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TM2, a novel strong matrix attachment region isolated from tobacco, increases transgene expression in transgenic rice calli and plants

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Abstract Nuclear matrix attachment regions (MARs) are thought to influence the expression of the flanking genes. TM2, a new DNA fragment isolated from tobacco, can bind with the rice nuclear matrix in vitro. In this study, we investigated the effect of TM2 on transgene expression under the control of three different promoters in stably transformed rice calli and plants. The presence of TM2 flanking the transgene increased the expression of constructs based on the constitutive CaMV 35S and maize *ubiquitin* gene promoters in both resistant calli and transformed plants. The GUS expression directed by the photosynthetic-tissue-specific PNZIP promoter was also increased in photosynthetic tissues of transformants. However, TM2 did not change the gene expression pattern controlled by the PNZIP promoter. The effect of TM2 in transgenic plants was stronger than that in transgenic calli based on all three promoters. Our results indicate that TM2, as a novel strong MAR, can be used to increase the transgene expression levels in the whole plant or in particular tissues of monocotyledons.

Introduction

Both practical applications and basic research of gene transfer technology are sometimes severely handicapped by transgene silencing and instability of gene expression (Allen et al. 2000). Gene silencing is caused by many factors, such as DNA methylation, position effect, co-suppression, chromatin structure, and trans-inactivation (Fagard et al. 2000). Matrix attachment regions (MARs) have recently been seen as fundamental tools to reduce or eliminate some forms of transgene silencing because of their capacity to increase transgene expression levels or reduce the variance between transgenic events when flanking a transgene cassette in both plants and animals (Breyne et al. 1994; Spiker et al. 1996; Allen et al. 2000).

Different kinds of MARs have been isolated and studied from a diverse range of eukaryotes such as yeasts, plants, and animals (Breyne et al. 1992; Allen et al. 1996; Michalowski et al. 1999). Nearly all characterized MARs are AT-rich and generally contain regions that tend to produce single-stranded or base-unpaired regions such as A boxes, T boxes, one- or multi-unwinding sites, and topoisomerase II-binding sites (Allen et al. 2000). MARs are classed as "strong", "medium," and "weak" sequences, based on the strength with which they bind to the nuclear matrix in vitro and upon how they function to improve transgene expression in vivo (Michalowski et al. 1999).

In higher plants, studies about MARs are mainly focused on the effect of enhancing transgene expressions and the influences on transformant variabilities (Mlynarova et al. 1996; Han et al. 1997; Butaye et al. 2004; Peterson et al. 2002). Various influences of MARs on transgene expression have been reported (Spiker et al. 1996). Flanking with the bean β-phaseolin gene MAR caused a modest increase in overall expression and a modest decrease in variability of the expression of a reporter gene in tobacco plants (Van der Geest et al. 1994). The presence of MAR isolated from a clone containing a soybean heat shock gene resulted in a fivefold-to-ninefold increase in the expression of the β-glucurindase (*gusA*) reporter gene, but no effect on the variability of expression was noted (Schoffl et al. 1993). A recent report showed that the maize 5′ADH1 MAR played a dual role in transgenic maize; it increased the gene expression in resistant calli, but the gene expression in transgenic roots was inhibited (Brouwer et al. 2002). Due to the diversity of the function of different MARs, it is necessary to obtain MARs that can be widely used to improve transgene expression in various crops (Spiker et al. 1996).

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We have been interested in isolating and identifying strong MARs that could be used to improve transgene expression. In our previous work, 19 DNA fragments (MARs) from tobacco nuclear matrixes were isolated, and most of them could bind with the rice nuclear matrix in vitro. The subsequent tobacco transformation experiment indicated that TM2, a novel MAR, could enhance transgene expression more efficiently than some other MARs (Zhang et al. 2002). After further investigating of its influence on transgene expression by different promoters and in other plant species, we describe here the functional analysis of TM2 in transgenic monocotyledon rice in different development stages and tissues, and discuss the prospects for the applications of TM2.

Materials and methods

Isolation and characterization of TM2

Nuclei were isolated from a tobacco (*Nicotiana tabacum* L. cv. NC89) cell line, and nuclear matrixes were isolated from the isolated nuclei according to Hall's method (Hall et al. 1994). The nuclear matrix was treated with the restriction enzymes *Eco*RI and *Hin*dIII. The remaining DNA fragments that were associated with the purified nuclear matrix were purified by overnight treatment with proteinase K, followed by phenol/chloroform extraction and ethanol precipitation. The DNA fragments obtained from matrixes made with *Eco*RI and *Hin*dIII were cloned into the pUC18 vector, and large fragments were further cleaved with additional restriction enzymes to produce subclones (Michalowski et al. 1999). To investigate the ability of the inserted fragments to bind with rice nuclear matrixes, we chose 19 clones to analyze by in vitro binding assay, using Hall's method (Hall et al. 1991). The pUC18 plasmids were cut with *Eco*RI and *Hin*dIII to release the inserts, and both vectors and inserts were end-labeled with α-[^{32}P]dCTP. About 200 ng labeled MARs and the corresponding linear plasmid vectors were incubated with a $3A_{260}$ nuclear matrix and with 50 g/l sonicated *Escherichia coli* genomic DNA as competitor in 400 µl binding buffer at 37°C overnight. After the incubation of a pellet (bound DNA fragment) fraction, which was formed by centrifuging at 12,000 *g* for 40 min at room temperature, DNA was recovered from supernatant and pellet fractions by treatment with proteinase K in the presence of 1% SDS for 1 h at 65°C and subsequently extracted with phenol. Then, one fifth of the DNA was gel-electrophoresed, dried, and exposed on X-ray films. In an assay involving quantification, the amounts of DNA in bands in the supernatant and pellet fractions were determined by using Labworks Analysis Software, version 3.0, to calculate the amount of probe DNA (vectors and MARs) in bands in the pellet and supernatant fractions. Then, the ratios of matrix-bound probe to unbound probe could be directly compared from the pellet lane to the supernatant lane. The known tobacco RB7 MAR was used as a control in the binding assays (Hall et al. 1991). Fifteen of the tested clones could bind with rice nuclear matrixes in vitro, and these clones were sequenced by the Nucleic Acids Facility (Sangon, Shanghai, China).

Plasmid constructs

Six constructs were used in this study (Fig. 3). The *gusA* reporter plasmid P1 is a derivative of pCAMBIA1300 (CAMBIA, Canberra, Australia) and the 3.0-kb *Eco*RI–*Hin*dIII fragment of pBI121. This fragment contains a chimeric *gusA* gene under transcriptional control of a Cauliflower mosaic virus (CaMV) 35S promoter and terminated by a poly-adenylation signal from the nopaline synthase (*nosT*) gene (Depicker et al. 1982).

A *Hin*dIII–*Bam*HI fragment containing the Ubi promoter (Christen et al. 1996) was linked with the P1 construct (*Hin*dIII-*Bam*HI cut) to produce the P3 construct. Another *Hin*dIII–*Bam*HI fragment containing the PNZIP promoter (Yang et al. 2003) was amplified from *pharbitis nil* via PCR (primers: 5′-ACTCA AAG CTT ACA TGG GGA TGA GGC AGG - 3′ and 5′-CATCA GGA TCC GGG TAG AGT GTA CTG T - 3′, in which the underlined sequences denote restriction sites of *Hin*dIII and *Bam*HI, respectively), and this fragment was inserted into the P1 construct (*Hin*dIII–*Bam*HI cut) to produce the P5 construct.

A *Hin*dIII–*Hin*dIII fragment containing the TM2 sequence was amplified from tobacco by PCR (primers: 5′- AAG CTT TCG ATT AAA AAT CCC ATT TA –3′ and 5′- AAG CTT TAT TTT CAG AAG AAG TTC CC-3′, in which the underlined sequences denote the restriction sites of *Hin*dIII), and the fragment was then inserted into the pGEM-T Easy vector to produce a pGEM-TM2′ plasmid.

The *Eco*RI fragment containing the TM2 sequence released from pGEM-TM2 was recovered by a gel extraction kit (Sangon) and then inserted into the dephosphated *Eco*RI site of the constructs P1, P3, and P5 to produce P1′, P3′, and P5′. The *Hin*dIII fragment containing the TM2 sequence released from pGEM-TM2′ was inserted into the dephosphated *Hin*dIII site of the constructs P1′, P3′, and P5′ to produce P2, P4, and P6, respectively.

Rice transformation

Mature seeds of the Zhonghua 11 (*Oryza sativa* L. cv. Zhonghua 11) were surface-sterilized with 10% sodium hypochlorite for 10 min. NB medium (N6 basic medium plus 0.5 g/l casin acid hydrolysates plus 2 mg/l 2,4-D plus 30 g/l sucrose plus 3 g/L Gelrite agrose) was used as a calli induction and subculture medium. Calli which were subcultured on a solid medium for no longer than

6 months were used as explants for transformation. The whole transformation procedure took 8 days.

- Day 1: Embryogenic calli 2–3 mm in diameter were subcultured on the NB solid medium at 25°C in the dark for 4 days.
- Day 3: A single clone of *Agrobacterium* was cultured in the liquid LB medium (10 g/l sodium chloride plus 5 g/l yeast extract plus 10 g/l tryptone) with the appropriate antibiotic at 28°C for 2 days.
- Day 5: Two milliliters of the *Agrobacterium* culture were centrifuged at 3,000 *g* for 10 min, and the pellet was resuspended in 10 ml liquid NB medium supplemented with 100 μM acetosyringone (As). The culture was shaken at 150 *g* for another 3 h. The calli were soaked in bacterial suspension for 3 min and dried with sterile filter paper to remove excess bacteria. Then the calli were transferred onto the co-culture medium (NB plus 100 μM As). Co-cultivation was carried out in the dark at 26°C for 3 days.
- Day 8: The infected calli were washed with liquid NB medium containing 300 mg/l cephalosporin to kill the *Agrobacterium*, dried with sterile filter paper, and transferred onto a selection medium (NB plus 300 mg/l cephalosporin plus 25 mg/l Hygromycin B) containing the appropriate antibiotic. The calli were subcultured every 2 weeks. After 6 weeks, the calli were transferred onto the regeneration medium (MS basic medium plus 25 mg/l Hygromycin B plus 3 mg/l 6-BA plus 0.2 mg/l NAA), where shoot formation occurred. The transformed plants were readily regenerated on the one-half MS medium.

GUS assays

Calli that had been transferred onto the regeneration medium 2 weeks previously and the leaves and roots of five-leaf stage transgenic rice plants were used to test the GUS activity according to the method of Jefferson (Jefferson et al. 1987). Numbers of calli we tested were shown in Table 1, and the mean GUS values were taken from all resistant calli we tested. We chose ten transformants of each construct to assay the GUS expression of leaves and roots. Samples (150 mg) were ground in extraction buffer. The extracts were centrifuged at 3,000 *g* for 10 min (4°C), and then, the supernatant was used for GUS assays. The GUS activity was assayed by adding 20 μl supernatant (about 20–30 μg protein) to the reaction buffer containing 1 nmol/l 4-methylumbelliferyl-β-D-glucuronide as a substrate, which was then incubated at 37°C for 30 min, and 100 μl reaction buffer was added to Na_2CO_3 solution to stop the reaction. GUS activity was measured at an excitation wavelength of 365 nm and an emission wavelength of 455 nm with a spectrofluorimeter (Hi-850, Hitachi, Japan). Protein contents were quantified using the standardized assay method according to the method of Bradford (Bradford et al. 1976). The GUS activity was expressed as nanomoles of methylumbelliferone per microgram of soluble protein per minute.

Northern blot analysis

On the same line, tissues were taken from ten independent transformants of each construct and mixed for RNA extraction. We performed RNA isolation and Northern blot hybridization according to the method described by Zheng et al. (1998). Total RNA was separated on an 0.8% formaldehyde agarose gel and blotted onto a Nytran membrane (Schleicher and Schuell, Dassel, Germany). Blots were hybridized with a *gusA* cDNA probe labeled by the random priming method. Blots were washed three times for 20 min at 55, 60 and 65°C with 0.2×SSC and 0.1% SDS solution, and then autoradiographed at −80°C, using Kodak XAR-5 film and an intensifying screen (Cronex Lightning Plus, DuPont, Wilmington, Del., USA).

Results

Isolation and characterization of TM2

According to the method described by Michalowski et al. (1999), we constructed a library of DNA sequences that were copurified with a tobacco nuclear matrix. From the library, a 1,001-bp DNA fragment was isolated, which was presumably attached to the nuclear matrix and was thus enriched by the purification procedure. This fragment, named TM2, had a 62.8% AT

Table 1 Effects of TM2 on transgene silencing in resistant calli

Promoter	Control (without TM2)			TM2 flanked		
	Num[a]	Non-exp[b]	Percent (%)[c]	Num[a]	Non-exp[b]	Percent (%)[c]
35S	19	9	47	20	4	20
Ubi	21	8	38	18	2	11
PNZIP	22	11	50	23	5	22

[a]Number of resistant calli tested
[b]Number of resistant calli with GUS activity of ≤ 0.1 nmol methylumbelliferone (MU)/min mg protein
[c]Percent of resistant calli that fail to express GUS among all the resistant calli tested (Non-exp[b]/Num[a])

Fig. 1 Sequence properties of TM2 (GenBank No. AF373415). *Underlined* nucleotides designate unwinding sites (AATATT); *double-underlined* nucleotides designate topoisomerase II-binding sites (CTTTATATTGTTGAC); the *panel* indicates the T box (ATTAATAAAA)

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AGGAAGAAGT TCCCAATCTT GAAATATCGA AGGTTCGCCT TTCTGTTGTA ATATTGCTCA 60
ATCCTCTACT TTTGTGCCGC CATCTGTATC AAAGTTGCTT CCCTTATCTC CTTTGTCAAA 120
TCTAAATTTG TTAAAAGTTC TTTCTCATTT GTTACTTCAT TTGTATGTAC GTATCTTGTT 180
CTTGGCTCTC CTATTTCCAC CGAAATTAAG GCCTCAGTAC CATAAATAAG TGAGAATGGT 240
GTTTCTCTTG TGCTTATCTT CGTCGTTGTC CTGTAATCCC ATACTAGCCC AAGTAGTACT 300
TCAGGGAACC TGCCTTTTGA TGATTCTAAC CGTTTCTTTA TATTGTTGAC AATAATTGTA 360
TTTGTCGATT ATGGTTTCCC ATTAGCTATG GGATGGCAGT GCGCCGAAAT AATTTGTTTA 420
ATTTCCCAGA TTTTGAGAAA ATCGGTAACT TTTGAACCTA CGAATTGTGG CCCATTATCG 480
CGTACTATTT TTCTTGGGAC ACAAAATCGA CATATTATGT TCCTCCAAAT AATATCAACA 540
ACCTCTTTCT CTCGATCTAT TTAAAGGCAC ATGCTTCTAC CCACATAGAA AAATAATCAG 600
TTAAGATTAA TAAAAGTCGT TCCTTTCCTG GTGCTTTAGG TAGAGGACCT ACAATACATA 660
ACCCACTTCA TAAACGGCCA CGGTGCTATG ATTGAATGAA GAAACACTGC TGGTTGATGC 720
ATATTGTTTG CACCCCGTTG ACATTTATCA CACCTTCTAA TAAAAATTTT CGCTTTGTCT 780
TCTATTTTTT GCAAATAATA CCGCCCCTTT TAGCGTCTTT AACAATGACC TTCCTCCAAC 840
GTGATTGCAA CAATGTTCTT TATGTACTTC TTGCATCACA TATTCAGTTT GTGATAGACC 900
AAGGCACCGT GCTAAGGGTC CCATGAACAT TTCGATACAA ATTTTCTTCT ATTAAGCAAT 960
ATTGAGCAGC TTTCACTCGC AATAATTGGG ATTTTTAATC G                    1001
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content. Michalowski's method (Michalowski et al. 1999) was used to define the motifs of the T box (TTWTWTTWTT), DNA unwinding sequence (AATATATTT or AATATT), and topoisomerase II-binding site (GTNWAYATTNATNNR); one T box, two unwinding sites, and one topoisomerase II-binding site were found in the TM2 DNA sequences (Fig. 1). To identify the binding ability of TM2 to the rice nuclear matrix, the in vitro binding assay was performed. The signal strength of the X-ray film is shown in Fig. 2. The binding efficiency ratios of the binding MARs and the corresponding vector were embodied the relative binding strength of MARs to the nuclear matrix. The ratios of TM2 and RB7 to the corresponding vectors were 5.81 and 3.08, respectively, indicating that both TM2 and RB7 were obviously bound to nuclear matrix much more strongly than the corresponding vectors.

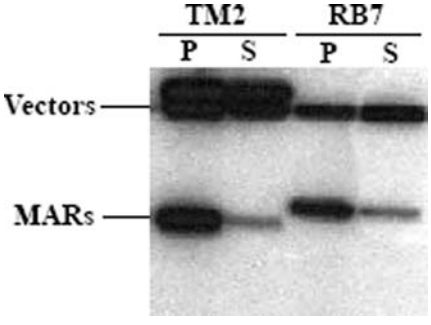

Fig. 2 In vitro binding assays of tobacco matrix attachment regions (*MARs*) to the rice nuclear matrix. Plasmid DNA was digested with restriction enzymes, and the released vector and MAR fragments were end-labeled with α-[^{32}P] dCTP. *P* Pellet, *S* supernatant, *RB7* a known strong MAR isolated from the 3′ flanking region of the RB7 root-specific gene of tobacco (used as control here)

TM2 enhances GUS expression in transformed rice calli

To investigate the influence of TM2 in transgenic rice, six plant expression vectors with or without TM2 were constructed (Fig. 3). By *Agrobacterium*-mediated transformation methods, the recombinant constructs were introduced into rice calli and were subsequently regenerated into plants. Hygromycin B was used as a selectable marker to obtain a population of independently transformed rice calli and the *gusA* gene under the control of three different promoters (35S, Ubi, and PNZIP) was used to evaluate the effect of TM2 on gene expression (Depicker et al. 1982; Christensen and Quail 1996; Yang et al. 2003). The transformed calli were further confirmed by a PCR analysis (data not shown). The results showed that when the expression cassettes were flanked by TM2, the average GUS activities were much higher than those without TM2 in transgenic rice calli (Fig. 3; Table 2). The GUS expression driven by the 35S, Ubi, and PNZIP promoters increased 3.2-, 4.1-, and 2.3-fold, respectively. Our results indicate that the presence of TM2 does have a large impact on enhancing gene expression and reducing the number of resistant calli that fail to express GUS (Tables 1, 2).

Interestingly, the PNZIP promoter, which was expressed specifically in photosynthetic tissues of transgenic tobaccos (Yang et al. 2003), exhibited a detectable expression level in transgenic rice calli with or without flanking TM2. The possible reasons will be considered in the "Discussion" section of this paper.

TM2 increases GUS activity at the whole-plant level

Regenerated plants were grown to maturity in a greenhouse, and young leaf tissues from these plants were

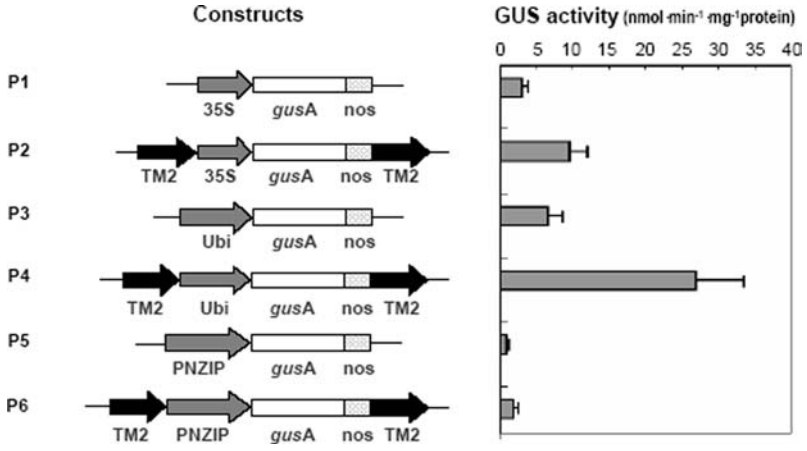

Fig. 3 Schematic representation of the constructs used in this study and their effects on the GUS expression of independently resistant rice calli. The various constructs, fused to different promoters, are indicated on the *left*. *35S* CaMV 35s promoter, *Ubi* promoter of the maize *ubiquitin* gene, *PNZIP* promoter of *pharbits nil PNZIP* gene, *gusA* cDNA for *β*-glucuronidase. The average GUS activity from 20 independent transformants of each cell line is given as nanomoles of methylumbelliferone generated per minute per milligram of protein. Each cell line was assayed three times. Standard errors are shown as *bars above the columns*

used for PCR analysis. The results presented here are based on the examination of a large number of rice plants from independent transgenic lines. To better compare the results, the leaf and root tissues from the same plant were used to perform GUS activity, Northern blot, and histochemical analysis. As shown in Fig. 4, the quantitative GUS activity, mRNA levels, and tissue-staining intensities are quite consistent. Three promoters used in this study resulted in different GUS expression levels in transgenic rice plants. Ubi, a constitutive promoter, is most active in both the leaves and the roots of transgenic rice. 35S, another constitutive promoter, has weak activity in both the leaves and roots of transgenic rice. As expected, the PNZIP, a tissue-specific promoter, has higher activity only in transgenic leaves. Moreover, all three of the promoter constructs showed significant increases in GUS activity when flanked by TM2. Constructs driven by the Ubi and 35S promoters showed 9.9- and 5.4-fold TM2 effects in leaves, and 7.8- and 6.2-fold TM2 effects in roots, respectively. In the PNZIP transformant rice leaves, GUS activity increased by 4.0-fold by TM2 (Table 2; Fig. 4).

Discussion

Previous reports about MARs were focused on gene expression with constitutive promoters (Allen et al. 1996; Han et al. 1997; Ülker et al. 1999; Vain et al. 1999; Butaye et al. 2004). Only one report about the effect of MARs with a heat shock promoter was presented recently. The presence of the Rb7 3′MAR increased transgene expression threefold under the control of an induced soybean heat shock protein promoter, and this MAR did not cause transgene expression in the absence of heat shock (Mankin et al. 2003). In this study, we introduced the *pharbits nil* PNZIP promoter, a strong promoter which was specifically expressed in photosynthetic tissues in transgenic tobacco plants, into rice to test its expression pattern. In transgenic rice plants, very high GUS activity, which was sixfold higher than with the 35S promoter, was detected in leaf tissue. However, the GUS activity was too low to detect in root tissues (Fig. 4; Table 2). This result accorded with our previous observations in *pharbitis nil* and transgenic tobaccos

Table 2 Effects of TM2 in different tissues of transformants

Plant tissue	35S			Ubi			PNZIP		
	GUS activity		Fold increase[c]	GUS activity		Fold increase[c]	Gus activity		Fold increase[c]
	(−)[a]	(+)[b]		(−)[a]	(+)[b]		(−)[a]	(+)[b]	
Calli	3.01	9.54	3.2	6.66	26.76	4.0	0.81	1.87	2.3
Leaves	5.02	27.06	5.4	17.53	175.07	9.9	32.13	121.18	4.0
Roots	6.91	43.04	6.2	26.15	210.01	7.8	–[d]	–[d]	–[d]

[a]Mean GUS activity (nmol MU/min mg protein) of all transformants without flanking TM2
[b]Mean GUS activity (nmol MU/min mg protein) of transformants with flanking TM2
[c]Mean GUS activity for TM2 transformants divided by mean GUS activity for control transformants
[d]Mean GUS activity of ≤ 0.1 nmol MU/min mg protein

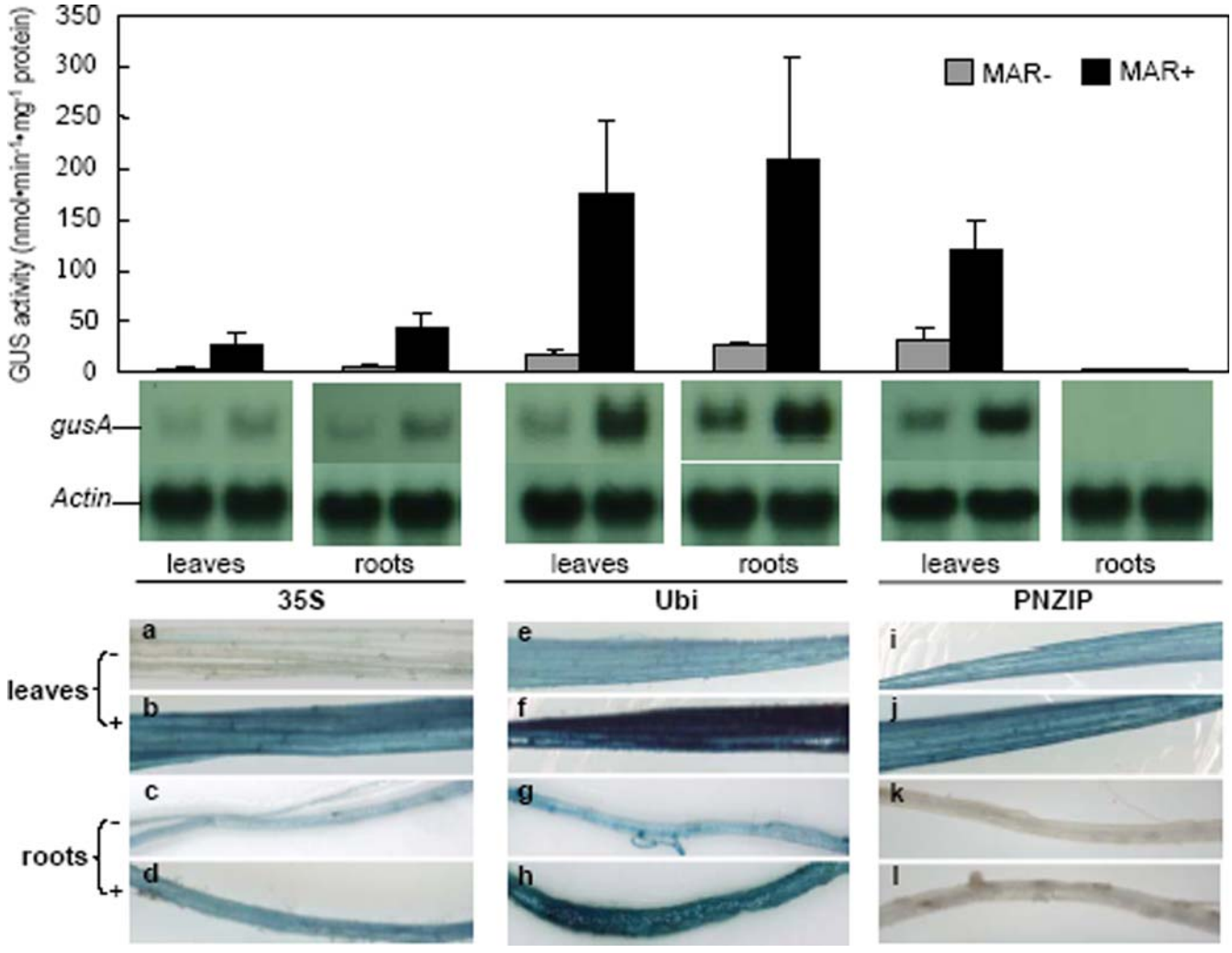

Fig. 4 GUS assays and RNA gel-blot analysis of transformant rice plants. The *reference frame* shows the average GUS expression of different transformants. The *abscissa* shows tissues assayed; the *y-axis* shows the average GUS activities (nmol/min mg protein) of each construct. We choose ten transformants of each construct transformant to assay GUS activity, and each line was assayed three times. The *β*-glucurindase gene (*gusA*) showed the expression of the *gusA* gene in transcription levels in different tissues. The expressions of the rice *actin* gene were used for equal RNA loading. *a –l* GUS dyeing pictures of different transformants. Tissues in the dyeing pictures were magnified 2.5-fold. A *minus sign* indicates materials without flanking TM2. A *plus sign* indicates materials with flanking TM2

(Zheng et al. 1998; Yang et al. 2003), indicating that the PNZIP promoter from dicotyledons has high activity not only in dicotyledons, but also in monocotyledon plants. Moreover, our assays based on the PNZIP promoter showed that the GUS expression at both the mRNA and the GUS activity levels were increased approximately fivefold by TM2 in transgenic rice leaves (Fig. 4), and TM2 did not cause GUS expression in non-photosynthetic tissues. To our knowledge, this is the first time a tissue-specific promoter has been used to test the influence of MARs on transgenic plants, and the result provides us with new ways to use MARs to increase gene expression in desired tissues with tissue-specific promoters.

To determine GUS activity in transgenic rice calli, the calli were grown in a 14-h photoperiod condition for 2 weeks. Some little green spots or buds were observed on the surface of the calli, indicating that the development of photosynthetic tissues had begun. It is logical that some GUS activity could be detected in this kind of resistant calli, which were transformed with the PNZIP promoter (Fig. 4). No GUS activity was detectable in resistant calli that were cultivated in constant darkness (data not shown).

Our results indicated that the presence of TM2 does have a large impact on reducing the number of resistant calli that fail to express GUS (Table 1). This effect of MARs on reducing silencing transgene numbers has been mentioned in some previous reports (Ülker et al. 1999; Mankin et al. 2003). It is possible that the transgenic cassette flanking MARs formed a loop domain through binding with the nuclear matrix, and this domain was an independent unit to eliminate the position effect in transformation (Allen et al. 2000).

In addition, different GUS expression levels were observed in different stages of transformants. The average GUS activity of transgenic calli was lower than that of transgenic plants (Table 2), which is accorded with other research groups' results (Hensgens et al. 1993; Cornejo et al. 1993), implying that GUS activities might accumulate with the development of transgenic plants. Fewer cells actively divide in calli compared to those in plants, resulting in lower GUS expression levels in calli. Another phenomenon was observed: even in the same transgenic plant which was based on the two constitutive promoters, the average GUS activity was normally higher in roots than in leaves (Table 2; Fig. 4). Some previous studies had mentioned that gene expression based on the 35S promoter exhibited a geotropic trait (Christensen and Quail 1996). Our results indicated that GUS expression based on the 35S and Ubi promoters also had this trend in transgenic rice plants.

Mankin et al. (2003) reported that the Rb7 3′MAR had a more effective influence in tobaccos 12 weeks post-transformation than in tobaccos 5 weeks post-transformation. Ronai et al. (1999) proposed that the probability of transgene silencing increases with the number of cell generations. Our results also showed clearly that TM2's impact on the enhancement of GUS expression levels was greater in leaves or roots than in calli (Table 2), suggesting that the effect of MARs accumulated with the growth and development of transformants or with the increase of cell numbers. Thus, it is possible that TM2 reduced the progressive silencing of transgenes during the course of growth, resulting in diverse influences in different growth stages.

Our previous results demonstrated that TM2 had beneficial effects on transgene expression in transgenic tobacco as a representative of a dicotyledonous plant (Zhang et al. 2002). We showed here that when different promoters were used, TM2 could increase the average expression levels in different stages and tissues in transgenic monocotyledon rice, suggesting that TM2 was a novel element to increase the transgene expression levels in both dicotyledons and monocotyledons. Further studies are in progress to investigate the mechanism of TM2 function and to identify its binding proteins. TM2 will be a very useful regulatory element in transgenic plant research and applications.

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References

Allen GC Jr, Hall G, Michalowski S, Newman W, Spiker S, Weissinger AK, Thompson WF (1996) High-level transgene expression in plant cells: effects of a strong scaffold attachment region from tobacco. Plant Cell 8:899–913

Allen GC, Spiker S, Thompson WF (2000) Use of matrix attachment regions (MARs) to minimize transgene silencing. Plant Mol Biol 43:361–376

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–293

Breyne P, Montagu MV, Depicker A, Gheyse G (1992) Characterization of a plant scaffold attachment region in a DNA fragment that normalizes transgene expression in tobacco. Plant Cell 4:463–471

Breyne P, Montogu M, Gheysen G (1994) The role of scaffold attachment region in the structure and functional organizational of plant chromatin. Transgenic Res 3:195–203

Brouwer C, Bruce W, Maddock S, Avramova Z, Bowen B (2002) Suppression of transgene silencing by matrix attachment regions in maize: a dual role for the maize 5′ *ADH1* matrix attachment region. Plant Cell 14:2251–2264

Butaye KM, Goderis IJ, Wouters PF, Delaure SL, Broekaret WF, De Bolle MF (2004) Stable high-level transgene expression in *Arabidopsis thaliana* using gene silencing mutants and matrix attachment regions. Plant J 39(3):440–449

Christensen AH, Quail PH (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res 5:213–218

Cornejo MJ, Luth D, Blankenship KM, Anderson OD, Blechl AE (1993) Activity of a maize ubiquitin promoter in transgenic rice. Plant Mol Biol 23(3):567–581

Depicker A, Stachel S, Dhaese P, Zambryski P, Goodman HM (1982) Nopaline synthase: transcript mapping and DNA sequence. J Mol Appl Genet 1:561–573

Fagard M, Vaucheret H (2000) Gene silencing in plants: how many mechanisms? Plant Mol Biol 51:167–171

Hall GE, Spiker S (1994) Isolation and characterization of nuclear Scaffolds. In: Gelvin S, Schilperoort R, Verma DP (eds) Plant molecular biology manual, vol D2. Kluwer, Dordrecht, pp 1–12

Hall G, Allen GC, Loer DS, Thompson WF, Spiker S (1991) Nuclear scaffolds and scaffold—attachment regions in higher plant. Proc Natl Acad Sci USA 88:9320–9324

Han KH, Ma CP, Strauss SH (1997) Matrix attachment regions (MARs) enhance transformation frequency and transgene expression in poplar. Transgenic Res 6:415–420

Hensgens LA, de Bakker EP, van de Mark F, Schilperoort RA (1993) Transient and stable expression of gusA fusions with rice genes in rice, barley and perennial ryegrass. Plant Mol Biol 23(3):567–581

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile fusion marker in higher plants. EMBO J 6:3901–3907

Mankin SL, Allen GC, Thomas P, Spiker S, Thomposon WF (2003) Elevation of transgene expression level by flanking matrix attachment regions (MAR) is promoter dependent: a study of the interaction of six promoters with the RB7 3′ MAR. Transgenic Res 12:3–12

Michalowski SM, Allen GC, Hall GE (1999) Characterization of randomly obtained matrix attachment regions and transgene expression in plants. Plant Physiol 110:15–22

Mlynarova L, Keizer LCP, Stiekema WJ, Nap JP (1996) Approaching the lower limits of transgene variability. Plant Cell 8:1589–1599

Peterson K, Leah R, Knudsen S, Cameron Mills V (2002) Matrix attachment regions (MARs) enhance transformation frequencies and reduce variance of transgene expression in barley. Plant Mol Biol 49:45–58

Ronai D, Berru M, Shulman MJ (1999) Variegated expression of the endogenous immunoglobulin heavy-chain gene in the absence of the intronic locus control region. Mol Cell Biol 19:7031–7040

Schoffl F, Schroder G, Kliem M, Rieping M (1993) An SAR sequence containing 395 bp DNA fragment mediates enhanced, gene-dosage-conelated expression of a chimaeric heat

shock gene in transgenic tobacco plants. Transgenic Res 2:93–100

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

Ülker B, Allen GC, Tompson WF, Spiker S, Weisinger AK (1999) A tobacco matrix attachment region reduces the loss of transgene expression in the progeny of transgenic tobacco plants. Plant J 18:253–163

Vain P, Worland B, Kohli A, Snape JW, Christou P, Allen GC (1999) Matrix attachment regions increase transgene expression levels and stability in transgenic rice plants and their progeny. Plant J 18:233–242

Van der Geest AHM, Hall GE, Spiker S, Hall TC (1994) The β-phaseolin gene is flanked by matrix attachment regions. Plant J 6:413–423

Yang YT, Yang GD, Liu SJ, Guo XQ, Zheng CC (2003) Isolation and functional analysis of a stronger specific promoter in photosynthetic tissues. Sci China 46:652–660

Zhang KW, Wang JM, Yang GD, Guo XQ, Wen FJ, Cui DC, Zheng CC (2002) Isolation of a strong matrix attachment region (MAR) and identification of its function in vitro and in vivo. Chin Sci Bull 47:23–29

Zheng CC, Port R, Lu PZ (1998) PNZIP is a novel mesophyll-specific cDNA that is regulated by phytochrome and a circadian rhythm and encodes a protein with a leucine zipper motif. Plant Physiol 116:27–35