLM (Figure 2), the orientation of this cassette is inverted with respect to the T-DNA borders. The effect found in the pLM population, however, indicated that the presence of the A elements insulated the genes within the presumed loop from influences of the surrounding DNA. In case this model proves correct, the precise orientation within the T-DNA would seem to be less important.

Copy Number-Dependent Gene Expression

In contrast to pPPG and pBA, the pLM population showed a good correlation between copy number and GUS gene expression levels for plants carrying one or two copies (Figure 5; Table 1). The few pLM plants identified that had a copy number of three or higher showed a GUS activity that leveled off to approximately the activity of plants with two copies. In contrast, in stably transfected animal cell cultures, a correlation of gene expression with copy number has been found for up to 270 copies (Phi-Van et al., 1990), but no such correlation is observed in transgenic mice (McKnight et al., 1992). The results from animal cell cultures would imply that those cells produce an excess of transcription factors, allowing more than 250 genes to be fully active. The presumed loop structure accomplished by the MAR elements may enhance the accessibility for transcription factors but is not likely to influence the total amount of transcription factors. In the pLM population, probably as a result of transcription factor dilution, either two to three genes were allowed to be expressed or more genes were suboptimally functional. These results were different from those of Allen et al. (1993), who report a positive gene dosage effect in MAR-containing plant cell lines in a window range of 20 to 50 copies. Yet the variability in gene expression in the plants carrying the same copy number appears too large to show any insulating effect of the MAR sequence (Allen et al., 1993). With so many copies in probably a wide variety of DNA configurations, it will be impossible to determine the relative contribution of each gene to the total level of gene expression.

Single-Copy Integration

The distribution of numbers of insertions was markedly different in the three populations studied; the pLM construct provided by far the largest number of plants carrying one complete copy. Generally it is assumed that differences in single- versus multiple-gene insertions result from differences in plant material, cocultivation conditions, notably the type and number of agrobacteria used, and the selection protocols employed (Heberle-Bors et al., 1988). In this case, the PPG and LM transformations were performed in parallel, using the same batch of plant material and the same protocol. The number of agrobacteria used was found to be similar for all three T-DNA constructs. The differences in copy number distribution would thus seem to reflect a property of the T-DNA configuration. Whether this property plays a role before or after integration in the plant genome is unclear. Any bias that will yield more intact single copies upon transformation would add to the overall appeal of pLM-like constructs in the study of plant gene expression.

METHODS

Plant Material

Tobacco (*Nicotiana tabacum* cv SR1) plants were grown sterilely on MS medium (Murashige and Skoog, 1962) as well as under normal greenhouse conditions.

Matrix Isolation and Binding Assay

Nuclei were obtained from mesophyll protoplasts isolated from leaves of 4- to 6-week-old in vitro–grown tobacco plants. Protoplasts were isolated according to the method described for potato protoplast isolation (Bokelmann and Roest, 1983). From ~10^8 protoplasts, routinely 10^7 nuclei were isolated using lysis conditions and Percoll gradient centrifugation as described previously by Slatter et al. (1991). Nuclei were checked for integrity, counted by flow cytometric analysis (Gilissen et al., 1993), and stored at −20°C in nuclei storage buffer (50 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$, 50% glycerol) at a concentration of 10^7 nuclei per mL until used. Nuclear matrices were isolated essentially

as described previously by Slatter et al. (1991). Nuclei were stabilized with 0.5 mM $CuSO_4$ and a heat treatment of 20 min at 37°C. Histones were removed by extraction with 10 mM lithium-3,5-diiodosalicylate (LIS) for 10 min at 25°C. After removal of LIS, the matrix preparation from 10^6 nuclei was digested (Luderus et al., 1992) for 3 to 4 hr at 37°C with 50 to 100 units of BglII and XbaI and without further centrifugation steps used for the matrix binding assay (Luderus et al., 1992). Plasmid pUC-B-1-X1 (Phi-Van and Strätling, 1988), carrying the A element as a 2953-bp XbaI-BamHI fragment, was digested with XbaI, BamHI, and AvaII and end labeled with α-^{32}P-dATP using standard techniques (Sambrook et al., 1989). Of the labeled plasmid, 50 ng was incubated overnight at 37°C with matrices isolated from 10^6 nuclei. As an aspecific competitor, sonicated *Escherichia coli* DNA was added. The incubation mix was centrifuged; the pellet fraction was treated for 1 hr at 65°C with 200 μg/mL proteinase K in the presence of 1% SDS and extracted with phenol/chloroform. Equal amounts of radioactivity from pellet and supernatant fractions were electrophoresed on a 1.2% agarose gel and visualized by autoradiography.

Matrix-Associated Region-Containing T-DNA Vector Construction

Standard cloning techniques (Sambrook et al., 1989) were used to create an XbaI fragment carrying the A element downstream of the β-glucuronidase (GUS) gene fragment present in plasmid pPPG that was described previously (Nap et al., 1993b). The 2953-bp A element from pUC-B-1-X1 was cloned as a BamHI-SalI fragment, together with an EcoRI-BamHI adaptor and the XbaI-EcoRI promoter–GUS gene fragment from pPPG in XbaI, SalI-digested pBluescript SK+ (Stratagene). To obtain pBA, the XbaI fragment was subsequently cloned in a pBin19 derivative in which the A element had been cloned in the multiple cloning site as an Asp718I-XbaI fragment that was isolated from pUC-B-1-X1. To obtain pLM, the same XbaI fragment was cloned in a pBin19 derivative in which the A element, as a blunted BamHI-XbaI fragment, had been cloned in the unique PmeI (New England Biolabs, Beverly, MA) site present at the extreme 5′ end of the nopaline synthase promoter driving the neomycin phosphotransferase II (NPTII) gene. For both pBA and pLM, plasmids were selected that had the upstream A element in the orientation with respect to the *Lhca3.St.1* promoter as was the orientation to the promoter of the chicken lysozyme gene (Phi-Van and Strätling, 1988).

Stable and Transient Plant Transformation

The binary plasmids were conjugated to *Agrobacterium tumefaciens* LBA4404 in a biparental mating using *E. coli* S17.1 (Simon et al., 1983) as the donor strain. Plasmid integrity was verified by restriction enzyme analysis upon reisolation from *A. tumefaciens* and transformation of *E. coli*. Tobacco (*N. tabacum* cv SR1) was transformed by the leaf disc transformation procedure (Horsch et al., 1985). To ensure independent transformation events, not more than two shoots were taken per leaf disc from opposite sites of the disc. Shoots that rooted in the presence of 50 μg/mL kanamycin were considered to be transgenic. Transgenic plants were transferred to soil and grown in a fully climatized greenhouse. For transient expression assays, 10 μg of binary plasmid was introduced by $Ca(NO_3)_2$-PEG–mediated transformation (Pröls et al., 1988) into 10^5 to 10^6 tobacco protoplasts that were isolated as described above. As an internal standard, 1 μg of pRT103cat (Pröls et al., 1988), carrying the chloramphenicol acetyltransferase (CAT) gene under control of the cauliflower mosaic virus 35S promoter, was added simultaneously to correct for the large batch-to-batch variation in protoplast preparations. Protoplasts were harvested after overnight incubation.

Reporter Gene and Photosynthesis Assays

For quantitative GUS assays of plants, 0.8-cm^2 leaf discs were punched from the third fully expanded leaf from the top using a cork borer, avoiding the midrib and major secondary veins. Each plant was sampled four times over a period of 7 weeks. Kinetic GUS assays were performed in duplicate as described previously by Nap et al. (1992). All GUS activities are expressed as picomoles of methylumbelliferone per minute per microgram of soluble protein. Statistical analysis was performed with the program Genstat 5 (Payne et al., 1987, 1990). CAT activity was determined using the digoxigenin-based CAT-ELISA kit (Boehringer Mannheim) according to the manufacturer's recommendations. The photochemical efficiency of photosystem II was measured by determining the ratio of variable-to-maximal in vivo fluorescence of detached dark-adapted tobacco leaves using the pulsed amplitude–modulated fluorescence technique (van Kooten et al., 1990).

Copy Number Determination

DNA was isolated from 2 to 3 g of tobacco leaf material by using a urea-phenol extraction procedure (Chen et al., 1992) with minor modifications. Material was frozen in liquid nitrogen and kept at −80°C until used. Extraction was performed for 15 min at room temperature, and ammonium acetate, pH 5.2, was used for the first two precipitation steps. Total DNA was digested with HindIII and blotted on GeneScreen Plus (Du Pont) membranes. Probes were labeled with ^{32}P-dATP using a random primed DNA labeling kit (U.S. Biochemical Corp.). DNA from all plants was hybridized with a GUS gene probe. DNA from pPPG plants was also analyzed with an NPTII gene probe. DNA from pBA plants was analyzed with the same NPTII gene probe and with a 560-bp BamHI-HindIII fragment from pUC-B-1-X1 containing part of the A element. The latter probe showed one faintly hybridizing fragment in DNA from untransformed tobacco plants. In pLM plants, the number of left border fragments was determined with the same A element probe as was used for the pBA plants. A 1.3-kb HindIII-HindIII fragment from pUC-B-1-X1 carrying another part of the A element was used to estimate the number of right border fragments. This second A element probe showed two faintly hybridizing fragments in DNA from untransformed tobacco plants. The error in the number of border fragments was estimated to be not larger than one border fragment. In a few cases, primary transformants were selfed, and the estimated copy number was confirmed by segregation of kanamycin resistance in seedlings and GUS activity in their offspring.

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