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High-Level Transgene Expression in Plant Cells: Effects of a Strong Scaffold Attachment Region from Tobacco

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We have previously shown that yeast scaffold attachment regions (SARs) flanking a chimeric β-glucuronidase (*GUS*) reporter gene increased per-copy expression levels by 24-fold in tobacco suspension cell lines stably transformed by microprojectile bombardment. In this study, we examined the effect of a DNA fragment originally identified in a tobacco genomic clone by its activity in an in vitro binding assay. The tobacco SAR has much greater scaffold binding affinity than does the yeast SAR, and tobacco cell lines stably transformed with constructs containing the tobacco SAR accumulated greater than fivefold more GUS enzyme activity than did lines transformed with the yeast SAR construct. Relative to the control construct, flanking the *GUS* gene with plant SARs increased overall expression per transgene copy by almost 140-fold. In transient expression assays, the same construct increased expression only approximately threefold relative to a control without SARs, indicating that the full SAR effect requires integration into chromosomal DNA. GUS activity in individual stable transformants was not simply proportional to transgene copy number, and the SAR effect was maximal in cell lines with fewer than ~10 transgene copies per tobacco genome. Lines with significantly higher copy numbers showed greatly reduced expression relative to the low-copy-number lines. Our results indicate that strong SARs flanking a transgene greatly increase expression without eliminating variation between transformants. We propose that SARs dramatically reduce the severity or likelihood of homology-dependent gene silencing in cells with small numbers of transgenes but do not prevent silencing of transgenes present in many copies.

INTRODUCTION

In recent years, it has become clear that a proteinaceous nuclear "matrix" or "scaffold" may play a central role in determining chromatin structure. Electron microscopy has shown that DNA is attached to this scaffold at intervals to produce a series of loops, varying in size from ~10 to several hundred kilobases (Bonifer et al., 1991; Cook, 1991; Getzenberg et al., 1991; Jackson, 1991; Zlatanova and Van Holde, 1992). Certain AT-rich DNA sequences, called scaffold attachment regions (SARs) or matrix attachment regions, are known to bind specifically to components of the nuclear scaffold (Gasser et al., 1989; Boulikas, 1993) and are therefore thought to form the bases of these loop domains. Several lines of evidence have led to proposals that SARs can insulate transgenes from surrounding chromatin. For example, when SARs are included on both sides of a transgene, the expression level in stably transfected mammalian cell lines may become proportional to transgene copy number, indicating that gene activity is independent of position in the chromosome (Grosveld et al., 1987; Stief et al., 1989; Bonifer et al., 1990; Phi-Van et al., 1990; McKnight et al., 1992). These and other data (reviewed in Elgin, 1991; Jackson, 1991; Pienta et al., 1991) have led to the idea that SARs define chromosomal domains within which higher order structure is regulated independently.

We initially set out to test the hypothesis that flanking a reporter gene with SARs should result in higher and less variable transgene expression in plant cells (Allen et al., 1993). Reporter gene constructs with or without yeast SARs (autonomously replicating sequence [ARS-1]) were introduced into tobacco suspension culture cells by microprojectile bombardment. Stably transformed cell lines in which the β-glucuronidase *gusA* reporter gene was flanked by SARs produced >20 times more GUS enzyme activity per gene copy than do control transformants without SARs. However, the yeast SAR did not dramatically reduce variation between different transformants, and high levels of expression were not observed in transformants containing many copies of the transgene (Allen et al., 1993).

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Although the yeast SAR shows specific binding to tobacco nuclear scaffolds, its binding activity is quite weak in comparison with a number of other SARs (S. Michalowski, unpublished data). Therefore, we asked whether a stronger SAR would cause even greater increases in gene expression and/or render expression more independent of genomic position effects. We now report results obtained with a SAR (Hall et al., 1991) isolated from a tobacco genomic clone containing the root-specific gene *RB7* (Conkling et al., 1990). Comparative studies have shown that this SAR binds much more strongly to tobacco scaffold preparations than does the yeast SAR used in our previous experiments. When the tobacco SAR was used to flank the same *gusA* reporter gene, average expression per gene copy was increased by nearly 140-fold in stably transformed cell lines. This increase is substantially greater than that seen with the yeast ARS-1 element (Allen et al., 1993). However, expression level still was not proportional to transgene copy number. Again, the SAR effect was maximal at relatively low transgene copy numbers. Thus, even a very strong SAR does not reduce variation among independent transformants. We discuss these results in the context of gene silencing phenomena known to affect multicopy genes.

RESULTS

Scaffold Binding

Although SARs are highly variable in sequence, several loosely defined SAR-related consensus elements or motifs have been identified from sequence comparisons in yeast and animal systems (Gasser and Laemmli, 1986; Gasser et al., 1989; Mielke et al., 1990; Dickinson et al., 1992). Figure 1 shows the distribution of some of these motifs in a SAR that we previously identified 3′ to the *RB7* gene of tobacco (Hall et al., 1991) as well as in the yeast SAR sequence that we used in our previous expression studies (Allen et al., 1993). The yeast SAR contains several A and T boxes. In addition, there is one ARS consensus element, two G exclusion regions or ATC tracts of 30 bp, and a 20-bp tract containing 90% A+T. However, it is striking that the plant SAR contains a much higher density of A and T box motifs, AT-rich tracts, and G exclusion regions, as well as three elements homologous with the Drosophila topoisomerase II consensus sequence. A systematic study of randomly cloned plant SARs (S. Michalowski, unpublished data) has not revealed a close correlation between any one of these motifs and binding activity in an in vitro assay. However, binding activity does correlate loosely with the total number or overall density of SAR-related motifs. From this analysis and the data summarized in Figure 1, we predicted that the *RB7* SAR binds to scaffold preparations much more strongly than does the yeast SAR.

To test this prediction, end-labeled restriction fragments from plasmids containing the two SAR sequences were mixed with tobacco nuclear scaffold preparations in the presence of restricted plant genomic DNA as a nonspecific competitor, as described in Methods. After incubation under binding conditions, bound and unbound DNA fragments were separated by centrifugation, and DNA was purified before gel analysis. In Figure 2B, equal percentages (20%) of the pellet and supernatant from each reaction, as well as an equivalent aliquot of the unfractionated probe, were run on adjacent lanes of an agarose gel and visualized by autoradiography. The same procedure was used for the gels shown in Figure 2C, except that in this case a 10-fold lower percentage (2%) of the total and supernatant fractions was loaded on the gel.

A low level of binding by the SAR-containing yeast ARS-1 fragment is discernible in Figures 2B and 2C. In repeated experiments under the same conditions, we consistently observed ~10% of the probe in the pellet. When the sensitivity of the assay was increased by differential loading, as shown in Figure 2C, it is clear that the ARS fragment binds, whereas pJKK plasmid vector sequences and a fragment containing the yeast TRP1 gene still do not bind detectably. These results confirm the specificity of the association between the yeast SAR and our plant scaffold preparations. Similar results can be obtained by loading the gel with equal numbers of counts from the pellet and supernatant fractions (Hall et al., 1991), a common practice that emphasizes the binding activity of weaker SAR elements.

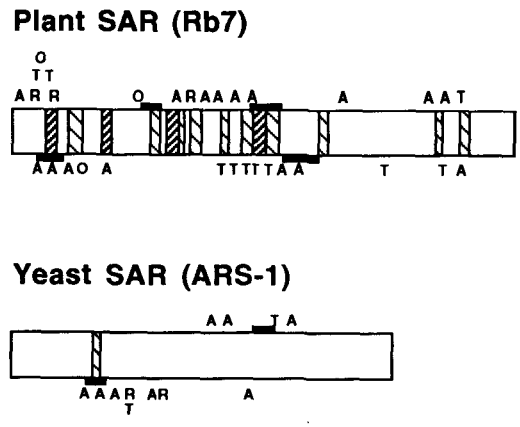

Figure 1. Schematic Comparison of Sequence Motifs in the Tobacco SAR (RB7) and Yeast SAR (ARS-1).

The occurrence and distribution of common SAR motifs are compared within the 1166-bp tobacco SAR (RB7) and the 838-bp yeast SAR (ARS-1), showing A boxes (A) (scored as an eight of 10 or better match with the consensus sequence AATAAAYAAA, where Y is pyrimidine), T boxes (T) (nine of 10 or better match with the consensus TTWTWTTWTT, where W is A or T), Drosophila topoisomerase II sites (O) (scored as a 13 of 15 or better match with the consensus GTNWAYATTNATNNG), ARS consensus sequences (R) (WTTTATATTTW), and G exclusion regions (ATC tract of 30 bp) represented by horizontal black bars. Local AT-rich regions (>20 bp) are indicated by the darkly hatched boxes that represent regions of >95% AT or the lightly hatched boxes that represent 90 to 95% AT residues.

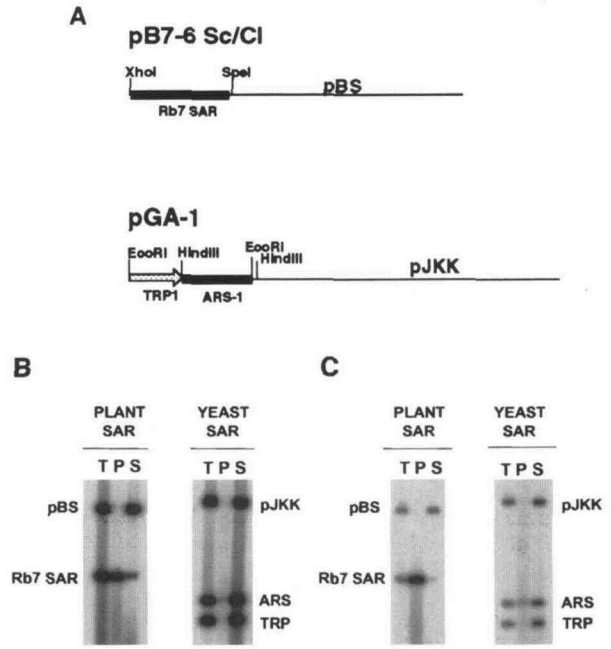

Figure 2. Comparison of Scaffold Binding by the Yeast SAR and the Tobacco SAR.

(A) Restriction maps for plasmids pB7-6 Sc/Cl and pGA-1 and the expected fragments. The direction of the arrow indicates the direction of transcription of the tryptophan synthase gene (*TRP1*).
(B) and **(C)** Autoradiography of a gel showing scaffold binding assay of the tobacco SAR (Rb7) and the yeast SAR (ARS), respectively, with nuclear scaffold prepared from NT-1 tobacco cells. Plasmid pGA1 (Hall et al., 1991), which contains the yeast SAR (ARS-1) and *TRP1*, was digested with EcoRI and HindIII, and plasmid pGH7-6Sca/Cla, which contains the tobacco SAR (RB7), was digested with SpeI and XhoI. Equal fractions (20%) of ^{32}P-labeled restriction fragments **(A)** from the scaffold binding assay were loaded into each lane, represented by T, total probe control; P, scaffold-bound fragments; and S, unbound fragments. **(C)** shows autoradiography of the same samples as given in **(B)**, except that the total and supernatant fractions are underrepresented by 10-fold (2%) to allow comparison of the weak binding yeast SAR (ARS-1) and the strong binding tobacco SAR.

Under the same binding conditions, the tobacco RB7 SAR consistently showed 80 to 90% binding. We do not know whether the differences in binding activities exhibited by these two SARs reflect differences in the number of potential binding sites, their relative binding affinity, or both. Future experiments are required to resolve this question.

We also have tested the possibility that elements other than known SARs might contribute to scaffold binding of the constructs used in our expression assays. Binding assays similar to those described in Figures 2B and 2C were conducted with restriction digests that separated fragments containing the cauliflower mosaic virus (CaMV) 35S promoter, the *GUS* gene, the nopaline synthase (*NOS*) polyadenylation signal, and vector sequences from the control plasmid pGHNC12. These binding assays gave uniformly negative results, even when the gel lanes were heavily overloaded with material from the pellet fraction (data not shown).

Expression Levels in Stable Transformants

In an earlier study (Allen et al., 1993), we demonstrated that even the weakly binding yeast SAR increased *GUS* expression in stably transformed cell lines, although we saw only minimal effects on variability between transformants. To test the hypothesis that a stronger SAR might have greater effect on variability, we transformed the same cell line with constructs made with the RB7 SAR. Figure 3 shows the constructs used. As before, we flanked a *GUS* reporter gene with direct repeats

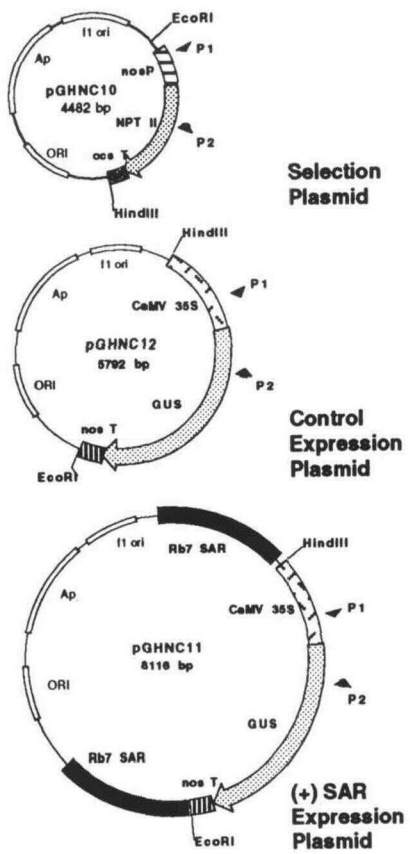

Figure 3. Schematic Diagrams of Plasmid Constructs.

Ap, β lactamase gene encoding ampicillin resistance; CaMV 35S, cauliflower mosaic virus 35S promoter; f1 ori, phage f1 origin of replication; GUS, coding region of the *E. coli* β-glucuronidase gene; nosP, promoter from the *NOS* gene; nos T, polyadenylation site/terminator from the *NOS* gene; NPT II, *NPTII* gene from Tn*5*; ocs T, polyadenylation site/terminator from octopine synthase gene; ORI, ColE1 origin of replication; Rb7 SAR, tobacco RB7 scaffold attachment region (the two copies are oriented as direct repeats). The arrowheads (P1 and P2) indicate the locations of the PCR primers used in estimating the copy number.

Table 1. Gene Copy Numbers for *GUS* and *NPTII* and Expression Levels for the Individual Transgenic Tobacco Lines Derived from Cotransformations of the Selection Plasmid with the Control Plasmid

Plasmid	Cell Line	*GUS* Gene Copy No.[a]	GUS Activity (pmol/min/mg of protein)[b]	*NPTII* Gene Copy No.[c]	NPTII Protein (pg/μg)[d]
Control without SARs	12-11	1	4.9	2	ND[e]
	12-9	1	12	2	43
	12-46	1	4.9	2	18
	12-48	2	7.7	2	ND
	12-2	2	94	3	83
	12-13	4	4.4	3	35
	12-23	4	1.6	3	38
	12-1	5	2.8	3	ND
	12-40	6	8.8	4	110
	12-36	11	6.6	3	64
	12-25	12	260	8	50
	12-10	29	3.8	4	80
	12-37	33	43	3	70
	12-18	63	1.1	34	86
	12-34	73	72	14	76
	12-41	77	190	10	46

[a] Samples were analyzed for *GUS* gene copy number by using the PCR assay, as described in Methods. The mean is 20.2; SE is 6.8; SD is 27; coefficient of variation (SD/mean; Sokal and Rohlf, 1969) is 1.3.

[b] Samples were analyzed for GUS specific activity by fluorometric assay. The NT-1 cell line was cotransformed, and 5-mL suspension cultures were started. Cultures were transferred weekly and harvested 56 days after transformation, as described in Methods. The mean is 44.8; SE is 19; SD is 76.5; coefficient of variation is 1.7.

[c] Samples were analyzed for *NPTII* gene copy number by using the PCR assay, as described in Methods. The mean is 6.2; SE is 2.1; SD is 8.1; coefficient of variation is 1.3.

[d] The same samples were analyzed for NPTII protein by ELISA, as described in Methods. The mean is 61; SE is 7.1; SD is 26; coefficient of variation is 0.4.

[e] ND, not determined.

of the SAR element and used a cotransformation protocol to avoid physical linkage between the assayable and selectable markers. Transformation was achieved by mixing the appropriate reporter plasmid with a selection plasmid, coprecipitating them onto microprojectiles and bombarding plates of tobacco suspension culture cells. Kanamycin-resistant microcalli were selected, and each callus was used to start an independent suspension culture cell line, as described in Methods. Histochemical staining of segments from the original microcalli showed that the staining intensity was much greater in cell lines transformed with SAR plasmids (data not shown). After 3 weeks of growth, with weekly transfers, cells were harvested for further analysis. DNA was extracted from each cell line for DNA gel blot analysis and quantitative polymerase chain reaction (PCR) assays, and portions of the same cell population were used to measure extractable GUS activity and neomycin phosphotransferase (NPTII) protein levels. Transgene copy number estimates and expression data are summarized in Tables 1 and 2.

Control GUS activities averaged 45 pmol of 4-methylumbelliferone (4-MU) min^{-1} mg of $protein^{-1}$, as compared with range of 1 to 54 nmol of a 4-MU min^{-1} mg $protein^{-1}$ commonly obtained for tobacco tissue transformed with similar constructs in Agrobacterium vectors (Jefferson et al., 1987; Hobbs et al., 1993; Frisch et al., 1995). When Rb7 SARs were included on both sides of the reporter gene, GUS activities averaged ~60-fold greater than those for the control construct lacking SARs. This effect on expression is approximately fivefold greater than that of the yeast SAR in our earlier experiments (Allen et al., 1993).

In cell lines transformed with the construct flanked by SARs, the average 35S::*GUS* gene copy number was reduced by approximately twofold compared with cell lines transformed with the control construct. This result is similar to that obtained in our earlier study using the yeast SAR. The lower *GUS* copy number in SAR transformants may reflect a loss of genes through recombination between the directly repeated SAR elements during or after the integration process, although we have no direct evidence on this point. However, the fact that SAR-containing lines have fewer copies of the *GUS* gene means that the average SAR effect on expression per gene copy is even greater than the 60-fold increase in overall expression. As shown in Tables 1 and 2, lines transformed with the RB7 SAR construct average nearly 140-fold more GUS enzyme activity per gene copy than do lines transformed with the same construct lacking SARs.

In sharp contrast to predictions from the "insulator" hypothesis mentioned in the Introduction, we observed only a modest reduction in our earlier experiments (Allen et al., 1993), and Tables 1 and 2 show that RB7 SAR had no significant effect on the coefficient of variation in the present work. Thus, even a strongly binding SAR that greatly increases average gene expression still does not decrease intertransformant variability as predicted by the insulator hypothesis. This point is considered further in Discussion.

Transient Expression

To distinguish effects that depend on chromosomal integration from those involving general transcriptional enhancer activity, we also tested our SAR constructs in a transient expression system. Such assays are used widely in studies of transcriptional enhancers. However, transiently transfected DNA is poorly organized into nucleosomes (Weintraub, 1985; Archer et al., 1992), and the fact that only a small minority of expressing cells go on to become stably transformed suggests that most transient expression occurs without chromosomal integration (Paszkowski et al., 1984; Davey et al., 1989; Saul and Potrykus, 1990; Christou, 1992). When the plant SAR plasmid was electroporated into tobacco NT-1 protoplasts before GUS assay 20 hr later, we observed an approximately threefold increase in *GUS* gene expression. The increase was 15 to 40 pmol min^{-1} mg of $protein^{-1}$, as compared with those transfected with the control plasmid lacking SARs. Because this effect is much less than the effect we observed in stably transformed lines, we conclude that the SARs are not simply acting as classical transcriptional enhancers.

Integration Patterns

Direct gene transfer procedures can result in complex integration patterns (e.g., Paszkowski et al., 1984; Tomes et al., 1990; Mittelsten Scheid et al., 1991; Christou, 1992; Koziel et al., 1993; Wan and Lemaux, 1994). Therefore, each cell line was compared by using DNA gel blot analysis after digestion of isolated genomic DNA with EcoRI and HindIII, which cut on either side of the 35S::*GUS*::*NOS* cassette (Figure 3). When probed with sequences from the 35S promoter, digests of the parent

Table 2. Gene Copy Numbers for *GUS* and *NPTII* and Expression Levels for the Individual Transgenic Tobacco Lines Derived from Cotransformations with the Selection Plasmid and the SAR Plasmid

Plasmid	Cell Line	*GUS* Gene Copy No.[a]	GUS Activity (pmol/min/mg of protein)[b]	*NPTII* Gene Copy No.[c]	NPTII Protein (pg/μg)[d]
With SARs	11-36	1	4.4	2	2
	11-13	1	88	2	35
	11-8	1	4,500	2	120
	11-12	2	21	3	12
	11-19	3	4,000	3	17
	11-43	3	17,000	4	54
	11-1	5	1,900	3	40
	11-2	7	610	4	20
	11-51	7	6,600	8	120
	11-37	8	5,500	5	91
	11-7	9	1,000	3	47
	11-41	10	600	3	25
	11-38	14	1,900	5	66
	11-18	15	2,100	28	120
	11-39	16	42	12	270
	11-23	20	25	3	50
	11-44	31	370	25	33

[a] Samples were analyzed for *GUS* gene copy number by using the PCR assay, as described in Methods. The mean is 9.0; SE is 2.0; SD is 8.1; coefficient of variation (SD/mean; Sokal and Rohlf, 1969) is 0.9.

[b] Samples were analyzed for GUS specific activity by fluorometric assay. The NT-1 cell line was cotransformed, and 5-mL suspension cultures were started. Cultures were transferred weekly and harvested 56 days after transformation, as described in Methods. Mean is 2,722; SE is 1,045; SD is 4,233; coefficient of variation is 1.6.

[c] Samples were analyzed for *NPTII* gene copy number by using the PCR assay, as described in Methods. Mean is 6.8; SE is 1.9; SD is 7.8; coefficient of variation is 1.1.

[d] The same samples were analyzed for NPTII protein by ELISA, as described in Methods. Mean is 66; SE is 16; SD is 65; coefficient of variation is 1.0.

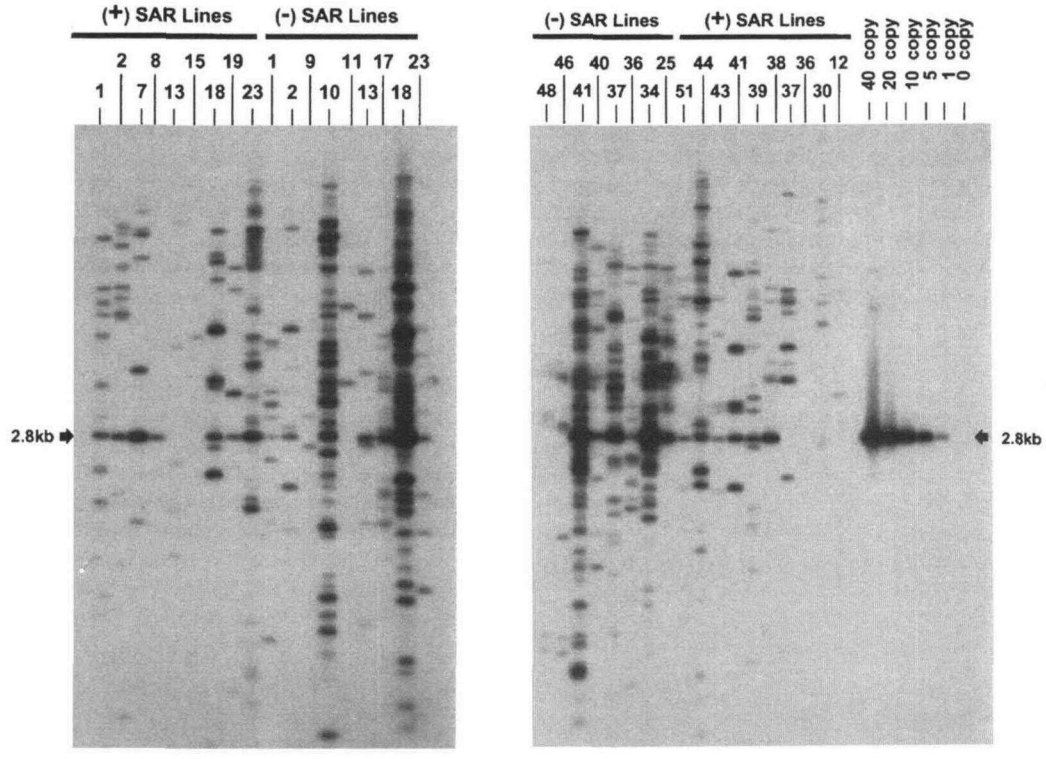

Figure 4. DNA Gel Blot Analysis of Transformed Cell Lines.

High molecular mass nuclear DNA was prepared and analyzed by gel blot hybridization with a ^{32}P-labeled 852-bp PstI-XbaI 35S promoter fragment, as described in Methods. Maps of the respective transforming plasmids with EcoRI and HindIII restriction enzyme sites are shown in Figure 3. Genomic DNA (10 μg) digested with HindIII and EcoRI genomic DNA was fractionated onto a 0.85% agarose gel. The DNA was blotted onto nylon membranes and probed, as described in Methods. Copy number reconstruction lanes contain 10 μg of nontransformed (control) genomic NT-1 tobacco DNA spiked with the equivalent of 40, 20, 10, 5, 1, and 0 copies of the 2.8-kb 35S::*GUS*::*NOS* terminator (arrow) per 1C DNA equivalent. The remaining lanes contain 10 μg of HindIII- and EcoRI-digested genomic DNA from the respective cell lines (indicated by the vertical lines and cell line number above each lane) transformed with (+) SAR plasmid or without (−) SAR control plasmid.

plasmids yielded a single band of 2.8 kb, as shown in the standard lanes of Figure 4. After integration into genomic DNA, complex hybridization patterns were observed, indicating extensive rearrangement during the integration process. Integration patterns for the control construct were somewhat more complex, on average, than those for the SAR plasmid. However, this difference probably reflects the higher average copy number in control lines (see above). There is no obvious difference in the complexity of integration patterns for SAR and control lines with similar copy numbers.

Intact 2.8-kb fragments containing the 35S::*GUS* gene were observed in most transformants, suggesting that most cell lines contained some nonrearranged gene copies. However, ~20 to 30% of the recovered lines lacked the 2.8-kb band indicative of intact 35S::*GUS* genes. Generally, this band was missing from lines with low overall copy number and with few, if any, bands at or above the intensity of a single-copy reconstruction standard. Expression levels were generally very low, and it seems likely that most of these lines contain genes with rearrangements that reduce or eliminate their activity. One exception was SAR line 37 (11-37), for which PCR analysis gave an estimate of eight copies and DNA gel blot analysis showed several high molecular weight bands of multicopy intensity. This cell line also had high *GUS* expression (Tables 1 and 2), indicating that in this instance, the rearrangement did not affect gene function dramatically.

Chen et al. (1994) reported that transgenes in wheat cell lines subjected to direct DNA transfer may sometimes contain N^6-methyladenine. This observation raises the possibility that transformation of an endophyte, such as a mycoplasma-like organism, occurred simultaneously with transformation of the wheat cells. To exclude this possibility, we carried out a methylation analysis with enzymes sensitive to adenine and cytosine methylation (data not shown). DpnI, which requires N^6-methyladenine for activity, does not cut transgene DNA but does completely digest the same sequence in the *Escherichia coli* plasmid DNA used for transformation. DpnII, an isoschizomer that differs from DpnI in that it is inhibited by adenine methyl-

ation, extensively cleaves transgene sequences in all tested cell lines but does not cut the plasmid DNA. Thus, the plasmid DNA is modified extensively by adenine methylation, but these modifications are eliminated in the transgene DNA, as would be expected if it is replicating as part of the plant genome. Another GATC-cleaving isoschizomer, Sau3A, completely digests plasmid DNA but shows only partial activity on transgene DNA. This enzyme is inhibited by cytosine methylation at the C residue in the GATC target sequence. Its failure to fully cleave transgene DNA indicates that cytosine methylation has occurred at some of the GATC sites in the transgene. Taken together, our data indicate that adenine methylation has been lost and that a plant-specific pattern of cytosine methylation has been established during replication of the transgene in our transformed cell lines.

We also probed gel blots of unrestricted DNA samples selected to represent a variety of copy numbers and expression levels (data not shown). In each case, all detectable *GUS* sequences migrated with high molecular mass chromosomal DNA, ruling out the possibility that they are maintained on extrachromosomal elements similar in size to the plasmids used in transformation. Similar results were obtained for lines with low and high overall copy numbers.

Copy Numbers and Expression Levels

Plotting *GUS* expression versus apparent gene copy number for individual cell lines produced the pattern shown in Figure 5. The largest SAR effects were obtained in cell lines with fewer transgene sequences, and expression of both SAR and control constructs was low in lines with high copy numbers. The overall degree of stimulation was much greater for the plant SAR, but the relationship to transgene copy number is quite similar to the one we observed in our previous experiments with a weaker SAR from the yeast ARS-1 element (Allen et al., 1993). One potentially interesting difference is that increased expression of the yeast SAR construct was seen in transformants carrying as many as 30 to 40 copies of the transgene, although a clear effect of the plant SAR was not seen in lines carrying >15 copies. However, the significance of this difference remains to be determined.

Three cell lines (11-12, 11-13, and 11-36) containing the SAR construct at low copy number also showed low GUS activity and therefore are apparent exceptions to the general rule that low copy numbers are associated with high expression (Tables 1 and 2). These lines were among those lacking the intact 2.8-kb 35S::*GUS* band (Figure 4) and thus probably contain only rearranged transgene sequences. Inclusion of these data has only a small effect on the overall analysis, however. By eliminating data from the SAR and control lines lacking the 2.8-kb band, we found an average GUS activity of 3300 pmol of 4-MU min^{-1} mg of $protein^{-1}$ for lines containing the SAR construct, as compared with 57 pmol of 4-MU min^{-1} mg of $protein^{-1}$ for control lines, which is a 58-fold difference. Corresponding values for the entire data set were 2700 and 45 pmol of 4-MU min^{-1} mg of $protein^{-1}$, a 61-fold difference.

Independent Expression

If the reporter and selection plasmids were cointegrated in a closely interspersed array, SARs on the plasmid containing the *GUS* reporter construct also might have stimulated *NPTII* gene expression. As shown in Tables 1 and 2, however, cotransformation with the SAR-containing vector had virtually no effect (1.1-fold) on average NPTII protein abundance. Figure 6A shows a plot of NPTII protein against gene copy number, showing that the pattern was largely unaffected by cotransformation with the SAR constructs. Figure 6B shows that there is, at best, only a weak correlation between NPTII protein and GUS activity in the SAR-containing transformants. Thus, the two plasmids introduced by our cotransformation procedure are expressed independently to a large extent, and there is no evidence for an effect of SARs in one plasmid on expression of a gene in the other.

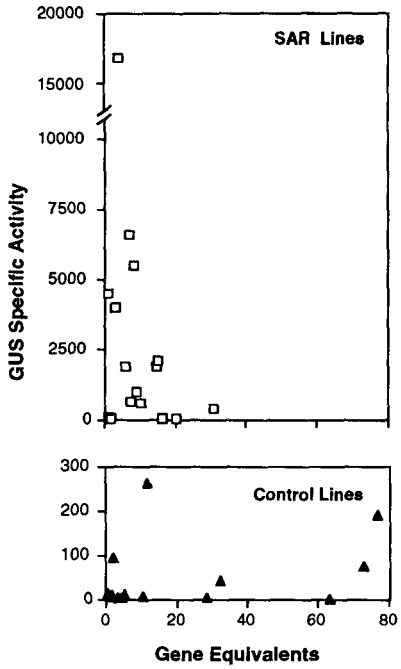

Figure 5. *GUS* Expression as a Function of Gene Copy Number in Stably Transformed Lines.

GUS activity and DNA from each transgenic cell line were analyzed 8 weeks after transformation. GUS specific activity was measured by flourometry and is reported as picomoles of 4-MU produced per minute per milligram of protein. Gene equivalents per haploid genome were estimated by quantitative PCR, as described in Methods, by using the primers indicated in Figure 3 and relating the resulting signals to standard curves prepared from reconstruction standards. SAR transformants are represented by open squares, and the control lines are represented by closed triangles.

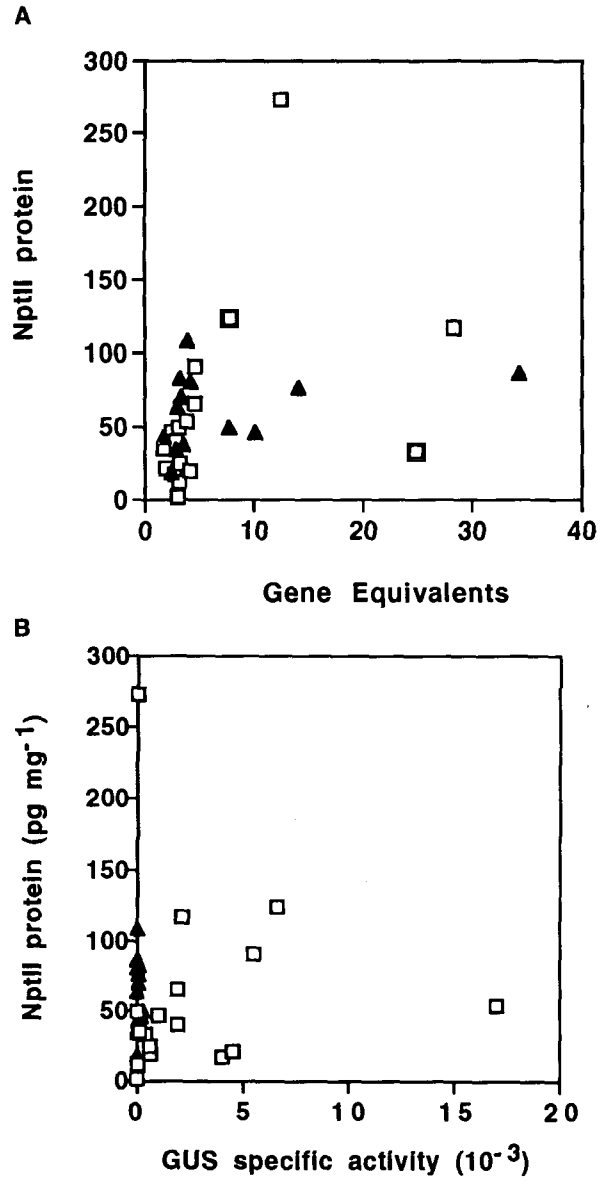

Figure 6. NPTII Expression in the Cotransformed Transgenic Lines.

(A) NPTII protein in picograms per microgram of total protein was determined by ELISA, and gene copy number was determined by the PCR procedure for the transgenic cell lines used for *GUS* analysis (see Figure 5). SAR transformants are represented by open squares, and the control lines are represented by closed triangles.

(B) The *GUS* and *NPTII* expression data from Figure 5 were replotted to compare the expression levels for each introduced gene. Double SAR transformants are represented by open squares, and the control lines are represented by closed triangles.

DISCUSSION

According to the loop domain model of chromatin organization, SARs should act as boundary elements, limiting the spread of condensed chromatin structures and blocking the influence of *cis*-regulatory elements in neighboring chromatin. If this model is correct and if variation in transgene expression is mainly attributable to genomic position effects, the presence of flanking SARs should normalize expression per gene copy and substantially reduce variability among independent transformants. Total gene expression then should vary in direct proportion to gene copy number. This prediction has been proven to be accurate in several previous experiments with animal cell systems (Grosveld et al., 1987; Stief et al., 1989; Bonifer et al., 1990; Phi-Van et al., 1990; McKnight et al., 1992).

In dramatic contrast to this expectation, even a strong SAR increases expression in tobacco cell lines but only at relatively low transgene copy numbers. At higher copy numbers, expression of both SAR and control constructs falls to low values. We conclude that SARs do not act as transcriptional insulators in plant cells and/or that variation from sources other than chromosomal position effects is largely responsible for variability in transgene expression. Recently published data from animal systems support a similar conclusion (Poljak et al., 1994; Kalos and Fournier, 1995), although differences between these recent results and earlier reports remain to be explained (for further discussion, see Thompson et al., 1996).

Variability in transgene expression was once thought to arise entirely from genomic position effects as described above. However, recent evidence indicates that one or more gene silencing phenomena also contribute to overall variability, especially in fungal systems (Selker, 1990a, 1990b; Rhounim et al., 1992; Barry et al., 1993; Selker et al., 1993) and higher plants (Matzke et al., 1989; Hobbs et al., 1990; van der Krol et al., 1990; Matzke and Matzke, 1991, 1993, 1995; Jorgensen, 1992; Assaad et al., 1993; Vaucheret, 1993; Finnegan and McElroy, 1994; Flavell, 1994). The distinction between position effects and gene silencing phenomena is not always a sharp one. In principle, however, position effects on transgene expression reflect preexisting features of the insertion site, such as proximity to genomic enhancers and degree of chromatin condensation, whereas gene silencing usually results from homology-dependent interactions involving the transgene itself (although chromosomal position may influence the severity of these interactions).

For a working hypothesis, we propose that a large portion of SAR effects reflects a reduction in the severity of gene silencing under conditions in which control transformants are severely affected. Homology-dependent gene silencing must be considered whenever multiple transgenes (or other homologous sequences, such as portions of transgenes) are present, as they are in most of our transformants. Although best known in fungi and higher plants, silencing of multicopy insertions has been reported recently in Drosophila as well (Dorer and Henikoff, 1994).

The predominance of multicopy insertions in our transformants may be one reason that our data on SAR effects differ from those reported by laboratories using Agrobacterium vectors. In four such reports, SARs caused a moderate increase in expression, along with a decrease in variation among transformants (Schöffl et al., 1993; Mlynárová et al., 1994, 1995; van der Geest et al., 1994). In a fifth set of experiments (Breyne et al., 1992), a slight decrease in average gene expression was reported. Most of these experiments involve heterologous SARs of animal (Mlynárová et al., 1994, 1995) or plant (Breyne et al., 1992; Schöffl et al., 1993) origin, whose affinity for host plant nuclear scaffolds cannot readily be compared with that of the tobacco SAR we tested. However, it is likely that all tested SARs bind at least as strongly as the yeast SAR used in our previous experiments (Allen et al., 1993), which still gives a >20-fold increase in expression per gene copy. In addition, van der Geest et al. (1994) found that the RB7 SAR and the phaseolin SARs used in their study had similar binding activities. Thus, it is likely that the major difference between our results and those of other laboratories is related to our method of transformation. In this regard, at least two major variables may be important.

First, in contrast to the relatively simple integration events commonly obtained with modern Agrobacterium vectors, most of our transformants exhibited complex integration patterns. These patterns are consistent with the known tendency for DNA introduced by direct transformation to integrate in clusters and undergo various kinds of rearrangement. Homology-dependent interactions among sequences at a locus are known to increase the frequency of silencing (Assaad et al., 1993; Hobbs et al., 1993); even gene fragments may contribute to this process. Thus, the tendency toward gene silencing may be more severe in our transformants than it normally is in transformants made with Agrobacterium. If SARs can reduce the interactions that lead to silencing (a hypothesis discussed below), we would expect them to have a greater effect in our system than in systems in which silencing is inherently less frequent.

Another major difference between our approach and that used in previous studies is our use of an unlinked selectable marker. Given the proximity of the selectable marker to the reporter gene on the T-DNA in most Agrobacterium vectors, drug selection probably eliminates many transformants with low T-DNA expression. Noncoordinate expression of genes in the same T-DNA has been reported, especially in cases when the two genes contain regions of sequence identity (An, 1986; Dean et al., 1988). However, a degree of coordination can be observed when nonhomologous genes are used (Dean et al., 1988). This tendency provides the basis for a "reference gene" procedure to normalize variation between transformants (Kuhlemeier et al., 1988; Ohme-Takagi et al., 1993) as well as for a technique in which strong selection for flanking drug resistance markers is used to eliminate most transformants with low levels of reporter gene expression (Bhattacharyya et al., 1994). Thus, it seems likely that selection for a physically linked drug resistance marker, included in virtually all T-DNA transformation procedures, would decrease the apparent magnitude of any SAR effect by differentially eliminating low expressers from transformant populations.

In contrast, our data show that expression of a selectable marker and a reporter gene can vary independently when the two genes are introduced on separate plasmids. In addition, we saw no difference in the average *NPTII* expression in populations of transformants produced by cotransformation with *GUS* plasmids either containing or lacking SARs (Table 2). That the SAR effect is not transferred from the *GUS* plasmid to the *NPTII* plasmid suggests that SARs are somehow functionally separated from the *NPTII* gene, but we can only speculate about actual integration patterns. Previous reports have shown that cotransformed plasmids frequently integrate near enough to one another to show tight genetic linkage (Chee and Slightom, 1992; Chen et al., 1994; Register et al., 1994; Peng et al., 1995), but few such events have been characterized on a molecular scale. It may be that a small amount of intervening DNA, such as might be provided by plasmid vector sequences, is all that is necessary to prevent the SAR effect from acting on an adjacent plasmid. Alternatively, the two plasmids might be arranged in separate clusters within a complex locus rather than in the type of regularly alternating pattern that would be required to place SARs adjacent to all the *NPTII* genes. Further analysis is required to clarify these possibilities. For our purposes, the important point is that the cotransformation technique allows us to identify transformants under conditions in which a wide range of reporter gene variation can be recovered for analysis.

Chromatin Structure

That SARs affect chromatin structure seems likely, given that they stimulate expression in stable transformants but not in transient expression systems. However, several hypotheses can be proposed in which effects on chromatin structure are mediated by mechanisms quite different from the conventional loop domain hypothesis. One possibility is suggested by the fact that SARS in tandemly integrated transgenes would be very closely spaced. If we assume that the SARs in our constructs bind to scaffold structures in vivo, the regions between binding sites will often be very short, perhaps too short to form the stably condensed chromatin structures required to repress transcription (Butler and Thomas, 1980). According to this model, the SAR effects we observe would involve a direct structural constraint on chromatin conformation rather than a domain boundary or insulator function proposed in many previous models (reviewed in Eissenberg and Elgin, 1991; Breyne et al., 1994; Spiker and Thompson, 1996; Thompson et al., 1996).

However, we need not suppose that SARs work only by binding to scaffold. The "chromatin-opening" model recently presented by Laemmli and colleagues (Kas et al., 1993; Zhao et al., 1993; Poljak et al., 1994) postulates that "displacement proteins," such as high-mobility group I/Y (HMG I/Y) proteins, interact with SARs to replace histone H1. Thus, when displacement proteins are abundant, the presence of SARs may favor

a more open chromatin conformation that allows access by RNA polymerase. Because HMG I/Y proteins are known to be more abundant in actively proliferating cells (Elton and Reeves, 1986; Johnson et al., 1990; Giancotti et al., 1993), the chromatin-opening model is consistent with the recent observation (Thompson et al., 1994) that the effects of SARs on heat shock protein *hsp70* gene expression in transgenic mice are evident mainly in rapidly growing embryonic tissues. If HMG I/Y homologs or other displacement proteins were more abundant in proliferating plant cells, a similar mechanism might help to explain why the SAR effects we observed in rapidly growing suspension cultures are greater than those reported for expanded leaves (Schöffl et al., 1993; Mlynárová et al., 1994, 1995; van der Geest et al., 1994) or solid callus cultures (Breyne et al., 1992).

By itself, the chromatin-opening model would not account for the decline in expression we observed with high doses of transgene DNA. However, the decline can be explained if we also assume that SARs reduce the probability of gene silencing. Given the existence of homology search mechanisms capable of scanning entire genomes (Haber et al., 1991; Camerini-Otero and Hsieh, 1993), it is attractive to speculate that at least some homology-dependent silencing events involve transient ectopic pairing interactions, followed by gene inactivation via changes in DNA methylation and/or chromatin condensation (Jorgensen, 1992, 1993; Assaad et al., 1993; Matzke et al., 1993, 1994; Flavell, 1994). This type of silencing would resemble processes in fungi known as repeat-induced point mutation and premeiotically induced methylation, which detect and modify duplicated DNA sequences (Selker 1990a, 1990b; Rhounim et al., 1992; Barry et al., 1993; Selker et al., 1993). Recent work on Drosophila indicates that ectopic transgene insertions may be affected by a similar mechanism and has lead to a model in which pairing between repeats leads to condensation and heterochromatinization (Dorer and Henikoff, 1994).

Considering SAR effects in relation to such models, we suggest that transgenes with SARs may be less vulnerable to ectopic pairing or other homology-sensing interactions that lead to gene silencing. For example, DNA closely associated with a strong SAR might be physically constrained in a way that reduces or prevents interactions with homologous sequences elsewhere in the genome. As the amount of transgene DNA increases, however, we assume there will be an increasing chance that some genes or gene fragments will escape from these constraints and undergo pairing. If ectopic pairing triggers a process leading to inactivation of other homologous genes, this model might explain the decline in expression we observed at high copy number. The model does not predict whether the inactivation process will be transcriptional or post-transcriptional. However, it does suggest that SARs reduce the probability that a silencing condition, rather than direct protection against inactivation, will be induced.

METHODS

Plasmid Constructs

The β-glucuronidase (*GUS*) reporter plasmids were made according to the following procedures. Plasmid pRB7-6 (Hall et al., 1991) was digested with ClaI and ScaI, and the resulting 1.1-kb SAR fragment was treated with the Klenow fragment of DNA polymerase I to create blunt ends. Plasmid pGHNC1 was created by ligating the 1.1-kb ClaI-ScaI SAR fragment into the XbaI site of pBluescript II SK+ (Stratagene) that had been treated previously with the Klenow fragment to create blunt ends. Similarly, plasmid pGHNC4 was created by ligating the 1.1-kb ClaI-ScaI SAR fragment from pRB7-6 (Hall et al., 1991) into the XhoI site in pBluescript II SK+ (Stratagene) that had been treated previously with the Klenow fragment to create blunt ends. The 1.1-kb ApaI-HindIII scaffold attachment region (SAR) fragment from pGHNC4 was then inserted into the ApaI and HindIII sites of pGHNC1 to give pGHNC5. The 2.8-kb HindIII-EcoRI fragment from pBI221 (Clontech, Palo Alto, CA), containing the cauliflower mosaic virus (CaMV) 35S promoter/*GUS* reading frame/nopaline synthase (*NOS*) terminator, was inserted into the HindIII and EcoRI sites of pGHNC5 or pBluescript II SK+ to yield pGHNC11 (with SARs) or pGHNC12 (without SARs), respectively.

The selection plasmid (pGHNC10) was created by ligating the HindIII-EcoRI fragment from pUCNK1 (Herrera-Estrella et al., 1988), containing the *NOS* promoter/neomycin phosphotransferase *NPTII* reading frame/octopine synthase (*OCS*) terminator, into the HindIII and EcoRI sites of pBluescript II SK+.

Transformation

The *Nicotiana tabacum* cell line NT-1 was obtained from G. An (Washington State University, Pullman). Suspension cultures were grown in a medium containing Murashige and Skoog salts (Gibco Laboratories, Grand Island, NY) supplemented with 100 mg/L inositol, 1 mg/L thiamine-HCl, 180 mg/L KH_2PO_4, 30 g/L sucrose, and 2 mg/L 2,4-D. The pH was adjusted to 5.7 before autoclaving. Cells were subcultured once a week by adding 3 mL of inoculum to 100 mL of fresh medium in 500-mL Erlenmeyer flasks. The flasks were placed on a rotary shaker at 125 rpm and 27°C with a light intensity of 47 μmol m^{-2} sec^{-1}.

Four-day-old cells, in early log phase, were transformed by microprojectile bombardment. Aliquots of 50 mL were centrifuged, and the pellet was resuspended in fresh culture medium at a concentration of 0.1 g/mL. Aliquots of 0.5 mL were spread as monolayers onto sterile lens paper that had been placed on culture medium solidified with 0.8% agar in 60-mm Petri plates. Plated cells were kept at 23°C for 3 hr before bombardment. Microprojectile bombardment was performed with a particle accelerator (model PDS-1000; Du Pont) by using a normal rupture disk value of 1500 psi, with the sample positioned 5.5 cm from the launch assembly.

Each batch of cells was cotransformed with a mixture of expression and selection plasmids. A *GUS* gene driven by the CaMV 35S promoter (Benfey and Chua, 1989) was used to measure expression, and an *NPTII* gene driven by the *NOS* promoter (Depicker et al., 1982) was used to select for cells that had stably integrated exogenous DNA. All plasmids were amplified in *Escherichia coli* DH5α and isolated by using

a plasmid maxiprep kit (Qiagen, Inc., Chatsworth, CA). Cotransformation mixtures contained a 4:1 molar ratio of *GUS* reporter plasmid to *NPTII* selection plasmid. Therefore, each 500 ng of SAR transformation mixture consisted of 432 ng of pGHNC11 and 68 ng of pGHNC10, whereas control mixtures contained 314 ng of pGHNC12 and 68 ng of pGHNC10. Each DNA preparation (in 5 μL of Tris-EDTA [TE] buffer) was mixed and precipitated with 50 μL of 2.5 M $CaCl_2$ and 20 μL of 0.1 M spermidine onto 1.0-μm gold microprojectiles.

After bombardment, the Petri plates were sealed with parafilm and incubated for 24 hr at 27°C under constant light. With lens paper, cells were then transferred to fresh plates containing medium supplemented with 100 μg/mL kanamycin. Isolated kanamycin-resistant microcalli began to appear in ~3 weeks, at which time they were transferred to fresh plates containing kanamycin medium. After 1 week of growth on plates, a suspension culture was started for each callus by inoculating 1 mL of broth supplemented with 50 μg of kanamycin per mL. Once established, the suspension cultures were transferred weekly by using 3% (v/v) inocula in 5 mL of broth supplemented with 50 μg/mL kanamycin.

Gene Copy Number Analysis

DNA was isolated as described by Allen et al. (1993). Estimates of *GUS* and *NPTII* gene copy number were obtained for all cell lines by a quantitative polymerase chain reaction (PCR) procedure and confirmed by genomic DNA gel blot analysis. Primers located in the 35S promoter (5′-TCAAGATGCCTCTGCCGACA-3′) and in the translated region of the *GUS* gene (5′-TCACGGGTTGGGGTTTCTAC-3′) were used for *GUS* copy number analysis. Primers located in the *NOS* promoter (5′-GGAACTGACAGAACCGCAAC-3′) and in the translated region of the *NPTII* gene (5′-GGACAGGTCGGTCTTGACAA-3′) were used for *NPTII* gene copy analysis. A "hot start" PCR procedure using Ampli Wax beads (Perkin-Elmer) was used according to the manufacturer's instructions. The lower reaction mixture (25 μL) contained 0.8 mM deoxynucleotide triphosphates, 6 mM $MgCl_2$, 0.4 mM each oligonucleotide primer, 50 mM KCl, 10 mM Tris-HCl, pH 8.8. The upper reaction mixture (75 μL) contained 50 mM KCl, 10 mM Tris-HCl, pH 8.8, 2.5 units of Taq polymerase, and 100 ng of genomic DNA in 10 μL of TE. Each cycle consisted of 2 min at 94°C, 2.5 min at 50°C, and 3 min at 72°C. Reactions were terminated after a final extension step of 7 min at 72°C.

PCR was limited to 18 cycles for both the *GUS* and *NPTII* copy number analysis to avoid substrate exhaustion, and amplification products were visualized by blotting and hybridization with a ^{32}P-labeled DNA probe. Reconstruction standards were prepared by serially diluting DNA from the pGHNC11 (with RB7 SAR) into wild-type NT-1 genomic DNA so as to introduce between one and 150 *GUS* genes per 1C (5 pg) equivalent of tobacco DNA (Arumuganathan and Earle, 1991). PCR reactions were done simultaneously for the reconstruction standards and the samples. Similarly, the *NPTII* reconstruction standards were prepared by serially diluting DNA from the pGHNC10 into wild-type NT-1 genomic DNA so as to introduce between one and 40 *NPTII* genes per 1C. Hybridization signals were quantified on a radioanalytical scanner (Ambis, San Diego, CA), and final copy number estimates were calculated using linear regression analysis.

DNA Gel Blot Analysis

DNA gel blot analysis was performed as described by Murray et al. (1992). Agarose gels were stained with 0.5 mg/mL ethidium bromide and photographed. The top one-third of the gels were treated with 0.25 N HCl for 10 min. The gels were then incubated twice for 15 min in 150 mM NaOH, 3 mM EDTA, and twice for 15 min in 150 mM $NaPO_4$, pH 7.4, and blotted to GeneScreen Plus (Du Pont–New England Nuclear, Wilmington, DE) by the method of Southern, as given in Sambrook et al. (1989), by using 25 mM sodium pyrophosphate. The membranes were blocked by incubating in 2% sodium lauryl sulfate, 0.5% BSA, 1 mM EDTA, 1 mM 1,10-phenanthroline, and hybridized in 100 mM $NaPO_4$, pH 7.8, 20 mM sodium pyrophosphate, 5 mM EDTA, 1 mM 1,10-phenanthroline, 0.1% SDS, 10% dextran sulfate, 500 μg/mL heparin sulfate, 50 μg/mL yeast RNA, and 50 μg/mL herring sperm DNA. Probes were prepared with a random prime DNA labeling kit (U.S. Biochemical Corp.). Washing conditions included one wash at room temperature with 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.5% SDS for 5 mins, one wash at room temperature with 2 × SSC, 0.1% SDS for 15 min, two washes at room temperature with 0.1 × SSC, 0.5% SDS for 15 min, and two washes at 37°C with 0.1 × SSC, 0.5% SDS for 30 min.

NPTII and GUS Assays

For NPTII protein assays, cells were ground in liquid nitrogen and suspended in 100 μL of 0.25 M Tris-Cl, pH 7.8. The mixture was centrifuged, and the supernatant was used for ELISA analysis, performed with an NPTII ELISA kit (5 Prime → 3 Prime, Boulder, CO) according to the instructions of the manufacturer.

For GUS fluorometric analysis, frozen cells were ground in liquid nitrogen, as described for the NPTII and DNA extraction. Approximately 50 mg of the resulting powder was resuspended in 600 μL of GUS extraction buffer containing 50 mM $NaPO_4$, pH 7.0, 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosine (w/v), and 0.1% Triton X-100 (w/v); this suspension was then sonicated twice for 10 sec. The extract was clarified by treatment with insoluble PVP and centrifuged. GUS activity was determined by means of the fluorometric assay described by Jefferson (1987), which used methylumbelliferone glucuronide as substrate. Total protein was measured using the Bio-Rad protein assay kit, and GUS specific activity is reported as picomoles of 4-methylumbelliferone (4-MU) formed per minute per milligram of protein from the initial velocity of the reaction. In an earlier paper (Allen et al., 1993), GUS specific activity units were inadvertently omitted. To determine the specific activity in pmols 4-MU/min/mg protein, multiply the values given in Allen et al. (1993) by a factor of 5.5.

Transient Expression

Protoplasts for electroporation were prepared from 4-day-old NT-1 suspension cultures by a procedure similar to that of Hall et al. (1991). Cells from 100 mL of culture were harvested by centrifugation (300*g* for 2 min), washed twice in 100 mL of 0.4 M mannitol, and resuspended in an equal volume of protoplasting solution containing 0.4 M mannitol, 20 mM Mes, pH 5.5, 1% cellulase (Onozuka RS; Kanematsu-Gosho, Los Angeles, CA), and 0.1% pectolyase Y23 (Onozuka). They were then incubated at 25°C for 30 to 60 min with shaking at 150 rpm. The resulting protoplasts were washed twice in protoplast buffer containing 0.4 M mannitol by centrifuging at 300*g* for 5 min in a Beckman

GPR centrifuge equipped with a GH3.7 rotor. A protoplast concentration of 4 × 10^6 per mL was obtained by diluting the mixture with 0.4 M mannitol. The resulting suspension was then diluted by adding an equal volume of 2 × electroporation buffer to a final concentration of 2 × 10^6 protoplasts per mL. The 2 × electroporation buffer contained 273 mM NaCl, 5.36 mM KCl, 2.94 mM KH_2PO_4, 15.5 mM Na_2HPO_4, 0.4 M mannitol, pH 6.5.

Each electroporation used 80 μg of sheared *E. coli* carrier DNA and 20 μg of the plasmid DNA mixture to be tested. One milliliter of protoplasts was added to the electroporation cuvette (Bethesda Research Laboratories), mixed with 100 μL of DNA mixture in TE buffer, and left on ice for 5 min. Electroporation was performed in a Bethesda Research Laboratory Cell-Porator at 250 V and 1180 μF. Cuvettes were placed on ice for 15 min immediately after treatment. Aliquots (400 μL) of electroporated protoplasts were then transferred to 60-mm Petri plates containing 4 mL of culture medium with 0.4 M mannitol. After incubation for various time periods, protoplasts were collected by centrifugation at 300*g* for 5 min at 4°C. Each pellet was suspended in 600 μL of GUS extraction buffer, and GUS activity was assayed by the fluorogenic procedure described above.

Scaffold Isolation and Binding Assays

Nuclei and nuclear scaffolds from NT-1 cells were isolated as described previously (Hall et al., 1991; Hall and Spiker, 1994). Nuclear halos were washed twice with digestion/binding buffer (D/BB; pH 6.5) containing 70 mM NaCl, 20 mM Tris, pH 8.0, 20 mM KCl, 0.1% digitonin, 1% thiodiglycol, 50 mM spermine, 125 mM spermidine with 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 2 μg mL^{-1} aprotinin (Hall et al., 1991; Hall and Spiker, 1994). The halos were washed again in the same buffer containing 10 mM $MgCl_2$. The halos were then diluted to 4 × 10^6 mL^{-1} in D/BB containing 0.5 mM PMSF, 2 μg mL^{-1} aprotinin, 10 mM phenanthroline, and 10 mM $MgCl_2$ and digested with 500 units mL^{-1} of the various restriction enzymes (New England Biolabs, Beverly, MA) at 37°C for 1 hr. Fresh enzymes were then added, and incubation was continued for an additional 1 hr. Aliquots (100 μL) containing scaffolds representing ~8 × 10^5 nuclei were centrifuged at 2600*g*, the supernatant was removed, and the scaffold pellets were resuspended in D/BB containing 0.5 mM PMSF, 2 μg mL^{-1} aprotinin, and 10 mM $MgCl_2$.

For binding assays, 4 fmol of ^{32}P-labeled fragments, previously digested with restriction enzymes (New England Biolabs), was added to the 100-μL scaffold aliquot and incubated at 37°C for 3 hr with frequent mixings. The scaffold aliquots were centrifuged at 2600*g*, and the pellet (containing scaffold-bound DNA fragments) and the supernatant containing nonbinding fragments were separated. The pellet fraction was washed in 200 μL of D/BB with 10 mM $MgCl_2$, resuspended in 100 μL of TE buffer (representing 100%) containing 0.5% SDS with 0.5 mg mL^{-1} proteinase K, and incubated at room temperature overnight. Equal fractions (usually 20%) of pellet and supernatant fractions were separated on a 1% agarose gel in TAE buffer (Sambrook et al., 1989). The gel was treated with 7% trichloroacetic acid for 20 min and dried onto filter paper followed by exposure to x-ray film.

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