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Original Paper

De novo DNA methylation of the 35S enhancer revealed by high-resolution methylation analysis of an entire T-DNA segment in transgenic gentian

Satoshi Yamasaki[1], Masayuki Oda[1], Nozomu Koizumi[1], Kazuhiko Mitsukuri[1], Masafumi Johkan[1,a], Takashi Nakatsuka[2], Masahiro Nishihara[2], Kei-ichiro Mishiba[1,*]

[1] Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Sakai, Osaka 599-8531, Japan;
[2] Iwate Biotechnology Research Center, Kitakami, Iwate 024-0003, Japan

* E-mail: mishiba@plant.osakafu-u.ac.jp Tel & Fax: +81-72-254-9416

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Abstract We have found that cauliflower mosaic virus (CaMV) 35S promoter-specific transgene silencing is mediated by DNA methylation in gentian (*Gentiana triflora* × *G. scabra*). *De novo* methylation of asymmetric cytosines (CpHpH; where H is A, C, or T) sequence has been detected at the enhancer region (−148 to −85) of the 35S promoter in transgenic gentians, and is thought to be responsible for the silencing mechanism. To clarify the concept of *de novo* methylation, the present study examined the detailed DNA methylation profile of the entire T-DNA sequence (ca. 4 kb) integrated into transgenic gentians. Although highly methylated cytosines at CpG and CpWpG (W is A or T) sequences were broadly distributed, except in the *sGFP* coding region, highly methylated cytosines at CpHpH and CpCpG sequences were mainly limited to the 35S enhancer region. In addition to the previously identified *de novo* methylation peak (−148 to −85), another peak was discovered at −298 to −241. Electrophoretic mobility shift assays showed that gentian nuclear extracts could bind to the corresponding probes (−149 to −124 and −275 to −250), and that the probes could compete with one another for binding. Thus, a nuclear factor might be involved in the *de novo* methylation of the two regions. In addition, the present data indicated that the methylation patterns at CpCpG sites could be categorized as CpHpH methylation rather than CpWpG methylation.

Key words: DNA methylation, gentian, 35S promoter, transgene silencing, T-DNA.

Genetic transformation is a promising approach for the improvement of various plant traits. Thus, we have attempted to establish an *Agrobacterium*-mediated genetic transformation system for gentian (Nishihara et al. 2006, 2008). Unexpectedly, we found that most of the transgenic gentian plants did not express the transgenes that were introduced into their genome (Mishiba et al. 2005). Although many studies have reported such an underperformance of transgene expression in higher plants, convincing explanations of the variations in transgene expression are only available for some model plant species, such as *Arabidopsis* and tobacco (Kooter et al. 1999; Matzke et al. 2000; Vaucheret and Fagard 2001). The copy number of the transgene is thought to be a major contributing factor, because transgenic plants harboring multicopy transgenes frequently display transgene silencing (Hobbs et al. 1990, 1993; Matzke et al. 1994). By contrast, a number of independent transgenic *Arabidopsis* lines containing single copy transgenes under the control of the cauliflower mosaic virus (CaMV) 35S promoter (35Spro; Benfey and Chua 1990) showed high and stable expression (Nagaya et al. 2005; Schubert et al. 2004).

Transgenic gentian plants exhibiting transgene silencing commonly used the 35Spro (Mishiba et al. 2005, 2010), whereas transgenic gentian plants containing the *rolC* promoter instead of the 35Spro did not exhibit transgene silencing (Mishiba et al. 2006; Nakatsuka et al. 2008, 2009, 2010). Transient expression of a 35S-*uidA* transgene could be detected in gentian leaf tissues (Mishiba et al. unpublished data); therefore, the silencing phenomenon must have occurred after integration of the transgene into the genome. Moreover, the introduced 35Spro regions were hypermethylated in the transgenic gentian plants. The silencing phenomenon was not found in transgenic tobacco plants containing the same T-DNA construct (Mishiba et al. 2005, 2010); therefore, the silencing is thought to be an intrinsic

Abbreviations: CaMV, cauliflower mosaic virus; CMT, chromomethylase; DRM, domains rearranged methyltransferase; EMSA, electrophoretic mobility shift assay; sGFP, synthetic green fluorescent protein; T-DNA, transfer DNA

[a] Present address: Graduate School of Horticulture, Chiba University, 648 Matsudo, Chiba 271-8510, Japan

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epigenetic feature in gentian. All 12 independent gentian lines containing single copy transgenes showed hypermethylation of the 35Spro, and the silencing occurred irrespective of T-DNA copy number and genomic location (Mishiba et al. 2010). In addition, transgenic plants carrying a modified 35Spro without the core promoter region (−90 to +8) also showed hypermethylation in the remaining 35S enhancer region, suggesting that the enhancer region is responsible for the methylation mechanism. We further analyzed highly *de novo* methylated regions in the 35S sequence by focusing on asymmetric cytosine sequences (CpHpH; H is A, C, or T), and identified a possible target region (−148 to −85) for *de novo* methylation (Mishiba et al. 2010). Consistent with that result, an electrophoretic mobility shift assay (EMSA) showed that gentian nuclear proteins bound to the −149 to −124 region (Mishiba et al. 2010). Small RNAs with homology to the 35Spro sequence could not be detected in gentian (Mishiba et al. 2005, 2010); therefore, we assumed that a gentian nuclear factor might contribute to the *de novo* methylation.

To test the hypothesis that a target region of *de novo* methylation can be predicted by the distribution of CpHpH methylation, we attempted to analyze the cytosine methylation status of the entire sequence of the T-DNA region in transgenic gentian plants, using bisulfite genomic sequencing. This single-base resolution mapping of DNA methylation pattern, spanning ca. 4 kb, reveals the methylation patterns at either CpHpH (with CpCpG) or CpG and CpWpG (W is A or T) methylation.

In addition to the *de novo* methylation analysis, we verified the methylation statuses of cytosine sequence contexts other than CpHpH sites in the T-DNA region. In higher plants, maintenance DNA methyltransferase (e.g. MET1 in *Arabidopsis*) recognizes hemimethylated CpG sites and methylates the cytosine in the unmethylated DNA strand (Finnegan and Kovac 2000). On the other hand, the domains rearranged methyltransferase (DRM) class and the chromomethylase (CMT) class of DNA methyltransferases in *Arabidopsis* are involved in methylation of non-CpG sequences (Tariq M and Paszkowski 2004). DRM class methyltransferases have homology to mammalian Dnmt3 *de novo* methyltransferase, whereas CMT class methyltransferases are unique to plants, and maintain non-CpG (primarily CpHpG) methylation (Vaillant and Paszkowski 2007; Wada 2005). The genome-wide bisulfite sequencing study revealed that CpHpG methylation on one strand was highly correlated with CpHpG methylation on the opposing strand (Cokus et al. 2008); however, the maintenance DNA methylation machinery for hemimethylated CpHpG sites is still unclear. Meanwhile, a sequence preference for CpHpG methylation has also been observed, indicating that methylation frequencies of CpCpG sites tend to be low (Cokus et al. 2008; Lister et al. 2008). In the present study, we were especially interested in CpCpG sites because of the sequence preference of the CMT class of methyltransferases.

Materials and methods

Transgenic plant materials and DNA isolation

Transgenic gentian plant lines #3, #15, and #19, into which a single copy of the T-DNA region of the binary vector pSMABR35SsGFP was introduced, were used in the present analysis. The T-DNA construct contains a CaMV-35S promoter-driven *sGFP* ORF (Niwa et al. 1999) with a nopaline synthase (*nos*) terminator, and a *nos* promoter-driven *bar* (bialaphos resistance gene) ORF with the *Arabidopsis rbcS* (Rubisco small subunit) terminator (Igasaki et al. 2002). Genomic DNAs were isolated from young leaves of plants grown *in vitro* using a GenElute Plant Genomic DNA Miniprep kit (Sigma-Aldrich, St. Louis, USA), following the supplier's instructions.

Isolation of genomic DNA sequence flanking the T-DNA insertion

Genome walking to isolate genomic DNA sequences flanking the T-DNA insertion was performed by Straight Walk (Tsuchiya et al. 2009) and Inverse-PCR (Ochman et al. 1988). For the former, a Straight Walk Kit (Bex, Tokyo, Japan) was used according to the supplier's instructions. Gene specific primers used for first and second amplifications are listed in Table S1. Inverse-PCR was used in cases where PCR amplification could not be obtained by Straight Walk. Primers for Inverse-PCR are also listed in Table S1. Amplified fragments were cloned into the pSTBlue-1 vector (Novagen, Madison, USA) and sequenced.

Bisulfite genomic sequencing

Bisulfite genomic sequencing (Frommer et al. 1992) was performed as described in our previous study (Mishiba et al. 2010). The primer sequences used for bisulfite-PCR are listed in Table S2. The amplicons from the bisulfite-PCR were designed to cover all the cytosine residues in the T-DNA region. The PCR reaction was performed as described in our previous study (Mishiba et al. 2010), except for the annealing temperatures, as shown in Table S2. At least 10 independent clones were sequenced for each amplicon.

EMSA (Electrophoretic mobility shift assay)

EMSA analysis was performed as described in our previous study (Mishiba et al. 2010). Five grams of young leaves from wild-type gentian plants cultured *in vitro* were used for the preparation of crude nuclear extracts. Production of DIG 3′ end-labeled probes and detection of shift bands were performed by the DIG Gel Shift Kit, 2nd Generation (Roche, Mannheim, Germany). The oligonucleotides used for EMSA are shown in Figure 3B. Five micrograms of the nuclear extracts were used for each reaction.

Results and discussion

Bisulfite sequencing of an entire T-DNA region introduced in gentian genome

For methylation analysis, we selected three lines (#3, #15, and #19) of transgenic gentian plants that had a single copy of the T-DNA region of the pSMABR35SsGFP binary vector introduced by *Agrobacterium*-mediated transformation (Mishiba et al. 2010). In these three lines, *sGFP* transgene mRNA expression was not detected and the mRNA level of *bar*, which was used as the selectable marker gene, was lower than that in transgenic tobacco plants (Mishiba et al. 2010).

To obtain a methylation map of the complete T-DNA region in the gentian genome, we first isolated T-DNA boundary sequences by genome walking PCR. Using Straight Walk and inverse-PCR techniques, genomic sequences (ca. 0.2 to 1.3 kb) from the three lines flanking the left- and right-border regions of the T-DNA were obtained (data not shown). For line #3, 10 bp of the left border (LB; 26 bp) and the entire right border (RB; 25 bp) plus 4 bp (29 bp in total) were truncated from the T-DNA region. For line #15, 109 bp of the LB side and 22 bp of the RB were truncated, whereas 14 bp of the LB and 88 bp of the RB side were truncated in line #19.

Bisulfite genomic sequencing was used to determine the methylation status of the approximately 4 kb T-DNA region, which was segmented into 12 bisulfite-PCR amplicons and used 30 primers (see Table S2). The T-DNA border sequences were amplified using specific primers designed using the T-DNA boundary sequences determined above. For the *nos* terminator (NOST) region, only the #15 template could amplify the NOST-RB instead of the NOST amplicon, which was used for #3 and #19 templates due to their truncated regions. In total, the methylation statuses of 1013, 999, and 995 cytosine sites in #3, #15, and #19, respectively, were determined (Table 1). Among them, 236, 232, and 233 cytosines were CpG; 118, 116, and 115 were CpWpG; and 659, 651, and 647 were other sequence contexts (i.e. CpHpH and CpCpG). To the best of our knowledge, this is the first study to investigate methylation status at the single-base resolution of an entire T-DNA region introduced into a transgenic plant. Even though the present data represents the status of a single strand, our previous results indicated that the complementary strand probably follows the same methylation pattern (Mishiba et al. 2010).

Methylation status of the T-DNA region at CpG/CpWpG sequence contexts

Figure 1 shows the methylation status of the entire T-DNA sequence introduced into the transgenic line #3. Methylation data of the other two lines (#15 and #19) are shown in Figure S1. The methylation status showed a nearly identical pattern among the three lines, although their integrated genomic positions were different from one another. Table 1 shows the frequencies of methylated cytosines in each region of the T-DNA. There is no correlation between hypermethylation and GC content; thus, the hypermethylation of the 35Spro does not depend on the density of C/G nucleotides. Hypermethylation at CpG sites is distributed over the whole T-DNA region, except for the *sGFP* region. The average methylation frequencies of the adjacent 35S promoter and *nos* terminator regions (87.0 and 54.9%, respectively) is apparently higher than the *sGFP* region (13.8%), suggesting that CpG methylation of *sGFP* region might be inhibited or reduced (Table 1). In a separate study, we observed that dimethylated lysine 4 on histone H3 was enriched at the *sGFP* 3′ region in the gentian silenced T-DNA locus; thus, histone modification at the region might coordinate CpG hypomethylation (Yamasaki et al. 2011).

CpG hypermethylation of the *nos* terminator region might be caused by factors other than 35S-specific methylation, such as spreading of the DNA methylation status from gentian genomic region adjoining the T-DNA boundary. This assumption is partly supported by the fact that only the *nos* terminator region showed significantly different frequencies between CpG and CpWpG sites (54.9 and 4.4%, respectively; Table 1).

Methylation mapping of non-CpG/CpWpG cytosine sequences

The wide range of CpG and CpWpG hypermethylation from the *nos* promoter to the 35S promoter region made it difficult to predict where hypermethylation had primarily occurred, even though the 35S promoter was the most frequently methylated region for both CpG and CpWpG sites (Table 1). We previously analyzed non-CpG/CpWpG methylation in many transgenic gentian plants, and found that cytosine sites were frequently *de novo* methylated in the −148 to −85 region of the 35S promoter. To confirm whether such a highly *de novo* methylated region exists elsewhere within the T-DNA region, the methylation statuses of asymmetric cytosine sequences (i.e. non-CpG/CpWpG) were extracted from the bisulfite data. Figure 2 shows a schematic representation of CpHpH and CpCpG (the reason for the inclusion of this sequence context is described in a later section) methylation of the entire T-DNA region in the gentian lines. In all three lines analyzed, there were two major peaks of frequently methylated cytosines: −298 to −241 and −148 to −85. There were some additional peaks in the 35S promoter 5′ region and the *bar* coding region to the *rbcS* terminator region, especially in lines #3 and #19. However, we consider that the two regions

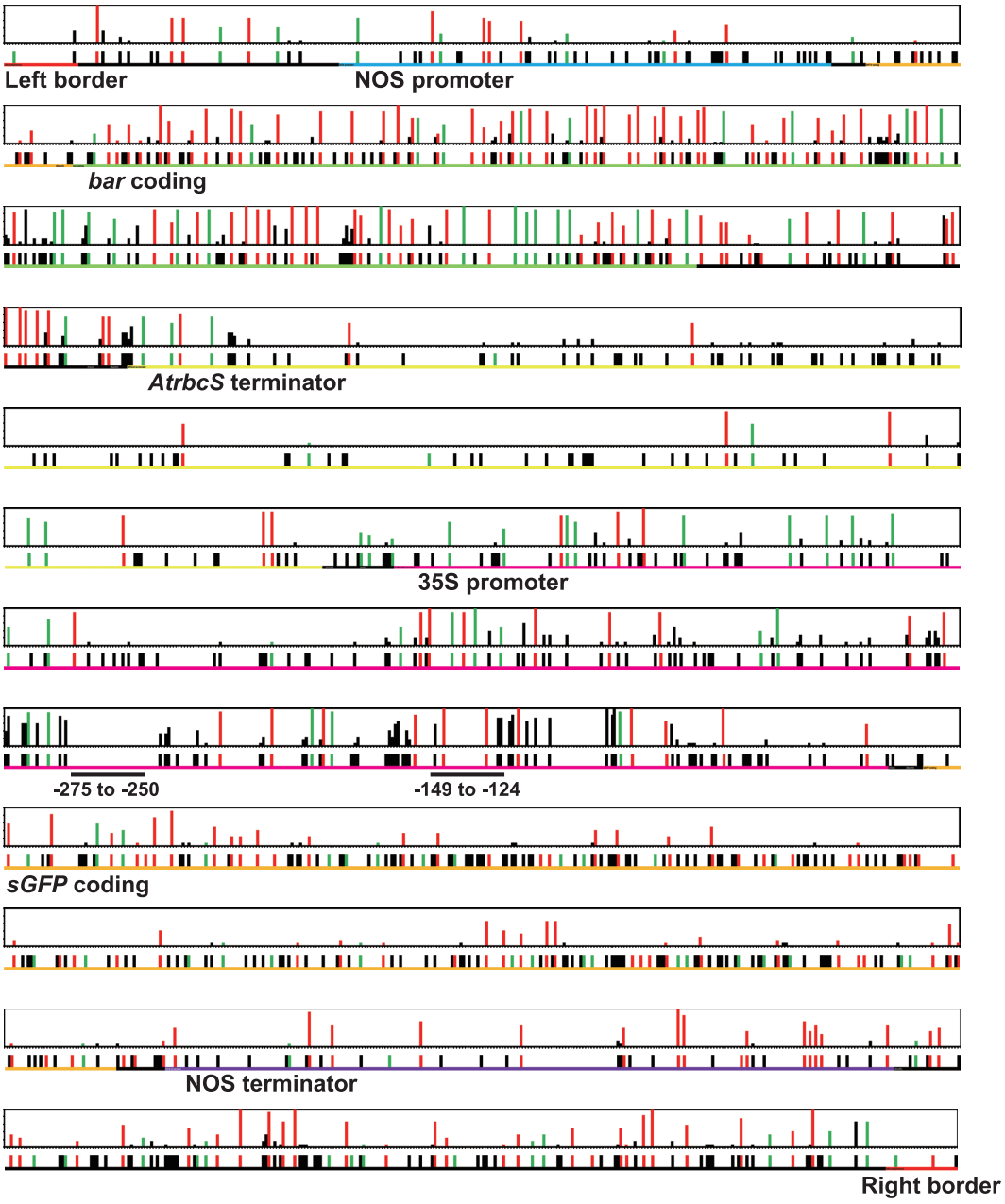

Figure 1. Single-base resolution DNA methylation map of the full-length T-DNA sequence of the binary vector pSMABR35SsGFP, integrated into the transgenic gentian plant line #3. The percentage of methylated cytosines is represented by bar charts (red, CpG; green, CpWpG; black, CpHpH and CpCpG), and the position of each cytosine is represented below. Each promoter, coding, terminator, and T-DNA border region is indicated by a different color. Crossbars at the bottom within the 35S promoter region indicate the region of the probes for EMSA.

(−148 to −85 and −298 to −241) are responsible for *de novo* methylation, because only these two peaks could be detected as distinct peaks in line #15.

Forming complexes of 35S enhancer probes with gentian nuclear extract

We previously demonstrated that complexes formed in gentian nuclear extract with the probe of the 35Spro −149 to −124 region, which is included in one of the *de novo* methylation peaks (−148 to −85). To confirm whether the newly found methylation peak (−298 to −241) also involves a sequence that can bind to a gentian nuclear factor, EMSA was performed using probes of the 35Spro −296 to −271 and −275 to −250 regions with the gentian crude nuclear extract. Consequently, the −275 to −250 probe showed distinct complexes with nuclear extracts from gentian and tobacco, which was used as a control, whereas no complexes were formed with the −296 to −271 probe in gentian (Figure 3A). Formation of the complex could be competed with by a 30-fold molar excess of unlabelled oligonucleotides of the 35Spro −280 to −255 and −275

Table 1. Frequencies of methylated cytosines at CpG, CpWpG, CpHpH, and CpCpG sequence contexts in different regions of the pSMABR35SsGFP T-DNA[a]

Line[b]	T-DNA[c] (48.54%)		NOSpro (41.28%)		*bar* (68.30%)		*AtrbcS*T (29.48%)		35Spro (43.76%)		*sGFP* (61.39%)		NOST (34.51%)	
	n[d]	% C[m]	n	% C[m]	n	% C[m]	n	% C[m]	n	% C[m]	n	% C[m]	n	% C[m]
CpG														
#3	236	56.5	6	58.3	68	73.5	9	78.3	23	91.0	60	21.1	15	57.2
#15	232	48.0		73.3		73.0		83.5		84.4		8.2		44.2
#19	233	53.4		49.4		67.6		78.8		85.6		12.0		63.3
ave.		52.6		60.3		71.3		80.2		87.0		13.8		54.9
CpWpG														
#3	118	43.1	4	31.3	25	81.7	9	45.7	23	73.5	31	4.6	2	4.2
#15	116	42.4		80.0		69.5		52.2		80.2		2.3		9.1
#19	115	44.6		47.5		70.2		59.3		79.6		2.2		0.0
ave.		43.3		52.9		73.8		52.4		77.8		3.0		4.4
CpHpH, CpCpG														
#3	659	8.2	33	1.4	107	10.7	92	4.2	144	20.2	149	0.9	21	2.4
#15	651	5.8		3.0		6.1		3.5		16.1		0.3		3.5
#19	647	12.0		4.6		9.9		12.3		29.7		0.6		2.8
ave.		8.7		3.0		8.9		6.6		22.0		0.6		2.9
CpCpG														
#3	69	7.5	0	—	25	9.3	1	0.0	4	16.7	20	0.4	2	4.2
#15	69	3.9		—		4.7		0.0		7.5		0.0		0.0
#19	67	8.3		—		6.8		7.7		30.8		0.9		4.2
ave.		6.6		—		6.9		2.6		18.3		0.4		2.8

[a] Parentheses indicate GC content of each region.
[b] Transgenic gentian lines.
[c] Entire T-DNA region.
[d] Number of cytosine residues in each region.

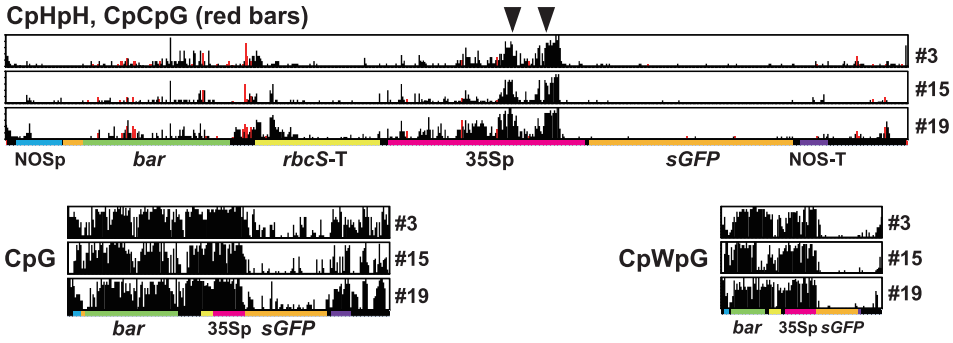

Figure 2. Distribution of methylation percentage at each CpHpH and CpCpG (top), CpG (bottom left), or CpWpG (bottom right) site of the T-DNA region in transgenic gentian lines (#3, #15, and #19). In the CpHpH and CpCpG methylation chart, red bars indicate cytosines at CpCpG sites. Each promoter, coding, terminator, and T-DNA border region is indicated by a different color on crossbars at the bottom. Arrowheads indicate the positions of probes (−275 to −250 and −149 to −124) for EMSA.

to −255 regions, but not by the −273 to −255 oligonucleotide (Figure 3B, C). Moreover, the same competition behavior was shown for complex formation by the −149 to −124 probe with gentian nuclear extract (Figure 3D). Both the −275 to −250 and −149 to −124 probes have two consensus sequences: 5′-GTGGAAA-3′ and 5′-GAAGA-3′ (Figure 3B; underlined bases).

Other probes (−254 to −229, −233 to −208, −212 to −187, −191 to −166, and −170 to −145; Mishiba et al. 2010), located between the *de novo* methylated regions, did not form any complexes, and complex formation by the −149 to −124 probe was competed with by the −275 to −250 probe. Therefore, it is conceivable that the two regions (−149 to −124 and −275 to −250) are targeted by a common nuclear factor(s). This assumption is strongly supported by the result that the two regions share the two consensus sequences, 'GTGGAAA' and 'GAAGA'. The former motif is similar to the core sequence ('GTGGAAAG') of the SV40 enhancer (Fang et al. 1989; Weiher et al. 1983), whose binding affinity to plant DNA binding factors is unclear. The fact that the −273 to −255 region did not effectively compete for the binding of −275 to −250 and −149 to −124 probes suggests that other sequence motifs are also required. Tobacco nuclear extract also formed complexes with the −275 to −250

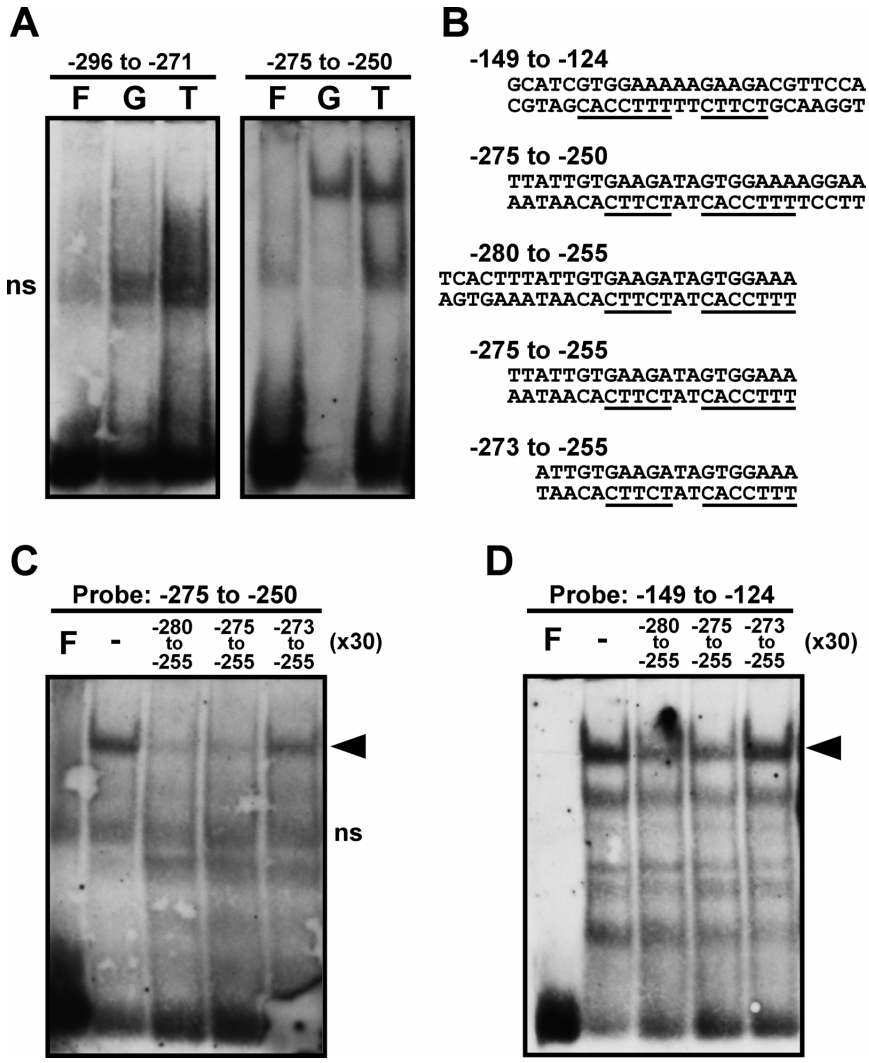

Figure 3. EMSA of the 35S enhancer sequence probes. (A) EMSA using 26 bp probes (−296 to −271 and −275 to −250) of the 35S enhancer region. F, free probe. G, probe with gentian nuclear extract. T, probe with tobacco nuclear extract. ns, positions of non-specific signals. (B) Sequences of the two probes (upper) and three competitors used in EMSA. (C, D) Determination of binding affinity of gentian nuclear extracts with the 35Spro −275 to −250 (C) and −149 to −124 (D) region probes. Thirtyfold molar excess of unlabeled 35Spro −280 to −255, −275 to −255 or −273 to −255 region oligonucleotides was incubated with the gentian nuclear extract. Arrowheads and ns indicate the positions of representative shifted bands and non-specific signals, respectively.

probe; therefore, only the gentian nuclear factor may specifically interact, either directly or indirectly, to direct *de novo* DNA methyltransferase. Accordingly, further studies are necessary to determine whether, and which, DNA binding factor(s) are involved in the *de novo* methylation machinery.

Methylation pattern at CpCpG sites

In the present study, cytosines of CpCpG sequence contexts were categorized together with CpHpH for methylation analysis, because the methylation patterns of CpCpG are closer to that of CpHpH than CpWpG. The methylation frequencies of CpCpG cytosine sites within the *bar* and *sGFP* coding region (4.7 to 9.3% and 0.0 to 0.9%, respectively) were nearly the same as those of CpHpH sites, but were substantially different from those of CpWpG sites (69.5 to 81.7% and 2.2 to 4.6%, respectively) (Table 1). Recently, whole genome bisulfite sequencing of *Arabidopsis* was performed using high-throughput sequencing technologies (Cokus et al. 2008, Lister et al. 2008). These results showed that methylation frequencies of CpCpG sites were significantly lower than those of CpApG and CpTpG. This trend might be due to prioritization of CpG methylation over CpHpG methylation. Accordingly, the methylation pattern (i.e. positions of peaks) of CpCpG sites (red bars) in the T-DNA region was congruent with that of CpHpH sites (Figure 2), suggesting that the CpCpG methylation is mainly caused by DRM-type DNA methyltransferases, just like CpHpH sites. We therefore assume that maintenance methylation activity by MET1-type CpG methyltransferase occurs exclusively on the second

cytosine of the CpCpG sites, where only DRM-type, but not CMT3-type, DNA methyltransferase can access the first cytosine.

In conclusion, the single-base resolution methylation map of an entire T-DNA region in gentian showed two major peaks of frequently methylated CpHpH and CpCpG sites. The corresponding probes within the peak regions bound to gentian nuclear extracts, suggesting that a nuclear factor might be involved in the *de novo* methylation that contributes to 35Spro-specific transgene silencing. An understanding of the transgene silencing machinery in a non-model plant species will improve the utility of transgene promoters for molecular breeding of gentian and other related horticultural plant species.

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