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Matrix attachment regions (MARs) enhance transformation frequency and transgene expression in poplar

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We tested the value of a matrix attachment region (MAR) fragment derived from a tobacco gene for increasing the frequency of Agrobacterium-mediated transformation. A binary vector that carried a GUS reporter gene containing an intron and an nptlI gene was modified to contain flanking MAR elements within the T -DNA borders. Vectors containing or lacking MARS were then used to transform tobacco, a readily transformable poplar clone (*Populus tremula* X *P alba*), and a recalcitrant poplar clone (*Populus trichocarpa* X *P deltoides*). MARS increased GUS gene expression approximately 10-fold in the two hybrid poplar clones and twofold in tobacco one month after cocultivation with Agrobacterium; MARS also increased the frequency of kanamycin-resistant poplar shoots recovered.

Keywords: GUS; matrix attachment regions; Populus; transformation; transgene expression; Agrobacterium

Introduction

The level of transgene expression varies widely among independent transformants (Hobbs *et al.,* 1990), which causes the majority of detectable transformants to express introduced genes at low levels (Peach and Velten, 1991). This phenomenon is usually attributed to `position effects', chromosomal differences among sites of integration that influence gene expression. In addition, gene silencing (Finnegan and McElroy, 1994; Flavell, 1994; Matzke and Matzke, 1995) may inactivate many transgenes shortly after their incorporation into plant chromosomes. As a consequence, many physically transformed plant cells are likely to be killed during antibiotic selection, resulting in the low recovery of transgenic tissues. Modification of transgenes to increase the uniformity and level of expression may therefore result in a substantial improvement in rate of transformation.

Matrix attachment regions (MARS) are DNA sequences that bind to the cell's proteinaceous nuclear matrix to form DNA loop domains (for a recent review, see Spiker and Thompson, 1996). Transgenes flanked with MARS are thought to be able to form their own chromatic domain and thus be insulated from the influences of factors in the chromatin adjacent to its site of insertion (Hall *et al.,* 1991). Because a large majority of plant chromatin is in an inactive conformation at any given time, insulating transgenes with MARS may reduce the incidence of gene silencing and enhance transgene expression.

MARS have been shown to increase reporter gene expression in tobacco cells, and reduce position effects in mature transgenic plants. When the (3-glucuronidase (GUS) reporter gene was flanked by MARS, variation in GUS expression among independent transformants (Mlynarova *et al.,* 1994, 1995) and their offspring (Mlynarova *et al.,* 1996) was reduced. Using biolistic transformation, Allen *et al.* (1993) presented evidence that MARS reduced co-suppression when the number of transgenes did not exceed 40. Mlynarova *et al.* (1995) found that MARS reduced the number of putatively transgenic shoots that were unable to root in the presence of antibiotic (`escapes'), and thus improved net transformation efficiency.

The goal of this study was to investigate the value of MARS for enhancing transformation efficiency and

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transgene expression in poplars. We studied poplars because of their economic importance, the recalcitrance to transformation of many economically important clones (Han *et al.,* 1997), and because the effects of MARS in their genomes was unknown. In forest trees, MARS may be important for maintaining strong and stable transgene expression during the many cycles of vegetative propagation and years of growth in plantations.

Materials and methods

Plasmid constructs

The MAR fragments used in this study were obtained from Dr Steven Spiker at North Carolina State University and derived from a tobacco genomic clone (Hall *et al.,* 1991; Allen *et al.,* 1996). An intermediate vector pKH20 was made by inserting the *KpnI/SacI* fragment from the North Carolina State University vector pGHNC9, containing the HPTII gene flanked by MARS, into the *KpnI/SacI* site of pKH. pKH is a vector derived from the binary vector pBINl9 (Bevan, 1984) by deletion of the 1.6 kb *PstI* fragment containing the NPTII gene. Then, a *HindIII* fragment with GUS-INT gene (Vancanneyt *et al.,* 1990) was inserted into the HindIII site of the intermediate vector pKH20 to generate the final vector pKH200 (pMAR+), carrying GUS and NPTII genes flanked by MARS. Likewise, a Kpn *IlSac I* fragment from pGHNC 10, containing the NPTII gene without flanking MARS, was inserted into pKH to make another intermediate vector pKH30. This vector was then used to make the control construct lacking MARS, pKH300 (pMAR-) by insertion of GUS-INT fragment (Fig. 1).

Plant materials *and transformations*

Shoot cultures were established from two hybrid poplars *(Populus tremula* x *P. alba* clone 717 and Populus *trichocarpa* x P *deltoides clone* 184-402). Stem internodes from in-vitro-grown poplars and leaf disks from growth room-grown *Nicotiana tabacum* var. Xanthi were used for transformation. The growth room was maintained at 23 °C with a 16-hr photoperiod using white fluorescent lamps. Binary vectors were introduced into the *Agrobacterium tumefaciens* strain C58/pMP90 (Knocz and Schell, 1986) by the freeze-thaw transformation method (An *et al.,* 1988). Transformation methods were largely as described previously (Strauss *et al.,* 1995; Han *et al.,* 1996). Bacterial cultures (OD6oo = 0.3-0.5) were induced for 1 hr in Murashige and Skoog (MS) medium supplemented with 200 [tM acetosyringone, 10 mM galactose, and 1.28 mM 2-[N-Morpholinol]ethanesulfonic acid (MES) (adjusted to pH of 5.0). Explants were cut in the bacterial suspension and placed under vacuum (50 mmHg) for 20 min at room temperature, followed by cocultivation for 2-3 days in the dark on a callus induction medium (CIM: MS + 0.5 [LM 6-benzylaminopurine (BA) + 0.5 p,M zeatin + 5 [tM a-naphthaleneacetic acid (NAA) + 5 g,M 2,4dichlorophenoxyacetic acid (2,4-D) + 0.3% Phytoagar + 0.1% Gelrite + 1.28 mM MES; pH 5.8). After cocultivation, the explants were rinsed with wash solution (WS: 1 /2 MS salts and vitamins + 250 mg 1-1 ascorbic acid -+ 1 pM NAA + 10 NM BA +I p,M N6-isopentenyl-adenine (2iP) + 1 ~tM indole-3-butyric acid (IBA); pH 5.8), and cultured on CIM supplemented with 500 mg 1-1 cefotaxime for 7 days. Then, the explants were cultured for 21 days on CIM containing 100 mg 1-1 kanamycin and 500 mg 1-1 cefotaxime. Shoots were regenerated on shoot induction medium (SIM: MS + 10 p,M BA + 10 W zeatin + 1 [t,M NAA +0.3% Phytoagar + 0.1% Gelrite + 1.28 mM MES; pH 5.8) supplemented with 100 mg 1-1 kanamycin. Regenerated shoots were excised from the explant and rooted on shoot culture medium (SCM: 1/2 MS medium supplemented with 0.5 [t,M IBA and 25 mg 1-~ kanamycin).

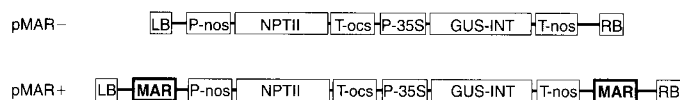

Fig. 1. Schematic maps of the T -DNA region of binary vectors. The relative positions of the GUS gene and the nptTI gene are shown with respect to the left border (LB) and right border (RB) of the T - DNA. The T -DNA regions are not drawn to scale.

Reporter gene expression

For quantitative GUS assays, total protein extracts were prepared from five stem explants for poplars and five leaf disks for tobacco. Protein content was measured using a standardized Bradford assay. (3-Glucuronidase activity was measured on extracts containing 30 ~tg of total protein using 2 mM methylumbelliferylglucuronide (MUG) after a 30-min incubation at 37 °C (Jefferson *et al.,* 1987). Typical GUS readings from these assays (before normalization - see below) were 400-4000 pmol mg-1 min-' for the MAR containing construct, 200-1200 for the MARlacking construct and 15-60 for the non-transgenic controls (data from genotype 717).

Statisitical design and analysis

We conducted up to 10 independent transformation experiments over several months, from which subsamples were taken at various times after cocultivation to assess the effect of the MAR construct on GUS trangene expression and recovery of kanamycin-resistant tissues. To reduce the variability in mean GUS expression levels observed among experiments, fluorometric GUS readings were normalized for each experiment and genotype by dividing GUS activity per unit protein by that from samples of nontransgenic control plants. All data were then analysed

using GLM procedure in SAS (SAS, 1990). Effects were plant genotype, experiment, MAR and the MAR x genotype interaction. Means were compared by leastsquares means tests when overall effects were significant in the models. Separate analyses were conducted to study MAR effects on gene expression at specific intervals after cocultivation, for separate genotypes, and when using means taken over a number of days of assay. Standard errors were derived from variance among experiments.

Results

Effects of MARS on transformation during the first 7 days

Histochemical GUS assays were used to examine the number of transformation events (GUS foci). Assays were performed on days 1, 2, 3, 5 and 7 after cultivation. Apart from a modest increase in expression at days 3-5 observed in 717, MAR effects only became evident during days 5 to 7. Pooled over all 7 days, the increase in the number of GUS foci was statistically significant only in tobacco ($p < 0.01$) and when data from all species were pooled ($p < 0.05$).

Fluorometric GUS assays during the same period differed substantially, however, from those based on number of GUS foci. The largest effect of MARS was observed with poplar 717; GUS activity conferred by the MAR-containing vector was 5.9-fold above that without the flanking MAR elements when avaraged over the 5 assay periods. Although the MAR vector also increased the average level of gene expression in the other two genotypes, the effect was small and not statistically significant.

Effects of MARS four weeks after transformation

To reduce possible influences of MARS on transient gene expression, we assayed GUS gene expression four weeks after cocultivation. During this time explants were cultured on CIM medium without kanamycin. As transformed foci enlarged, GUS-positive regions tended to overlap making counting difficult (Fig. 2). Pooled over species, MARs caused a significant ($p < 0.05$) 1.2-fold increase in the mean number of foci per explant.

Fluorometric GUS activity, however, was strongly and significantly increased by the MAR vector in all species. GUS activity was increased 8.7-fold in 717, 7.7-fold in 184-402, and 1.6-fold in tobacco (Fig. 3). The absolute level of GUS gene expression from the MAR vector was more than 20-fold higher in 717 compared to that in 184402 and tobacco.

Effects of MARS on the recovery of kanamycin-resistant tissues

We used kanamycin selection to examine the effects of MARS on transformation efficiency. As verified by GUS

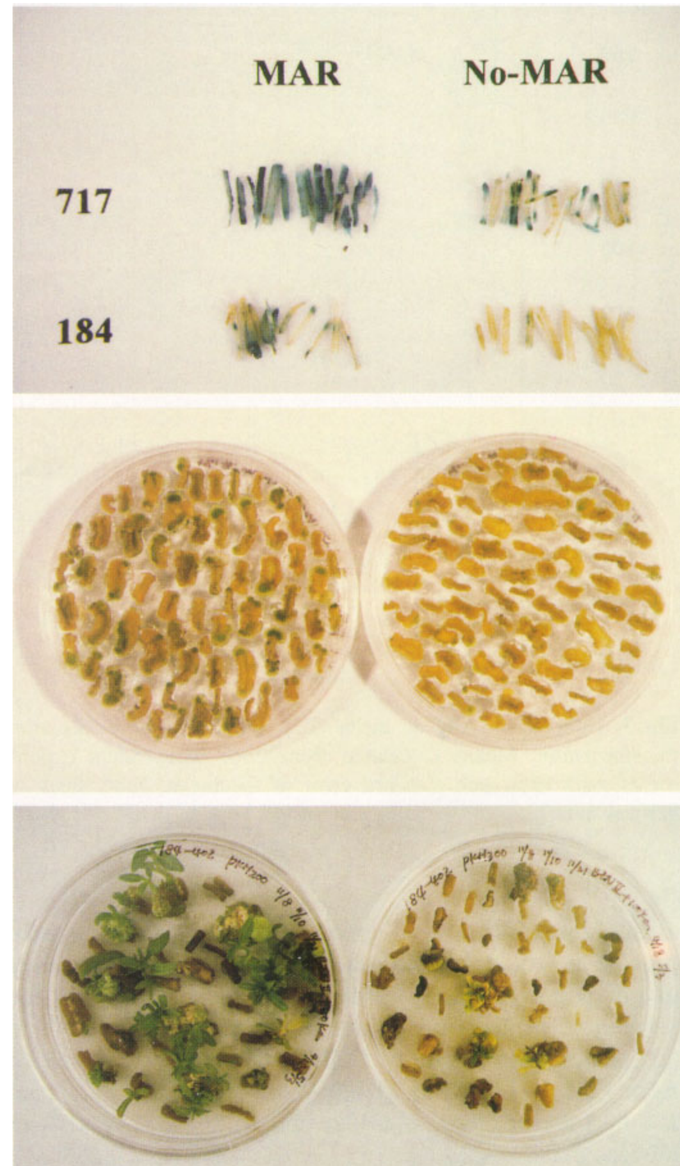

Fig. 2. Effect of MARS on GUS expression and antibiotic resistance of representative examples of transformed tissues. (Top) GUS staining of representative stem explants from clones 717 and 184-402 transformed with binary vectors with or without flanking MARS. GUS expression was assayed four weeks after culture on a callus induction medium lacking kanamycin. (Middle) MARS increased the frequency of kanamycin-resistant (green) calli on stems from clone 717 cultured for two months on callus induction medium supplemented with 100 mg 1-l kanamycin. Explants on left plate were inoculated with Agrobacterium carrying pMAR+, explants on the right plate with pMAR-. Photo taken from underside of the plates. (Bottom) MARs increased the frequency of regeneration of kanamycin-resistant shoots from stems of clone 184-402 cultured on a shoot induction medium containing 100 mg 1` kanamycin. Explants on left plate were inoculated with Agrobacterium carrying pMAR+, explants on the right plate with Agrobacterium carrying pMAR-. The photograph was taken 6 months after cocultivation.

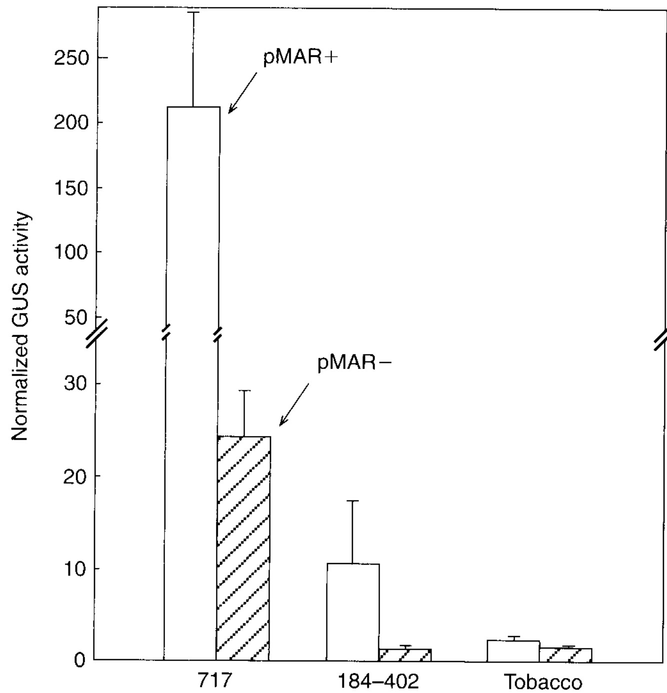

Fig. 3. Effect on MARs on mean GUS activities four weeks after transformation. Means calculated from three independent experiments; bars show one standard error of the mean. Note break in vertical axis.

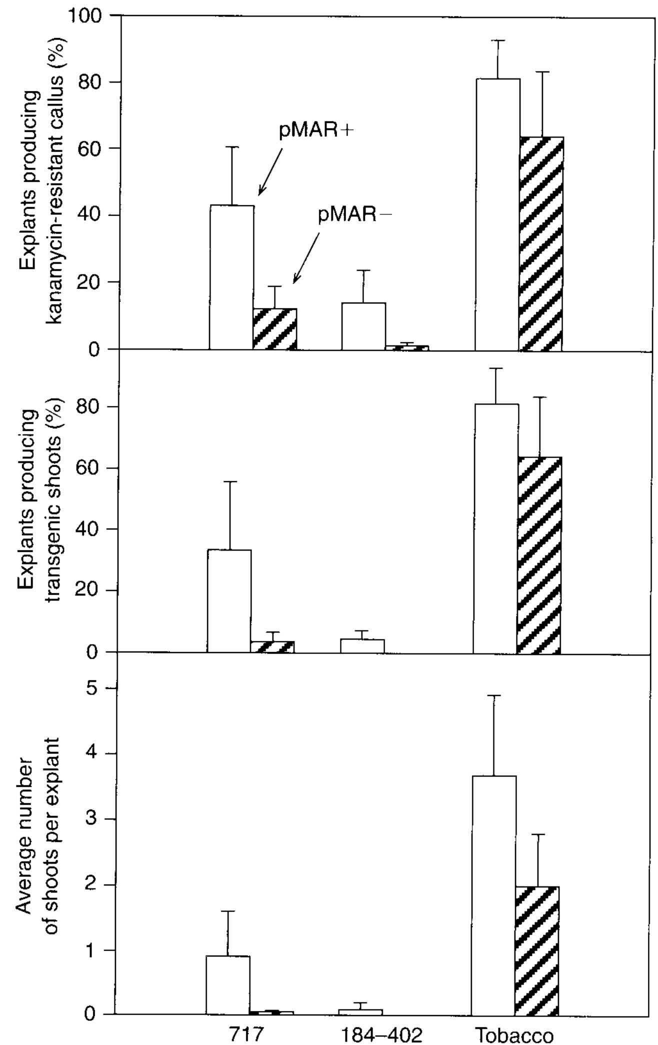

Fig. 4. Effect of MARS on apparent transformation frequency. Average values were calculated from three independent experiments which contained 17-68 explants per treatment. (Top) The MARcontaining construct increased the frequency of kanamycin-resistant calli in the three plant genotypes studied. Explants were cultured for two months in callus induction medium supplemented with 100 mg 1-l kanamycin. (Middle) The MAR-containing construct increased the frequency of explants regenerating putative kanamycin-resistant shoots in the three plant genotypes studied. Explants were cultured for two months on callus induction medium and then for three months on shoot induction medium containing 100 mg 1-1 kanamycin. (Bottom) The MAR-containing construct increased the frequency of regeneration of putative kanamycin-resistant shoots per explant in the three plant genotypes. Experiment as described above for regeneration of kanamycin resistant shoots.

assays, the formation of green, vigorously growing calli provide a reliable indication of transformation frequency in our culture system. MARS significantly increased the number of explants producing kanamycin-resistant calli in all species (Figs 2 and 4). Callus frequency was increased from 12% (pMAR-) to 43% (pMAR+) in clone 717, from 1 % to 14% in clone 184-402, and from 64% to 81 % in tobacco.

Regeneration of putative kanamycin-resistant shoots was also significantly enhanced by the MAR vector (Fig. 4). MARS increased shoot regeneration frequency from 4% (pMAR-) to 34% (pMAR+) in clone 717, from 0% to 4% in clone 184-402, and from 64% to 81 % in tobacco. Based on subsequent rooting in kenamycincontaining medium, the advantage of pMAR+ compared with pMAR-- for frequency of rooted shoots per cocultivated explant was 12% versus 1% for 717, 0.6% versus 0% for 184-402, and 14% versus 6% for tobacco. MARs also appeared to increase the rate of shoot regeneration per explant in all species; however, this effect was not statistically significant.

Discussion

To our surprise, we observed larger effects of the MAR construct in both poplar species than we did in tobacco.

This may represent differences in how these MARs function in the different genera, or could simply result from differences in tissue development in our experimental system; leaf disk materials from non-sterile, growth room-derived plants were used for tobacco, while

sterile, tissue-cultured stem segments were used for the poplars. Given the functional conservation of MAR elements, where animal MARS have been shown to function well in plants (Mlynarova et al., 1994, 1995), differential activity between two families of discotyledonous plants was unexpected.

We assessed transformation based on both kanamycin resistance and expression of an intron containing GUS gene. In our transformation system, shoot/root regeneration from a kanamycin-resistant callus in the presence of kanamycin is proportional to the rate of actual transformation. When judged by subsequent GUS-INT expression, we had only a 3% rate of escape (1 out of 35 shoots were able to root in kanamycin at 100 mg *1-1* with weekly subculture, but did not show GUS expression). Because of the possibility of differential gene silencing, all plants rooting in kanamycin may in fact be transformed, but the GUS gene inactivated in some; thus, our escape rate may be even lower. We believe that this low rate of escape results from frequent subculture (7-10-day interval), which avoids significant depletion of antibiotic from the medium.

We attempted to measure the number of transformed cells by histochemical GUS assays; however, there was little correlation between the number of GUS foci and total GUS expression. MARs stimulated the largest increase in the number of GUS foci in tobacco, yet they caused a smaller increase of net GUS expression there than they did in the poplars. This may indicate that the mechanism of MAR effects can differ between species, perhaps because the MAR we used was in homologous cellular environment and stimulated co-suppression in tobacco. In addition, number of GUS foci is likely to have been a weak measure of the number of transformed cells in our study, mainly because GUS foci were difficult to accurately score on our small in-vitro-grown stem segments. The rate of transformation was sometimes so high that it was difficult to recognize individual spots (e.g. in clone 717 with pMAR+, which may account for its nonsignificant increase in number of GUS foci yet very large increase in GUS activity).

The effect of MARS on temporal patterns of gene expression varied widely among genotypes. Early effects (3-5 days) were observed in clone 717, while the other species did not display substantial effects until four weeks after cocultivation. Clone 717 also showed the largest relative increase in transgene expression at 4 weeks (Fig. 3). MARS typically have little effect on transient gene expression when measured in animal and plant systems (Allen et al., 1993; Klehr et al., 1991), suggesting that MARS do not work as simple enhancers but must be incorporated into the host genome to have their primary effect. Thus, we interpret the strong response of clone 717, which is highly susceptible to Agrobacterium transformation, to be due to rapid incorporation and expression of transgenes in this genotype.

We have demonstrated an increase of both transformation rate and transgene expression in a woody plant shortly after Agrobacterium cocultivation. Studies are in progress to determine whether improved transgene expression also occurs in regenerated trees. Our work adds to the growing literature that shows that MARS are useful tools for elevating and stabilizing the expression of transgenes in plants and animals. Further, we have shown that MAR effects, while generally beneficial, differ importantly between genotypes and species. Use of MARS should expand the range of genotypes amenable to standard Agrobacterium-transformation protocols, and may allow scientifically and commercially useful transgenic plants to be generated from small transformation programmes.

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