Plant Biotechnology 22(2), 137–140 (2005)

Short Communication

TJ1 is an orientation-independent transformation enhancer sequence

Hiroo Hokazono[1], Misa Takahashi[1], Atsushi Sakamoto[1], Hiromichi Morikawa[1, 2]*

[1] Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8526, Japan; [2] Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation (JST), Kawaguchi 332-0012, Japan
* E-mail: hmorikaw@sci.hiroshima-u.ac.jp Tel: +81-82-424-7449 Fax: +81-82-424-0749

Received January 26, 2005; accepted February 17, 2005 (Edited by H. Sano)

Abstract A 507-bp nuclear matrix attachment region (MAR) sequence (designated TJ1) was flanked at both at 5′ and 3′ ends of the expression cassette for a modified green fluorescent protein (GFP) gene of the 35 Ω-sGFP(S65T) in four different orientations. A plasmid p507TH bearing a 507-bp non-MAR sequence from yeast at both ends of the cassette also was constructed. These five plasmids and 35 Ω-sGFP(S65T) were separately delivered into cultured tobacco BY-2 cells by particle bombardment. The yield of stable transformants that showed a uniform green fluorescence was determined. Southern blot analysis confirmed the integration of the GFP gene in tobacco genome. Transformant yields of the two TJ1-lacking plasmids were not significant. Transformant yields appeared to be increased by more than 2 times by the presence of TJ1-MAR sequences. The orientation of TJ1-MAR sequences did not influence the transformant yield.

Key words: GFP, MAR, particle bombardment, tobacco BY-2 cells.

Genomic DNA in eukaryotic nucleus is currently considered to be organized as a three-dimensional structure such that it is anchored through the nuclear matrix attachment regions (MARs) at every 50–200 kb to the nuclear matrix (Lewin 1997). MARs are reportedly involved in transcriptional regulations (Allen et al. 1993; Sandhu et al. 1998), and chromosome condensation (Strick and Laemmli 1995), replication (Gasser 1991) and transgene integration (Sawasaki et al. 1998). Information on chromosomal elements that control the integration of DNA will be pivotal for improving genetic transformation technology.

MAR-based transformation vectors have been used to improve the efficiency of transgenics in plants (Galliano et al. 1995; Shimizu et al. 2001) and animals (Menck et al. 1998; Gutiérrez-Adán and Pintado 2000). However, the orientation dependence of the MAR sequences on the transformation frequency or on the expression efficiency (Hormes-Davis and Comai 1998) has not been addressed yet.

TJ1 is a 507-bp MAR sequence that originates from the junction region in a transgene locus of transgenic tobacco BY-2 cells (*Nicotiana tabacum* L. cv. Bright Yellow-2). This sequence improved transformant yields by 10 times when it was flanked to the 5′ and 3′ ends of the expression cassette of the NPT II gene. However, whether this effect of TJ1 is attributable to the enhancement of the transgene integration *per se* or of the expression of integrated transgene (i.e., survival against pressure by the antibiotics), or both remains to be elucidated (Shimizu et al. 2001).

In this study, we therefore investigated the effect of orientation of TJ1 sequence on the transformation using a non-selectable, green fluorescent protein (GFP) gene from jellyfish (Chiu et al. 1996) as a reporter. Thanks to the use of the non-selectable gene, all of the transformant cells can directly be reflected on the estimation of transformation frequency.

Figure 1 shows plasmid constructs used in this study. The 35 Ω-sGFP (S65T) that contains the cDNA of the modified GFP gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (NOS) terminator (Chiu et al. 1996) was a gift from Dr. Y. Niwa, Shizuoka Prefectural University. Plasmid p507TH that contains a 507-bp non-MAR sequence at both 5′ and 3′ ends of the GFP expression cassette is used as a control. This stuffer DNA sequence was taken from yeast (accession no. AB012284; from 5372 to 5878), whose AT content is 63.4% which is comparable to that of TJ1 (68.3%). Plasmids pTJ1ATH, pTJ1BTH, pTJ1CTH and pTJ1DTH were derivatives of 35 Ω-sGFP (S65T), in which 507-bp TJ1 sequence is flanked at both 5′ and 3′ ends of the GFP expression cassette in different orientations (Figure 1). Details of

Abbreviations: CaMV, cauliflower mosaic virus; CCEG, cell clumps expressing GFP gene; GFP, green fluorescent protein; MAR, nuclear matrix attachment region; NOS, nopaline synthase; NPT II, neomycin phosphotransferase II; PCR, polymerase chain reaction.

Figure 1. Diagrams of plasmid constructs used. Plasmid 35 Ω-sGFP (S65T) contains the GFP expression cassette (indicated as GFP in square), in which the cDNA of a modified GFP gene is under the control of the CaMV 35S promoter and the NOS terminator (Chiu et al. 1996). p507TH (used as a control) contains a 507-bp non-MAR sequence (indicated as 507 in double arrow) at both 5′ and 3′ ends of the GFP expression cassette. pTJ1ATH, pTJ1BTH, pTJ1CTH and pTJ1DTH contain the 507-bp TJ1 sequence flanked to the GFP expression cassette at the 5′ and 3′ end in the orientation indicated. The *Eco*RI site in pTJ1ATH, pTJ1BTH, pTJ1CTH and pTJ1DTH is shown by broken line. TJ1 was amplified by PCR using a TJ1-carrying plasmid (pTJ1/GUS, Morikawa, Takahashi and Okamura, unpublished) as the template, Pfu DNA polymerase (Promega, Madison, WI, USA) and a set of primers; forward (5′GTAAAATTGCATACCAAAATTCT-GCAGATG-3′) and reverse (5′-CGAAACATACTTGCTATCAAAGT-TGTTTAT-3′), after which the PCR mix was supplemented with TaKaRa Taq™ DNA polymerase (TaKaRa Bio Inc., Ohtsu, Japan) and incubated further so that a deoxyadenosine residue was added at 3′ ends of the amplified TJ1 fragments. The TJ1 fragments were then gel purified and ligated into pGEM®-T Easy Vector (Promega) to give pTJ1 that contains *Eco*RI sites at 5′ and 3′ ends of TJ1. Another plasmid, pTJ1-H that contains *Hin*dIII sites at 5′ and 3′ ends of TJ1, was constructed similarly except that a set of primers; forward (5′-aagcttGTAAAATTGCATACCAAAATTCTGCAGATG-3′) and reverse (5′-aagcttCGAAACATACTTGCTATCAAAGTTGTTTAT-3′) was used. TJ1 sequence was excised from pTJ1-H by digestion with *Hin*dIII and inserted into the unique *Hin*dIII site just upstream of the CaMV 35S promoter in 35 Ω-sGFP (S65T). Another TJ1 sequence, which was excised from pTJ1 by digestion with *Eco*RI, was inserted into the resultant plasmid at the unique *Eco*RI site located immediately downstream of the NOS terminator. This cloning strategy yielded four plasmids (pTJ1ATH, pTJ1BTH, pTJ1CTH and pTJ1DTH) that differed in the orientation of TJ1 relative to the GFP-expression cassette. The 507-bp non-MAR sequence that is derived from yeast (accession no. AB012284; from 5372 to 5878) was amplified by PCR using a set of primers; forward (5′-aagcttAATTTTATAAATTTTTGAAATAAA-3′) and reverse (5′-aagcttAAGTTGGAAGCAAAAGAATTGTCT-3′). p507TH that contains this non-MAR sequence at both 5′ and 3′ ends of GFP expression cassette was then constructed in the same manner as described above. Plasmids for bombardment were purified using a QIAGEN Plasmid Maxi kit (Qiagen, Valencia, CA, USA).

construction of plasmids are described in the legend of Figure 1.

Suspension-cultured BY-2 cells that had been subcultured weekly in modified LS medium (Nagata et al. 1981) and maintained at 25±1°C in the dark with shaking at 130 rpm were used. BY-2 cells (100 mg fresh weight; 5×10^5 cells) were bombarded with gold particles (1.1-μm diameter, Tokuriki Honten, Tokyo, Japan) that had been coated with appropriate plasmid DNA (0.8 μg) by use of a particle gun as reported elsewhere (Yamashita et al. 1991). Six replicate cell samples were shot in an experiment, and a single shot was given to a replicate.

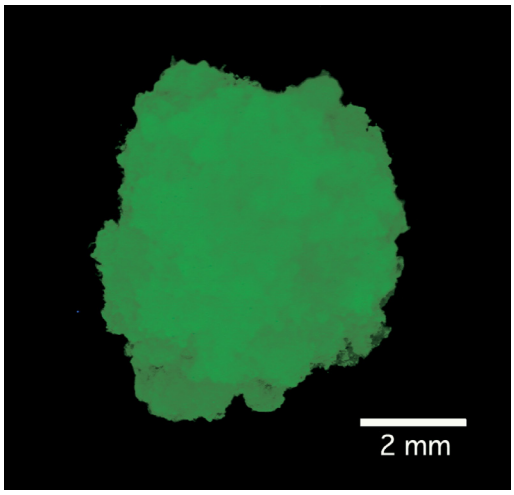

Figure 2. A typical fluorescence micrograph of cell clump expressing GFP (CCEG) on day 30. This CCEG was picked up from BY-2 cells that were bombarded with pTJ1ATH, and cultured for 30 days. Note that CCEG shows a uniform green color.

Three days after bombardment (day 3), BY-2 cells were suspended in 4 ml of the modified liquid LS medium, and dispensed into four Petri dishes containing the same medium solidified with 0.8% agar (20 ml each). They were then cultured at 25±1°C in the dark.

The number of cell clumps expressing GFP gene (CCEG) coincided within experimental errors among cell samples bombarded with different plasmids; for example, 474±212, 466±140, 508±253, 521±184 and 521±116 (average of 6 replicates±SD) per 100 mg initial cells at day 5 for 35 Ω-sGFP (S65T), pTJ1ATH, pTJ1BTH, pTJ1CTH and pTJ1DTH, respectively. Thus, the efficiency of gene delivery between bombardments with different plasmids in the present study was concluded to be statistically the same.

At day 10, the number of CCEG was approx. 1/20 of the respective number on day 5. No further change or only a slight change in the number of CCEG was observed by day 20 or 30. By day 30, all of the CCEG appeared to fluoresce uniformly green under the microscope (as shown in Figure 2), and further culture of them resulted in a “reen fluorescing lawn”, an indication that the foreign gene was integrated into the host genome by day 30. The CCEG contained 1–10 and 10–30 fluorescent cells at days 5 and 10, respectively. They measured 1–3 mm and 1–10 mm in diameter at days 20 and 30, respectively. The CCEG were picked up with a needle, subcultured onto a fresh medium at days 10 and 20 and cultured up to day 30. Number counting and

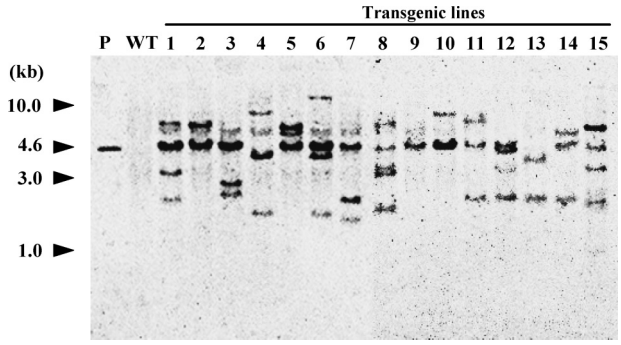

Figure 3. Southern blot analysis of 15 independent transgenic lines of CCEG 30 days after bombardment with pTJ1ATH. P and WT correspond to pTJ1ATH and non-transformed cells, respectively. BY-2 cells were bombarded with pTJ1ATH and CCEG were picked up and cultured for 30 days. A 15 CCEG were randomly chosen, and from them and non-transformed cell, total DNA were isolated according to Doyle and Doyle (1990). Genomic DNA (20 μg) or plasmid DNA (2.4 pg) was restricted with *Eco*RI (NEW ENGLAND BioLabs, Beverly, USA), separated on 0.8% agarose gels and transferred onto Biodyne Bnylon membrane (PALL, New York, USA) Labeling a 0.8-kb full length GFP cDNA (used as the gene probe) and Southern blot hybridization (at 55°C over night) were made using AlkPhos Direct kit (Amersham Bioscience, Buckinghamshire, U.K.). Hybridization signals were detected by a chemiluminescent reaction with CDP-Star™ (Amersham) and visualized by a 1-h exposure in the VersaDoc (Bio-Rad).

picking up were done under a stereoscopic fluorescence system (×16 magnification, model MZ FL III, Leica, Heerbrugg, Switzerland).

A 15 CCEG at day 30 was randomly chosen and subjected to the Southern blot analysis using a full length GFP cDNA as the gene probe as shown in Figure 3. Clearly, all of the 15 independent transgenic lines showed hybridization signals. The 4.6-kb signal corresponds to the full length of plasmids containing a TJ1 sequence since the other TJ1 was cut out by the *Eco*RI digestion (see legend for Figure 3). These results confirm that the integration of the GFP gene in tobacco genome occurred by day 30. Supporting this conclusion is the fact that no hybridization signals at 5.1 kb but high molecular weight (>20 kb) signals were observed in the Southern blots of total DNA extracted from the CCEG at day 30 without digestion with restriction enzymes (data not shown). As reported elsewhere (Iida et al. 1990), the stable transformant cells obtained by the particle bombardment of pCaMVNEO also showed, when their total DNA was digested with a unique restriction enzyme and subjected to Southern blot analysis, a hybridization band corresponding to the full length of this plasmid, which was attributed to rearranged copies of the plasmid DNA integrated in the genome.

Table 1 summarizes the yield of transformant cells at day 30. The constructs pTJ1ATH, pTJ1BTH, pTJ1CTH and pTJ1DTH always more than two times greater transformant yields than 35 Ω-sGFP (S65T) or p507TH that lack TJ1 sequences. This difference was significant

Table 1. The effect of the orientation of TJ1 sequences relative to the GFP expression cassette on the yield of stable transformant cells

Plasmid	TJ1 orientation relative to the GFP expression cassette[1)]	Yield of stable transformant cells per 100 mg initial cells[2)]	
35 Ω-sGFP(S65T)	—	13.2±5.2	(100)
p507TH	—	15.8±5.7	(120)
pTJ1ATH	⇨▭⇨	33.2±11.3*	(251)
pTJ1BTH	⇦▭⇨	33.8±6.8*	(257)
pTJ1CTH	⇨▭⇦	33.2±8.7*	(252)
pTJ1DTH	⇦▭⇦	27.7±6.6*	(210)

[1)] Details of the orientation of TJ1 sequences relative to the GFP expression cassette are shown in Figure 1. Square (▭) and arrow (⇨) respectively correspond to GFP gene and TJ1 sequence.

[2)] Data represent average of 6 replicates with SD. The value as a percentage of the 35 Ω-sGFP (S65T) value is shown in parenthesis. Level of significance when compared to the 35 Ω-sGFP (S65T) value: * $P<0.01$.

at the 99% level. The fact that the transformant yield between 35 Ω-sGFP (S65T) and p507TH was not significant means that simple increase in the vector size by adding a non-MAR sequence does not increase the transformation efficiency. Thus, the difference in the transformant yield between TJ1-bearing and TJ1-lacking plasmids is attributable to the effect of TJ1 sequence itself. Interestingly, there was no significant difference among the four TJ1-bearing constructs. Thus, it is concluded that TJ1 MAR enhances stable transformation in an orientation-independent manner. It should be noted that total of four sets of the present experiment consisting of 6 replicates gave a very similar result. To our knowledge, this is the first report on the effect of orientation of transformation enhancer sequence.

In our previous report on the effect of TJ1 on transformation of BY-2 cells using a selectable NPT II gene, the presence of TJ1 sequences on the transgene expression cassette increased the transformant yield by a factor of 10 (Shimizu et al. 2001). A plausible explanation for the discrepancy in the effect of TJ1 on the transformation yield between the present (factor of 2 using non-selectable gene) and previous (factor of 10 using selectable gene) study may be that the non-selectable gene directly reflects the transformation or integration of complete expression cassette into the host genome.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research (no. 11460156) from the Ministry of Education, Culture and Sports, Japan. Financial support from the Research for the Future Program of the Japanese Society for the Promotion of Science (JSPS-RFTF97R16001) is greatly appreciated.

References

Allen GC, Hall GE Jr, Childs LC, Weissinger AK, Spiker S, Thompson WF (1993) Scaffold attachment regions increase reporter gene expression in stably transformed plant cells. *Plant Cell* 5: 603–613

Chiu W, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineered GFP as a vital reporter in plants. *Curr Biol* 6: 325–330

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. *Focus* 12: 13–15

Galliano H, Muller AE, Lucht JM, 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

Gasser SM (1991) Replication origins, factors and attachment sites. *Curr Opin Cell Biol* 3: 407–413

Gutiérrez-Adán A, Pintado B (2000) Effect of flanking matrix attachment regions on the expression of microinjected transgenes during preimplantation development of mouse embryos. *Transgenic Res* 9: 81–89

Hormes-Davis R, Comai L (1998) Nuclear matrix attachment regions and plant gene expression. *Trends Plant Sci* 3: 91–97

Iida A, Morikawa H, Yamada Y (1990) Stable transformation of cultured tobacco cells by DNA-coated gold particles accelerated by gas-pressure-driven particle gun. *Appl Microbiol Biotechnol* 33: 560–563

Lewin B (1997) *Genes VI*. Oxford University Press. Tokyo, Japan

Menck M, Mercier Y, Campion E, Lobo RB, Heyman Y, Renard JP, Thompson EM (1998) Prediction of transgene integration by noninvasive bioluminescent screening of microinjected bovine embryos. *Transgenic Res* 7: 331–341

Nagata T, Okada K, Takebe I, Matsui C (1981) Delivery of tobacco mosaic virus RNA into plant protoplasts mediated by reverse-phase evaporation vesicles (liposomes). *Mol Gen Genet* 184: 161–165

Sandhu JS, Webster CI, Gray JC (1998) A/T-rich sequences act as quantitative enhancers of gene expression in transgenic tobacco and potato plants. *Plant Mol Biol* 37: 885–896

Sawasaki T, Takahashi M, Goshima N, and Morikawa H (1998) Structure of transgene loci in transgenic *Arabidopsis* plants obtained by particle bombardment: junction regions can bind to nuclear matrices. *Gene* 218: 27–35

Shimizu K, Takahashi M, Goshima N, Kawakami S, Irifune K, Morikawa H (2001) Presence of an SAR-like sequence in junction regions between an introduced transgene and genomic DNA of cultured tobacco cells its effect on transformation frequency. *Plant J* 26: 375–384

Strick R, Laemmli, UK (1995) SARs are cis DNA elements of chromosome dynamics: synthesis of a SAR repressor protein. *Cell* 83: 1137–1148

Yamashita T, Iida A, Morikawa H (1991) Evidence that more than 90% of β-glucuronidase-expressing cells after particle bombardment directly receive the foreign gene in their nucleus. *Plant Physiol* 97: 829–831