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Transgenic Note

The tobacco CHN50 matrix attachment region enhances transformation frequency

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Abstract The nuclear matrix attachment region (MAR) located upstream from the tobacco gene *CHN50*, designated CHN50MAR, consists of two MAR elements: S/M I and S/M II. In a direct gene transfer system using tobacco BY-2 cells, CHN50MAR produced a several-fold increase in colony formation of hygromycin-resistant (hyg^R) cells when arranged adjacent to both the 5′ and 3′ termini of a hygromycin-selective marker gene. S/M I and S/M II enhanced hyg^R colony formation to the same degree as CHN50MAR, suggesting that these MAR elements are functionally redundant for enhancing transformation. The presence of the marker genes in the hyg^R cells was confirmed using the polymerase chain reaction. These results imply that CHN50MAR and its MAR elements are available as transformation enhancers in direct gene transfer systems for plants.

Key words: BY-2 cell, direct gene transfer, nuclear matrix attachment region (MAR), *Nicotiana tabacum*, transformation frequency.

The genetic transformation of plants has primarily been accomplished using two systems: *Agrobacterium*-mediated gene transfer and direct gene transfer via chemical and physical techniques (Newell 2000). In direct gene transfer systems, transgenes tend to integrate as multiple copies, which are often fragmented and rearranged (Kohli et al. 2003). Such complex insertion events are thought to lead to transgene silencing, and the consequent transformation frequency may be decreased.

Nuclear matrix attachment regions (MARs) are chromosomal DNA elements that are thought to organize eukaryotic chromatin via interactions with the nuclear matrix that constitutes the intra-nuclear framework (reviewed by Davie 1995; Holmes-Davis and Comai 1998). Numerous MARs have been identified in plants, and studies of MARs have shown that adjacent MAR elements enhance and stabilize gene expression (reviewed by Allen et al. 2000). In addition, genomic fragments that enhance transformation have been isolated from petunia (transformation booster sequence, TBS; Meyer et al. 1988) and tobacco (TJ1; Shimizu et al. 2001). The DNA sequences of these fragments are rich in adenine and thymine residues and show high affinity to the nuclear matrix *in vitro* (Galliano et al. 1995; Shimizu et al. 2001), which are typical features of MARs (Holmes-Davis and Comai 1998). These findings suggest that MAR elements should improve the transformation frequency in direct gene transfer systems, although few attempts have been made to utilize them as transformation enhancers. The tobacco CHN50 matrix attachment region (CHN50MAR) was originally isolated as a 2.1-kb DNA segment upstream from the tobacco basic class I chitinase gene *CHN50*, and sequence analysis using biochemical techniques revealed that this segment includes two tandem MAR elements: S/M I and S/M II (Fukuda 1999). In this study, we examined the suitability of CHN50MAR as a transformation enhancer, using a direct gene transfer system with tobacco cells.

Plasmids carrying the gene constructs shown in Figure 1 were produced with the Enforcement Cloning System pKF3 (Takara Bio, Japan). The control plasmid pIGY-H was constructed by inserting a hygromycin phosphotransferase gene (*hpt*) cassette (35S-Hyg in the pGreen website, http://www.pgreen.ac.uk/; Hellens et al. 2000) into the *Bam*HI and *Kpn*I sites of the backbone plasmid pKF3. To construct derivatives of pIGY-H, the 1933-bp sequence of CHN50MAR (corresponding to nucleotide positions 12 to 1944 of DDBJ entry AJ006034) and partial sequences were inserted into 5′- and 3′-flanking sites of the *hpt* cassette in pIGY-H as

Abbreviations: CaMV, cauliflower mosaic virus; *hpt*, hygromycin phosphotransferase gene; hyg^R, hygromycin-resistant; MAR, nuclear matrix attachment region; PCR, polymerase chain reaction; PEG, polyethylene glycol.
This article can be found at http://www.jspcmb.jp/

*Hind*III-*Bam*HI fragments and *Kpn*I-*Sac*II fragments, respectively.

The tobacco (*Nicotiana tabacum*) cell line BY-2 was maintained by subculture in MS^{BY} medium (MS salts [Murashige and Skoog 1962], 100 mg L^{-1} myo-inositol, 2 mg L^{-1} Lglycine, 1 mg L^{-1} thiamine-HCl, 200 mg L^{-1} KH_2PO_4, 30 g L^{-1} sucrose, and 0.2 mg L^{-1} 2,4-dichlorophenoxyacetic acid, pH 5.8) at 26°C in the dark on a gyratory shaker at 110 rpm. After transferring 3 ml of BY-2 cells to 100 ml of fresh MS^{BY} medium and culturing them for 4 days, the cells were transformed using the polyethylene glycol (PEG) fusion method, essentially according to the procedure of Negrutiu et al. (1987). To induce plasmolysis, the cells were suspended in 20 ml of M/MS^{BY} (MS^{BY} medium containing 0.4 M mannitol, pH 5.8) for 10 min, and then digested in 20 ml of enzyme mixture (1% cellulase Onozuka RS [Yakult Pharmaceutical, Japan] and 0.1% pectolyase Y23 [Kikkoman, Japan] in M/MS^{BY}) at 26°C for 70 min with gentle shaking. Protoplasts were harvested by centrifugation at 150×*g* for 2 min and washed twice with W5 buffer (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, 0.4 M mannitol, 1.5 mM 2-[N-morpholino]ethanesulfonic acid [MES], pH 5.8). After incubation on ice for 1 h, the protoplasts were suspended in Ma-Mg buffer (0.4 M mannitol, 15 mM $MgCl_2$, 5 mM MES, pH 5.8) at a density of 10^7 ml^{-1}, and divided into 100-μl aliquots (10^6 protoplasts). Plasmid DNA (0.2 pmol) was added to each sample as 10 μl of aqueous solution, and this was mixed with 110 μl of 40% PEG 6000 (Calbiochem, USA) in CM buffer (0.1 M $Ca(NO_3)_2$, 0.4 M mannitol). After incubation at room temperature for 20 min, the sample was gradually diluted to 2.5 ml with W5 buffer and then washed once with 2.5 ml of M/MS^{BY}. The 10^6 treated protoplasts in each sample were embedded in an M/MS^{BY} gel (40-mm diameter and 1.5-mm thickness, solidified with 0.8% low-melting-point agarose [Invitrogen, USA]) and cultured at 26°C in the dark for 4 weeks. During the first week, the M/MS^{BY} gel was soaked in 10 ml of liquid M/MS^{BY} in a 90-mm Petri dish, after which half of the liquid M/MS^{BY} was replaced with fresh MS^{BY} medium supplemented with hygromycin B (Sigma-Aldrich, USA) at a final concentration of 100 μg ml^{-1}. After an additional week of soaking, the M/MS^{BY} gel was transferred onto semisolid MS^{BY} medium containing 200 μg ml^{-1} hygromycin B. After 2 weeks, visible colonies were counted as transformants (Figure 2).

The presence of the transgene *hpt* in tobacco cells was examined by the polymerase chain reaction (PCR), using the following sequences as primers: 5′-CTATGAAAA-AGCCTGAACTCACCGCGACGT-3′ (forward) and 5′-GAGACTGGTGATTTCAGCGTACCGAATTAATTCT CC-3′ (reverse). Genomic DNA was extracted from tobacco cells using a Nucleon PhytoPure kit (Amersham Biosciences, USA), and purified in SUPERC-01 columns (Takara Bio). Approximately 0.2 μg of DNA was used as template for PCR with TaKaRa LA *Taq* with GC buffer I (Takara Bio). The heating program for DNA amplification consisted of denaturation at 94°C for 1 min, 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 110 s, and an additional extension at 72°C for 5 min.

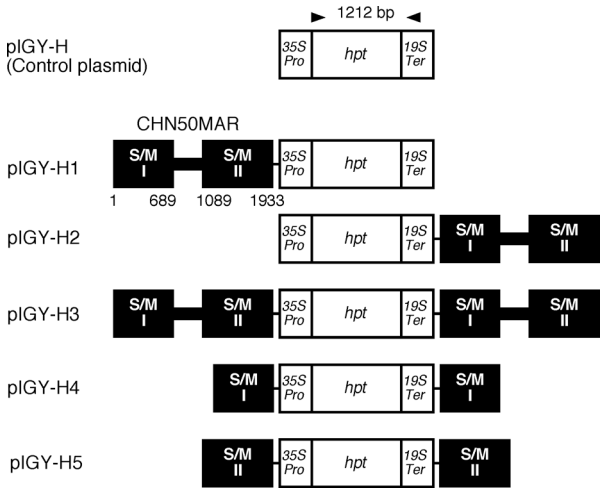

Figure 1. Schematic representation of the gene constructs in the plasmid vectors. The control plasmid pIGY-H carries a hygromycin phosphotransferase gene *hpt*, driven by the cauliflower mosaic virus (CaMV) 35S RNA gene promoter (*35SPro*) with the CaMV 19S RNA gene terminator (*19STer*). The derivative plasmids of pIGY-H (pIGY-H1, 2, 3, 4, and 5) carry the *hpt* cassette flanked by CHN50MAR and its MAR elements, S/M I and S/M II. The numbers under the shaded boxes indicate the nucleotide positions corresponding to the 1933-bp sequence of CHN50MAR. Arrowheads indicate the positions of the PCR primers that were used to amplify the *hpt* cassettes introduced into the tobacco cells.

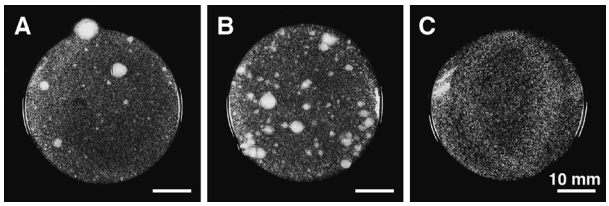

Figure 2. Colony formation of hygromycin-resistant tobacco cells. A 2-week culture in the presence of 200 μg ml^{-1} hygromycin gave rise to colony formation from tobacco cells treated with plasmids pIGY-H (A) and pIGY-H3 (B), but not from the recipient cells of these plasmids (C).

Colony counts of hygromycin-resistant (hyg^R) tobacco cells that were obtained from 10^6 protoplasts treated with 0.2 pmol of plasmid DNA are shown in Table 1. The plasmid pIGY-H3 (CHN50MAR::*hpt*::CHN50MAR) yielded an average of 29.8 hyg^R colonies, which is 2.8-times higher than the mean of the control plasmid pIGY-H. The plasmids pIGY-H4 (S/M I::*hpt*::S/M I) and pIGY-H5 (S/M II::*hpt*::S/M II) yielded an average of 25.6 and 26.2 hyg^R colonies respectively, which is approximately 2.5-times higher than the mean of the control plasmid

Table 1. Effect of CHN50 MAR on the transformation frequency of tobacco BY-2 cells

Plasmids[1)]	Number of colonies[2)]	Increase[3)]
pIGY-H (Control)	10.6±3.0[a]	1.0
pIGY-H1	13.8±2.9[a]	1.3
pIGY-H2	12.0±3.7[a]	1.1
pIGY-H3	29.8±9.5[b]	2.8
pIGY-H4	25.6±5.9[b]	2.4
pIGY-H5	26.2±8.5[b]	2.5

[1)] The plasmids shown in Figure 1 were transferred into BY-2 cells using a PEG-fusion method.

[2)] Number of hygromycin-resistant colonies obtained from 10^6 protoplasts treated with 0.2 pmol of plasmid DNA. Means with standard deviations were calculated from at least five replicates. Different lowercase letters indicate significant differences at $P<0.05$ using the Tukey-Kramer multiple comparison test.

[3)] N-fold increase relative to the control.

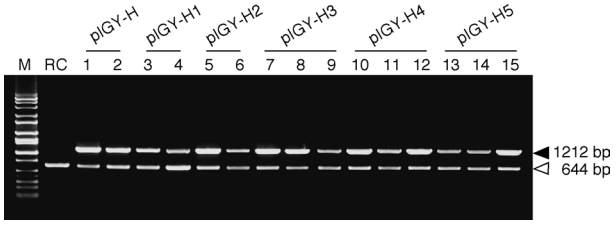

Figure 3. Detection of the marker genes introduced into tobacco cells by PCR. Approximately 0.2 μg of tobacco DNA was amplified by PCR primers specific to the *hpt* cassette sequence (Figure 1). To confirm the PCR performance of each sample, the sequence spanning nucleotide positions 1251–1894 of the tobacco gene *CHN50* (the DDBJ entry X51599) was concomitantly amplified. The PCR products were separated by electrophoresis on a 0.8% agarose gel and stained with ethidium bromide. The predicted DNA fragments of *hpt* (1212 bp) and *CHN50* (644 bp) are indicated with the black and white arrowheads, respectively. Lane M, All-purpose Hi-Lo DNA marker (Bionexus, USA); Lane RC, the PCR products of the recipient tobacco cells; Lanes 1-15, the PCR products of the hygromycin-resistant colonies obtained from the tobacco protoplasts treated with the indicated plasmids.

pIGY-H. Statistical analysis showed that the colony yields resulting from the transformation with pIGY-H3, pIGY-H4, and pIGY-H5 did not differ significantly. The plasmids pIGY-H1 (CHN50MAR::*hpt*) and pIGY-H2 (*hpt*::CHN50MAR) did not enhance hyg^R colony formation.

In 15 hyg^R colonies obtained from the protoplasts treated with the plasmid pIGY-H and its derivatives (pIGY-H1, 2, 3, 4, and 5), the presence of the marker gene *hpt* was examined by PCR. Using the *hpt* cassette sequence as primers, the predicted 1212-bp fragments were amplified from the DNA template of these hyg^R colonies (Figure 3, lanes 1-15), whereas no 1212-bp fragments were observed in the PCR products of the recipient cells (Figure 3, lane RC). These results suggest that the series of pIGY-H plasmids introduced the marker gene *hpt* into the tobacco genome.

CHN50MAR activates transcription of a downstream transgene (Fukuda and Nishikawa 2003), while the transformation frequency was not enhanced by 5′-flanking CHN50MAR alone (Table 1). This suggests that the transcriptional activation of transgenes does not involve the enhancement of transformation. In plants, illegitimate recombination is thought to be the main route for transgene integration (Somers and Makarevitch 2004). Müller et al. (1999) noted that AT-rich and palindromic sequences are important for promoting illegitimate recombination events. Moreover, genomic analysis of transgenic plants has revealed that transgenes tend to integrate into AT-rich regions, such as MAR (Takano et al. 1997; Sawasaki et al. 1999; Makarevitch et al. 2003). Considering these findings, we speculate that CHN50MAR enhances the transformation frequency by promoting transgene integration into host genomes.

Here, we demonstrated that CHN50MAR is available as a transformation enhancer in a direct gene transfer system for tobacco cells. The transformation frequency was enhanced by both 5′- and 3′-flanking CHN50MAR, and its MAR elements S/M I and S/M II act redundantly to enhance transformation. Although the full potential of CHN50MAR as a transformation enhancer is still unknown, CHN50MAR most likely has potential for transforming various plant materials, especially those recalcitrant to *Agrobacterium*-mediated gene transfer.

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