Doctor's Thesis : 0481039

Functional analysis of NtMET1, a type I DNA methyltransferase from tobacco plants (*Nicotiana tabacum*)

Hyun-Jung KIM

Research and Education Center for Genetic Information

Graduated School of Biological Sciences

Nara Institute of Science and Technology

(Professor Sano Hiroshi)

Submitted on 29th January, 2007

ABSTRACT

Plants possess three types of DNA methyltransferase, among which methyltransferase type 1 (MET1) is widely believed to be a major maintenance CpG-specific DNA methyltransferase. However, few information have been available as to its enzymatic activity, interacting proteins and spatial and temporal behaviors during DNA replication.

In the present study, I selected one example, NtMET1 from tobacco plants, and analyzed its biochemical properties and cellular localization. In transgenic tobacco plants over-expressing *NtMET1*, hypermethylation of genomic DNA was not observed. Contrary, hypomethylation of genomic DNA was evident. When NtMET1 was expressed in Sf9 insect cells, and purified sample was subjected to a standard methylation assay, methylation activity was not detectable with both hemimethylated and unmethylated DNA samples as the substrate. A lack of any detectable methylation activity in both *in vitro* and *in planta* suggested that NtMET1 activity is highly negatively controlled at protein level. Subsequently, I examined the possibility of intramolecular interaction within NtMET1 by the yeast two-hybrid and pull-down assays, and found that the N-terminus clearly interacted with the C-terminus. These results suggest that the inability of methylation was due to a tight intramolecular interaction, thereby the catalytic domain residing on the C-terminus being completely masked by N-terminus domains.

Cellular localization was then examined by fluorescence protein fusion, which was expressed in tobacco bright yellow 2 cells. Results indicated that NtMET1 localized to the nucleus in the resting stage, migrating to cytoplasm during mitosis, particularly at metaphase. The observed pattern resembled that of Ran GTPase, and *in vitro* pull-down assays showed a clear interaction between NtMET1 and AtRAN3, an *Arabidopsis* ortholog of tobacco Ran GTPase, NtRan-A1. These results suggest that enzymatic activity of NtMET1 is well adjusted by its own intra/inter molecular interaction and perhaps by interactions with other proteins, one of which was found to be Ran GTPase. Results also revealed that NtMET1 becomes localized to the vicinity of chromatin with the aid of Ran GTPase during cell division, and may play an important role in progress through mitosis independently of methylation activity.

The present study revealed unexpected feature of DNA methyltransfease. It may function in DNA methylation in combination with many other interacting proteins, and also in chromatin movement independently of DNA methylation activity.

TABLE OF CONTENTS

ABBREVIATIONS

AdoMet	*S*-adonosyl-L-methionine
BY2	Bright Yellow 2
CFP	cyan fluorescent protein
CMT	chromomethylase
DAPI	4’,6-diamidino-2-phenylindole
Poly (dI-dC) (dI-dC)	poly (eoxyinocinic-deoxycytidylic) acid
Dnmt	DNA methyltransferase
DRM	domains rearranged mehtyltransferase
GFP	green fluorescent protein
GST	glutathione-*S*-transferase
GUS	β-glucuronidase
HDAC	histone deacetylase
MET1	methyltransferase 1
m^5C	5-methylcytosine
NLS	nuclear localization signal
YFP	yellow fluorescent protein

INTRODUCTION

Covalently modified bases in nucleic acid (DNA) were discovered from quantitative recovery of purified bases using paper chromatography (Hotchkiss 1948). The most commonly modified base in higher eukaryotes is 5-methylcytosine (m^5C), which is produced by post-replicative methylation of cytosine residues (Fig. 1). The concentration of m^5C to the total cytosines varies depending on the organism, species, tissue and organelle. In vertebrates, 3 to 8 out of cytosines are methylated, and located almost exclusively in CpG, while in plants it increased up to 30% in both CpG and CpNpG and also in non-symmetric CpNpN cytosines (Finnegan and Dennis 1993). The methylation of these sequences is catalyzed by distinct DNA methyltransferases, which transfer a methyl-group from S-adenosyl-L-methionine (AdoMet) to position 5 of a cytosine (Yoder *et al*., 1997b).

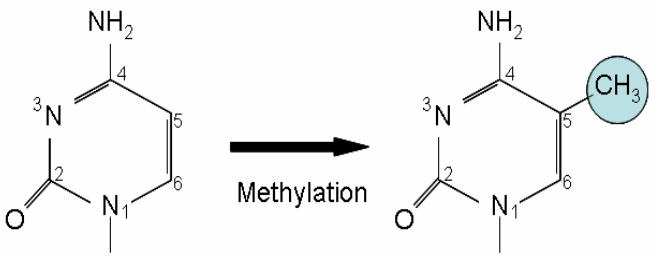


Figure 1. Structure of 5-methylcytosine in DNA. Unmethylated cytosine (left) is methylated at the C-5 position by a cytosine methyltransferase, yielding m^5C (right).

Cellular DNA methylation mechanism was established by two different activities of methyltransferase, maintenance and *de novo* (Holliday and Push 1975, Riggs 1975). The initial establishment of DNA methylation requires *de novo* methylation that occurs predominantly during early development and gametogenesis (Okano *et al*., 1999). The methylation of hemimethylated symmetrical sequences (CpG and CpNpG) after DNA replication was faithfully maintained by maintenance DNA methyltransferase through cycles of cell division. However, methylation in nonsymmetrical sequences (CpNpN) is not methylated by maintenance methyltransferase, and currently it is not clear whether or not nonsymmetrical methylation patterns can be maintained (Finnegan *et al*., 1998, Wada 2005) (Fig. 2).

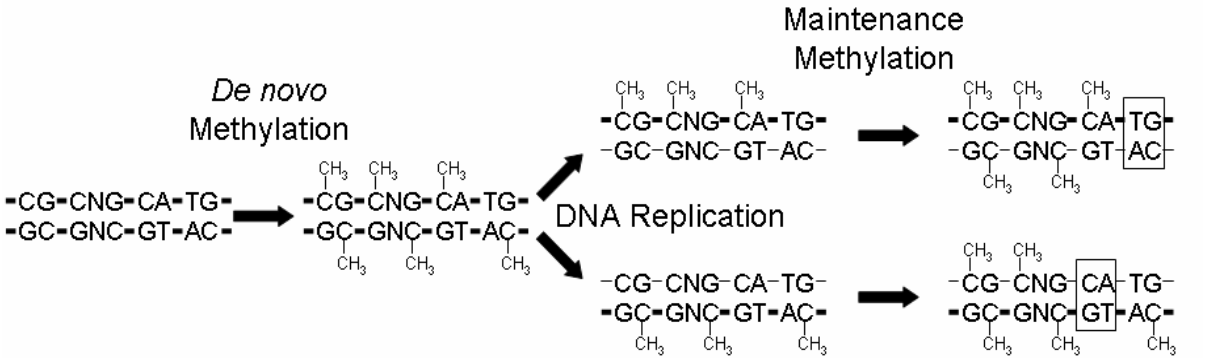


Figure 2. Mechanism for transmitting methylation patterns. Patterns of methylation in symmetric sequences (CpG and CpNpG where N is A, T, or C) are transmitted to both daughter strands following replication by maintenance methytransferase, which preferentially methylates hemimethylated DNA. Methylation of cytosines in nonsymmetric sequences (CpNpN) is not transmetted to the newly synthesized daughter strand. Thick and thin lines indicate the parental and daughter strand, respectively. Boxes indicate loss of methylation at nonsymmetric sites on the daughter strand.

In prokaryotic cells, DNA methylation primarily acts to protect the host DNA from cleavage by the restriction endonuclease that referred as restriction-modification systems (Noyer-Weidner and Trautner 1993). Methylation by dam methytransferase in *E. coli* also plays regulatory roles in the cell, including control of DNA replication, transcription and virulence genes (Reisenauer *et al*., 1999, Boye and Lobner-Olesen 1990, Heithoff *et al*., 1999). Prokaryote DNA methyltransferases have a common structure consisting of two-domain proteins comprising one large and one small domain. The large domain contains 10 conserved amino acid motifs, including binding site for the AdoMet and plays the most important role in catalysis. The small domain of different DNA methyltransferases is dissimilar in amino acid sequence, and contains variable region of the target recognition domain (TRD), which directs the enzyme to the target cytosine (Chen *et al*., 1991, Jeltsch 2002).

Eukaryotic cytosine DNA methyltransferase is first identified by sequence similarity of bacterial restriction methyltransferases, and named as Dnmt1 (Bestor *et al*., 1998). The Dnmt1 cDNA encodes a 190 kDa protein of 1620 amino acid residues that had a C-terminal domain with similarities to the bacterial restriction methltransferase and a large N-terminal domain, which is not present in bacterial enzymes. Dnmt1 prefers hemimethylated substrates at an initial rate 30-fold higher activity than unmethylated substrate *in vitro* assay (Yoder *et al*., 1997a). The preference of Dnmt1 for hemimethylated substrates is predicted to be involved in semiconservative inheritance of the methylation pattern of the parental DNA, and is referred

as maintenance methyltransferase. In mammals, Dnmt1 has been shown to directly interacting with histone deacetylases HDAC1 (Fuks *et al*., 2000), tumor suppressor protein retinoblastoma (Robertson *et al*., 2000), corepressor DMAP1 (Rountree *et al*., 2000) and histone modification enzymes (Fuks *et al*., 2000). This suggests that type I DNA methyltransferase (MET1) possesses transcriptional repressing activity and plays an important role in gene regulation by influencing the status of DNA and chromatin structures (Robertson *et al*., 2000, Milutinovic *et al*., 2004). The generation of a null allele of the Dnmt1 by homologous recombination in mouse embryonic stem (ES) cells showed that DNA mathylation level in genomic DNA was significantly reduced, but maintained stable. Also *de novo* methylation of retroviral DNA was not effect or little (Okano *et al*., 1998). These results provide the evidence for the existence of a second DNA methyltransferase in mammalian cells. The second DNA methyltransferase, *de novo* methyltransferase, Dnmt3 family (Dnmt3a and Dnmt3b), was cloned and characterized in 1998 (Okano *et al*., 1998). Mouse knockouts of the Dnmt3 family by targeting in ES cells have revealed that they are required for genome-wide *de novo* methylation and are essential for mammalian development (Okano *et al*., 1999). *In vitro* assay showed that Dnmt3 enzymes were equally active when both hemimethylated and unmethylated DNA, with lower preference for CG sites than Dnmt1, suggesting a role of this enzymes in *de novo* methylation (Okano *et al*., 1999, Gowher and Jeltsch 2001).

In the case of plants, three types of DNA methyltransferases are found, one of which is

similar in structure and function to mammalian Dnmt1 and named MET1(Finnegan and Dennis 1993). MET1 introduces methyl groups specifically into cytosines in CpG sequences, and has been suggested to function in maintenance of global genomic methylation (Finnegan *et al*., 1996, Ronemus *et al*., 1996, Nakano *et al*., 2000) Chromomethylase (CMT) was suggested to methylate CpNpG sequence to inactivate the invader DNA such as transposons (Tompa *et al*., 2002), and domains rearranged methylase (DRM) was shown to preferentially methylate CpNpG and CpNpN in both hemimethylated and unmethylated DNA (Wada *et al*., 2003). In addition, the green alga, *Chlamydomonas reinhardtii* was shown to possess a DNA methyltransferase, whose structure resembles the MET1, but exhibits catalytic activities similar to DRM (Nishiyama *et al*., 2004).

To data, MET1 homologues have been identified from wheat (Theiss *et al*., 1987), pea (Pradhan *et al*., 1998), carrot (Bernacchia *et al*., 1998), tomato (Bonnema *et al*., 1996), maze (Steward *et al*., 2000) and Tobacco (Nakano *et al*., 2000). MET1s are composed of two domains, the C-terminal catalytic domain which contain eight of ten conservative regions of prokaryotic cytosine DNA methyltransferases, and the long N-terminal domain for regulatory functions. However, despite the similar molecular size, few common motifs with plant MET1 were found in the N-terminal of *Chlamydomonas* enzyme, suggesting a complicated function of the N-terminal regions (Nishiyama *et al*., 2004).

MET1 transcripts are found only in meristematic tissues and can be detected only during the S-phase in a tobacco cells, suggesting them to be associated with cell division (Nakano *et al*., 2000, Steward *et al*., 2000). The physiological functions of MET1 have been examined using antisense-*MET1* transgenic *Arabidopsis* and tobacco plants, which exhibited a genome-wide demethylation at symmetrical sequences (Finnegan *et al*., 1996, Kakutani *et al*., 1996, Nakano *et al*., 2000). These plants showed abnormal phenotypes including small leaves, short internodes, abnormal flower structures and defects in vernalization responses, suggesting MET1 play an important role in plant development (Finnegan *et al*., 1996, 1998, Ronemus *et al*., 1996, Nakano *et al*., 2000). Studies on *Arabidopsis met1* mutants suggested a role in RNA-directed *de novo* DNA methylation, which may be critical for epigenetic gene silencing (Aufzats *et al*., 2004).

Although physiological role of MET1 in plants has intensively been studied, its biochemical properties and interacting proteins have not yet completely been understood. In chapter I of this thesis, I selected one example, NtMET1, and isolated full-length cDNA from tobacco plants as a model system. In order to understand enzymatic property of type I DNA methyltransferas (MET1) *in planta*, I constructed over-expressing transgenic plants. In spite of producing excess NtMET1, *in planta* assay showed no hypermethylation but rather hypomethylation of genomic DNA. I also screened pathogen responsive genes by differential expression, and observed that these genes were accumulated in hypomathylated transgenic

plants without pathogen attack. In chapter II, I analyzed its biochemical properties and cellular localization. The results showed that NtMET1 did not exhibit any detectable enzymatic activity under *in vitro* assay. NtMET1 was well adjusted by its own intramolecular interaction between the N- and C-terminal regions. Subsequent cellular localization analyses indicated that NtMET1 localized to the vicinity of chromatin with the aid of Ran GTPase during cell division, and may play an important role in progress through mitosis independently of methylation activity.

CHAPTER I

Analysis of tobacco plants with hypomethylated genomic DNA

Introduction

The cytosine residues of genome DNA in plants are methylated up to 30 % of all cytosine residues at carbon 5. It occurs predominantly in symmetric sequences such as CG and CNG, also at non-symmetric CNN sites in many cases. Methylation is enzymatic reaction, catalyzed by three types of DNA methyltransferases (Finnegan *et al*., 1996, Ronemus *et al*., 1996). DNA methyltransferase type I (MET1) preferentially methylates cytosine residues in hemimethylated symmetrical CG after DNA replication, referred to as a maintenance methyltransferase (Finnegan and Denis 1993). *De novo* DNA methylation is catalized by domains rearranged methyltransferase (DRM), which targets CNG and CNN in both hemimethylated and unmethylated DNA (Wada *et al*., 2003). Chromomethylase (CMT) has a chromodomain of chromatin-associated proteins, and is responsible for maintenance of cytosine methylation at CNG sequence of invading DNA such as retrotransposons (Tompa *et al*., 2002).

Physiological function of cytosine methylation is essentially to repressing transcription

of gene by blocking of transcriptional machinery attached to promoter regions of genes and influencing of nucleosome conformation and stability (Bird and Wolffe 1999, Steward *et al*., 2000). Recent studies showed *de novo* methylation at almost all cytosine residues in the presence of RNA signals, and this methylation may contribute to the silencing of viral and transposon sequences (Aufsatz *et al*., 2004).

Role of DNA methylation in plants has been studied by knock-out transgenic of DNA methyltransferases in Arabidopsis and tobacco. In our previous study, anti-sense MET1 transgenic tobacco plants showed a genome-wide demethylation and abnormal developmental phenotypes (Nakano *et al*., 2000). Results of screening using the differential display method in anti-sense MET1 transgenic tobacco exhibited that transcripts of biotic and abiotic stress responses genes were specifically induced in transgenic plants (Wada *et al*., 2004). We also found that genomic DNA methylation status changed by cold treatment in maize seedlings (Steward *et al*., 2002). These observations proposed that DNA methylation level changes dynamically in response to environmental stress, resulting in activation of many genes involved in resistance.

Despite enormous evidence, enzymatic property of type I DNA methyltransferase (MET1) is yet to be characterized. In this chapter, I first described isolation of full-length NtMET1 cDNA from tobacco plants, and construction of over-expressing transgenic plants. Then I analyzed genomic DNA methylation levels and differentially expressed genes in

transgenic plants. Unexpectedly, over-expressing NtMET1 plants exhibited hypomethylation of genomic DNA, indicating enzymatic inability *in planta*. I also observed that transcripts of pathogen responsive genes were accumulated by hypomethylation without pathogen attack. These observations support the idea that DNA methylation plays a role in regulation of plant defense genes against stress and pathogen attack.

Materials and Methods

Isolation of NtMET1 cDNA

Since a full-length cDNA of previously reported *NtMET1* (accession no. AB030726) has not been available, the entire coding region spanning over 4.5 kb was amplified by PCR using tobacco (*N. tabacum*) cDNA (accession no. AB280788), and a set of primers containing *att*B sites and specific nucleotide sequences of *NtMET1* (forward, 5'-AAAAAGCAGGCTTAATGGGTTCCCTGGCGGGGTTG-3'; reverse, 5'-AGAAAGCTGGGTCCTAAGTGGACCTCTTCTTGCT-3'; *NtMET1* specific sequences are underlined). PCR products were subjected to the second PCR with another set of primers (forward, 5'-GGGGACAAGTTTCTACAAAAAAGCAGGC-3'; reverse, 5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-3'), and resulting 4.5 kb products were cloned into the pDONR201 vector plasmids, and used for further experiments including

protein expression, GFP-fusion and transformation.

Plant materials and Transformation

Full-length *NtMET1* was cloned into pGWB5 (CaMV35S-NOS3') binary vector using GATEWAY cloning system (Invitrogen) as previously described (Wada *et al*., 2003). The construct was introduced into *Agrobacterium tumefaciens* strain EHA105 by heat-shock method. Transformation of tobacco leaf discs was performed as previously described (Nakano et al., 2000). Wild-type and transgenic tobacco plants (*Nicotiana tabacum* cv. Xanthi nc) were grown in a growth cabinet at 23^{o}C under a 14 h/10 h light/dark photo cycle.

Phenotypic observation

Seeds of wild type and transgenic plants were sown on 1/2 Murashige and Skoog agar (MS) (Murashige and Skoog 1962) and allowed to grow at 23^{o}C under a 16 h/8 h light/dark photo cycle. Phenotype was observed 3 weeks later to determine primary root growth parameters. Root length of indicated plants was measured from more than 10 samples and mean values presented with standard deviation from triplicate experiments. Root tips were stained with 5 μg mL^{-1} propidium iodide (Sigma), and observed using an LSM510 confocal microscope (Carl Zeiss, Germany).

Southern hybridization

Genomic DNA was extracted from fresh leaves by the cetyl-trimethyl ammonium bromide method (Murray and Thompson 1980). A 20-μg aliquot was digested with either *Hpa*II or *Msp*I, and fractionated by 1% agarose gel electrophoresis, transferred onto nylon membrane (Hybond N+, Amersham Pharmacia Biotech, Piscataway, NJ, USA), and subjected to hybridization with ^{32}P-labeled cDNA fragment of tobacco retrotransposon 1 (*Tto1*; accession no. D83003) (Hirochika, 1993) or *BamH*I tandem repeat element (*HRS60.1*; accession no. X12489). Hybridization was performed at 65^{o}C for 14h. Membranes were washed in 0.5×SSC containing 0.1% SDS at 65 ^{o}C for 30min, and then washed in 0.1×SSC containing 0.1% SDS for 30min, and subjected to autoradiography with X-ray film as described previously (Steward et al. 2002)

RT-PCR analyses and Northern hybridization

Total RNA was isolated from young leaf tissues by the acid guanidinium-phenol-chloroform (AGPC) method (Verwoerd *et al*., 1989) and RNA gel-blot analysis as previously described (Steward *et al*., 2002). A 1-μg aliquot of total RNA was used for reverse transcription using RNA PCR kit (Takara). Resulting cDNA was amplified with NtMET1 specific primer pairs: M1F, 5’-ATGGGTTCCCTGGCGGGGTT-3’ and M1733R, 5’-CTGAAACCCTTTTAATGACA-3’. PCR was carried out under the condition of 28 cycles

of 94^{o}C 15 sec, 57^{o}C 30 sec, 72^{o}C 1 min, and final extension at 72^{o}C 7 min (GeneAmp 2400, PerkinElmer), and the products were fractionated on 1.5% agarose gel and visualized by ethidium bromide staining.

High performance liquid chromatography

The amount of 5-methylcytosine (m^5C) in total genomic DNA were measured by high performance liquid chromatography (HPLC) according to protocols described previously (Wada *et al*., 2003). A 15-μg aliquot of DNA was digested with 2 units of nuclease P1 (Sigma) in 100-μl buffer containing 3 mM sodium acetate (pH 5.4) and 0.5 mM $ZnSO_4$ at 37 ^{o}C for 16 h. Nucleotides were dephosphorylated with 20 units of calf intestine alkaline phosphatase (Takara, Kyoto, Japan) and filtered through membrane with pore size 0.2 μm. Samples (10 μl) were injected into a Supelcosil LC-18-S colum (Supelco, Bellafonte, PA, USA), and separated with 2.5 - 20% methanol gradient in the presence of 50 mM KH_2PO_4 (pH 4.3).

Immuno-blot analysis

Total protein extract (20 μg) was prepared from 0.5 g young leaf tissues, and suspended in 1 ml cold lysis buffer containing 50 mM Tris-HCl, pH 7.5, 0.1% SDS, 10% glycerol, 100 mM DTT and 1 mM PMSF. Proteins were fractionated by 12% SDS-PAGE, and after blotted

to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA), NtMET1 protein was detected by immunoblotting using rabbit anti-ZmMET1 antibodies raised against specific peptide in catalytic regions (Steward *et al*., 2000) and peroxidase-conjugated goat anti-rabbit secondary antibodies (BioRad, Hercules, CA, USA).

Pathogen infection

Pseudomonas syringae pv. *glycinea* 801 was grown in King's B (KB) medium at 25 ^{o}C. Healthy leaves of wild type and transgenic plants were inoculated with *P. syringae* pv. *glycinea* 801 (absorbency at 600 nm was 0.5) by injection using a syringe without a needle and incubated at 23 ^{o}C for an appropriate time period up to 2 days. Bacterial growth was estimated by counting bacterial numbers in leaf discs, which were collected immediately after infection, and periodically up to 2 days, and homogenized in 10 mM $MgSO_4$ solution. After dilution, bacteria were plated onto KB agar, incubated at 25 ^{o}C for 1 day in the dark, and numbers of colonies were counted.

Results

Isolation and structure of NtMET1

A cDNA encoding a DNA methyltransferase type 1 (MET1) was isolated by PCR using tobacco cDNA. The full-length cDNA was 4,677 bp in size containing an open reading frame for 1556 amino acids (175 kDa), and named *NtMET1*. NtMET1 is consisted of the N-trerminal regulatory domain and the C-terminal catalytic domain (Fig. 1A). The N-terminal domain contains several putative signal motifs, including nuclear localization, bromo-adjacent homology (BAH) domains, zinc fingers and serine-rich regions (Fig. 1A). Similar signal motifs are also found in mammalian DNA methyltransferase 1 (Dnmt1), the ortholog of plant MET1 proteins, possessing S-phase targeting to replication foci, BAH, nuclear localization and cysteine-rich regions, being consistent with a proposed common role in CpG maintenance methylation. In contrast, the C-terminal domain contains highly conserved protein motifs, including the binding site for AdoMet, the active site for methyl-transfer and the recognition sites for cytosine residues (Fig. 1B). These motifs are common among DNA methyltransferases of different types from different organisms (Buryanov and Shevchuk 2005). However, NtMET1 was well conserved all amino acid motifs, which critical domains necessary for maintenance DNA methyltransferase activity (Fig. 1B).

Construction of transgenic tobacco plants

I constructed transgenic tobacco plants, in which *NtMET1* was over-expressed under the control of cauliflower mosaic virus (CaMV) 35*S* promoter. More than 10 lines were initially selected and grown on soil. At maturity, their phenotype was apparently normal, except that growth rate was slow, resulting in small statures in comparison with the control wild type plants (representative two lines are shown in Fig. 2A). Four lines were finally selected and high *NtMET1* expression was confirmed by RT-PCR (Fig. 2B), and produced NtMET1 proteins by immuno-blot staining (Fig. 2C). Phenotypically, these transgenic plants exhibited retardation of root growth and reduction of cell number of root meristem at 3 weeks after germination (Fig. 2D and E).

Methylation analysis of transgenic plants

Total DNA was isolated, hydrolyzed to mononucleotides and m^5C contents were estimated by HPLC. In wild type plants, up to 12% of cytosines were m^5C, while in transgenic lines, m^5Cs were only 8% (line #1), 7.3% (line #2) and 6.3% (line #4) of total cytosines (Fig. 3A). Thus global methylation in these transgenic lines was clearly reduced up to nearly 50%. Methylation status at particular loci was then analyzed by DNA blot hybridization using a pair of methylation-sensitive restriction endonucleases (Fig. 3B and C). When probed with cDNA of *Tto1*, a multicopy retrotransposon of tobacco, genomic DNA

from wild type plants showed a clear resistance to cleavage by *Hpa*II, which is sensitive to Cm^5CGG, whereas it was susceptible to *Msp*I, which is insensitive to Cm^5CGG (Fig. 3B). In contrast, genomic DNA from all transgenic lines was efficiently cleaved by *Hpa*II, showing similar patterns with those by *Msp*I (Fig. 3B). This indicated that the second cytosines in CCGG sequence in *Tto1* loci were highly methylated in wild type control, but demethylated in transgenic lines. Similar results were obtained with a tandem repeat element, *HRS60.1*, which comprises 2.3% of the total tobacco genome (Kovarik *et al*., 1994). In wild type plants, this sequence was sensitive to cleavage by *Msp*I, but highly resistant to *Hpa*II, indicating a high frequency of the second cytosine methylation in CCGG sequence (Fig. 3C). In transgenic tobacco lines #1, #2 and #4, the locus was apparently demethylated, showing cleavage pattern by *Hpa*II similar to that by *Msp*I (Fig. 3C).

Transcript accumulation of pathogen-responsive genes

In previous report, we found that methylation level in genomic DNA dynamically changed in response to cold stress and pathogen attack (Steward *et al*., 2002, Wada *et al*., 2004). Interestingly, in the absence of pathogens, NtMET1-overexpressing transgenic plants expressed high levels of *PR1a* and *PI-II* in tobacco, which normally are activated during defense response (Fig.4A). Wild type plant grown under identical conditions did not express these genes. These result suggested that transgenic lines are in a hypersensitive state even

under non-stressed conditions, and we examined their response to pathogen infection. Detached healthy leaves of wild type and transgenic plants were inoculated with *P. syringae* pv. *glycinea*. In wild type, disease symptoms were visible 24 h later, and tissue death was evident after 48 h. In contrast, disease symptoms were observed in transgenic lines at 24 h, and tissue death was still not observed even after 48 h (Fig. 4B). This was confirmed by counting numbers of propagated bacteria in the inoculated leaves (Fig. 4C). Bacterial number in wild type leaves increased up to 5×10^3 after 48 h, while in transgenic leaves, the increase was much slow, and reduced to nearly 30% compared with the wild type.

Discussion

Methylation of cytosine residues is commonly observed in DNA of all vertebrates and flowering plants. DNA methylation is involved in the regulation of genes expression including transposable elements, repeated sequences and in imprinted genes (Ferguson-Smith and Surani 2001). To data, three types of genes encoding putative DNA methyltransferases were founded in tobacco plants, MET1, CMT and DRM. NtMET1 is homologous to the mammalian Dnmt1, and responsible for maintenance of DNA methylation pattern by adding methyl groups to new replicated DNA strands from physiological studies (Nakano *et al*., 2000,

Wada 2005).

The present study documents properties of NtMET1 from tobacco plants. The full-length cDNA is 4.6 kb in size and encodes a 1556 amino acids polypeptide with a relative molecular mass of approximately 175 kDa. The entire region of NtMET1 is composed of two domains, the C-terminal catalytic domain which contains 10 conserved motifs of MET1s, and the N-terminal domain for regulatory functions. When MET1 was over-expressed in tobacco a clear hypomethylation of genomic DNA was observed, in spite of efficient expression of NtMET1 protein *in planta*. This unexpected feature might indicate that the excess NtMET1 protein interferes with normal methylation process. Although, the possibility cannot be excluded that NtMET1 is inherently deficient in enzymatic activity, it appears to be low, since the catalytic domain of NtMET1 highly resembles mammalian Dnmt1 and *Chlamydomonas* CrMET1, both have been confirmed to possess high activity (Okano *et al*., 1999, Nishiyama *et al*., 2004). Genetic analyses also point to a clear involvement of MET1 in genomic CpG methylation (Ronemus *et al*., 1996), although the possibility remains that the effects are indirect.

The hypomethylated transgenic plants showed a dwarf phenotype and ectopic expression of pathogen responsive genes that are commonly expressed without pathogen attack. When wild-type and transgenic leaves were inoculated with *P. syringae* pv. *glycinea*, resistance was increased in hypomethylated transgenic plants against pathogen. This was conceivably due to

activation of resistant genes by demthylation of their genomic DNA loci. Identification of such genes and their methylation status must be determined in future study.

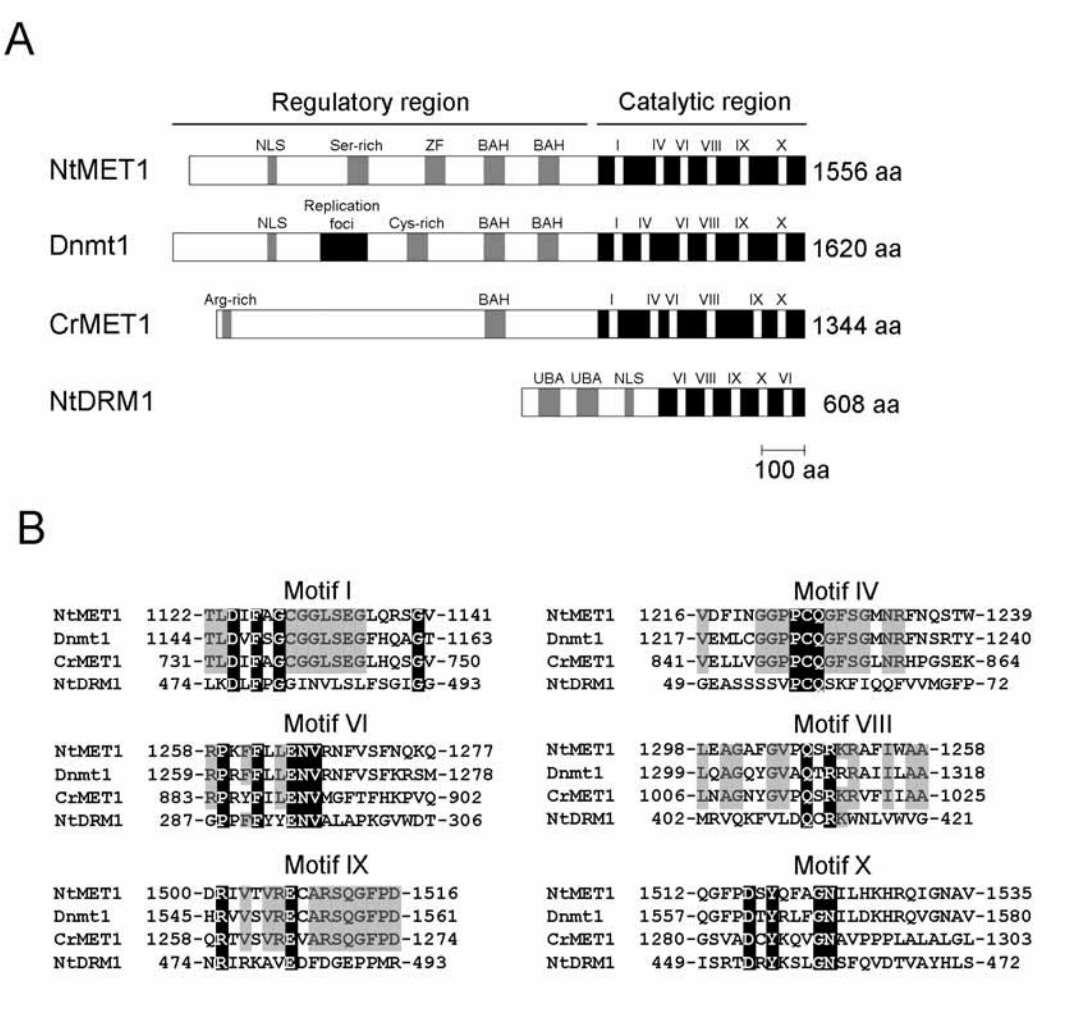


Figure 1. Properties of DNA methyltransferase. (A) Schematic illustration of DNA methyltransferases. Structure of NtMET1 (tobacco; accession no. AB280788), DNMT1 (murine; NM010066), CrMET1 (*Chlamydomonas*; AB073989) and NtDRM1 (tobacco; AB087883) are compared. Their in vitro enzymatic activity was confirmed except for NtMET1. NLS, nuclear localization signal; Ser-rich, serine-rich region; ZF, zinc-finger region; BAH, bromo-adjacent homology domains; cys-rich, cysteine-rish region; Arg-rich, arginine-rish region, UBA, ubiquitin association domain. Roman letters indicate conserved domains for catalytic activity. Bar indicates the range of 100 amino acids. (B) Motif alignment. Catalytic motifs illustrated in A are aligned. Common amino acids among four proteins are boxed, those among three are shaded.

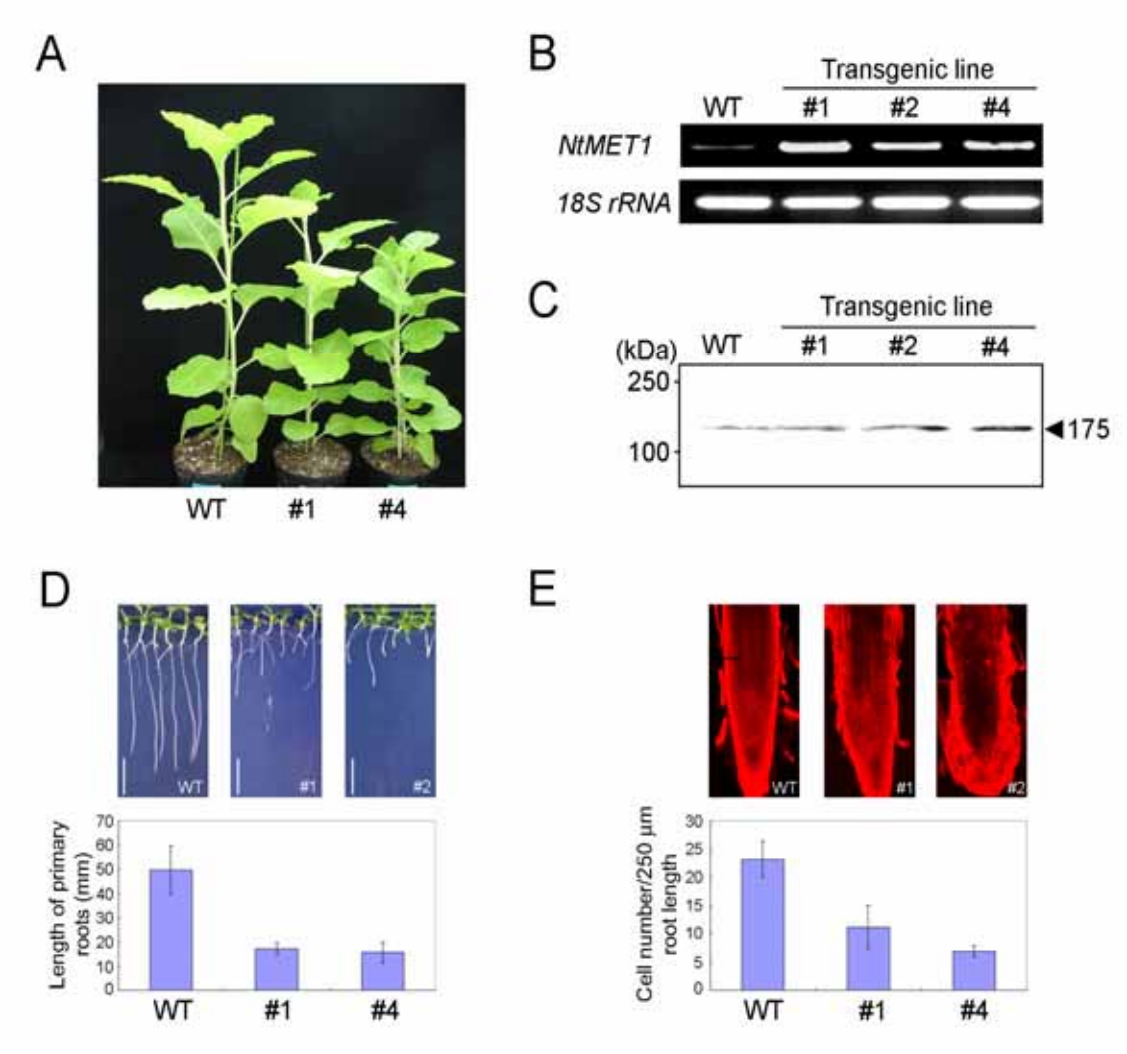


Figure 2. Transgenic tobacco plants. (A) Phenotype of two-month old mature plants. Samples were wild-type (WT) and over-expressing transgenic lines #1 and #4. (B) Transcript accumulation assay by RT-PCR. Total RNA was extracted from young leaves of wild type (WT) or transgenic lines #1, #2 and #4, reverse transcribed using oligo$(dT)_{15}$, and amplified with a set of specific primers for NtMET1. Products were fractionated on agarose gel electrophoresis and visualized with ethidium bromide staining. As the PCR control, rRNA was amplified (18S rRNA). (C) Protein accumulation assay by immuno-blotting. Total proteins were prepared from leaves of wild type (WT) or transgenic lines #1, #2 and #4, separated by SDS-PAGE and transferred to cellulose membrane, which were subjected to immuno-blot assay using rabbit anti-MET1 antibodies. Location of the antigen was visualized by using HRP-conjugated goat anti-rabbit antibody and the ECL system. (D) Root growth. Seeds were plated on vertical agar plates and incubated for 3 weeks. Vertical bar stands for 1 cm. (E) Phenotype of root meristem. Root tips were stained with propidium iodide and observed using an LSM510 confocal microscope.

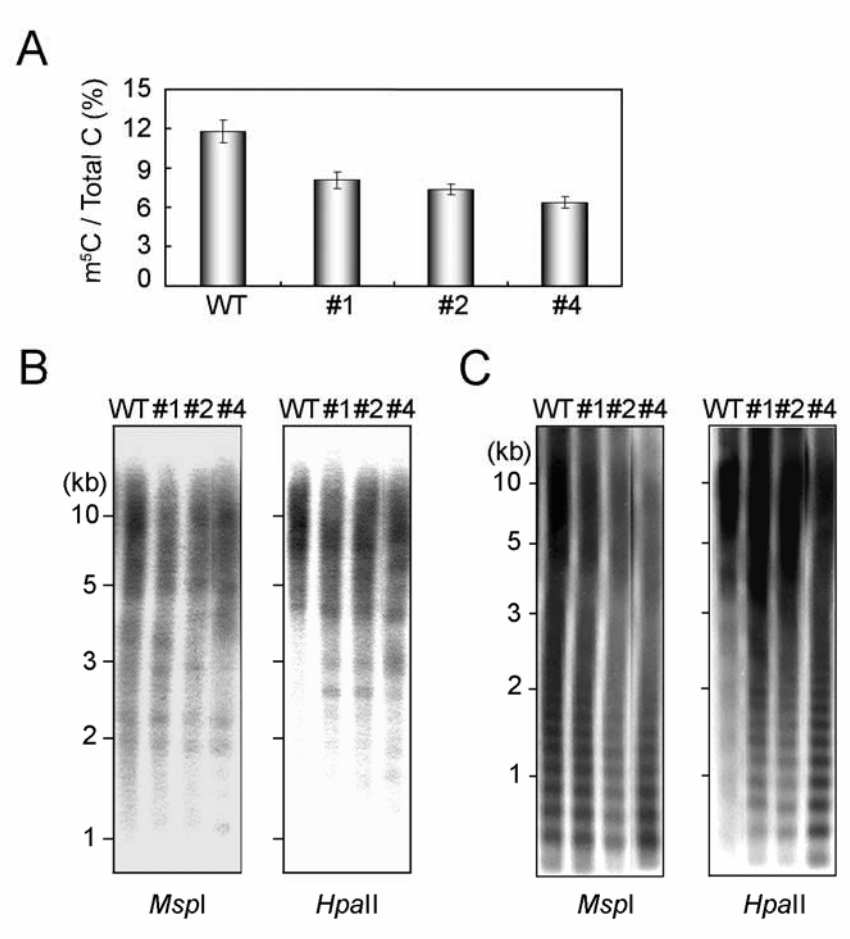


Figure 3. Methylation analysis of transgenic plants. (A) Contents of m^5C in total genomic DNA. Total DNA was isolated from leaves of wild type (WT) or transgenic lines #1, #2 and #4. DNA samples were digested to mononuleosides, dephosphorylated and subjected to HPLC analysis. The ratio of m^5C to total cytosine was calculated relative to concentration of nucleotide standard authentic cytosine and m5C. (B) Methylation status at *Tto1* locus. A 20-μg aliquot of genomic DNA was digested with *Msp*I (left panel) or *Hpa*II (right panel) and subjected to DNA blot hybridization using tobacco retrotransposon *Tto1*. (C) Methylation status at tandem repeat locus. Genomic DNA from indicated samples was processed as described above, and DNA blot hybridization was carried out using *Bam*HI tandem repeat element (*HSR60.1*).

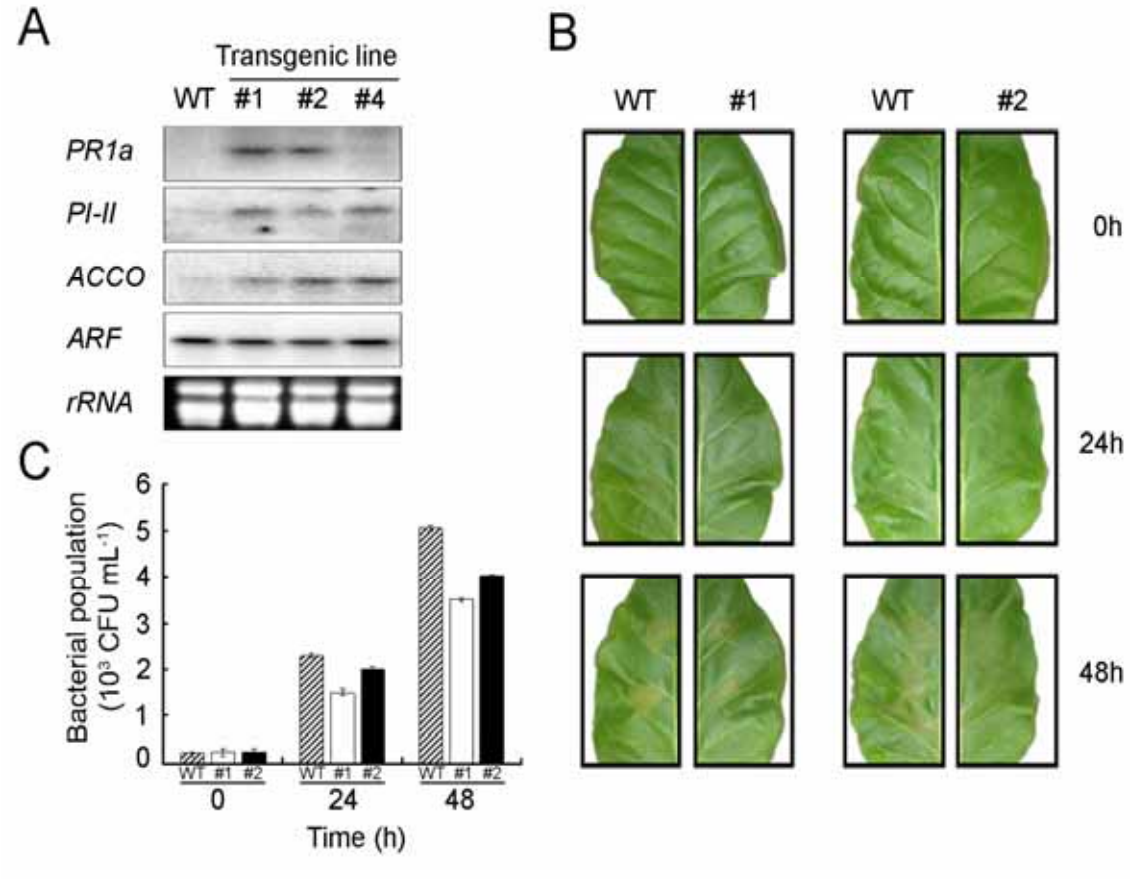


Figure 4. Differential expression of pathogen-responsive genes in transgenic lines. (A) Constitive expression of defense related genes in transgenic lines. Total RNA was isolated from detached healthy leaves of wild type and three independent transgenic lines, and 20-μg aliguot per lane was fractionated on agarose gel electrophoresis, transferred to nylon membrane and subjected to hybridization with indicated cDNA probes. Probes were *PR1a* (Pathogen-related 1a, X12737), *PI-II* (Protein inhibitor II, Z29537), *ACCO* (aminocyclopropane carboxylic acid oxidase, AB012857) and *ARF* (ADP ribosylation factor, AAD17207). (B) Disease symptoms. Leaves of wild type (WT) and transgenic lines (#1 and #2) were inoculated with *P. syringae* pv *glycinea*, and observed at indicated time points. (C) Quantification of P. syringae pv Glycinea cells propagated in wild type (gray bar), #1 (open bar) and #2 (black bar). Leaf discs were collected at indicated time point after infection and subjected to bacteria counting.

CHAPTER II

Properties of a tobacco DNA methyltransferase, NtMET1 and its involvement in chromatin movement during cell division

Introduction

Methylation of cytosine residues is commonly observed in DNA of most eukaryotes, this often being called DNA methylation, and has been considered to play certain roles in controlling gene expression (Yoder *et al*., 1997b). Cytosine methylation is enzymatically catalyzed by DNA methyltransferases, which transfer a methyl group from *S*-adenosylmethionine to the 5-position of cytosines in DNA. In plants, three types of DNA methyltransferases have been identified based on sequence analyses: DNA methyltransferase type I (MET1), chromomethylase (CMT) and domains rearranged methylase (DRM), among which MET1 is believed to predominantly catalyze methylation of hemimethylated symmetrical CpG, thereby maintaining methylation patterns after DNA replication (Finnegan and Dennis 1993).

Curiously however, biochemical studies on catalytic properties of MET1 enzymes have been hitherto limited. To our knowledge, only two preliminary reports are available; one is a

study on maize MET1 (ZmMET1, AF229183), of which N-terminal truncated protein was reported to exhibit a low methylation activity *in vitro* (Steward *et al*., 2000). The other case is pea MET (C-5 MTase, AF034419), which showed methylation activity towards Cp(A/T)pG and poly(dI-dC) synthetic oligomers (Pradhan *et al*., 1998). However, lack of enough knowledge on catalytic properties has made it somehow difficult to fully understand the biological functions of MET1 proteins. Indeed, most functional studies, including *Arabidopsis* enzymes, have been carried out on genetic bases without substantial activity assays (Finnegan and Kovac 2000, Goll and Bestor 2005).

In addition to methylation activity, DNA methyltransferase proteins have been proposed to directly participate in regulatory function (Rountree *et al*., 2000). The N-terminus of mammalian DNA methyltransferase 1 (Dnmt1) was shown to possess transcriptional repressing activity by directly interacting with histone deacetylase (Rountree *et al*., 2000), histone methyltransferase (Fuks *et al*., 2000) and retinoblastoma tumor suppressor proteins (Robertson *et al*., 2000). These observations suggest that Dnmt1 plays an important role in gene regulation network independent on DNA methylation (Milutinovic *et al*., 2004). In accordance with this idea, intracellular localization analyses revealed that mouse Dnmt1 moved between nuclei and cytoplasm during embryo development (Mertineit *et al*., 1998). It was speculated that Dnmt1 family proteins might be involved in sister chromatid segregation

during the mitotic phase (Hung *et al*., 1999). As to plant MET1 proteins, no such information has so far been available.

In chapter II, I characterized enzymatic property, and observed cellular localization of NtMET1. I found that NtMET1 apparently does not exhibit DNA methylation actitivity *in vitro* assay, conceivably due to tight intra/inter-molecular interactions. I also report that NtMET1 interacts with Ran GTPase, thereby reversibly changing the cellular localization during mitosis of tobacco cells.

Materials and Methods

Plant materials

Tobacco cultured bright yellow 2 (BY2) cells, both from wild type and transgenic lines, were grown in Murashige and Skoog medium on a rotary shaker (115 rpm, 25°C) in the dark. Cultured BY2 cells were transformed via *Agrobacterium*-mediated method as described (Yamaguchi *et al*., 2003).

Fusion proteins

Full-length *NtMET1* was fused to the gene for glutathione *S*-transferase (GST) at the N terminus and cloned into the pDEST20 vector plasmid, which was transformed into *E. coli*

DH10Bac^{TM} cells (Invitrogen) as described (Wada *et al*., 2003). Insect Sf9 cells were maintained in Grace's insect medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 500 μg mL^{-1} gentamycin. Approximately 6 × 10^{9} cells per dish were infected with the recombinant baculovirus stock (500 μl) at suitable titer using Cellfectin Reagent (Invitrogen) and incubated at 27^{o}C for 3 days. Infected cells were suspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride, and 100 μg mL^{-1} aprotinin) and sonicated for 10 sec twice. Resulting solution was used as the crude enzyme solution, or subjected to purification through glutathione-Sepharose column (Amersham Biosciences, Piscataway, NJ, USA) as described (Wada *et al*., 2003). GST-fused NtH4 was constructed using pGEX4T-2 (Pharmacia Biotechnology, Piscataway, NJ, USA) according the described method (Seo *et al*., 1995). His-tagged AtRAN3 was prepared as described (Yano *et al*., 2006) Protein concentrations were estimated using the Bradford assay with bovine serum albumin as standard.

DNA methyltransferase assay

DNA methyltransferase activity was assayed by measuring ^{3}H-labeled methyl group transfer from *S*-adenosylmethionine (AdoMet) to substrates as described (Wada *et al*., 2003) with modification. For the initial assay, 2 μg of poly(dI-dC)/poly(dI-dC) (Sigma, St Lois, MI, USA) was used as the substrate. For sequence specificity assays, the synthetic

oligonucleotides 5’-ACGATCGTACGATCGTACGATCGT-3’ (for CpG), 5’-ACTGCAGTACTGCAGTACTGCAGT-3’ (for CpNpG, where N is A or T), and 5’-AGCATGCTAGCATGCTAGCATGCT-3’ (for CpNpN) were prepared. All these sequences are palidromic, which form duplexes. For hemi-methylation analysis, synthetic 28-mer oligonucleotides containing five 5-methylcytosines (m^5C) and its complementary strand without m^5C were independently prepared, and annealed to form duplexes. The substrates for CpG were 5’-ATTCGATCGAATCGTATACGTACGTATT-3’ and 3’-TAAGCTAGCTTAGCATATGCATGCATAA-5’ (m^5C is underlined), and those for CpNpG were 5’-ATTCAGTCAGATCTGATCAGTACTGATT-3’ and 3’-TAAGTCAGTCTAGACTAGTCATGACTAA-5’. The reaction mixture contained 20 mM MOPS-NaOH (pH 7.0), 5 mM EDTA, 200 μg mL^{-1} BSA, 25% (vol/vol) glycerol, 1 mM dithiothreitol, 100 μg mL^{-1} RNase A, 2 μmole of AdoMet (methyl-^{3}H, specific activity 307.1 GBq $mmol^{-1}$) (PerkinElmer, Wellesley, MA, USA), appropriate amounts of substrate DNA and purified protein fractions. After incubation for 90 min at 37^{o}C, the reactions were stopped by adding 500 μl of proteinase K-SDS buffer (1 mg mL^{-1} proteinase K, 1% SDS, 2 mM EDTA, 125 mM NaCl, and 0.5 mg mL^{-1} salmon sperm DNA) and further incubating at 50^{o}C for 1 h. The substrate DNA was extracted with phenol-chloroform, recovered by ethanol precipitation, and spotted on DEAE paper, dried, washed with 0.5 M sodium phosphate followed by 70% ethanol. Papers were placed in scintillation mixture, and DNA methylation

activity was determined by scintillation counting of radioactivity. The amounts of transferred methyl group were calculated based on the specific activity of [^{3}H]AdoMet (7.7×10^{4} cpm/pmol).

Fluorescence microscopy

For green fluorescence (GFP)-fusion, the *att*B-PCR product of *NtMET1* was cloned into the pGWB6 (CaMV35S-sGFP-NOS3') vector to fuse to green fluorescence (GFP) using GATEWAY cloning system (Invitrogen). A cDNA for tobacco histone H4 (NtH4) (accession no. AB280787) was obtained by PCR using specific primers containing *Sal*I and *Nco*I sites. Cyan fluorescence protein (CFP)-fused histone H4 (NtH4) was constructed by inserting the cDNA into the CaMV35S-CFP-NOS3' vector as previously described (Ito *et al*., 2003). GFP-AtMBD5, yellow fluorescence protein (YFP)-AtRan3 and CFP-RCC1 vectors were prepared as described (Yano *et al*., 2006). All constructs were transformed into *A. tumefeciens* stranin EHA105 and resulting strains were used to transform tobacco BY2 cells (Wada *et al*., 2003). Transgenic BY2 cells were observed with an AX70 fluorescence microscope (Olympus, Tokyo, Japan) with the U-MGFPHQ cube for GFP, YFP and CFP individually, and captured with a cooled charge-coupled device camera (CoolSNAPHQ, Photometrics, Tucson, AZ, USA). DNA staining was performed with an aliquot of LS medium containing 1 mg/ml 4'6-diamidino-2-phenylindole (DAPI) as described (Yano *et al*., 2006).

Construction of Ran GTPase mutants

Specific point mutant was introduced into AtRAN3 cDNA by PCR site-directed mutagenesis. The active form was constructed by substituting Gly at position 22 with valine (G22V), and inactive form with Thr at position 27 with Asn (T27N) (Dascher and Balch 1994, Haizel *et al*., 1997). Briefly the 5'-oligonucleotide primer, 5'-CACCATGGCTCTACCTAACCAGCAAAC-3' was used to facilitate directional cloning into the pBAD102/D-TOPO expression vectors (Invitrogen). The anti-sense mutagenic oligonucleotides, 5'-GGTCTTCCCTGTGCCTACATC-3' for G22V (mutation point is underlined), and 5'-ACA AATGTGTTCTTCCCTGTG-3' for T27N were prepared to serve as primers for amplification of the 5'-fragments. The 3'-fragments were generated using oligonucleotides complementary to the above mutagenic primers in combination with the 3'-end anti-sense oligonucleotide primer 5'-CTCGAAGGTGTCATCATCGTCAT-3'. After second overlap extension, the recombinant PCR products were cloned into pBAD102/D-TOPO vector and expressed in *E. coli* strain LMG194 according to the manufacturer's instructions (Invitrogen).

Pull-down binding assay

Pull-down assay was performed as described (Yap *et al*., 2005). Briefly, approximately 5 μg of purified GST, GST-fused NtMET1 or GST-fused NtH4 proteins were immobilized on

glutathione (GSH)-Sepharose (Amersham Biosciences) at 4^{o}C for 2 h. After blocking, beads were incubated with 25 μg of His-tagged fusion proteins at 4^{o}C for 16 h. Beads were collected and washed, and proteins were eluted with a buffer containing 10 mM reduced glutathione in 50 mM Tris-HCl at pH 8. Eluted proteins were fractionated on 12% SDS-polyacryl amide gel and subjected to immuno-blot staining using mouse anti-His-tag monoclonal antibodies and horse radish peroxidase (HRP)-conjugated anti-mouse IgG antibodies (MBL, Nagoya, Japan). Antibody-antigen complex was detected using ECL system (Amersham Biosciences). For far-western assays, a 20-μl aliquot of antibodies raised against the C-terminal region-specific peptide of ZmMET1 (Steward *et al*., 2000) was bound to protein A-Sepharose (Pharmacia Biotechnology), and washed three times with 500 μl of 50 mM Tris-Cl buffer (pH 7.0). A 25-μg ΔNtMET1/N protein was preincubated with 25-μg full-length NtMET1 or ΔNtMET1/C at 4^{o}C for 2 h on a rotator. Antibody-protein A complex was then mixed with appropriate amounts of proteins, and incubated at 4^{o}C for 16 h on a rotator. After washed with the buffer, samples were fractionated on SDS-PAGE, transferred to a membrane and subjected to immuno-staining by anti-GST antibodies.

Yeast two-hybrid assay

Yeast tow-hybrid assays were performed as described previously (Yap *et al*., 2005). GAL4 DNA-binding domain (BD) was fused to full-length NtMET1 (NtMET1), or

C-terminal truncated NtMET1 (ΔNtMET1/N), and activation domain (AD) was fused to N-terminal truncated NtMET1 (ΔNtMET1/C) using the MATCHMAKER GAL4 system (Clonetech, Palo Alto, CA, USA) Yeast AH109 cells were co-transformed with the indicated combinations. The co-transformants for interacting constructs were screened on Quadruple Dropout Medium (SD/-Ade/-His/-Leu/-Trip) and subjected to the β–galactosidase colony-lift filter and activity assays as described in the yeast protocols handbook (Clonetech).

Results

Methylation activity

Full-length or N-terminus truncated NtMET1 as glutathione S-transferase (GST) fusion protein was expressed in baculovirus-mediated expression system in *Spodoptera frugiperda* (Sf9) insect cell line, which lacks endogenous DNA methyltransferase activity. After 72 h post-infection, cell extracts were sampled, and protein was purified through a glutathione-Sepharose column, and immunoblotted with anti-GST antibodies (Fig. 1). It is evident that full-length (200 kDa, NtMET1) and N-terminus truncated (117 kDa, ΔNtMET1/C) proteins were successfully synthesized (Fig. 1). DNA methyltransferase activity

was then estimated using purified protein samples. As positive and negative controls, tobacco DRM1, a *de novo* DNA methyltransferase (Wada *et al*., 2003), and bacterial β–glucuronidase (GUS), respectively, were similarly expressed in insect cells and subjected to the same assay. Initially, *de novo* DNA methyltransferase activity was estimated by measuring ^{3}H-labeled methyl group transfer from AdoMet into synthetic poly(dI-dC), which provides a large number of potential dinucleotide sites for methylation. Results indicated that, while GST-NtDRM1 was clearly active, both GST-NtMET1 and GST-ΔNtMET1/C were totally inactive, showing the same level as the control GUS protein (Fig. 2A). The maintenance methyl transfer activity was then analyzed. Substrates were constructed using two types of synthetic 28-mer oligonucleotide substrates containing either CpG or CpNpG (N is A or T). In one sample, all cytosines were substituted with $m^{5}C$, and in the other, all cytosines remained intact. By mixing and annealing these two types, hemimethylated substrate was created. Unmethylated substrate was made from unmethylated oligomers. Results showed that GST-NtMET1 was inactive towards both unmethylated and hemimethylated substrates in CpG and CpNpG sequence context (Fig. 2B). Time-course analyses also exhibited no detectable activity up to 2 h incubation (data not shown). That the reaction system was appropriate was confirmed by a clear activity of GST-NtDRM1, which preferentially methylates both unmethylated and hemimethylated CpNpG and CpNpN (Wada *et al*., 2003) (Fig. 2B).

Intramolecular interaction

I examined the possibility of intramolecular interaction within NtMET1 molecule. Using the N-terminus with 895 amino acids (ΔNtMET1/N) and the C-terminus with 798 amino acids (ΔNtMET1/C) (Fig. 3A), *in vitro* far-western assay was performed by specific antibodies that recognized the C-terminal but not the N-terminal polypeptides. Results showed that N-terminus (ΔNtMET1/N) was efficiently precipitated only in the presence of the C-terminus (ΔNtMET1/C) (Fig. 3B). This interaction was abolished when the full-length NtMET1 was used instead of the C-terminus (Fig. 3B). The inability of coprecipitation might be due to that the C-terminus was already masked by the intact N-terminus, thus being prevented from further interaction with exogenous N-terminal fragments. Yeast two-hybrid assays confirmed these results, showing an effective interaction between ΔNtMET1/N and ΔNtMET1/C, but no interaction between full length NtMET1 and ΔNtMET1/C (Fig. 4A). These experiments proved a specific intra-molecular interaction between the N- and C-terminal regions in NTMET1 protein.

Intracellular localization

The cellular localization of NtMET1 protein was examined with GFP-fused constructs. Plasmid containing cauliflower mosaic virus (CaMV) 35*S* promoter *NtMET1-GFP* was constructed and introduced into tobacco cultured BY2 cells by the *Agrobacterium*-mediated

transformation method. When cells during the resting stage were examined, *NtMET1-GFP* constructs clearly gave fluorescent signals in nuclei (Fig. 5, middle panel), while the control GFP fluorescence was observed throughout cell (Fig. 5, upper panel). Upon merging with DAPI stained images, NtMET1 protein was apparently localized to regions where DNA is condensed. This was distinct when an image of nucleus was enlarged, showing well matching of DAPI and GFP images (Fig. 5, lower panel). Localization of NtMET1 was then examined in cells undergoing mitosis. At inter- and prophases, NtMET1-GFP was only localized in nucleus (Fig. 6A, top and second panels). At early metaphase, however, NtMET1-GFP was mostly found in cytoplasm (Fig. 6A, third panel), and in both cytoplasm and chromatins at middle and late metaphases (Fig. 6A, fourth panel). At anaphase, the protein began to align on chromatin (Fig. 6A, fifth panel), and at telophase, NtMET1 was again localized only in nucleus (Fig. 6A, bottom panel). These localization patterns were inherent in NtMET1 protein, since during all phases of mitosis, histone H4 protein distinctly remained with chromatin (Fig. 6C), and the control GFP protein was ubiquitously observed throughout cell (Fig. 6D).

Localization patterns of NtMET1 during mitosis appeared to resemble that of Ran GTPase, AtRAN3, which is an ortholog of tobacco NtRan-A1 showing a 96% homology (Yano *et al*., 2006). Subsequently, localization of YFP-AtRAN3 fusion protein was examined in parallel with NtMET1. At inter- and prophases, AtRAN3 was mainly localized to nucleus with weak dispersion into cytoplasm (Fig. 6B, top and second panels). At metaphase,

AtRAN3 was found in periphery of nucleus, particularly at the position of the spindle microtubules (Fig. 6B, third panel). At anaphase, AtRAN3 was localized in and between chromosomes (Fig. 6B, fourth panel), and at telophase, it was localized not only into nucleus, but also into the region of growing cell plate (Fig. 6B, bottom panel). When comparing the localization patterns of NtMET1 and AtRAN3, it is evident that they behave concertedly at pro- and metaphases, but differently at ana- and telophases. These results indicated that NtMET1 changes its localization during mitosis, and that such changes partly coincide with that of AtRAN3. Since Ran GTPases are considered to play critical roles in spindle formation (Kahana and Cleveland, 1999), colocalization of NtMET1 with AtRAN3 proteins may suggest that both proteins are positively involved in chromatin movement during cell division.

Physical interaction

Physical interaction between NtMET1 and AtRAN3 was assessed by *in vitro* pull-down assay, using GST-tagged NtMET1 (GST-NtMET1) and His-tagged AtRAN3 (His-AtRAN3) proteins. His-AtRAN3 was applied to a glutathione-Sepharose column containing GST-NtMET1 or GST proteins, eluted with a buffer containing reduced GST, separated by SDS-PAGE and subjected to immuno-blot assays with anti-His-tag antibodies. The results showed that AtRAN3 was only detectable upon incubation with GST-NtMET1 but not with GST protein alone, indicating specific binding of AtRAN3 to NtMET1 (Fig. 7). Ran GTPase

often changes its location and targeting of interacting proteins depending on whether it is in the GTP-bound active or GDP-bound inactive forms (Zheng 2004). In order to determine which preferentially binds to GST-MET1, pull down assays were performed with active and inactive AtRAN3 mutants, which were constructed by single amino acid substitution. Binding tests showed that GST-MET1 equally interacted with both forms of AtRAN3 (Fig. 7).

Ran GTPase was previously shown to directly bind to histone H4 in *Xenopus* cell (Bilbao-Cortes *et al*., 2002). To test this possibility in plant cell, tobacco histone H4 fused to GST was bacterially expressed and subjected to pull-down assay with His-AtRAN3 in a similar manner with GST-NtMET1 assays. Results showed that interaction indeed takes place between AtRAN3 and NtH4 (Fig. 8A), and that this interaction was equally effective with mutant proteins (Fig. 8A). The possibility of NtMET1 to interact with NtH4 was finally examined, but the results were apparently negative, showing no direct binding on pull-down assay (Fig. 8B).

Discussion

Despite of using intensively purified recombinant protein expressed in insect cells, a tobacco type 1 DNA methyltransferase, NtMET1 did not exhibit any detectable enzymatic

activity *in vitro* towards both unmethylated and hemi-methylated DNA substrates. The inability was not due to experimental procedures, such as amino acid mutation, since the total cDNA sequence was confirmed to be correct after the genomic sequence (unpublished observation). Also all amino acid motifs necessary for activity were well conserved. Transgenic tobacco producing excess NtMET1 protein exhibited hypomethylation of genomic DNA, indicating enzymatic inability *in planta*. Several causes are conceivable. First, NtMET1 needs modification to be activated, including glucosylation, phosphorylation and/or acetylation/methylation; second, it needs counterpart factors such as protein(s) for activation; and third, the activity is blocked by protein structure.

The first idea that posttranslational modification is prerequisite for conferring activity is also possible. In silico analysis indicated that NtMET1 can potentially be phosphorylated at 84 Ser/Thr sites throughout the molecule. In mouse DNA methyltransferase, phosphorylation of serine at position 514 was shown to be critical for bringing the enzyme to the replication foci (Glickman *et al*., 1997). It was suggested that phosphorylation may affect subcelluar localization and attenuate substrate inhibition due to allosteric effect of the enzyme (Glickman *et al*., 1997). A similar modification could occur in NtMET1, thereby modulating the activity. The second point suggesting NtMET1 to require a protein factor for *in vivo* activity is highly probable. To date, mammalian Dnmt1 have been shown to form stable complexes with histone deacetylase (HDAC) 1 and 2, tumor suppressor gene product (Rb), histone

methyltransferase (SUV39H1) and DNA methyltransferase associated protein (DMAP1) (Robertson *et al*., 2000, Rountree *et al*., 2000, Fuks *et al*., 2003). These proteins were supposed to modulate Dnmt1 function by, for example, mutually affecting methylation activity of DNA and histones (Fuks *et al*., 2003), or recruiting it to transcription complex (Rountree *et al*., 2000). For plant MET1, however, no protein factors have so far been identified that directly regulates its activity. The third idea suggesting that intra-molecular interaction might interfere with the activity was partly supported by our present finding showing a tight interaction between the N- and C-terminal regions. A similar but opposite case was reported for mouse Dnmt1, of which full activity required the N-terminus interaction with the C-terminus (Margot *et al*., 2003). It was concluded that a physical interaction between the N- and C-terminal domains was prerequisite for activation of the catalytic domain. Hence intramolecular interaction might be critical for regulation of Dnmt1 and NtMET1 activities. However, structural relaxation alone appears not to be sufficient for activation of NtMET1, as judged from our observation. For example, the N-terminus truncated construct, which does not interact with the C-terminus, did not exhibit detectable methylation activity (Fig 1B), and treatments of purified enzyme with mild detergents such as Tween-20 and Nonidet P-40 did not recover the activity (unpublished observation). I can thus safely conclude that NtMET1 activity is finely regulated by complex formation with various proteins, which may be important to relax and/or tighten its higher conformation, and to assist

in activity expression.

A question then arises as to how and which factors activate the enzyme *in vivo*. The best clue might be obtained through identification of interacting protein(s) by the yeast two-hybrid method. Subsequent intensive screening of a cDNA library containing 1.2×10^9 clones by the NtMET1 bait yielded clones encoding such as ubiquitin carrier and DnaJ heat shock proteins together with several unknown proteins, but we decided not to further characterize them (unpublished observation). Another clue could be obtained through NtMET1 behaviour during cell division, since this enzyme is supposed to function during DNA replication to maintain the CpG methylation (Goll and Bestor 2005). Mammalian Dnmt1 was shown to be localized in cytoplasm during interphase, and translocated to nucleus at S-phase to associate with replication foci (Leonhardt *et al*., 1992, Goll and Bestor, 2005), so that the enzyme specifically acts on newly replicated DNA strands. In contrast, NtMET1 appeared to stay within nucleus throughout cell cycle, except during M-phase, when it diffuses into cytoplasm. This apparently opposite localization between mammalian and plant enzymes may be correlated with their activity. Mammalian Dnmt1 was shown to be constitutively active when the N-terminus interacts with the C-terminus catalytic domain (Margot *et al*., 2003). NtMET1 could be constitutively inactive when the N-terminus interacts with the C-terminus. Thus, in mammalian cells, methylation can be performed simply by bringing the active enzyme to the replication foci, while in plants, reactivation of inactive enzyme is necessary. This can be

accomplished by other protein factor(s), and such an example will be identified in a future study.

Translocation of NtMET1 into cytoplasm during metaphase is notable. Since no DNA replication takes place during M-phase, such a translocation is probably not involved in maintenance DNA methylation. In the case of *Drosophila* Dnmt1-like protein, DmMTR1, a cell cycle-specific switch of its localization in cytoplasm and nucleus was noted (Hung *et al*., 1999). During interphase, DmMTR1 was located outside the nucleus, and rapidly translocated into nucleus during mitosis. Since genomic DNA of *Drosophila* is not methylated, this protein was suggested to play an essential function in the cell-cycle regulated condensation of chromosomes (Hung *et al*., 1999). Although the translocation phase differs between DmMTR1 and NtMET1, these observations suggested that, independently on DNA methylation, both proteins may participate in progress of mitosis together with many other proteins. One such protein was found to be a Ran GTPase for NtMET1. Ran GTPase was shown to promote microtubule nucleation during mitotic spindle assembly, and nuclear envelop assembly (Clarke and Zhang 2001). It takes two alternative GTP- and GDP-bound forms, among which the former has been considered to be biologically active due to its localization on chromatin surface (Kahana and Cleveland 1999). Recent studies, however, revealed that Ran directly binds to nucleosomes and to histones H3 and H4 independently of GTP/GDP forms (Bilbao-Cortes *et al*., 2002). We found here that both forms of Ran GTPase

equally bind to NtMET1, and also to histone H4, consistent with above observation. This suggests that NtMET1 is one of chromatin proteins, attaching to H4 via Ran GTPase, since NtMET1 did not directly bind to H4. Previously we showed that active form of Ran GTPase efficiently interacts with a methyl CpG-binding protein (MBD), which migrates around chromatin during mitosis (Yano *et al*., 2006). Since behaviour of NtMET1 resembles this pattern, it is tempting to speculate that one of the protein complexes surrounding chromatin structure specifies in recognition of, and interaction with methylated DNA, and that dynamic movement of such a complex during mitosis is driven by Ran GTPase.

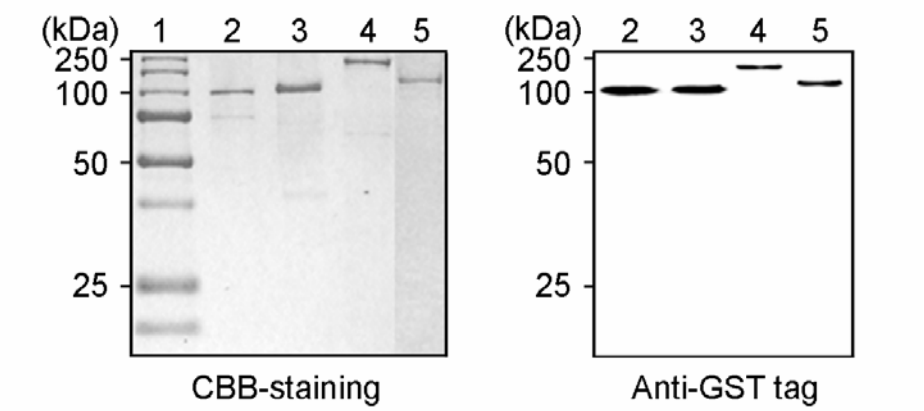


Figure 1. Production of NtMET1 protein. Recombinant proteins were expressed in Sf9 insect cells. Three days after infection, cells were collected, and proteins were extracted and purified through GST column. Purified proteins were fractionated on 12% SDS-PAGE, and after staining with Coomassie brilliant blue (CBB) (left panel), blotted onto cellulose membrane and subjected to immuno-blot staining using anti-GST antibodies (right panel). Samples were size markers as indicated at the left side in kDa (lane 1), β-glucuronidase as the control protein expressed in Sf9 cells (lane 2), NtDRM (lane 3), full length NtMET1 with 1556 amino acids (lane 4) and N-terminal-truncated NtMET1 (ΔNtMET1/C) with 795 amino acids (lane5).

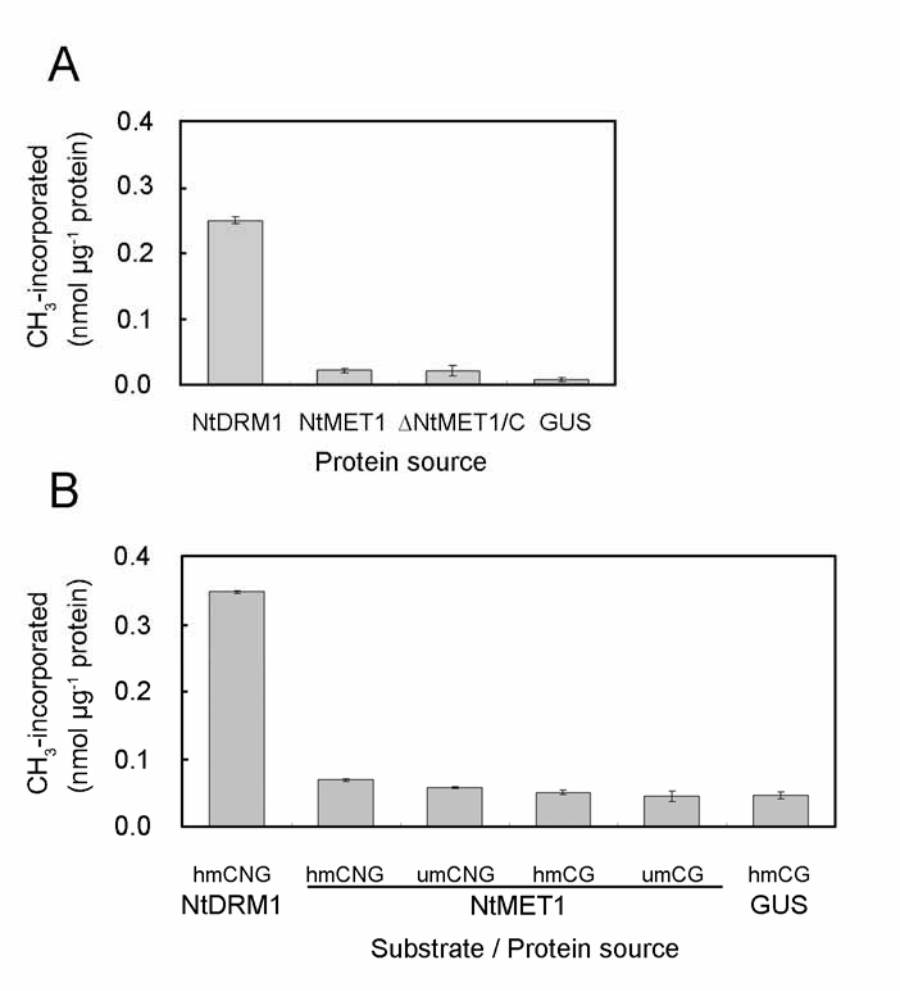


Figure 2. Activity assay. (A) *De novo* DNA methyltransferase activity assay. A standard methylation reaction mixture containing 50 ng of indicated purified protein and 2 µg of poly(dI-dC)/poly(dI-dC) was incubated at 37°C for 6 h and ^{3}H incorporation was counted. β-glucuronidase was used as the control. Values are mean of triplicate incubations for each protein and error bars indicate standard deviations. (B) Maintenance methytransferase activity assay. Synthetic duplex sequence of 28-mer oligonucleotiedes contained five methylatable cytosines per strand, offering 5 and 10 sites in hemimethylated and unmethylated substrates, respectively. A standard reaction mixture containing 50 ng of indicated purified protein and 125 nM of indicated substrate was incubate at 37°C for 1 h. Substrates were hemimethylated CpG (hmCG), unmethylated CpG (umCG), hemimethylated CpNpG (hmCNG) and unmethylated CpNpG (umCNG). Values are mean of triplicate incubations for each protein and error bars indicate standard deviations.

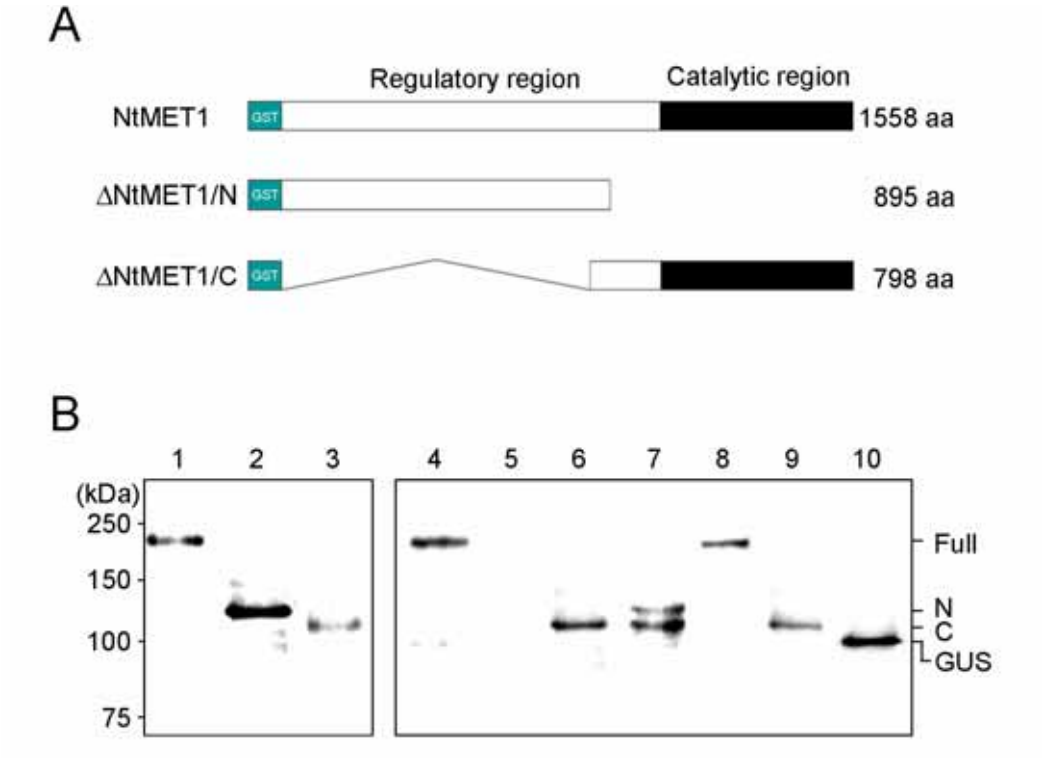


Figure 3. Intramolecular interaction. (A) Schematic representation of used proteins. GST-fused full-length NtMET1 (NtMET1), GST-fused C-terminal truncated NtMET1 (ΔNtMET1/N) and GST-fused N-terminal truncated NtMET1 (ΔNtMET1/C) were expressed in Sf9, purified through GST-column and used for binding assays. The N-terminal regulatory and the C-terminal catalytic regions and corresponding amino acid numbers (aa) are indicated. (B) Far-western assay. After 25-μg ΔNtMET1/N protein was preincubated with 25-μg full-length NtMET1 or ΔNtMET1/C, anti-ZmMET1/protein-A complex was mixed, fractionated on SDS-PAGE, and subjected to immuno-staining by anti-GST antibodies. Samples were approximately 0.1 μg of input full-length NtMET1 (lane 1), input ΔNtMET1/N (lane 2), input ΔNtMET1/C (lane 3), full-length NtMET1 with antibodies (lane 4), ΔNtMET1/N with antibodies (lane 5), ΔNtMET1/C with antibodies (lane 6), ΔNtMET1/N and ΔNtMET1/C with antibodies (lane 7), Full-length NtMET1 and ΔNtMET1/N with antibodies (lane 8), ΔNtMET1/C and GUS proteins with antibodies (control) (lane 9), and input GUS proteins (lane 10).

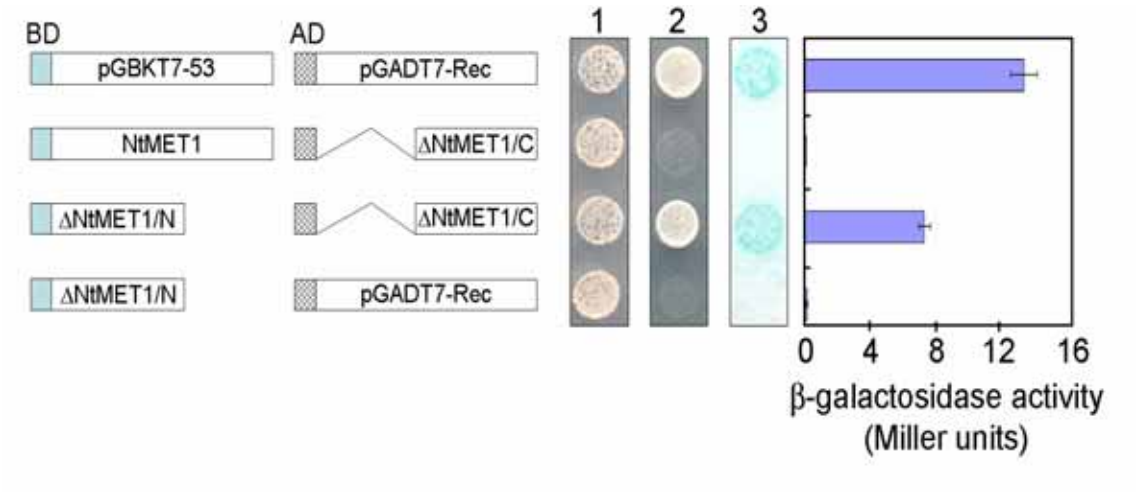


Figure 4. Yeast two-hybrid assay. Yeast AH109 cells were co-transformed with the AD-ΔNtMET1/C and BD-NtMET1 or BD-ΔNtMET1/N. As the positive and negative controls, BD-pGBKT7-53 or BD-ΔNtMET1/N were co-transformed with AD-pGADT7-Rec, respectively (left panel). The transformants were planted on SD agar supplemented with an amino acid mixture depleted of tryptophan and leucine (SD/-Trp/-Leu). Colonies cultured in SD/-Trp/-Leu/ plate were spotting on YPDA (lane 1) and SD/-Ade/-His/-Trp/-Leu (lane 2), and assayed for β-galactosidase by the filter lift method (lane 3). The β-galactosidase activity was estimated using *O*-nitriphenyl-β-$_{D}$-galactopyranoside as the substrate and expressed in Miller units (right panel). Values are from triplicates with standard deviation.

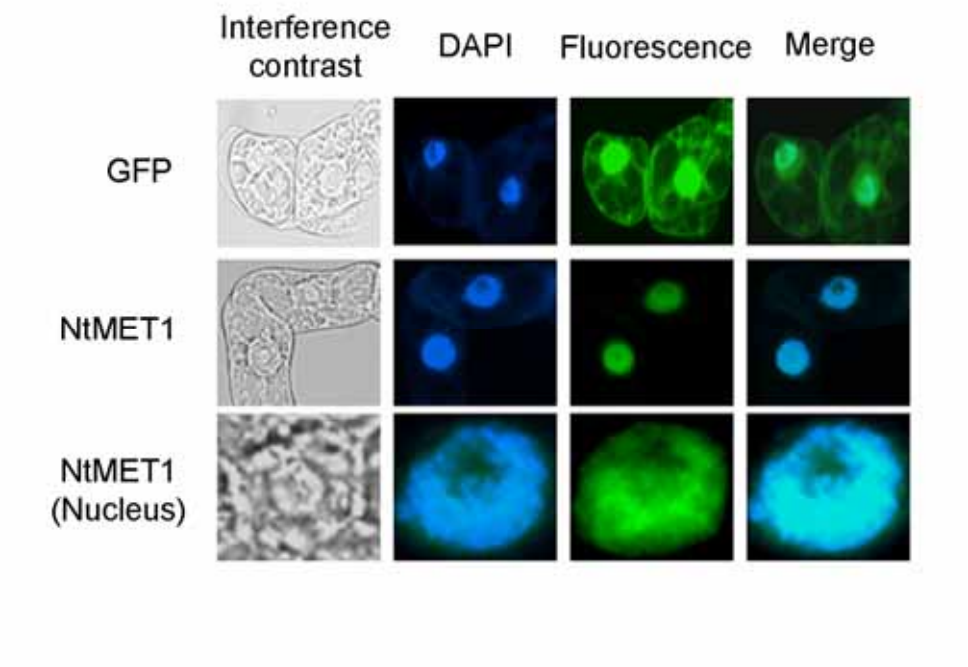
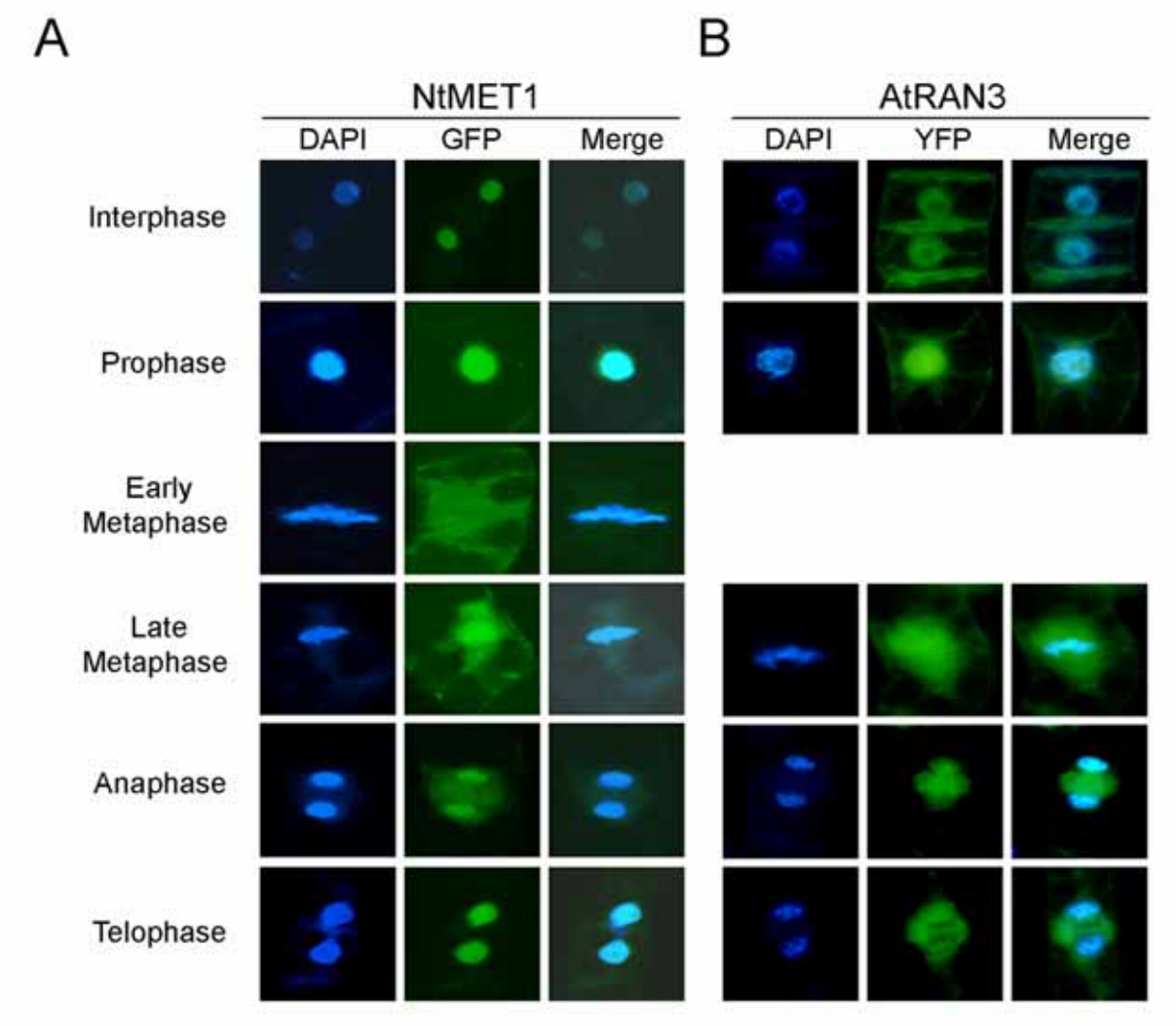


Figure 5. Subcellular localization at resting stage. GFP-NtMET1 fusion protein was stably expressed in tobacco BY2 cells. Cells at resting stage were observed by microscope under light (Interference contrast), or under fluorescence for GFP (Fluorescence). Cells were also stained with DAPI (DAPI) for DNA localization, and images from DAPI and GFP were merged (Merge). Samples were cells expressing CaMV35S-GFP alone (control) (top panel) and those expressing NtMET1 (middle panel). Nucleus of NTMET1 expressing cells are enlarged (lower panel).


A
B
NtMET1
AtRAN3
DAPI
GFP
Merge
DAPI
YFP
Merge
Interphase
Prophase
Early Metaphase
Late Metaphase
Anaphase
Telophase

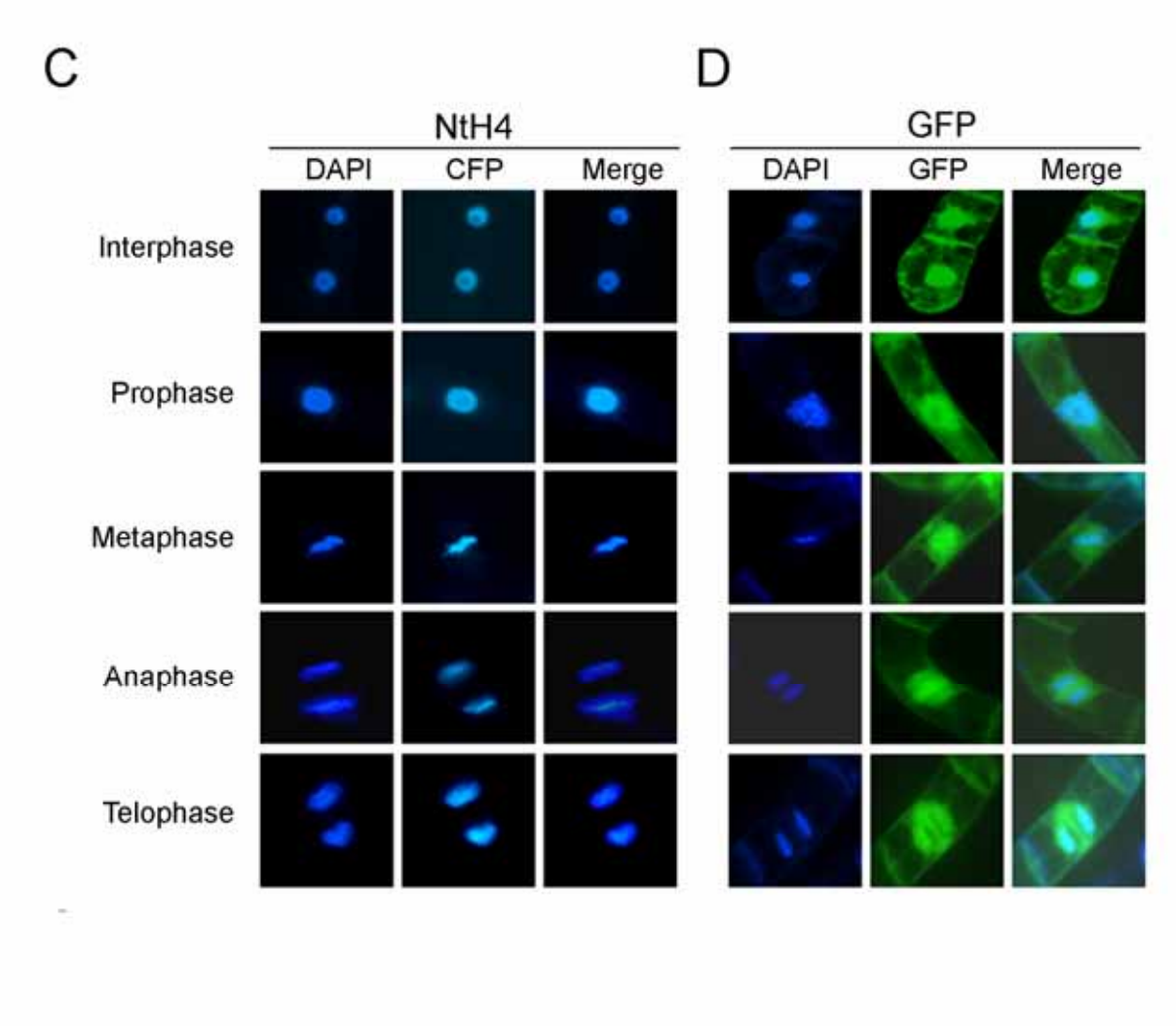


Figure 6. Subcellular localization during mitosis. BY2 cells were stably transformed with expression vectors for GFP-NtMET1 (A), YFP-AtRAN3 (B), CFP-NtH4 (C) and GFP alone (D). Transgenic cells were fixed on a slide glass and observed using specified filters for each fluorescence at indicated division phase. For identification of chromatin, cells were stained with DAPI (DAPI), and observed using specified filters. DAPI and epifluorescence images are merged (Merge).

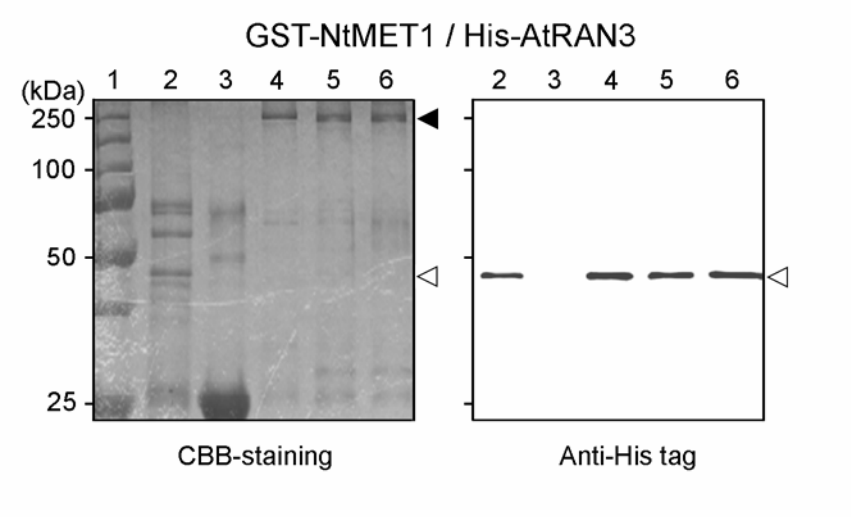


Figure 7. Interaction of NtMET1 with AtRAN3. A 25 μg GST-fusion NtMET1 was bound to a glutathione-column and 25 μg His-tagged AtRAN3 were applied to the column. After elution with reduced glutathione, proteins were fractionated on SDS-PAGE (CBB staining, left panel) and subjected to immuno-blotting assay with antibodies against anti-His-tag (right panel). Closed and open arrowheads indicate the position of GST-NtMET1 and His-tagged AtRAN3 proteins, respectively. Samples were molecular markers (lane 1), input wild type His-tagged AtRAN3 (lane 2), control GST protein (lane 3), GST-NtMET1 with wild type His-tagged AtRAN3 (lane 4), GST-NtMET1 with active form of His-tagged AtRAN3^{G22V} (lane 5) and GST-NtMET1 with inactive form of His-tagged AtRAN3^{T27N} (lane 6).

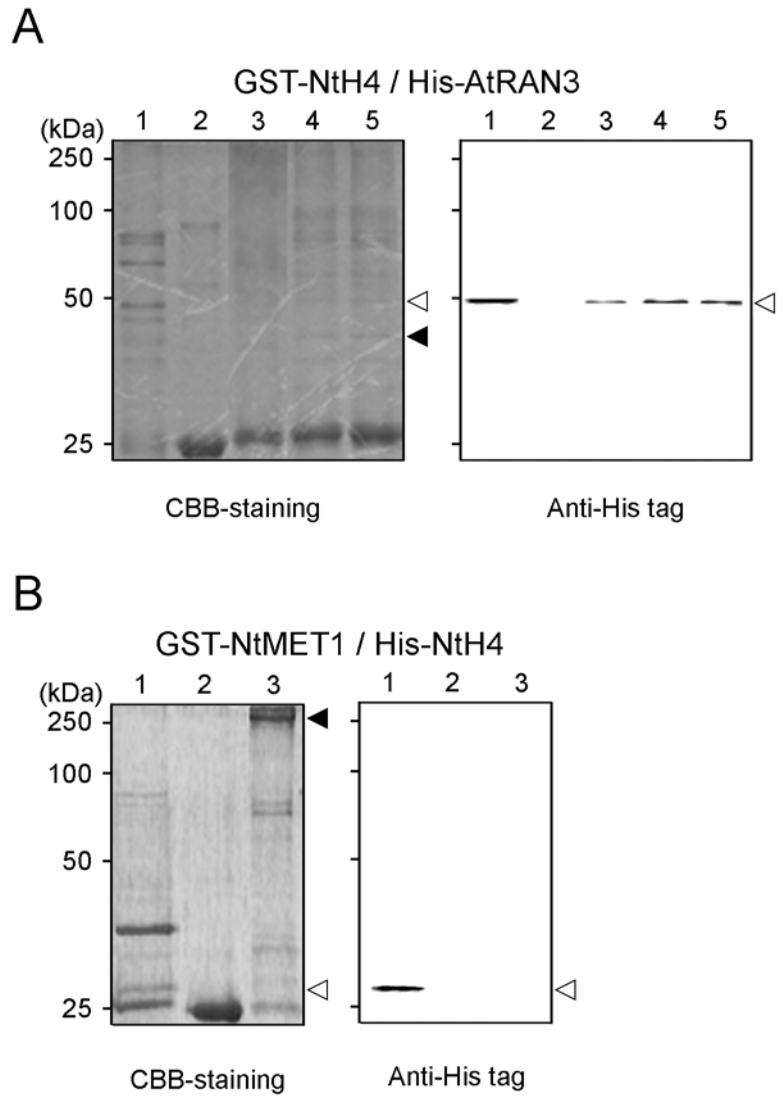


Figure 8. Pull-down assay. (A) Interaction of NtH4 with AtRNA3. A 25 μg GST-fusion NtH4 and 25 μg His-tagged AtRAN3 were examined for interaction as described above. Closed and open arrowheads indicate the position of GST-NtH4 and His-tagged AtRAN3 proteins, respectively. Samples were input wild type His-tagged AtRAN3 (lane 1), control GST protein (lane 2), GST-NtH4 with wild type His-tagged AtRAN3 (lane 3), GST-NtH4 with His-tagged AtRAN3^{G22V} (lane 4) and GST-NtMET1 with His-tagged AtRAN3^{T27N} (lane 5). (B) Interaction of NtMET1 with H4. Assay was performed as described above using 25 μg each of GST-NtMET1 and His-tagged NtH4. Samples were input His-tagged NtH4 (lane 1), control GST protein (lane 2) and GST-NtMET1 with His-tagged NtH4 (lane 3).

CONCLUDING REMARKS

In the first part of this thesis, I discussed functions of methyltransferase type 1 (MET1) in tobacco plants. In plants, three types of DNA methyltransferase are known, among which MET1 was expected to play a major role by maintaining the CpG methylation patterns from physiological studies. *In planta* assay with over-expressing transgenic lines showed no hypermethylation of genomc DNA, but rather hypomethylation to have occurred. These results indicated that, in spite of efficient expression of NtMET1 protein, genomic DNA was not over-methylated but rather demethylated, perhaps due to excess NtMET1 protein interfering normal methylation process. This suggests that NtMET1 is highly controlled to function at protein level, so that unnecessary proceeding of genomic methylation could be avoided.

In chapter II, I expressed recombinant GST-NtMET1 fusion protein in Sf9 insect cells, and purified sample was subjected to standard methylation assay. *In vitro* assay showed no detectable methylation activity when both hemimethylated and unmethylated DNA samples were used as the substrate. The inability of NtMET1 to methylate DNA *in vitro* was puzzling, since its critical domains necessary for activity were highly conserved among DNA methyltransferases, of which enzymatic activities have been confirmed. A lack of enzymatic activity in both *in vitro* and *in planta* suggested that NtMET1 activity is highly negatively

controlled at protein level. One of such controlling factors could be higher conformation of protein itself, since the N-terminal region of mammalian DNMT1 was reported to efficiently bind to the C-terminus (Margot *et al*., 2003). Subsequently, intramolecular interaction was examined by the yeast two-hybrid and pull-down assays. The inability of methylation was conceivably due to a tight intra-molecular interaction between the N- and C-terminal regions, thereby the catalytic domain residing on the C-terminus being completely masked.

Cellular localization analyses by GFP-fusion proteins using tobacco BY2 cells indicated that NtMET1 localized to the nucleus in the resting stage, migrating to the vicinity of chromatin during mitosis, particularly at metaphase. The observed pattern resembled that of Ran GTPase, and in vitro pull-down assays indeed showed a clear interaction between NtMET1 and AtRAN3, an Arabidopsis ortholog of tobacco Ran GTPase, NtRan-A1. These results suggest that NtMET1 becomes localized to the vicinity of chromatin with the aid of Ran GTPase during cell division, and may play an important role not only in maintenance of CpG methylation, but also in progress through mitosis by detaching from chromatin structure.

Overall, the present study revealed that enzymatic activity of NtMET1 is finely regulated by intramolecule interaction and combination with interacting other proteins through movement in the vicinity of chromatin during cell division.

REFERENCES

Aufsatz, W., Mette, M. F., Matzke, A. J. M., and Matzke, M. (2004). The role of MET1 in RNA-directed de novo and maintenance methylation of CG dinucleotides. Plant Molecular Biology 54, 793-804.

Bernacchia, G., Primo, A., Giorgetti, L., Pitto, L., and Cella, R. (1998). Carrot DNA-methyltransferase is encoded by two classes of genes with differing patterns of expression. The Plant Journal 13, 317-329.

Bestor, T., Laudano, A., Mattaliano, R., and Ingram, V. (1988). Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases. Journal of Molecular Biology 203, 971-983.

Bilbao-Cortes, D., Hetzer, M., Langst, G., Becker, P. B., and Mattaj, I. W. (2002). Ran binds to chromatin by two distinct mechanisms. Current Biology 12, 1151-1156.

Bird, A. P., and Wolffe, A. P. (1999). Methylation-induced repression--belts, braces, and chromatin. Cell 99, 451-454.

Bonnema, G., Hontelez, J., Verkerk, R., Zhang, Y. Q., van Daelen, R., van Kammen, A., and Zabel, P. (1996). An improved method of partially digesting plant megabase DNA suitable for YAC cloning: application to the construction of a 5.5 genome-equivalent YAC library of tomato. The Plant Journal 9, 125-133.

Boye, E., and Lobner-Olesen, A. (1990). The role of dam methyltransferase in the control of DNA replication in E. coli. Cell 62, 981-989.

Buryanov, Y. I., and Shevchuk T. V. (1995). DNA methyltransferases and structural-functional specificity of eukaryotic DNA modification. Biochemistry 70, 730-742

Chen, D. F., Liu, Q. A., Chen, X. W., Zhao, X. L., and Chen Y. W. (1991). The inhibition of restriction endonuclease PuvII cleavage activity by methylation outside its recognition sequence. Nucleic Acids Research 19, 5703-5705.

Clarke, P. R., and Zhang, C. (2001). Ran GTPase: a master regulator of nuclear structure and function during the eukaryoitic cell division cycle? Trends in Cell Biology 11, 366-371.

Dascher, C., and Balch, W. E. (1994). Dominant inhibitory mutants of ARF1 block endoplasmic reticulum to Golgi transport and trigger disassembly of the Golgi apparatus. Journal of Biological Chemistry 269, 1437-1448.

Ferguson-Smith, A. C., Surani, M. A. (2001). Imprinting and the epigenetic asymmetry between parental genomes. Science 293, 1086-1089.

Finnegan, E. J., and Dennis, E. S. (1993). Isolation and identification by sequence homology of a putative cytosine methyltransferase from Arabidopsis thaliana. Nucleic Acids Research 21, 2383–2388.

Finnegan, E. J., Genger, R. K., Peacock, W. J., and Dennis, E. S. (1998). DNA methylation in

plants. Annual Review of Plant Physiology and Plant Molecular Biology 49, 223-247.

Finnegan, E. J., Peacock, W. J., and Dennis, E. S. (1996). Reduced DNA methylation in Arabidopsis thaliana results in abnormal plant development. Proceedings of National Academy of Science USA 93, 8449-8454.

Finnegan, E. J., and Kovac, K. A. (2000). Plant DNA methytransferase. Plant Molecular Biology 43, 189-201.

Fuks, F., Burgers, W. A., Brehm, A., Hughes-Davies, L., and Kouzarides, T. (2000). DNA methyltransferase Dnmt1 associates with histone deacetylase activity. Nature Genetics 24, 88-91.

Glickman, J. F., Pavlovich, J. G., and Reich, N. O. (1997). Peptide mapping of the murine DNA methyltransferase reveals a major phosphorylation site and the start of translation. Journal of Biological Chemistry 272, 17851-17857.

Goll, M. G., and Bestor, T. H. (2005). Eukaryotic cytosine methyltransferases. Annual Review of Biochemistry 74, 481-514.

Gowher, H., and Jeltsch, A. (2001). Enzymatic properties of recombinant Dnmt3a DNA methyltransferase from mouse: the enzyme modifies DNA in a non-processive manner and also methylates non-CpG [correction of non-CpA] sites. Journal of Molecular Biology 309, 1201-1208.

Haizel, T., Merkle, T., Pay, A., Fejes, E., and Nagy, F. (1997). Characterization of proteins that

interact with the GTP-bound form of the regulatory GTPase Ran in Arabidopsis. Plant Journal 11, 93-103.

Heithoff, D. M., Sinsheimer, R. L., Low D. A., and Mahan M. J. (1999). An essential role for DNA adenine methylation in bacterial virulence. Science 5416, 967-970.

Hirochika, H. (1993). Activation of tobacco retrotransposons during tissue culture. EMBO Journal 12, 2521-2528.

Holliday, R., and Pugh, J. E. (1995). DNA modification mechanisms and gene activity during development. Science 187, 226-232.

Hotchkiss, R. D. (1948). The quantitative separation of purines, pyrimidines, and nucleosides by paper chromatography. Journal of Biological Chemistry 175, 315–332.

Hung, M. S., Karthikeyan, N., Huang, B., Koo, H. C., Kiger, J., and Shen, C. K. J. (1999). Drosophila proteins related to vertebrate DNA (5-cytosine) methyltransferases. Proceedings of National Academy of Science USA 96, 11940-11945.

Ito, M., Koike, A., Koizumi, N., and Sano, H. (2003). Methylated DNA-binding proteins from Arabidopsis. Plant Physiology 133, 1747-1754.

Jeltsch, A. (2002). Beyond Wastson and Crick: DNA methylation and molecular enzymology of DNA methyltransferases. ChemBioChem 3, 274-293.

Kahana, J. A., and Cleveland, D. W. (1999). Beyond nuclear transport: Ran-GTP as a determinant of spindle assembly. Journal of Cell Biology 146, 1205-1209.

Kakutani, T., Jeddeloh, J. A., Flowers, S. K., Munakata, K., and Richards, E. J. (1996). Developmental abnormalities and epimutations associated with DNA hypomethylation mutations. Proceedings of National Academy of Science USA 93, 12406-12411.

Kovarik, A., Koukalova, B., Holy, A., and Bezdek, M. (1994). Sequence-specific hypomethylation of the tobacco genome induced with dihydroxypropyladenine, ethinine and 5-azacytidine. FEBS Letters 353, 309-311.

Leonhardt, H., Page, A. W., Weier, H. U., and Bestor, T. H. (1992). A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. Cell 71, 865-873.

Margot, J. B., Ehrenhofer-Murray, A. E., and Leonhardt, H. (2003). Interactions within the mammalian DNA methyltransferase family. BMC Molecular Biology 4, 1-9.

Mertineit, C., Yoder, J. A., Taketo, T., Laird, D. W., Trasler, J. M., and Bestor, T. H. (1998). Sex-specific exons control DNA methyltransferase in mammalian germ cells. Development 125, 889-897.

Milutinovic, S., Brown, S. E., Zhuang, Q., and Szyf, M. (2004). DNA methyltransferase 1 knock down induces gene expression by a mechanism independent of DNA methylation and histone deacetylation. Journal of Biological Chemistry 279, 27915–27927.

Murray, M. G., and Thompson, W. F. (1980). Rapid isolation of high molecular weight plant DNA. Nucleic Acids Research 8, 4321-4325.

Nakano, Y., Steward, N., Sekine, M., Kusano, T., and Sano, H. (2000). A Tobacco NtMET1 cDNA Encoding a DNA Methyltransferase: Molecular Characterization and Abnormal Phenotypes of Transgenic Tobacco Plants. Plant and Cell Physiology 41, 448-457.

Nishiyama, R., Wada, Y., Mibu, M., Yamaguchi, Y., Shimogawara, K., and Sano, H. (2004). Role of a nonselective de novo DNA methyltransferase in maternal inheritance of chloroplast genes in the green alga, Chlamydomonas reinhardtii. Genetics 168, 809-916.

Noyer-Weidner, M., and Trautner, T. A. (1993). Methylation of DNA in prokaryotes. Experientia Supplementum 64, 38-108.

Okano, M., Bell, D. W., Haber, D. A., and Li, E. (1999). DNA methyltransferases Dnmt3b are essential for de novo methylation and mammalian development. Cell 99, 247-257.

Okano, M., Xie, S. P., and Li, E. (1998) Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransfeases. Nature Genetics 19, 219-220.

Pradhan, S., Cummings, M., Roberts, R. J., and Adams, R. L. P. (1998). Isolation, characterization and baculovirus-mediated expression of the cDNA encoding cytosine DNA methyltransferase from Pisum sativum. Nucleic Acids Research 26, 1214-1222.

Reisenauer, A., Quon, K., and Shapiro, L. (1999). Bacterial DNA Methylation: a Cell Cycle Regulator? Journal of Bacteriology. 181, 2430-2439.

Riggs, A. D. (1975). X inactivation, differentiation, and DNA methylation. Cytogenetics and Cell Genetics 14, 9-25.

Robertson, K. D., Ait, S. A., Yokochi, T., Wade, P. A., Jones, P. L., and Wolffe, A. P. (2000). DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. Nature Genetics 25, 338-42.

Ronemus, M. J., Galbiati, M., Ticker, C., Chen, J., and Dellaporta, S. L. (1996). Demethylation-induced developmental pleiotropy in Arabidopsis. Science 273, 654-657.

Rountree, M. R., Bachman, K. E., and Baylin, S. B. (2000). DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. Nature Genetics 5, 269-277.

Seo, S., Okamoto, M., Seto, H., Ishizuka, K., Sano, H., and Ohashi, Y. (1995). Tobacco MAP Kinase: A Possible Mediator in Wound Signal Transduction Pathways. Science 270, 1988-1992.

Steward, N., Ito, M., Yamaguchi, Y., Koizumi, N., and Sano, H. (2002). Preiodic DNA methylation in maize nucleosomes and demethylation by environmental stress. Journal of Biological Chemistry 277, 37741-37746.

Steward, N., Kusano, T., and Sano, H. (2000). Expression of ZmMET1, a gene encoding a DNA methyltransferase from maize, is associated not only with DNA replication in actively proliferating cells, but also with altered DNA methylation status in cold-stressed quiescent cells. Nucleic Acids Research 28, 3250-3259.

Theiss, G., Schleicher, R., Schimpff-Weiland, R., and Follmann, H. (1987). DNA methylation in wheat. Purification and properties of DNA methyltransferase. European Journal of

Biochemistry 167, 89-96.

Tompa, R., MaCallum, C. M., Delrow, J., Henikoff, J. G., van Steensel, B., and Henikoff, S. (2002). Genome-wide profiling of DNA methylation reveals transposon targets of CHROMOMETHYLASE3. Current Biology 12, 65-68.

Verwoerd, T. C., Dekker, B. M., and Hoekema, A. (1989). A small-scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Research 17, 2362.

Wada, Y. (2005). Physiological functions of plant DNA methyltransferases. Plant Biotechnology 22, 71-80.

Wada, Y., Miyamoto, K., Kusano, T., and Sano, H. (2004). Association between up-regulation of stress-responsive genes and hypomethylation of genomic DNA in tobacco plants. Molecular Genetics and Genomics 271, 658-666.

Wada, Y., Ohya, H., Yamaguchi, Y., Koizumi, N., and Sano, H. (2003). Preferential de novo methylation of cytosine residues in non-CpG sequences by a domains rearranged DNA methyltransferase from tobacco plants. Journal of Biological Chemistry 278, 42386-42393.

Yamaguchi, Y., Yoda, H., Akiyama, K., and Sano, H. (2003). Induction of transcripts encoding a novel transmembrane protein during the hypersensitive response upon tobacco mosaic virus infection in tobacco plants. Planta 218, 249-253.

Yano, A., Kodama, Y., Koike, A., Shinya, T., Kim, H. J., Matsumoto, M., Ogita, S., Wada, Y.,

Ohad, N., and Sano, H. (2006). Interaction between methylated-DNA binding protein and Ran GTPase during cell division in tobacco cultured cells. Annals of Botany 98, 1179-1187.

Yap, Y. K., Kodama, Y., Waller, F., Chung, K. M., Ueda, H., Nakamura, K., Oldsen, M., Yoda, H., Yamaguchi, Y., and Sano, H. (2005). Activation of a novel transcription factor through phosphorylation by WIPK, a wound-induced mitogen-activated protein kinase in tobacco plants. Plant Physiology 139,127-137.

Yoder, J. A., Soman, N. S., Verdine G. L., and Bestor T. H. (1997a). DNA (cytosine-5)-methyltransferases in mouse cells and tissues. Studies with a mechanism-based probe. Journal of Molecular Biology 270, 385-395.

Yoder, J. A., Walsh, C. P., and Bestor, T. H. (1997b). Cytosine methylation and the ecology of intragenomic parasites. Trends in Genetics 13, 335-340.

Zheng, Y. (2004). G Protein Control of Microtubule Assembly. Annual Review of Cell and Developmental Biology 20, 867-894.

PABLICATION LIST

Kim, H. J., Yano, A., Wada, Y., and Sano, H. (2006). Properties of a tobacco DNA methyltransferase, NtMET1 and its involvement in chromatin movement during cell division. *Annals of Botany* (in press).

Yano, A., Kodama, Y., Koike, A., Shinya, T., Kim, H. J., Matsumoto, M., Ogita, S., Wada, Y., Ohad, N., and Sano H. (2006). Interaction between methylated-DNA binding protein and Ran GTPase during cell division in tobacco cultured cells. *Annals of Botany* 98, 1179-1187.

ACKNOWLEDGEMENTS

First, I would like to express my greatest gratitude for my advisor, Prof. Hiroshi Sano, who instructed me patiently and encouraged heartfully throughout my study. I would also like to give my thanks to Profs. Ill-Sup Nou (Sunchon National University) and Kwon-Kyoo Kang (Hankyong National University) for continuous encouragement that enable me to carry out study in Japan. I wish to thank Drs. Nozomu Koizumi, Yuko Wada and Yun-Soo Kim (NAIST) for their helpful advice, and all other member of the Laboratory of Plant Molecular Breeding (Nara Institute of Science and Technology) for their useful discussion and an enjoyable laboratory life. I am greatly in debt to the Japanese government for its financial support. Finally, I would like to dedicate this work to my family and partner for their support and encouragement throughout my study.