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Matrix attachment regions (MARs) enhance transformation frequencies and reduce variance of transgene expression in barley

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Abstract

Nuclear matrix attachment regions (MARs) are defined as genomic DNA sequences, located at the physical boundaries of chromatin loops. They are suggested to play a role in the *cis* unfolding and folding of the chromatin fibre associated with the regulation of gene transcription. Inclusion of MARs in transgene cassettes enhances their expression and reduces position-effect variations in the transgenic host. The present study is the first to investigate the influence of MAR sequences on transformation frequencies and transgene expression in barley, which is highly relevant to the future improvement of this crop by biotechnology. Two plant MAR sequences were tested both for their ability to bind to the nuclear matrix of barley leaf nuclei and to regulate the expression of a reporter gene in transgenic barley. Competitive *in vitro* MAR binding assays with the 520 bp P1-MAR from soybean and the 516 bp TBS-MAR from petunia revealed that only the P1-MAR had specific binding affinity for barley nuclear matrices. The barley transformation frequency with the *uidA* reporter gene was increased 2-fold when the gene was flanked with either the P1-MAR or TBS-MAR, while the gene copy number was strongly reduced. The presence of P1-MAR sequences increased the mean activity and reduced the variance in expression of a co-integrated reporter gene in barley consistent with the proposed model of MAR activity.

Introduction

It has long been observed that the level of transgene expression in transformants generated in higher eukaryotes can vary over a wide range (Herrera-Estrella *et al.*, 1994). This phenomenon has been ascribed to the random integration pattern of transgenes, as well as to variation in the transcriptional potential of different chromosomal insertion sites (Peach and Velten, 1991).

The activation and expression of genes during the life cycle of a cell is dependent on the controlled unfolding and looping out of specific regions of chromosomal DNA. Within the nucleus, DNA is packed around histones in 30 nm chromatin fibres, which in turn are organized into loops of 5 to 200 kb, attached at their base to a proteinaceous nuclear matrix. Matrix attachment regions (MARs), which are believed to form the boundary between these loops, have been identified by their association with the nuclear matrix from *in vitro* studies (Mirkovitch *et al.*, 1984). MARs are between 300 and 1000 bp in length and have a high AT content. MARs contain several sequence motifs of 5–20 bp, dispersed throughout their length, but lack any unique consensus sequences (Boulikas, 1995). The nuclear matrix-binding affinity of MARs appears to be conserved throughout evolution, since MARs from one species often bind to nuclear matrices from other species (Mielke *et al.*, 1990; Breyne *et al.*, 1992; Allen *et al.*, 1993). Besides having a structural role in DNA packaging, MARs are proposed to contribute to the unfolding of chromatin, facilitating the transcription of genes within loop domains (Laemmli *et al.*, 1992; Zhao *et al.*, 1993). According to this model,

transgenes flanked by MAR sequences would form an independent loop domain upon integration into the host genome, and their expression would be insulated from the influence of the surrounding chromatin (Stief *et al.*, 1989; Phi-Van *et al.*, 1990). The loop domain model predicts that all single-copy transformants, in the absence of position effects, would transcribe a given transgene at the same rate. If additional copies of the transgene in the genome were transcribed at the same rate, total transgene expression level would depend on the transgene copy number (Spiker and Thompson, 1996). However, data from both animals and plants on the ability of MARs to confer position-independent and copy number-dependent control of gene expression have been contradictory (Stief *et al.*, 1989; Phi-Van *et al.*, 1990; Breyne *et al.*, 1992; Allen *et al.*, 1993, 1996; Poljak *et al.*, 1994; Breyne *et al.*, 1992; van der Geest *et al.*, 1994; Mlynárová *et al.*, 1994, 1995; Chinn and Comai, 1996; Han *et al.*, 1997; Liu and Tabe, 1998; Vain *et al.*, 1999; Ülker *et al.*, 1999). Some of these discrepancies may be due to the intrinsic properties of the MARs tested or to other factors, such as gene silencing, which are known to influence transgene expression (Thompson *et al.*, 1996).

In plants, the soybean P1-MAR has been shown to reduce variability in gene expression among independent tobacco transformants (Breyne *et al.*, 1992), while the petunia TBS-MAR (transformation booster sequence-MAR) was found to enhance transformation frequencies in petunia, tobacco and maize (Buising and Benbow, 1994; Meyer *et al.*, 1988). In the present study, the properties of these two plant MARs were investigated with regard to their binding affinity for barley nuclear matrices and their ability to stabilize gene expression levels and reduce position effects in transgenic barley. An *in vitro* MAR binding assay, established for barley nuclei, revealed that the P1-MAR has a strong binding affinity for barley matrices, while none could be detected for the TBS-MAR. Inclusion of either P1-MAR or TBS-MAR in transgene cassettes was found to increase barley transformation frequencies 2-fold, while only the P1-MAR effected levels of transgene expression in a manner consistent with MAR activity.

Materials and methods

Preparation of barley nuclear matrices and in vitro *MAR binding assay*

Barley nuclei were isolated from *Hordeum vulgare* L. cv. Bonus seedlings dark-grown for 6–7 days as described by Green *et al.*, (1989) with the following modifications. Seedling leaf material (200 g) was chopped into 2–3 mm segments, washed in cold distilled H_2O and incubated in 1 litre homogenization buffer (10 mM PIPES-KOH pH 7.0, 10 mM $MgCl_2$, 1 M hexylene glycol, 5 mM 2-mercaptoethanol, 5 mM PMSF, 0.5% Triton X-100) for 12 h under slow stirring. The leaf suspension was homogenized to a coarse brei in a Warring blender for 20 s at medium speed, and then filtered through 1000 μm, 200 μm and 40 μm nylon mesh. Nuclei in the filtrate were pelleted by centrifugation at 1000 $\times$ *g* for 10 min and re-suspended in nuclear wash buffer (NWB: 10 mM PIPES-KOH pH 7.0, 10 mM $MgCl_2$, 0.5 M hexylene glycol, 5 mM 2-mercaptoethanol, 5 mM PMSF, 0.2% Triton X-100). The nuclei were purified on a Percoll gradient by layering the suspension on a step gradient comprising 5 ml each of 40%, 60%, and 80% v/v Percoll in NWB on top of a 5 ml 85% sucrose cushion, and centrifuged at 450 $\times$ *g* for 30 min. Nuclei banding on the sucrose cushion were collected and washed first in 50 ml NWB (minus Triton X-100), pelleted at 365 $\times$ *g* for 10 min, and then in 50 ml nuclear isolation buffer (NIB: 20 mM HEPES-HCl, 20 mM KCl, 0.5 M hexylene glycol, 1% thiodiglycol, 0.05 mM spermine, 0.125 mM spermidine, 0.5 mM EDTA, 0.1 mM PMSF, 2 μg/ml aprotinin pH 7.4), followed by pelleting at 160 $\times$ *g* for 10 min. Washed nuclei, re-suspended in NIB containing 50% glycerol at 5 $\times$ 10^6 nuclei per ml, provided the starting material for the isolation of nuclear matrices.

Barley nuclear matrices were prepared as described by Hall *et al.* (1994) and Mirkovitch *et al.* (1984) with minor modifications. Nuclei for 5 assays (106 per assay) were thawed on ice, washed once in NIB (without EDTA) and resuspended gently in 200 μl NIB (without EDTA) with 1 mM $CuSO_4$. After stabilization for 10 min at 42 °C, 10 ml halo isolation buffer (HIB: 5 mM LIS (lithium diiodosalicylate), 100 mM lithium acetate, 20 mM Hepes-HCl; pH 7.4, 0.1% digitonin, 2 mM EDTA pH 7.4, 0.1 mM PMSF, 2 μg/ml aprotinin) was slowly added and non-matrix proteins were extracted for 15 min at room temperature, while occasionally inverting the tube. Nuclear

halos were collected by centrifugation at room temperature for 10 min at 1000 × *g* and washed 3 times with digestion/binding buffer (D/BB: 70 mM NaCl, 20 mM Tris-HCl; pH 8.0, 20 mM KCl, 10 mM $MgCl_2$, 1% thiodiglycol, 0.1% digitonin, 0.05 mM spermine, 0.125 mM spermidine, 0.1 mM PMSF, 2 μg/ml aprotinin). Optimal extraction was reached when the nuclear mass appeared flaky and was pelletable at 1000 × *g*. Nuclear halos were resuspended in 500 μl of D/BB, 250 units of restriction enzymes *Eco*RI and *Hin*dIII were added, and the suspension was incubated for 3 h at 37 °C on a rotating wheel. After 90 min, another 250 units of each restriction enzyme were added.

Aliquots of nuclear matrices, representing 106 nuclei, were centrifuged at 1000 × *g* for 5 min and the pelleted nuclear matrices resuspended in 100 μl of D/BB. Plasmids containing the P1-MAR (pMAR-1) and TBS-MAR (pTBS2) were digested with the indicated restriction enzymes and the generated restriction fragments were then end-labelled with [^{32}P]-dCTP by Klenow polymerase in the presence of unlabelled dATP, dGTP and dTTP. To assay for MAR binding to nuclear matrices, 18 ng of [^{32}P]-labelled DNA fragments was mixed with 100 μl aliquots of nuclear matrices together with different amounts of unlabelled *Escherichia coli* competitor DNA and incubated for 3 h at 37 °C on a rotating wheel. The nuclear matrices, together with bound DNA fragments, were pelleted by centrifugation at 4500 × *g* for 5 min, leaving unbound DNA fragments in the supernatant. EDTA was added to the supernatant fractions to a final concentration of 20 mM, while the pellet fractions were washed in 200 μl D/BB (without protease inhibitors) and resuspended in 100 μl 10 mM Tris-HCl, 1 mM EDTA buffer containing 0.5% SDS and 0.5 mg/ml proteinase K. After an overnight incubation at 20 °C, the digested pellet fractions were extracted with an equal volume of phenol/chloroform (2:1) and DNA fragments in the aqueous phase were precipitated with sodium acetate/ethanol according to standard protocols. Aliquots of the two separated fractions containing 1500 cpm of [^{32}P]-labelled DNA fragments were subjected to electrophoresis on 1% agarose gels, which were fixed in 7% TCA, dried and analysed by autoradiography.

Plasmid constructions

Standard procedures for PCR and DNA manipulations were as detailed by Sambrook *et al.* (1989). The reporter plasmid (pCUIDAR) contains a reference gene unit previously described by Fang *et al.* (1989), comprising the *E. coli* β-glucuronidase gene (*uidA*; Jefferson *et al.*, 1986) driven by the CaMV 35S promoter (Odell *et al.*, 1985) with the *rbcS*-3c terminator sequence from *Pisum sativum* (Fluhr *et al.*, 1986). Four *uidA* reporter gene constructs flanked by either soybean P1-MAR (Breyne *et al.*, 1992) or petunia TBS-MAR (Meyer *et al.*, 1988; Galliano *et al.*, 1995) sequences, illustrated in Figure 2, were constructed in the following manner. The 520 bp *Eco*RI-*Apa*I P1-MAR fragment and the 516 bp *Hin*dIII TBS-MAR fragment were PCR-amplified with primers to introduce either *Hin*dIII or *Eco*RI restriction sites, and facilitate their subsequent cloning 5′ (*Hin*dIII fragment) or 3′ (*Eco*RI fragment) to the GUS reporter gene. Either one or two MAR fragments were introduced into the reporter gene constructs, in the orientation indicated in Figure 2.

The selection plasmid, pUBARN, previously described by Jensen *et al.* (1998), contains the herbicide resistance *bar* gene from *Streptomyces hygroscopicus* driven by the constitutive maize ubiquitin (Ubi-1) promoter and first intron (Christensen *et al.*, 1992) with the *nos* terminator sequence (Bevan and Favell, 1983).

Stable transformation

Hordeum vulgare L. cv. Golden Promise was grown in a growth chamber under a 16 h light (15 °C)/8 h dark (10 °C) period. Immature zygotic embryos were isolated from spikes, 12–16 days after anthesis (DAA), bisected longitudinally and transformed by microprojectile bombardment as previously described (Wan and Lemaux, 1994; Brinch-Pedersen *et al.*, 1996). The half-embryos were co-transformed with a mixture (1:1) of the *uidA* reporter gene and *bar* selection plasmids. Bombarded half-embryos were incubated in the dark at 25 °C for 24 h on MSWL callus induction-selection medium (Murashige and Skoog medium modified by Wan and Lemaux, 1994) and then transferred to MSWL-5B (containing 5 mg/l bialaphos) for two weeks. Growing calluses were broken into smaller pieces and sub-cultivated onto new MSWL-5B medium every two weeks. Each callus cluster was then sub-cultivated as an individual cell line and harvested after about 4 sub-cultivations.

PCR-based detection of transgenes in barley callus

Barley callus tissue was screened for integration of bar and *uidA* sequences by PCR. Genomic

DNA was isolated from 0.5 g of barley callus from each independent line using a Fast-DNA kit (BIO 101, Vista, CA 92083) as described by the manufacturer. The *bar* gene was detected by PCR amplification of a 429 bp fragment with-using forward 5′-GCAGGAACCGCAGGAGTGGA-3′ and reverse 5′-ATCTCGGTGACGGGCAGGAC-3′ primers, corresponding to bases 150–169 and 559–578 of the *bar* gene (Jensen *et al.*, 1998). The *uidA* gene was detected by PCR amplification of a 306 bp fragment with the forward 5′-GCCAAAAGCCAGACAGAGTG-3′ and reverse 5′-AAACTGCTGCTGTCGGCTTT-3′ primers, corresponding to bases 1066–1085 and 1352–1371 of the *uidA* gene. The 25 μl PCR mixtures comprised 0.2 μg DNA, 0.2 mM dNTPs, 50 pmol of each primer, 0.65 U *Taq* polymerase (Perkin Elmer), 1× PCR buffer (Perkin Elmer), supplemented with 10% DMSO for amplification of the *bar* gene and 20% glycerol for amplification of the *uidA* gene. Forty amplification cycles (94 °C for 30 s; 67 °C (*bar* gene)/52 °C (*uidA* gene) for 30 s; 72 °C for 2 min) were performed.

GUS assay

Callus tissue samples (ca. 0.5 g) were suspended in 250 μl 100 mM K_2HPO_4/KH_2PO_4, pH 7.8, 1 mM DTT and disrupted by the Fast-Prep equipment (BIO 101). The soluble extracts were cleared by centrifugation and 150 μl aliquots were assayed for GUS activity according to Jefferson *et al.* (1987), using the substrate 4-methylumbelliferyl β-D-galactoside (MUG). GUS activity was measured by the amount of fluorescent 4-methyl umbelliferone produced, detected in a fluorometer (Fluoroscan II scanner, Labsystem, Finland), and expressed as fluorescent units (minus the control extract values) generated in 24 h at 37 °C. GUS activity is given in arbitrary units per μg total protein in the callus extract, measured with a Bradford protein assay kit (BioRad, Hercules, CA 94547). Statistical analysis of the data was performed with the Microsoft Excell 97 SR-1 spread sheet program (t-test and F-test), Analyse-it for Microsoft Excel (Analyse-it software, Leeds, UK; Shapiro-Wilk test), and VassarStats (Statistical Computations Web Site; Mann-Whitney test (http://faculty.vassar.edu/∼lowry/utest.html).

Gene copy number analysis

Estimates of *uidA* gene copy number were obtained by a combination of a quantitative polymerase chain reaction (PCR) procedure and Southern blot hybridization analysis (Allen *et al.*, 1996). The PCR procedure for amplification of the *uidA* gene in the genomic DNA samples, described above, was modified to allow a 'hot-start' by inclusion of Ampli Wax beads (Perkin-Elmer) according to the manufacturer's instructions. PCR cycles were limited to 18, to avoid exhaustion of substrates, followed by a final extension step of 10 min at 72 °C. Reconstruction standards were prepared by serially diluting the *uidA* reporter gene plasmid DNA into wild-type genomic barley DNA to introduce between 1 and 20 *uidA* genes per 1C chromosome complement of barley DNA.

A Southern blot was prepared of the PCR products of the genomic DNA samples and the reconstruction standards on Hybond-N+ membrane (Amersham Pharmacia Biotech, Little Chalfont, HP7 9NA, UK) according to the manufacturer's instructions. The blot was hybridized with a 306 bp *uidA* gene fragment (corresponding to 1066–1371 bp), random-prime-labelled with ^{32}P using the Megaprimer DNA labelling system (Amersham Pharmacia Biotech). The hybridized blot was washed under high stringency (3 times 0.1× SSC, 0.1% SDS for 15 min at 68 °C) and the hybridizing signals were analysed and quantified on a phosphorimager (Molecular Dynamics 3.3). The copy number estimates were calculated by linear regression analysis.

Transient transformation

Hordeum vulgare L. cv. Alexis plants were grown in a growth chamber with 16 h light (15 °C) and 8 h (10 °C) dark periods and developing grains were harvested 25–27 DAA. The grains were surface-sterilized in 3.5% sodium hypochlorite, rinsed in sterile water and the embryo and husk/pericarp/testa layers were removed from the dorsal grain surface under sterile conditions. The five different *uidA* reporter gene plasmids (Figure 2) were each mixed in a 2:1 molar ratio with a luciferase control plasmid (pAHC18; Bruce *et al.*, 1989), precipitated onto gold particles (ca. 1.6 μm in diameter) and introduced into the aleurone layer of the dissected grains by particle bombardment according to the method of Klein *et al.* (1988). Five replicate bombardments of five grains were performed for each of the five *uidA* reporter gene plasmids and a minus-DNA control, and the series was repeated three times. The bombarded grains were incubated for 2 days at 25 °C in a humidified chamber and then each sample was homogenized in 1 ml 100 mM K_2HPO_4/KH_2PO_4, pH 7.8, 1 mM DTT. The grain extract was cleared by

centrifugation and the GUS and luciferase activities in the supernatant were measured as described by Leah *et al.* (1994). The relative GUS activity was calculated by dividing the GUS fluorescent unit value by the luciferase light unit value obtained for each sample. This relative value minimizes the variation in activity due to differences in efficiency of the sample bombardments and extractions.

Results

In vitro *binding affinity of P1-MAR and TBS-MAR to the barley nuclear matrix*

In vitro MAR binding assays were performed to test the affinity of the soybean P1-MAR (Breyne *et al.*, 1992) and the petunia TBS-MAR (Meyer *et al.*, 1988) to barley nuclear matrices. The proteinaceous nuclear matrices were prepared from nuclei isolated from barley leaves according to a procedure developed for barley. The nuclear matrices were incubated with end-labelled endonuclease restriction fragments of the P1-MAR or TBS-MAR plasmids, shown in Figure 1C, in the presence of 0–50 μg *E. coli* DNA, which was added as a competitor to eliminate non-specific DNA binding to the nuclear matrix.

All three restriction fragments of the P1-MAR plasmid, namely the 520 bp P1-MAR fragment, the 1030 bp P1 fragment and the 3 kb vector, all bound to barley matrices in the absence of competitor DNA (Figure 1A, lane 2). However, non-specific binding was abolished in the presence of 50 μg competitor DNA and only the P1-MAR fragment retained a strong affinity for the barley nuclear matrices (Figure 1A, lane 4). The 50 μg *E. coli* DNA corresponds to a 25 000-fold excess of competitor DNA with respect to the amount of P1-MAR fragment, indicating that the interaction between the P1-MAR fragment and the barley nuclear matrix was specific. The P1 fragment, located immediately 3′ to the P1-MAR fragment, also bound to the nuclear matrix in the presence of competitor, but with a weaker affinity than the P1-MAR (Figure 1A, lane 4 vs. lane 5).

The five restriction fragments of the TBS-MAR plasmid all showed partial binding to the nuclear matrices in the presence of 5 μg competitor DNA (Figure 1B, lane 2 vs. lane 3). When the concentration of added competitor DNA was increased to 15 and 50 μg (Figure 1B, lanes 4–7 vs. lane 2) the level of DNA binding to the matrices was negligible. An additional band, migrating above the 516 bp TBS-MAR, was weakly detectable in each of the pellet fractions. Since this band failed to co-migrate with any of the restriction fragments in the probe, it is probably an artefact, possibly resulting from the incomplete stripping of proteins non-specifically bound to one of the probe fragments, as observed previously for the 3′ MAR of the tomato HSC80 gene (Chinn and Comai, 1996). Accordingly, these data indicate that the petunia TBS-MAR had no specific *in vitro* binding affinity for barley nuclear matrices, while the soybean P1-MAR has a strong *in vitro* binding affinity. In order to correlate the observed nuclear matrix binding properties of the P1-MAR and TBS-MAR fragments with their respective structural properties, their DNA sequences were analysed for the occurrence of sequence motifs commonly associated with MARs (Singh *et al.*, 1997). MAR sequences are frequently AT-rich and may include ATTA, ATTTA and ATTTTA motifs which are found at origins of replication and homeotic protein-binding sites. TG-rich spans, abundant in the 3′-untranslated regions of genes and associated with sites of DNA recombination, and topoisomerase II binding and cleavage sites are also clustered in MARs. Furthermore, MARs may contain regions of intrinsically curved DNA or kinked DNA caused by the repetition of specific sequence motifs. Rather than performing a manual search for MAR associated sequence motifs, the P1-MAR and TBS-MAR sequences, fused in tandem, were submitted to www.futuresoft.org/modules/MarFinder/index for analysis using the MarFinder algorithm (Singh *et al.*, 1997). Since matrix association is a property of a span of a sequence, the analysis is performed on a sliding window which in this case was set to a width of 500 nt and a slide distance of 50 nt. The algorithm calculates a MAR potential value, normalized to fall between 0 and 1, which is based on the density of the sequence motifs/patterns described above. Analysis of the complete fused sequence (1–4300), shown in Figure 1C, defines the P1-MAR fragment as a region with high MAR potential and the P1 fragment as a region with lower MAR potential, while the TBS-MAR sequence was found to be devoid of MAR potential. Since peaks of MAR potential can be attenuated by adjacent peaks with particularly high MAR potential, the analysis window was shifted to the sequence downstream of the P1-MAR (521–4300). The second MarFinder scan confirmed the peak of MAR potential in the P1 fragment and the absence of MAR potential in the TBS-MAR.

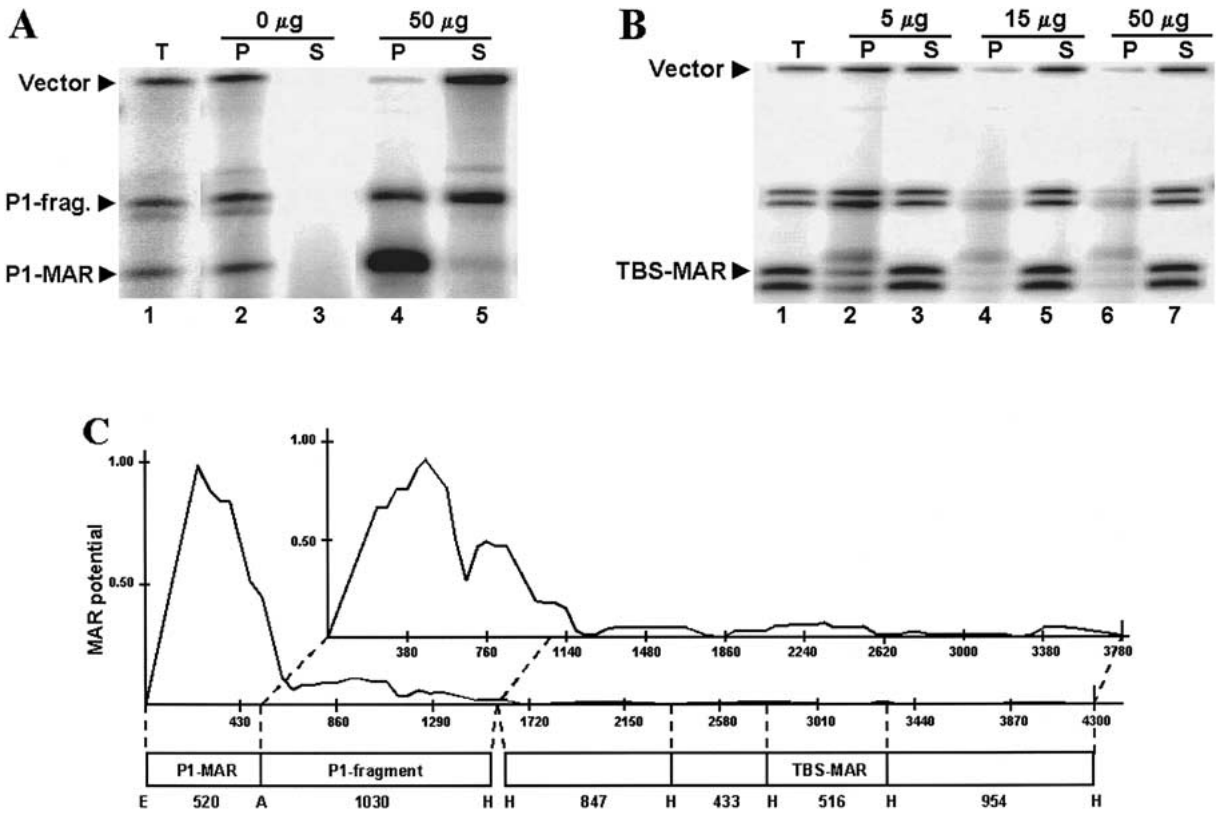

Figure 1. A and B. *in vitro* nuclear matrix binding assay of the soybean P1-MAR and petunia TBS-MAR with nuclear matrices from barley leaves. A. The P1-MAR plasmid was digested with the restriction enzymes *Eco*RI, *Apa*I and *Hin*dIII to give a 520 bp P1-MAR fragment, a 1030 bp P1 fragment and a 3 kb vector fragment. B. The TBS-MAR plasmid was digested with the restriction enzyme *Hin*dIII to give 954 bp, 847 bp, 516 bp and 433 bp fragments and a 3 kb vector fragment. Matrices prepared from 10^6 barley nuclei were incubated with ca. 18 ng of ^{32}P-labelled fragments together with different concentrations of *E. coli* competitor DNA (0, 5, 15 or 50 μg/assay). About 1500 cpm of ^{32}P-labelled fragments from the matrix binding assay were separated on agarose gels and analysed by autoradiography. T, total probe control; P, matrix-bound fragments; S, unbound fragments. C. Analysis of P1-MAR and TBS-MAR using the MarFinder algorithm (Singh *et al.*, 1997). The two sequences (P1-MAR and TBS-MAR) were fused in tandem and the MAR potential is indicated on the abscissa along with the relative DNA position (bp). The width of the sliding window and the slide distance are 500 nt and 50 nt, respectively. In order not to attenuate the MAR potential for the P1 fragment and the TBS-MAR, a second scan was performed as indicated in the smaller window. A, *Apa*I; E, *Eco*RI; H, *Hin*dIII.

Generation of transgenic barley callus harbouring MAR constructs

The influence of the P1-MAR and the TBS-MAR on the integration pattern and expression levels of transgenes introduced into the genome of barley was investigated. A series of plasmids were prepared which all contained a reporter gene cassette, the *uidA* gene driven by the CaMV 35S promoter (Figure 2). The control plasmid comprised the reporter gene cassette alone (control plasmid), while the test plasmids contained the reporter cassette flanked by either one or two copies of P1-MAR (denoted 1-MAR and 2-MAR, respectively) or one or two copies of TBS-MAR (denoted 1-TBS and 2-TBS, respectively). Each plasmid was co-transformed into immature barley embryos, together with a physically unlinked selectable marker gene, *bar*, carried on the selection plasmid pUBARN. The *bar* gene, specifying phosphinothricin acetyltransferase, confers resistance to the herbicide bialaphos, which is included in the tissue culture medium to select for transformed barley tissue. In five independent transformation experiments, each of the five *uidA* reporter constructs were transformed into two sets of 30 embryos by particle bombardment.

A total of 180 independent bialaphos-resistant callus lines were obtained from 1500 bombarded embryos. Bialaphos-resistant callus lines were analysed by PCR to confirm the integration of the *bar* gene and to study the co-transformation frequency of the

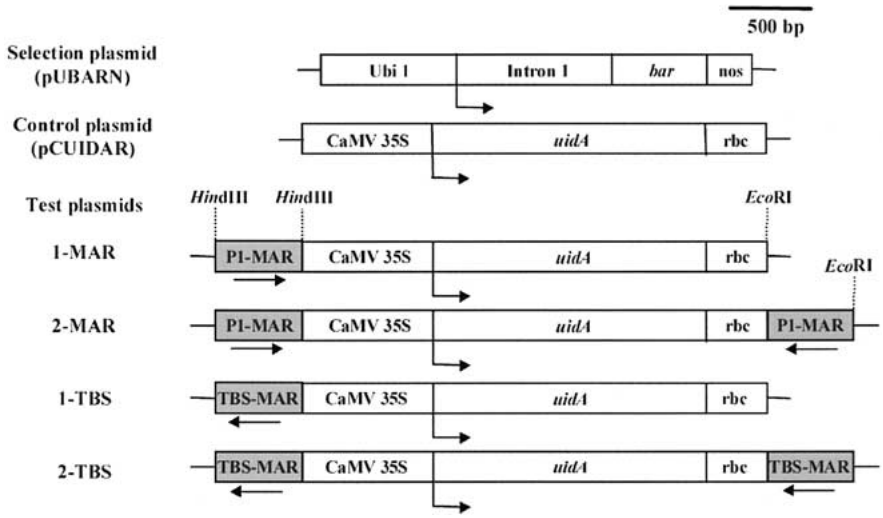

Figure 2. Schematic illustration of the *uidA* reporter gene and the selectable *bar* gene constructs used for barley transformation. Test plasmids comprised the P1-MAR and TBS-MAR, inserted 5′ (*Hin*dIII) to, or 5′ (*Hin*dIII) and 3′ (*Eco*RI) to the *uidA* reporter gene. Construct with one P1-MAR is denoted 1-MAR and with two P1-MARs, 2-MAR, and constructs with one TBS-MAR are denoted 1-TBS and with two TBS-MARs, 2-TBS. Arrows beneath the MARs indicate the orientation (forward or reverse) of the MARs. MAR activity has not been found to be influenced by their orientation (Schöffl *et al.*, 1993). Bar represents 500 bp.

uidA reporter genes. The *bar* gene was present in all bialaphos-resistant callus lines, indicating that there were no escapes in the transformation selection regime. However, the *uidA* gene was only detected in 104 of the 180 bialaphos-resistant callus lines. Comparison of the five transformed populations (Table 1) revealed that the co-transformation frequency of the *uidA* gene was increased two-fold when the reporter gene cassette was flanked by either a P1-MAR (1-MAR or 2-MAR) or a TBS-MAR (1-TBS or 2-TBS).

MARs enhance transgene expression in stable callus lines

The barley callus lines transformed with both *bar* and *uidA* genes were harvested after 4 sub-cultivations, and analysed for GUS activity. Initial analysis of the transgene GUS activity in the five populations indicated that most of the data sets were positively skewed, having a wide range bounded by zero at the low end and outliers at the high end. Since the most powerful statistical tools are based on the assumption of a normal distribution, we have followed the statistical guidelines of Nap *et al.* (1993) for handling transgenic plant data. A logarithmic transformation of the GUS activity data gave data sets with a closer approximation to normality, as judged by comparing their actual and predicted normal cumulative distribution functions (not shown). The correlation coefficients of the lnGUS activity data to a normal distribution (Shapiro-Wilk test) were 0.9254, 0.9818, 0.9583, 0.8399 and 0.8774 for the control,1-MAR, 2-MAR, 1-TBS and 2-TBS populations respectively.

A statistical analysis of the GUS activity data for the five populations of transgenic lines is set out in Table 1. All four populations transformed with MAR-flanked sequences had a higher mean lnGUS activity with respect to the control population (Table 1, Figure 3), but only in the 1-MAR and 2-MAR populations was the difference statistically significant (t-test, $P<0.001$). Since the normal distribution correlation coefficient was poorer for the 1-TBS and 2-TBS populations, a distribution-free test was also used to compare the untransformed GUS activities. The Mann-Whitney test confirmed that the increased GUS activity in the 1-MAR and 2-MAR populations was highly significant, while the increased GUS activity in the 1-TBS population was just significant ($P<0.05$). A reduction in variance, which has been attributed to MAR-flanked gene expression, was only found for the 1-MAR and 2-MAR populations, significant at $P<0.001$ (F-test).

In order to study the influence of MAR flanking sequences on the number of gene copies integrated during a transformation event and their expression levels, the transgene copy number was analysed in 59 of the *bar/uidA*-transformed lines generated from

Table 1. Statistical analysis of GUS activity expressed in five transgenic barley populations.

Population	Lines[a] (*n*)	Mean[b] GUS	*P* M-W test[c]	Mean[d] lnGUS	Variance[d] lnGUS	*P* *t*-test[e]	*P* *F*-test[f]
Control	11	212		3.92	2.24		
1-MAR	27	699	***	6.39	0.71	**	***
2-MAR	23	719	***	6.47	0.49	**	***
1-TBS	23	679	*	5.37	2.26	NS	NS
2-TBS	20	759	NS	5.25	2.39	NS	NS

[a]Number of *bar/uidA* transformed lines.
[b]Means calculated of GUS activity expressed as arbitrary units of GUS per mg protein.
[c]Probability according to the Mann-Whitney test for location with respect to the corresponding control population, calculated from untransformed GUS activity.
[d]Mean and standard deviation (= variance$^{1/2}$) calculated from natural logarithms of GUS activity (lnGUS).
[e]Probability according to the *t*-test for location for a two-tailed distribution with unequal variance, with respect to the corresponding control population (calculated from lnGUS activity).
[f]Probability according to the *F*-test for homogeneity of variances with respect to the control population (calculated from lnGUS activity).
NS, not significant; * $P<0.05$; ** $P<0.01$; * $P<0.001$.

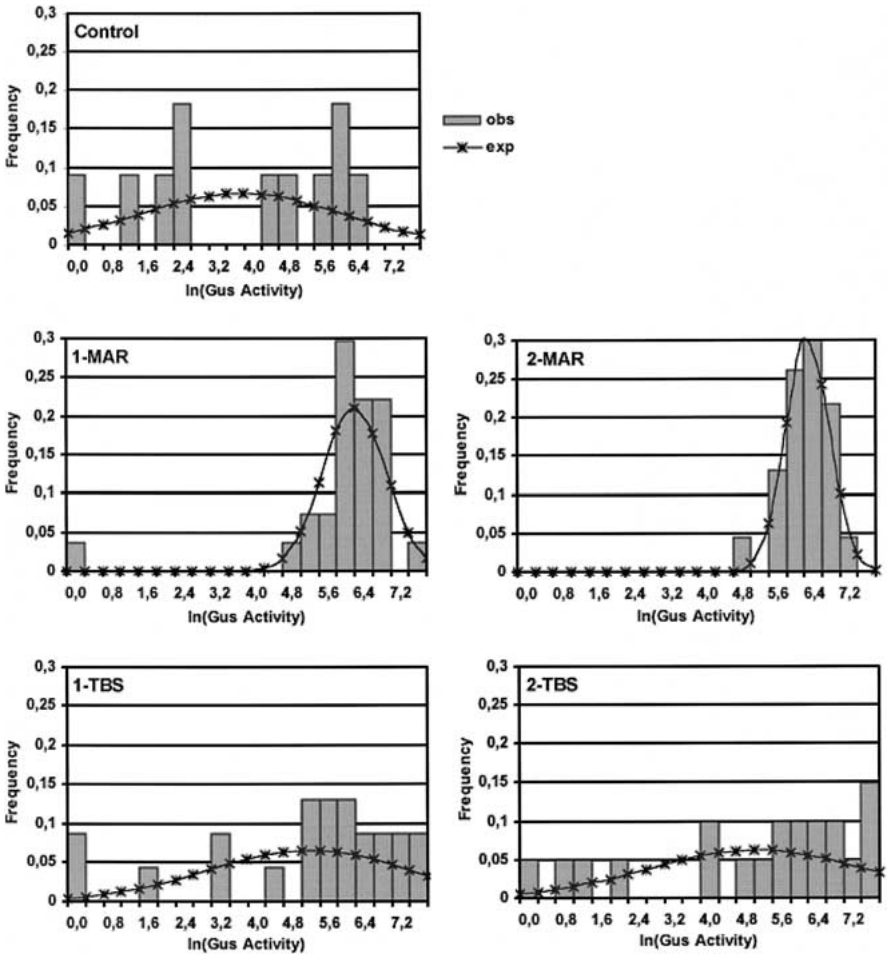

Figure 3. Frequency distribution plots of the logarithmic transforms for GUS activity for the different transgene constructs. Expected distribution (***) is the normal distribution curve based on the mean and standard deviation of the ln(GUS activity) data, which are shown as histograms.

Table 2. Transient expression.

Plasmid[a]	GUS/LUC ratio[b]
Control	1.00
1-MAR	1.15
2-MAR	0.94
1-TBS	1.10
2-TBS	0.88

[a]The five *uidA* reporter gene plasmids were mixed with pAHC18 (luciferase) in the proportion 2:1.
[b]Relative GUS activity is reported as GUS fluorescent units per LUC light unit and normalized to the control plasmid. Mean values were calculated from three independent experiments.

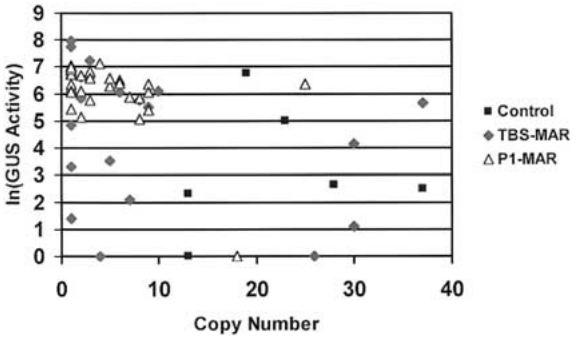

Figure 4. GUS activity as a function of *uidA* gene copy number. The 1-MAR and 2-MAR transformed lines are represented by triangles, 1-TBS and 2-TBS lines by diamonds and the control lines by squares. For each of the populations, the number of *uidA* reporter gene copies are plotted against the natural logarithm (ln) of GUS activity, which is reported as arbitrary units per μg total protein.

two of the 5 transformation series. The *uidA* copy number in the 59 lines was analysed with a combination of transgene PCR amplification and quantitative Southern hybridization (data not shown). The lines transformed with the control *uidA* plasmid had a high mean copy number of 22.2 copies per transformant, while lines transformed with a MAR-flanked *uidA* plasmid had lower mean copy numbers, of 3.7 (1-MAR population), 5.7 (2-MAR population), 9.2 (1-TBS population) and 9.6 (2-TBS population) copies. The relationship between the GUS activity and *uidA* gene copy number for each of the 59 transformed lines are shown in Figure 4. The level of GUS activity in those lines having a high copy number was low, relative to lines with fewer than 10 copies. The lines transformed with a MAR-flanked reporter gene were characterized by both a lower *uidA* copy number and a higher level of GUS activity. There is no indication, even in lines with low copy numbers, that the level of reporter gene expression is positively correlated with copy number.

Genomic integration is a prerequisite for MAR enhancement

The MAR sequences in each of the test plasmids were placed in close proximity to the *uidA* reporter gene cassette, with the potential to influence directly the rate of *uidA* transcription. A transient expression assay was performed to detect potential transcriptional enhancement effects of the different MAR sequences. In this assay the transgenes are not integrated in the genome, thereby eliminating the influence of local chromatin structure at the site of integration on reporter gene expression levels. Each test plasmid was introduced into developing barley aleurone tissue by particle bombardment together with a luciferase (LUC) gene plasmid as an internal control to measure transformation efficiency. After 24 h incubation, extracts were prepared from the transformed tissues and assayed for GUS and LUC activities, and the calculated relative GUS activities are given in Table 2. Three independent experiments revealed that the presence of neither of the two MAR elements significantly increased GUS expression levels relative to the control construct. Accordingly, the two MAR sequences in this study do not function as *cis*-acting transcriptional regulators of the *uidA* gene during transient expression.

Discussion

Soybean P1-MAR binds specifically to barley nuclear matrices

Barley nuclear matrices have been isolated from purified leaf nuclei, using procedures developed for higher plants (Hall *et al.*, 1994), allowing the matrix-binding properties of MAR sequences (P1-MAR and TBS-MAR) to be examined in barley for the first time.

P1-MAR was originally identified as one of six matrix-binding fragments (S1) present in a 17.1 kb lectin L4p1 clone from soybean (Breyne *et al.*, 1992). The 1.55 kb P1-MAR yields two fragments on digestion with *Apa*I, where the smaller fragment of 520 bp exhibits stronger binding properties to tobacco matrices than the 1030 bp fragment. The

two P1-MAR fragments were also found to have a binding affinity for barley nuclear matrices, where the 520 bp fragment similarly bound more strongly. Breyne *et al.* (1992) performed a structural analysis of the P1-MAR sequence and reported a high A+T content for the 520 bp fragment (78%) as well as a 190 bp region (70%) of the 1030 bp fragment. Furthermore, several sequence motifs characteristic of animal MARs, including A-boxes and T-boxes and poly(dAdT) stretches, were localized to these regions. We have used the MarFinder algorithm to analyse the pattern density of MAR sequence motifs in the P1-MAR and identify DNA regions with high MAR potential (Singh *et al.*, 1997). The 520 bp fragment and the 5′ region of the 1030 bp fragment showed strong MAR-potential scores consistent with the reported structural analysis and with the observed matrix binding of the two P1-MAR fragments (Figure 1A).

The transformation booster sequence, TBS-2, is a 2 kb *Eco*RI fragment from a *Petunia hybrida* genomic fragment, which was found to enhance the transformation frequency of selectable marker plasmids introduced into petunia by PEG-mediated, direct transfer (Meyer *et al.*, 1988). TBS-2 contains a MAR with binding affinity for petunia nuclear matrices, which has been localized to a 516 bp *Hin*dIII fragment within the 2 kb sequence (Galliano *et al.*, 1995). *Petunia* TBS showed no specific affinity for barley nuclear matrices in the *in vitro* binding assay. The failure of TBS to bind selectively to barley nuclear matrices was unexpected in view of the extent of heterologous MAR binding observed in *in vitro* assays (Breyne *et al.*, 1992). Sequence analysis of the TBS has previously identified the presence of MAR-associated elements along the length of the TBS sequence (Buising and Benbow, 1994). However the A+T content is not strongly elevated in any region (53–56%) and the MarFinder analysis failed to detect MAR-potential within any part of the TBS sequence (Figure 1C).

Barley transformation frequencies are enhanced by both P1-MAR and TBS-MAR

Barley embryos were co-transformed by particle bombardment with two plasmids, one carrying the selectable marker gene *bar* and a second carrying the reporter gene *uidA*. In the control plasmid the reporter gene was flanked by plasmid DNA sequences, while in the test plasmids it was flanked by one or two TBS-MAR as a direct repeat, or one or two P1-MAR as inverse repeats. The barley co-transformation frequency was increased about 2-fold when the reporter gene was flanked with either one or two copies of the 520 bp fragment of P1-MAR or the 516 bp *Hin*dIII of TBS (TBS-MAR). The significance of the orientation of two flanking MAR sequences is not known, but the similar affects of one or two MAR sequences on transformation frequency argues against a major effect.

Although the effect of P1-MAR on transformation frequency has not previously been examined, the ability of the TBS-MAR to enhance transformation rates is well documented. Transformation frequencies of both petunia and tobacco protoplasts were enhanced ca. 20-fold when the selectable marker gene was flanked by the 2 kb TBS fragment, placed in either forward or reverse orientation relative to a reporter gene (Meyer *et al.*, 1988). Subsequent studies have confirmed that the TBS fragment can enhance transformation frequencies in tobacco (8- to 16-fold), as well as in maize (1.7- to 2.4-fold) at a level similar to that seen in barley (Buising and Benbow, 1994).

From our own observations in barley, it is likely that those sequence elements in TBS required to enhance transformation frequency are present in the smaller 516 bp *Hin*dIII fragment included in our test plasmids. TBS is enriched in elements which facilitate DNA unwinding, increase sensitivity to single-strand-specific nucleases or are sites for topoisomerase II cleavage, which generates double-stranded breaks, autonomous replication elements and pyrimidine tracts (Buising and Benbow, 1994). These elements have been suggested to contribute to the increased transformation frequency associated with TBS, perhaps by enhancing illegitimate recombination between the introduced transgenes and genomic DNA. Integration of foreign DNA into random chromosomal sites has been shown to occur by illegitimate recombination (Takano *et al.*, 1997). The ability of TBS to promote recombination within plant protoplasts between homologous sequences in extrachromosomal plasmid DNA is consistent with the effect of TBS of transgene integration (Galliano *et al.*, 1995).

Transgenes flanked by P1-MAR have higher expression levels with reduced variance in barley

GUS activity in the four populations of barley callus lines transformed with the *uidA* reporter gene flanked with MAR sequences was compared with the control population transformed with the *uidA* gene. The two populations transformed with P1-MAR constructs

(1-MAR and 2-MAR) had a higher mean GUS activity than the control population which was statistically significant, while the increased GUS activity in the population transformed with a single TBS (1-TBS) was only weakly significant according to the Mann-Whitney test (Table 1). A wide variance in transgene expression levels, characteristic of plants transformed by direct transfer, was seen in the control population as well as the two TBS populations. However, the variance in GUS expression levels in the 1-MAR and 2-MAR populations was significantly reduced (Figure 3).

MAR sequences flanking integrated genes are proposed to enhance transgene expression levels by binding to the nuclear matrix and insulating the intervening DNA from transcription-negative properties conferred by the surrounding chromatin, which may be transcriptionally inactive. Furthermore, the gene sequences between two matrix-bound MARs are believed to form an independent chromatin loop, which is then directly accessible to the transcription machinery responsible for gene expression. While the ability of P1-MAR flanking sequences to reduce variance in transgene expression in barley, as seen in our own experiments, and in tobacco (Breyne *et al.*, 1992) is consistent with this model, it is important to consider the impact of MARs on the number of genes integrated and their relative expression levels.

Transgene copy number in barley is reduced by P1-MAR-flanking sequences coincident with higher expressionn

Analysis of the *uidA* gene copy number in the five transgenic populations, generated by two transformation experiments, revealed that the copy number was reduced by the presence of MAR-flanking sequences, particularly by the 1-MAR and 2-MAR where the mean copy numbers were 3.7 and 5.7, respectively, compared to 22.2 in the control population. When GUS expression levels are compared with gene copy number in the individual lines within the five populations (Figure 4) it is immediately apparent that GUS expression per gene copy falls sharply with increasing copy number. Furthermore, a copy number of above two to three in the majority of transformants irrespective of the presence of MARs is accompanied by a reduction in total detectable GUS activity, a phenomenon suggestive of gene silencing.

Plant transformation by direct transfer using supercoiled plasmids commonly leads to the integration of multiple copies of plasmid genes which may be inserted in clusters at a few loci. T-DNA-mediated transformation, as well as direct transfer of linearized gene cassettes, tends to generate low-copy-number transformants (van der Geest *et al.*, 1994; Fu *et al.*, 2000). Despite this, the presence of *Arabidopsis* MAR sequences flanking the *uidA* in T-DNA further reduces transgene copy numbers in tobacco transformed with *Agrobacterium* (Liu and Tabe, 1998). Similarly, a reduction in copy number after transformation of tobacco by direct transfer has been seen for reporter genes flanked by either a MAR sequence from yeast (ARS-1) or from tobacco (Rb7) (Allen *et al.*, 1993, 1996). Gene silencing, due to reversible epigenetic gene inactivation between multigene copies, is widely recognized as a cause of low transgene expression in plants (Cogoni and Macino, 1999). However, recent studies have shown that the presence of MAR sequences cannot always protect co-integrated transgenes from gene silencing at either the transcriptional or post-transcriptional level (Vaucheret *et al.*, 1998).

Increased expression of MAR-flanked transgenes may not solely due to the low copy number providing a lower risk of gene silencing. In a comparison of low-copy-number transformants, the presence of MAR-flanking sequences (ARS-1 or Rb7) is associated with significantly higher transgene expression in both tobacco and rice, consistent with the MAR chromatin hypothesis (Allen *et al.*, 1993, 1996; Vain *et al.*, 1999). In our own studies in barley, P1-MAR greatly reduced the variance in GUS expression among low-copy-number transformants in comparison to the TBS-MAR transformants, similar to the reduced variance seen among P1-MAR transformants in tobacco (Breyne *et al.*, 1992). Since all of our control population of barley transformants contained multiple gene copies, where gene silencing may have occurred, we are not able to assess the extent to which the P1-MAR may increase the expression of a single-copy co-integrated reporter gene. Indeed the increased mean GUS expression in the P1-MAR populations may primarily be attributed to the reduced copy number, together with reduced variance in expression levels.

Two copies of the P1-MAR increased mean GUS expression and reduced variance among the transformants only slightly more than one copy. At first sight this observation is inconsistent with the chromatin loop hypothesis which requires two MAR sequences surrounding the transcribed region. However, the integration of <2 transgenes copies at one locus will effectively provide flanking MAR sequences around

at least one of the reporter genes, which may partly explain the similarity between 1-MAR and 2-MAR populations. Recombination events between the introduced plasmids prior to or accompanying the transformation event may also lead to the inclusion of one or more MAR sequences at the integration site. As a consequence of the co-integration events occurring during transformation it would also seem likely that P1-MAR flanking sequences may enhance the expression of both the *bar* selectable marker and the GUS gene. If the level of *bar* gene expression in barley cells was a major limiting factor during the selection of transformed tissue, then the frequency of transformation should be particularly enhanced for the P1-MAR constructs. However, this mechanism cannot account for the enhanced transformation frequency of TBS-MAR constructs, where reporter gene expression is not significantly increased. Alternatively, DNA recombination elements present in both TBS-MAR and P1-MAR may be responsible for the increased transgene integration in barley.

Since the MAR sequences in our test constructs were placed in close proximity to the *uidA* gene and its promoter, elements within the MAR region could act as enhancers or repressors of the downstream CaMV 35S promoter driving *uidA* expression. However, transient expression assays of the control and test constructs in aleurone cells confirmed that neither P1-MAR nor TBS-MAR acted as enhancers/repressors of *uidA* expression in barley.

In conclusion, the soybean P1-MAR sequence shows a specific affinity for barley nuclear matrices in an *in vitro* binding assay and reduces the variance in expression of transgenes co-integrated into the barley genome in a manner consistent with the proposed properties of MARs. TBS-MAR does not bind specifically to barley nuclear matrices and fails to exhibit any significant effect on the level or variance in transgene expression in barley, which is consistent with the results of similar studies in tobacco and maize (Buising and Benbow, 1994). However, both P1-MAR and TBS-MAR increased the frequency of transformation and the number of low-copy-number transformants, suggesting that both sequences contain elements that enhance the recombination events leading to gene integration in the barley genome and which may occur independently of matrix binding.

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References

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

Allen, G.C., Hall, G. Jr., Michalowski, S., Newman, W., Spiker, S., Weissinger, A.K. and Thompson, W.F. 1996. High-level transgene expression in plant cell: effect of a strong scaffold attachment region from tobacco. Plant Cell 8: 899–913.

Allen, G.C., Hall, G. Jr., Michalowski, S., Newman, W., Spiker, S., Weissinger, A.K. and Thompson, W.F. 1996. High-level transgene expression in plant cell: effect of a strong scaffold attachment region from tobacco. Plant Cell 8: 899–913.

Bevan, M.W. and Flavell, R.B. 1983. A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. Nature 304: 184–187.

Boulikas, T. 1995. Chromatin domains and prediction of MAR sequences. Int. Rev. Cytol. 162A: 279–388.

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

Brinch-Pedersen, H., Galili, G., Knudsen, S. and Holm, P.B. 1996. Engineering of the aspartate family biosynthetic pathway in barley (*Hordeum vulgare* L.) by transformation with heterologous genes encoding feedback-insensitive aspartate kinase and dihydrodipicolinate synthase. Plant Mol. Biol. 32: 611–620.

Bruce, W.B., Christensen, A.H., Klein, T., Fromm, M. and Quail, P.H. 1989. Photoregulation of a phytochrome gene promoter from oat transferred into rice by particle bombardment. Proc. Natl. Acad. Sci. USA 86: 9692–9696.

Buising, C.M. and Benbow, R.M. 1994. Molecular analysis of transgenic plants generated by microprojectile bombardment: effect of petunia transformation booster sequence. Mol. Gen. Genet. 243: 71–81.

Chinn, A.M. and Comai, L. 1996. The heat shock cognate 80 gene of tomato is flanked by matrix attachment regions. Plant Mol. Biol. 32: 959–968.

Christensen, A.H., Sharrock, R.A., and Quail, P.H. 1992. Maize polyubiquitin genes: structure thermal perturbation of expression and transcript slicing and promoter activity following transfer to protoplasts by electroporation. Plant Mol. Biol. 18: 675–689.

Cogoni, C. and Macino, G. 1999. Homology-dependent gene silencing in plants and fungi: a number of variations on the same theme. Curr. Opin. Microbiol. 2: 657–662.

Fang, R.X., Nagy, F., Sivasubramaniam, S. and Chua, N.H. 1989. Multiple *cis* regulatory elements for maximal expression of the cauliflower mosaic virus 35S promoter in transgenic plants. Plant Cell 1: 141–150.

Fluhr, R., Moses, P., Morelli, G., Coruzzi, G. and Chua, N.H. 1986. Expression dynamics of the pea *rbcS* multigene family and organ distribution of the transcripts. EMBO J. 5: 2063–2071.

Fu, X., Duc, L.T., Fontana, S., Bong, B.B., Tinjuangjun, P., Sudhakar, D., Twyman, R.M., Christou, P. and Kohli, A. 2000. Linear transgene constructs lacking vector backbone sequences generate low-copy-number transgenic plants with simple integration patterns. Transgenic Res. 9: 11–19.

Galliano, H., Müller, A.E., Lucht, J.M. and Meyer, P. 1995. The transformation booster sequence from *Petunia hybrida* is a retrotransposon derivative that binds to the nuclear scaffold. Mol. Gen. Genet. 247: 614–622.

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

Green, P.J., Kay, S.A., Lam, E. and Chua, N.H. 1989. *In vitro* DNA footprinting. In: S.B. Gelvin and R.A. Schilperoort (Eds.) Plant Molecular Biology Manual, Kluwer Academic Publishers, Dordrecht, Netherlands, pp. B11: 1–22.

Hall, G. Jr. and Spiker, S. 1994. Isolation and characterisation of nuclear scaffolds. In: S.B. Gelvin and R.A. Schilperoort (Eds.) Plant Molecular Biology Manual, Kluwer Academic Publishers, Dordrecht, Netherlands, pp. D2: 1–12.

Han, K., Ma, C. and Strauss, S.H. 1997. Matrix attachment regions (MARs) enhance transformation frequency and transgene expression in poplar. Transgenic. Res. 6: 415–420.

Herrera-Estrella, L., van den Broeck, G., Maenhaut, R., Van Montagu, M., Schell, J., Timko, M. and Cashmore, A. 1984. Light-inducible and chloroplast-associated expression of a chimaeric gene introduced into *Nicotiana tabacum* using a Ti plasmid vector. Nature 310: 115–120.

Jefferson, R.A., Burgess, S.M. and Hirsh, D. 1986. Bbeta-glucuronidase from *Escherichia coli* as a gene-fusion marker. Proc. Natl. Acad. Sci. USA 83: 8447–8451.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. 1987. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6: 3901–3907.

Jensen, L.G., Politz, O., Olsen, O., Thomsen, K.K. and von Wettstein, D. 1998. Inheritance of a codon-optimized transgene expressing heat stable (1,3-1,4)-β-glucanase in scutellum and aleurone of germinating barley. Hereditas 129: 215–225.

Klein, T.M., Gradziel, T., Fromm, M.E. and, Sanford, J.C. 1988. Factors influencing gene delivery into *Zea mays* cells by high-velocity microprojectiles. Bio/technology 6: 559–563.

Laemmli, U.K., Käs, E., Poljak, L. and Adachi, Y. 1992. Scaffold-associated regions: *cis*-acting determinants of chromatin structural loops and functional domains. Curr. Opin. Genet. Dev. 2: 275–285.

Leah, R., Skriver, K., Knudsen, S., Ruud-Hansen, J., Raikhel, N.V. and Mundy, J. 1994. Identification of an enhancer/silencer sequence directing the alurone-specific expression of a barley chitinase gene. Plant J. 6: 579–589.

Liu, J.W. and Tabe, L.M. 1998. The influences of two plant nuclear matrix attachment regions (MARs) on gene expression in transgenic plants. Plant Cell Physiol. 39: 115–123.

Meyer, P., Kartzke, S., Niedenhof, I., Heidmann, I., Bussmann, K. and Saedler, H. 1988. A genomic DNA segment from *Petunia hybrida* leads to increased transformation frequencies and simple integration patterns. Proc. Natl. Acad. Sci. USA 85: 8568–8572.

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

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

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

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

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

Odell, J.T., Nagy, F. and Chua, N.H. 1985. Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313: 810–812.

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

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

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

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, NY.

Schöffl, F., Schröder, G., Kleim, M. and Rieping, M. 1993. A SAR sequence containing 395 bp DNA fragment mediates enhanced gene-dosage-correlated expression of a chimaeric heat shock gene in transgenic tobacco plants. Transgenic Res. 2: 93–100.

Singh, G.B., Kramer J.A. and Krawetz, S.A. 1997. Mathematical model to predict regions of chromatin attachment to the nuclear matrix. Nucl. Acids Res. 25: 1419–1425.

Spiker, S. and Thompson, W.F. 1996. Matrix attachment regions and transgene expression in plants. Plant Physiol. 110: 15–21.

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

Takano, M., Egawa, H., Ikeda, J.-E. and Wakasa, K. 1997. The structures of integration sites in transgenic rice. Plant J. 11: 353–361.

Thomson, W.F., Allen, G.C., Hall, G. Jr. and Spiker, S. 1996. Matrix attachment regions and transgene expression. In: P. Gustafson and R.B. Favell (Eds.) Genomes of Plant and Animals (Proceedings of the 21stst Stadler Genetics Symposium), Plenum, New York, pp. 243–270.

Vain, P., Worland, B., Kohli, A., Snape, J.W., Christou, P., Allen, G.C. and Thompson, W.F. 1999. Matrix attachment regions increase transgene expression levels and stability in transgenic rice plants and their progeny. Plant J. 18: 233–242.

Vaucheret, H., Elmayan, T., Thierry, D., van der Geest, A., Hall, T., Conner, A.J., Mlynárová, L. and Nap, J.P. 1998. Flanking matrix attachment regions (MARs) from chicken, bean, yeast or tobacco do not prevent homology-dependent *trans*-silencing in transgenic tobacco plants. Mol. Gen. Genet. 259: 388–392.

Wan, Y. and Lemaux, P.G. 1994. Generation of large numbers of independently transformed fertile barley plants. Plant Physiol. 104: 37–48.

Ülker, B., Allen, G.C., Thompson, W.F., Spiker, S. and Weissinger, A.K. 1999. A tobacco matrix attachment region reduces the loss of transgene expression in the progeny of transgenic tobacco plants. Plant J. 18: 253–263.

Zhao, K., Käs, E., Gonzalez, E. and Laemmli, U.K. 1993. SAR-dependent mobilization of histone H1 by HMG-I/Y *in vitro*: HMG-I/Y is enriched in H1-depleted chromatin. EMBO J. 12:, 3237–3247.