バイオサイエンス研究科　博士論文要旨

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要旨

In higher organisms, cytosine methylation is essential for transcriptional silencing by condensing chromatin structure. Methylated cytosines are frequently observed in retroelements and in CpG islands within a promoter region of particular genes. In plants, cytosine methylation is also important for development and for repression of transposable elements. Genetic analyses suggested that pattern of methylation is created by '*de novo*' DNA methyltransferases and is maintained by 'maintenance' DNA methyltransferases. However, their enzymatic activities and physiological functions remain to be determined.

The first chapter of this study discusses physiological meaning of maintenance DNA methylation in tobacco plants. The global methylation pattern is maintained through generations by the type I methyltransferase (MET1), which is homologous to mammalian maintenance methyltransferase (Dnmt1). In order to identify genes that are regulated by DNA methylation, the differential display was performed using transgenic tobacco plants expressing an anti-sense construct for tobacco MET1, *NtMET1*. Among 31 identified genes, transcripts of a pathogen responding gene (*NtAlix1)* were found to be accumulated. The methylation status of the genomic locus was obviously changed at CCG sites upon tobacco mosaic virus infection in inoculated leaves of wild-type plants.

The results suggest that the level of DNA methylation at CCG sites changes in response to external stresses, and that this is closely related to activation of stress responsive genes.

In chapter II, I characterized a domains-rearranged methyltransferase (DRM), whose enzymatic activity has not been determined so far, although sequence analysis suggested it to be responsible for *de novo* methylation. Using GFP fusion technique, I found that the protein localized exclusively in the nucleus. Upon expression in insect cell, Sf9, it exhibited methylation activity towards unmethylated DNA samples. Methylation mapping directly demonstrated preferential methylation toward cytosines in CpNpN and CpNpG sequences (N is A, T, or C), but apparently excluded CpG. Transcripts of *NtDRM1* ubiquitously accumulated in all tissues and during the cell cycle in tobacco cultured BY2 cells. These results indicated that NtDRM1 is a *de novo* non-CpG methyltransferase, which is the first clear example of enzyme involved in asymmetric cytosine methylation.

In chapter III, I examined the molecular function of *in vivo* methylation by NtDRM1. To this end, I isolated an NtDRM1 complex from BY-2 cells, and screened for NtDRM1 interacting factors.

Results of the current study will provide new insights into physiological function of maintenance and *de novo* DNA methylation in higher plants.

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Biochemical Properties and Physiological Function of DNA Methyltransferases from Tobacco Plants

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TABLES OF CONTENT

INTRODUCTION

The presence of 5-methylcytosine (m^5C) as a minor base in DNA was established as early as 1951 (Wyatt 1951). The ratio of m^5C to the total bases varies among organisms, ranging between less than 0.25% in bacteria up to 7% in plants (Hall 1971), and its physiological functions have long been a matter of debate. In bacteria, m^5C and 6-methyladenine have been proposed to serve as negative markers for DNase targeting, protecting the host DNA from degradation (Kuhnlein and Arber 1972). Their generation for this system, referred as restriction-modification, has been shown to be catalyzed by DNA methyltransferases in combination with corresponding restriction endonucleases in many bacteria, including *Escherichia coli* (Smith and Kelly 1984).

In the case of eukaryotes, m^5C occurs in retroelements, and locates in the CpG islands within a promoter region of many genes (reviewed in Bird 1986). This is essential for transcriptional silencing and condensed inactive chromatin structure. In mammals, it is also associated with development of cancer (reviewed in Jones and Baylin 2002), X-chromosome inactivation (Panning and Jaenisch 1996), tissue specific gene expression (Futscher et al. 2002), and heterochromatin formation (Urnov and Wolffe 2001).

A DNA modification mechanism was proposed by Holliday and Pugh (Holliday and Pugh, 1975), and Riggs (Riggs 1975). Patterns of methylated bases were proposed to be heritable, assuming that once established by a *de novo* DNA methyltransferase activity, methylation

could be faithfully maintained by a maintenance DNA methyltransferase activity which recognize newly replicated, hemimethylated DNA, a DNA duplex methylated in one strand but unmethylated in the other. In this proposal, two different activities of methyltransferase were predicted. Recent progress in analyses of DNA methylation research has provided much of evidence for this model. The CpG doublets, which is methylated to a high level in mammalian cells, are frequently seen in retroelements and within a promoter region of particular genes, which are called CpG islands. The CpG doublet is symmetrical, being often methylated in both strands.

Mammalian DNA methyltransferase 1 (Dnmt1) is the first eukaryotic cytosine methyltransferase characterized, which relates to bacterial type-II cytosine restriction methyltransferases (Bestor et al. 1988). Dnmt1 displays a strong preference for hemimethylated DNA with a 30-fold higher activity than for unmethylated DNA *in vitro* (Yoder and Soman et al. 1997). With this specificity, Dnmt1 completes half-methylated sites after semiconservative DNA replication, restoring symmetry of methylated cytosines, and is referred as maintenance methyltransferase.

Ten years later of the discovery of maintenance methyltransferase, another type of DNA methyltransferase, which methylates unmethylated DNA, was discovered. This was referred as *de novo* methyltransferases, 3a (Dnmt3a) and 3b (Dnmt3b) (Okano et al. 1998). Mutants of Dnmt3a or Dnmt3b failed to establish methylation pattern during embryogenesis and showed impaired development (Okano et al. 1999). Mammalian cells exhibit cell type specific DNA

methylation patterns, being established by *de novo* and maintenance DNA methyltransferase activities during every DNA replication. However, patterns of methylation are completely erased during gametogenesis, allowing a creation of new patterns during early developmental stage in progeny.

Epigenetic inheritance is defined as a heritable change in gene function, that cannot be explained by changes in DNA sequence (Russo et al. 1996). The mechanism of epigenetic regulation of gene expression has been intensively investigated in a last decade, and cytosine methylation is now generally accepted to play a critical role in it. m^5C is considered as the hallmark for transcriptional inactivation being bound by methyl-CpG-binding domain protein (MBDs) complex. MBDs function in recruiting various protein complexes that consists of histone deacetylase and ATP-dependent chromatin remodeling factors. Deacetylation of histone N-terminus leads to condensation of chromatin and transcriptional repression. It was reported that histone methylation is essential for DNA methylation in *Neurospora crassa* (Tamaru and Selker 2001), and a similar relationship between histone methylation and DNA methylation was found in mammals (Lehnertzet al. 2003) and *Arabidopsis* (Jackson et al. 2002).

The level of plant DNA methylation is generally higher than in mammals. The location of m^5C also differs from mammals, found not only in CpG dinucleotides, but also in CpNpG trinucleotides and other sequence contexts. Both CpG and CpNpG sequences are symmetric, whose methylation is transmitted through meiosis by the action of maintenance

methyltransferase. Methylation of nonsymmetrical cytosines was demonstrated to be associated with inactivated transgenes (Meyer and Heidmann 1994), but its role in plants is not well understood.

Subsequently, genetic and biochemical studies have been intensively performed to clarify mechanism and physiological significance of DNA methylation in plants. In mammals, in which DNA methyltransferase is silenced, showed embryonic lethality (Okano et al. 1999). The mutants of higher plants, in which DNA methylation drastically decreased due to defects of DNA methyltransferase and chromatin remodeling factors, exhibit clear morphological abnormalities, suggesting that DNA methylation is essential for plant development (Vongs et al. 1993, Finnegan et al. 1996, Cao and Jacobsen 2002a, Nakano et al. 2000, Saze et al. 2003). DNA methylation has also been proven to be essential for inactivation of transposable elements (Miura et al. 2001). Methylated cytosines are often observed in inactivated transgenes (Wassenegger 2000) and epigenetically silenced endogenes (Jacobsen and Meyerowitz 1997).

Plants possesses genes encoding three different types of DNA methyltransferases. Methyltransferase1 (MET1) is homologous to mammalian Dnmt1 (Finnegan and Dennis 1993), which introduces methyl groups specifically into cytosines in CpG sequences (Finnegan and Kovac 2000). Proteins of chromomethylase (CMT) group have a chromodomain in catalytic motifs which is thought to be a chromatin interaction domain and/or an RNA binding motif. Domains rearranged methyltransferase (DRM) targets

non-CpG (Wada et al. 2003). Most characteristic feature of DRMs is the rearrangement in catalytic motifs between I to VI and V to X. Genetic analyses using *Arabidopsis* mutants for DNA methylation proposed at least two methylation pathways: CpG methylation and non-CpG methylation. Chromatin remodeling factor DDM1 is thought to be responsible for both pathways. MET1 is needed for CpG methylation. Histone methyltransferase KRYPTONITE (KYP) and DNA methyltransferase CMT3 are predicted to be involved in non-CpG methylation (Jackson et al. 2002).

In chapter I of this thesis, I described that a maintenance DNA methyltransferase gene from tobacco plants (*NtMET1*) was suppressed by introducing the antisense construct, which resulted in ectopic gene transcriptions. Transcriptionally activated genes in *NtMET1* antisense plants were identified and methylation levels of those loci were analyzed. Physiological role of maintaining DNA methylation for regulating gene expression is discussed. In chapter II, I characterized enzymatic property of a tobacco DRM (NtDRM1). The results showed that NtDRM1 apparently non-selectively methylates any cytosines except for that in CpG, indicating that NtDRM1 is a *de novo* asymmetric cytosine methyltransferase. In chapter III, I initiated examination on the molecular function of *in vivo* methylation by NtDRM1. To this end, I isolated an NtDRM1 complex from BY-2 cells, and screened for NtDRM1-interacting factors.

CHAPTER I

Association between Up-Regulation of Stress-Responsive Genes and Hypomethylation of Genomic DNA in Tobacco Plants

Introduction

The physiological function of m^5C has long been a matter of debate, and a recent survey established that it plays a critical role in gene expression and epigenetic control (Bird 2002). In plants, methylation of symmetric CpG is considered to be related to control of gene expression, as patterns are faithfully maintained through generations. Methylation of CpNpG and CpNpN may respectively function in transposon inactivation and in epigenetic silencing (Cao and Jacobsen 2002). This speculation has partly been substantiated by transgenic analyses, showing MET1 suppression to result in a reduction of global CpG methylation and abnormal phenotypes in *Arabidopsis* and tobacco plants (Finnegan et al. 1996, Ronemus et al. 1996, Nakano et al. 2000).

Plants have evolved a set of defenses to cope with severe environmental stresses. For example, several hundred genes are predicted to be activated in response to cold stress (Thomashow 1998). However, the molecular switch system for their concerted regulation is currently not known. In our previous study, we found that cold treatment of maize seedlings resulted in a global demethylation of root genomic DNA, particularly in nucleosome core regions (Steward et al. 2002). Based on this finding, we proposed that the DNA methylation

level dynamically changes in response to environmental stimuli, and that this is one of factors that simultaneously regulate many genes involved in plant defense (Steward et al. 2002).

In the present study, we screened genes whose expression is specifically affected by DNA methylation by the differential display method, using transgenic tobacco plants in which DNA methylation levels were suppressed by expression of anti-sense *NtMET1*. The predicted functions of more than half of the identified gene products were found to be related to stress responses. We also established that pathogen attack simultaneously induced demethylation of a particular gene and its transcripts. These observations support the idea that DNA methylation changes upon exposure to environmental stress, and that it may globally regulate expression of stress responsive genes.

Materials and Methods

Plant Materials and TMV Treatment

Transgenic tobacco plants (*Nicotiana tabacum* cv. Xanthi nc) expressing anti-sense *NtMET1* were constructed as described earlier (Nakano et al. 2000), and appropriate lines were selected and used. Both wild-type (*N. tabacum* cv. Xanthi nc) and transgenic plants were grown in a growth cabinet at 23^{o}C under a 14 h/10 h light/dark photo cycle. For pathogen response analysis, about 8-week-old mature leaves from wild-type plants were detached, inoculated with or without tobacco mosaic virus (TMV) (10 μg/ml) in phosphate buffer (pH 7.2) using carborundum (Mesh 600), incubated at 30^{o}C under continuous light for 48 h, and then

at 20°C, allowing plants to initiate the hyper-sensitive response (HR) by recognizing TMV under continuous light. Leaves were harvested after appropriate time intervals, immediately frozen in liquid nitrogen and stored at -80°C until used for extraction of RNA and DNA.

Differential Display and cDNA Cloning

The simplified differential display method was carried out as described (Yoshida et al. 1994) with modification. Total RNA was isolated from sample leaves by the acid guanidium thiocyanate-phenol-chloroform (AGPC) method (Chomczynski and Sacci 1987), and cDNA was synthesized as described (Yoda et al. 2002). Eighty independent PCRs were performed using an arbitrary 10-mer primer (one of OPA1-OPA80, Operon) for each sample applying 40 cycles of 92°C 1 min, 35°C 1 min and 72°C 2 min with additional extension at 72°C for 5 min. Products (45 μl out of 50 μl) were fractionated on 1% agarose gels, and appropriate fragments were isolated, cloned into pGEM T-Easy vector (Promega) and subjected to DNA dot hybridization for confirmation. In brief, the residual PCR products (5 μl each) and aliquots of PCR products amplified with the same 10-mer primer from randomly selected colonies were spotted onto nylon membrane filters (Hybond N, Amersham-Pharmacia Biotech) and hybridized with one of the cloned fragments. This method efficiently removed false clones, and results were consistent with those obtained using northern hybridization.

RNA Blotting and RT-PCR Analyses

Total RNA was isolated and used for RNA gel blot analysis as previously described (Yoda et al. 2002). RT-PCR was performed with gene specific primers; *HMA7*, 5'-CTACGCCTAAA AAGAACGGAGGATGAG-3' and 5'-GATAGAGAGACCAGATTTCTGCAGTTAC-3'; *HMA9*, 5'-GAAAGACTTATGTTGGCTCAGGC-3' and 5'-ACTGAGACCTGAGATT TGCCTCTG-3'; *HMA15*, 5'-GATGCTTATCGAGACTTGCCACGC-3' and 5'-CAACAG CTTCATGTGAAGAGC-3'. PCR was carried out under the condition of a 35-cycle of denaturation at 94ºC for 30 sec, annealing at 55ºC for 30 sec, and extension at 72ºC for 1 min. After fractionation by agarose gel electrophoresis, products were visualized by ethidium bromide staining.

DNA Methylation Analysis with Restriction Enzymes

DNA methylation was analyzed by Southern hybridization employing methylation sensitive restriction enzymes. Genomic DNA was extracted from fresh leaves of transgenic and stress-exposed plants by the cetyl-trimethyl ammonium bromide method (Murray and Thompson 1980). A 20 μg-aliquot was digested with *Hap*II, *Msp*I (Takara, Japan) and digestion products were fractionated by agarose gel electrophoresis. Samples were then transferred to nylon membranes (Hybond N^+, Amersham-Pharmacia Biotech) and subjected to hybridization using appropriate probe DNA. The *NtAlix1* probe was generated by PCR using gene specific primer sets (forward, 5'-GAAAGACTTATGTTGGCTCAGGCGCAGG-3' and reverse,

5'-AACTGAGACCTGAGATTTGCCTCTGTACCTCTTC-3'). Probe DNAs were radioactively labeled using [α-^{32}P]dCTP and Bcabest labeling kit (Takara). After hybridization at 65ºC for 16 h, filters were washed in 0.5 x SSC containing 0.1% SDS at 65ºC for 45 min, and then washed in 0.1 x SSC containing 0.1% SDS for 45 min as described (Steward et al. 2002).

Results

Screening of Hypomethylation-Associated cDNAs

To isolate genes that are specifically expressed under hypomethylated conditions, we used transgenic tobacco plants, in which DNA methylation was suppressed by anti-sense *NtMET1*. The affected plants showed a clear dwarf phenotype (Figure 1A) consistent with our previous observations (Nakano et al. 2000). In order to obtain appropriate starting materials, we initially examined more than twenty independent transgenic lines for their global methylation status by Southern hybridization assay, using methylation-sensitive restriction enzymes and an anonymous cDNA clone as the probe. Subsequently we selected six lines, among which three (#33, #49 and #83) showed distinct hypomethylation resulting in several restricted fragments after digestion with *Hap*II, which cleaves only unmethylated CCGG sites (Figure 1B). In contrast, methylation of the other three (#13, #20 and #34) was apparently the same as in the wild-type control, showing resistance to *Hpa*II (Figure 1B). These results are consistent with our previous findings (Nakano et al. 2000), and suggest that the antisense construct was effective in the former three lines, but inactive in the others.

Using these lines together with wild-type, we performed differential display-PCR with arbitrary primers. Transcripts that were commonly expressed in three hypomethylated lines, but not in three normally methylated lines were regarded as hypomethylation-specific genes. After confirmation with co-migration (Hara et al. 2000) and dot hybridization tests, 31 clones were finally identified. Sequences of all clones were determined, and in some cases, full length cDNAs were isolated by screening 2 x 10^5 plaques of a cDNA library constructed from mRNA of TMV-infected tobacco plants (Yoda et al. 2002). A homology search indicated that 16 clones encoded putative proteins with known function, and these were tentatively designated as *HMA* (hypomethylation associated) with serial numbers (Table 1). The remaining 15 also encoded putative proteins, but as their function could not be assigned, we do not refer to them in this article. The transcript levels of several selected genes were further examined by RNA blot hybridization analysis or by RT-PCR, and confirmed to be differentially regulated in hypomethylated lines (Figure 2).

Identity of Isolated Genes

Among the 16 genes with known functions, 10 were found to be related to stress responses, and 6 to other cellular functions (Table 1). The putative identities of the stress responsive genes are as follows; *HMA1* encodes a polypeptide showing a similarity with glutathione *S*-transferase, which is involved in detoxification of xenobiotics; *HMA2* and *HMA5* encode cellulose synthase and extension, respectively, functioning in cell wall repair; *HMA3,*

HMA4 and *HMA12* encode proteins induced by abiotic stress due to aluminium, salt and dehydration, respectively; *HMA7* and *HMA8* are associated with disease resistance, encoding proteins related to a leusine-rich repeat (LRR) protein and a tomato disease resistant protein (Prf), respectively; *HMA9* encodes a protein similar to ALG-2 interacting protein X (Alix), which functions in apoptosis (Chatellard-Causse et al 2002). The genes involved in fundamental cellular function were: *HMA13*, encoding a proton pump component; *HMA11* and *HMA16* encoding proteins involved in cell division; *HMA17* encoding clathrin, a fundamental protein in intracellular transportation; and *HMA15* encoding a proteasome-related protein.

Among the identified genes, *HMA9* drew our particular interest, because its encoded polypeptide, showing a similarity to Alix, is a first such example in plants. Subsequently we isolated a full length clone and designated this as *NtAlix1* (Figure 3A). Its characterization will be described elsewhere, and we simply used this clone as the marker in the current study. To determine hypomethylation of genomic loci of identified genes in transgenic plants, restriction analyses were performed using *NtAlix1* as the representative probe (Figure 3B). *Hap*II yielded several distinct fragments in line #49, nearly identical fragments with those generated by *Msp*I in line #83. The results indicated clear hypomethylation in lines expressing *NtAlix1* but not in non-expressing lines.

Expression of NtAlix1 upon Pathogen Infection

Results obtained from transgenic plants suggested that many stress responsive genes can

be up-regulated by demethylation. In order to determine whether this occurs under natural stress conditions, we analyzed methylation status and expression profile of *NtAlix1*, because of its possible relationship to programmed cell death during pathogen attack, one of the severest stresses plants suffer. Experimentally, wild-type tobacco plants were inoculated with TMV, maintained at 30^{o}C for 2 days, during which the host does not recognize the pathogen due to incapacitation of the *N*-gene (resistance gene), resulting in pathogen propagation (Yoda et al. 2002). When inoculated samples were transferred to 20^{o}C, the *N*-gene was activated and resistance took place, showing necrosis around the infected region 24 h after temperature shift (Figure 4A). This is referred to as the hypersensitive response (HR). Transcripts of *NtAlix1* began to accumulate within 6 h of temperature shift and reached maximal levels after 12 h (Figure 4B), then maintained up to 24 h. As mock treatment did not induce the transcripts, the response is apparently due to the HR.

Demethylation of NtAlix1 locus

Methylation of the *NtAlix1* locus was then examined by restriction assay using *Msp*I and *Hap*II, and the resulting hybridization profile was densitometrically quantified (Figure 5). These patterns, suggesting high and partial methylation at Cm^{5}CGG and m^{5}CCGG sites, respectively, were maintained throughout the treatment period. In TMV infected leaves, the digestion pattern by *Hap*II was the same as in the control for the initial 12 h after temperature shift. However, a 6-kb fragment was clearly produced at 24 h, indicating one or two Cm^{5}CGG

sites to be demethylated (Figure 5A). Methylation at m^5CCGG also changed, as demonstrated by different migration profiles (Figure 5B). For example, two small *Msp*I fragments (1.6 kb and 2.3 kb) disappeared 24 h after the onset of HR, suggesting these sites to have acquired resistance to the enzyme, or be remethylated. Similarly a 5-kb fragment disappeared while a new fragment of 7-kb appeared and the amount of the 3.5-kb fragment increased (Figure 5B). It is difficult to judge from these patterns whether the relevant sites were demethylated or re-methylated, but it is clear that a dynamic change in DNA methylation occurred during the HR. Notably, changed status in methylation at 24 h appeared to be restored, with gradual recovery of the initial methylation pattern over 96 h (Figure 5A, B).

Discussion

DNA methylation has long been considered to function in control of gene expression. Indeed, physiological abnormalities due to abnormal gene expression have been shown to be frequently associated with the methylation status of the genes in question (Bird 2002). For example, chromosomal DNA of cancer cells was found to be locally hypermethylated but globally hypomethylated (Ehrlich 2000). Genomic imprinting is another case in which appropriate differential methylation in germ cells from each parent is prerequisite for normal development (Ferguson-Smith and Surani 2001).

Despite this importance, information on the number and classes of genes that are regulated in concert by DNA methylation are limited. In cultured mouse cells in which Dnmt1 was in-

hibited by an antisense construct, approximately 10% of all genes were shown to be transcriptionally activated by DNA microarray analysis (Jackson-Grusby et al. 2001). Affected genes included examples related to apoptosis, imprinting, cell-cycle control, growth and mobilization of retroelements (Jackson-Grusby et al. 2001). In plants, hypomethylation caused by antisense processing (Finnegan et al. 1996; Ronemus et al. 1996; Nakano et al. 2000), mutations (Kankel et al. 2003; Miura et al. 2001) and gene destruction (Saze et al. 2003) result in abnormalities in development, growth and phenotype. However, identities of the responsible genes have mostly not been determined.

In the present study, we randomly screened genes whose transcripts were accumulated in hypomethylated tobacco plants and found more than 30, among which 16 could be assigned a function. Ten of them were directly related to stress responses and the remainder to other cellular functions. These findings are essentially consistent with those found earlier in a mammalian system (Jackson-Grusby et al. 2001), and strongly suggest a critical role of DNA methylation in controlling gene expression during development and in response to stress.

The question then arises whether or not the DNA methylation status changes upon exposure to external stimuli or environmental stresses. In order to address this question, we took advantage of the tobacco-TMV interacting system, in which the induction of necrosis and pathogen-related gene expression is synchronously controlled. Subsequent analysis using *NtAlix1*, found to be expressed in hypomethylated plants, provided clear evidence that methylation indeed changed and transcripts were induced after virus infection. However, time

course analysis showed that, while transcripts were induced 6 h after the onset of HR, methylation change was only detectable after 24 h. This time gap does not necessarily indicate that change of methylation occurred after the initiation of transcription. Since we analyzed methylation only at CCGG sites, it is possible that we did not detect global changes that occurred at many other sites. Judging from our previous results of direct methylation mapping, which showed plant DNA to be highly methylated at cytosines located not only in symmetrical sequences such as CpG and CpNpG, but also in asymmetrical CpNpN sites (Steward et al. 2002; Wada et al. 2003), it is conceivable that changes in methylation status in TMV-infected leaves might occur much earlier than 24 h during the HR.

Restriction analysis suggested that demethylation occurred at CpG and CpCpG (CpNpG) sites. In addition, some CpCpG (CpNpG) sites appeared to be remethylated. This is consistent with the observation that, in MET1-suppressed plants, methylation at CpNpG in *SUPERMAN* locus is increased despite a global decrease in CpG sites (Finnegan et al. 1996). The molecular mechanism of demethylation is not well understood. Recent studies on *Arabidopsis* suggested DNA glycosylase to be one of the responsible enzymes, with participation in gene silencing by demethylating target genes (Gong et al. 2002). Remethylation is catalyzed by DNA methyltransferases, MET1 for CpG and DRM (and possibly CMT) for CpNpN (Wada et al. 2003). The observed differential methylation at the *NtAlix1* locus may thus reflect crosstalk among these enzymes, whereby fine tuning of the state of global methylation is achieved.

The other question to be answered is whether or not the stress-induced change in DNA

methylation correlates with, or triggers off transcriptional activity. In the present study, we demonstrated that *NtAlix1* is only expressed in hypomethylated plants, and that its methylation status changed upon pathogen attack with its concomitant expression. In our previous work, we showed that a genome-wide demethylation of genomic DNA in maize seedlings occurs upon chilling, with simultaneous activation of a particular gene, *ZmMI1* (Steward et al. 2002). Taken together, our observations strongly suggested that demethylation due to environmental stress triggers activation of many genes involved in resistance. However, further direct evidence is necessary to prove this theory, for example using transgenic plants expressing all three methyltransferases, thereby preventing any type of demethylation.

Table 1 Clones identified by differential display analysis

Function	Clone	Size of fragment (bp)	Accession	Best match	Species	Accession
Stress response	HMA1	451	AB120516	Glutathione S-transferase	*N. tabacum*	Q03662
	HMA2	1200	AB120517, AB120518	Cellulose synthase	*A. thaliana*	BAB10307
	HMA3	1100	AB120519, AB120520	Aluminium- tolerance associated- like protein	*A. thaliana*	NM125190
	HMA4	463	AB120521	Salt- inducible protein	*A. thaliana*	AAB60736
	HMA5	439	AB120522	Extensin	*A. thaliana*	T14192
	HMA6	1100	AB120523	Retroelement reverse transcriptase	*O. sativa*	BAB03416
	HMA7	2200	AB120524	NBS-LRR resistance protein RGH1	*M. esculenta*	AY188523
	HMA8	1100	AB120525	Prf (disease resistant protein)	*L. esculentum*	AAF76312
	HMA9	2100	AB120526	ALG-2 interacting protein X	*A. thaliana*	AC007591
	HMA12	910	AB120529	Dehydration-induced protein	*A. thaliana*	NM174468
Cellular function	HMA10	1200	AB120527	Putative DEAD/DEAH box helicase	*A. thaliana*	NP177830
	HMA11	317	AB120528	Cell division-linked gene	*A. thaliana*	AAF00659
	HMA13	1250	AB120530, AB120531	Plasma membrane ATPase 4	*N. plumbaginifolia*	Q03194
	HMA14	1085	AB120534	Proteasome- related	*A. thaliana*	AAF22524
	HMA15	1500	AB120535	CDK5-activator binding protein	*A. thaliana*	Z99708
	HMA16	1800	AB120536	Putative Clathrin heavy chain	*A. thaliana*	AAF01510

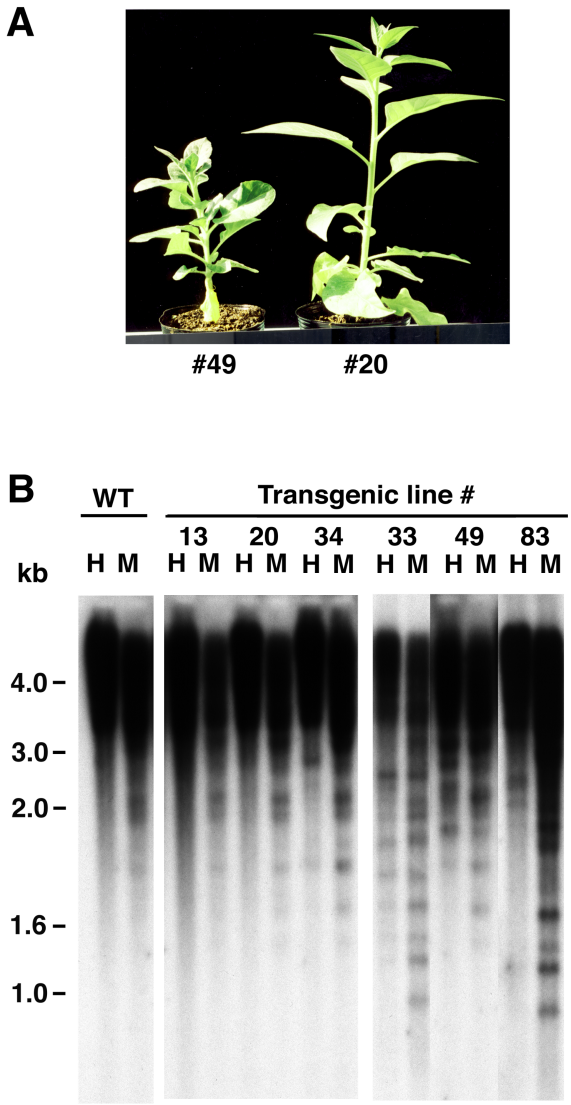

Figure 1. Transgenic tobacco plants transformed with antisense *NtMET1*. A Phenotype of 2-month old mature plants. Line #20 (right) is apparently normal while line #49 (left) is clearly dwarf. **B** Southern hybridization with Tobacco retrotransposon Tto1. A 20 μg of total DNA from the indicated plant was digested with methylation sensitive restriction enzymes, *Hap*II (H) or *Msp*I (M) and subjected to Southern hybridization analysis. Lines #20 and #34 show the same resistant pattern to *Hap*II as the wild-type control, whereas lines #49 and #83 appear sensitive to the enzyme demonstrating more restriction fragments as with *Msp*I.

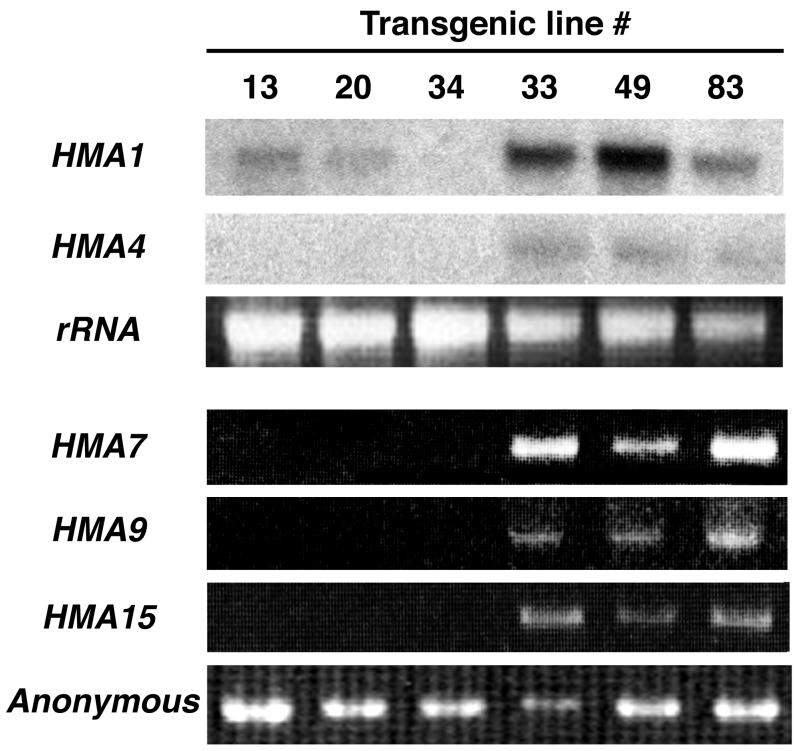

Figure 2. Differential expression of *HMA* genes among transgenic lines. Accumulation of transcripts of indicated genes was estimated by northern hybridization and RT-PCR as described in the text. For the internal standard for northern and RT-PCR assays, rRNA and an anonymous clone (*G17*) were used, respectively.

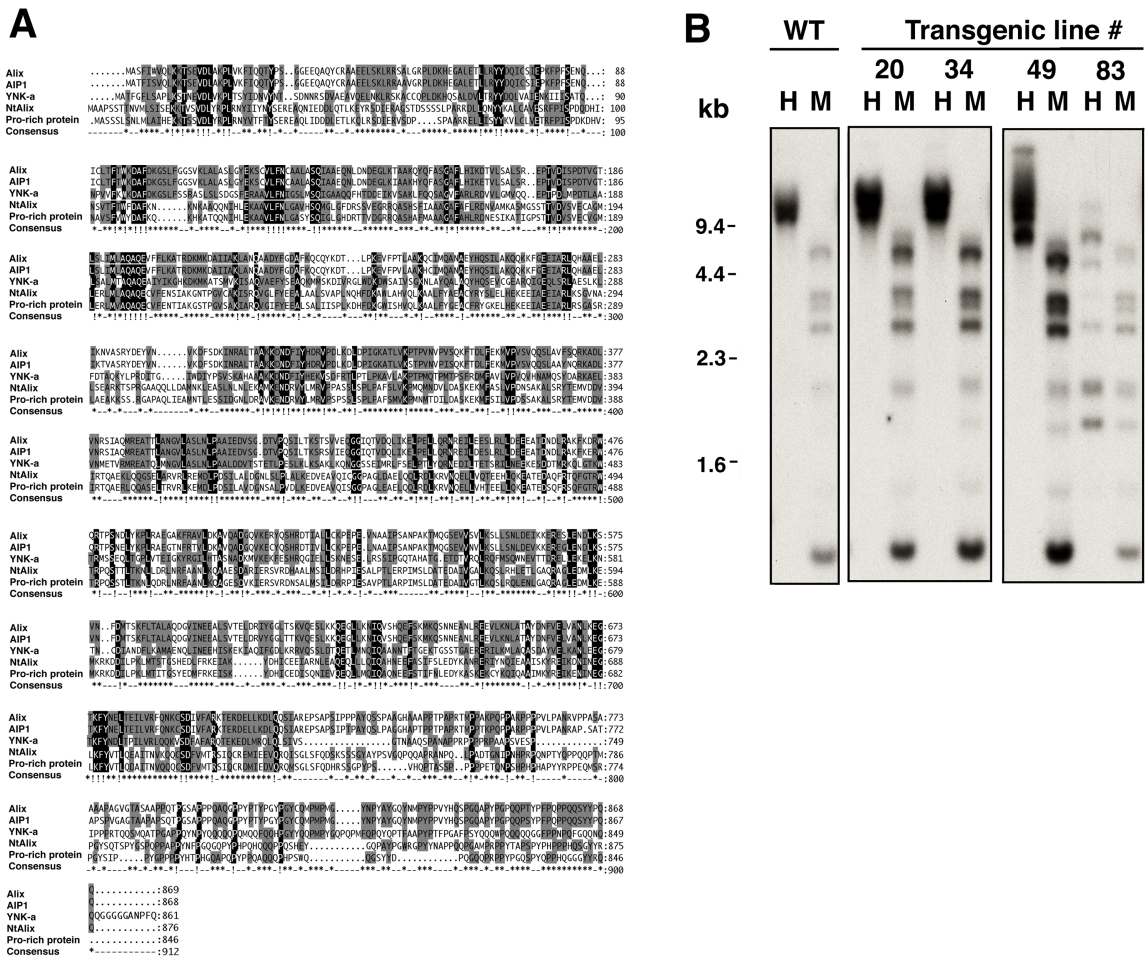

Figure 3. Identification of *NtAlix1* and its methylation status. A Amino acid sequence and alignment to other proteins. Genes, products and accession numbers are as following: Alix, mouse ALG-2 interacting protein X (AJ005073); AIP1, human ALG-2 interacting protein 1 (AF151793); YNK-a, *C.elegance* A putative element in signal transduction (U73679); NtAlix1, *Nicotiana tabacum* Alix homology protein 1; Pro-rich protein, *Arabidopsis thaliana* Proline-rich protein (BT005821). **B** Southern hybridization. A 20 μg DNA from the indicated plant was digested with *Msp*I (M) or *Hap*II (H), fractionated on agarose gel electrophoresis and subjected to Southern hybridization using *NtAlix1* as the probe.

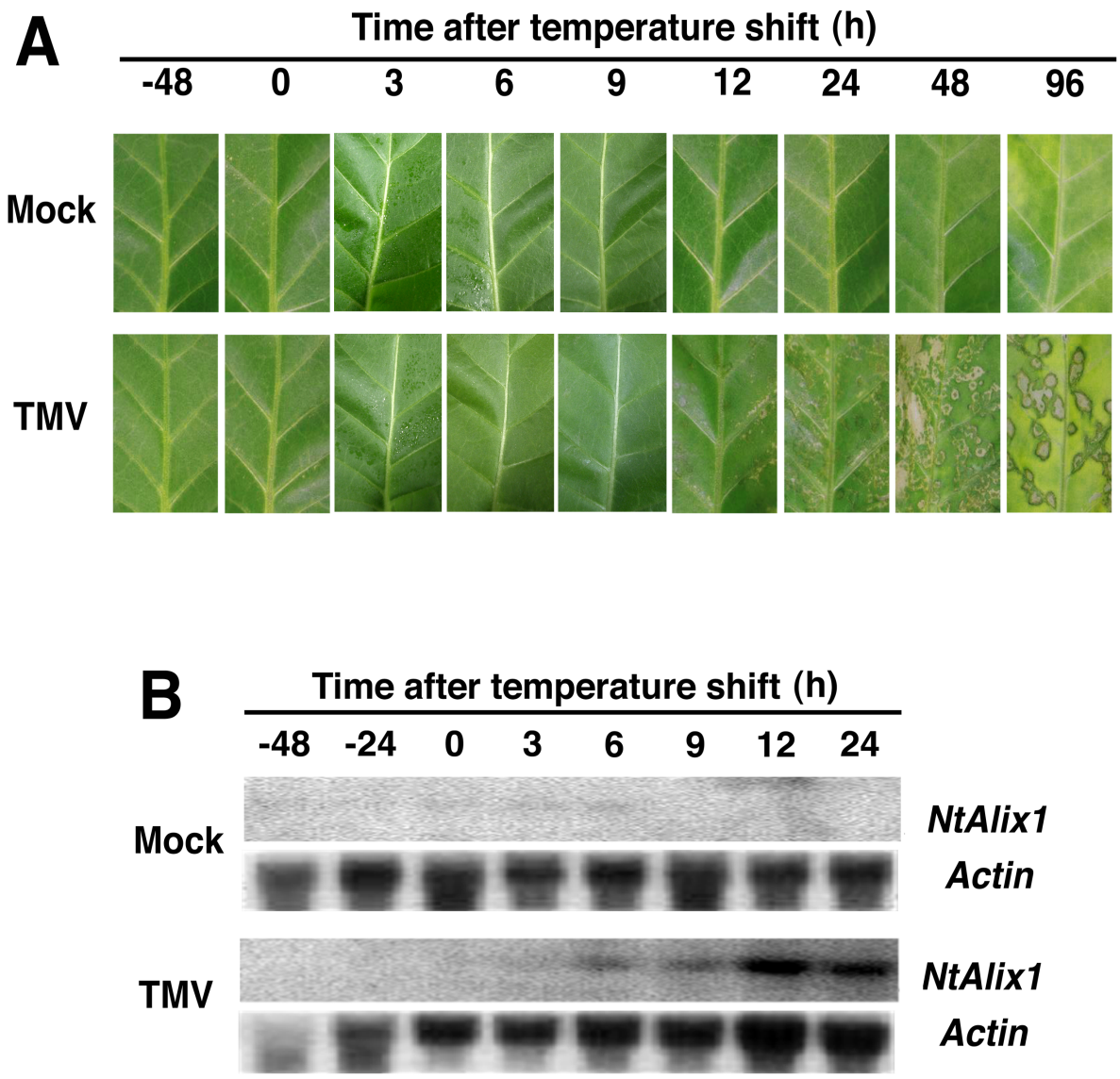

Figure 4. **Induction of *NtAlix1* transcripts upon TMV infection.** **A** Time course of the HR. Healthy fresh leaves from wild-type tobacco plants were detached, treated with TMV (TMV) or buffer solution (Mock), and kept at 30°C for 48 h. Samples were then transferred to 20°C and lesion formation was observed at the indicated time points, indicated in terms of post-temperature-shift. **B** Accumulation of *NtAlix1* transcripts upon temperature shift. Total RNA was isolated from leaves which were similarly treated with TMV (TMV) or buffer (Mock), and a 10-μg aliquot from each sample was fractionated by agarose gel electrophoresis. Northern hybridization was performed using *NtAlix1* as the probe. For the internal standard, actin cDNA was used.

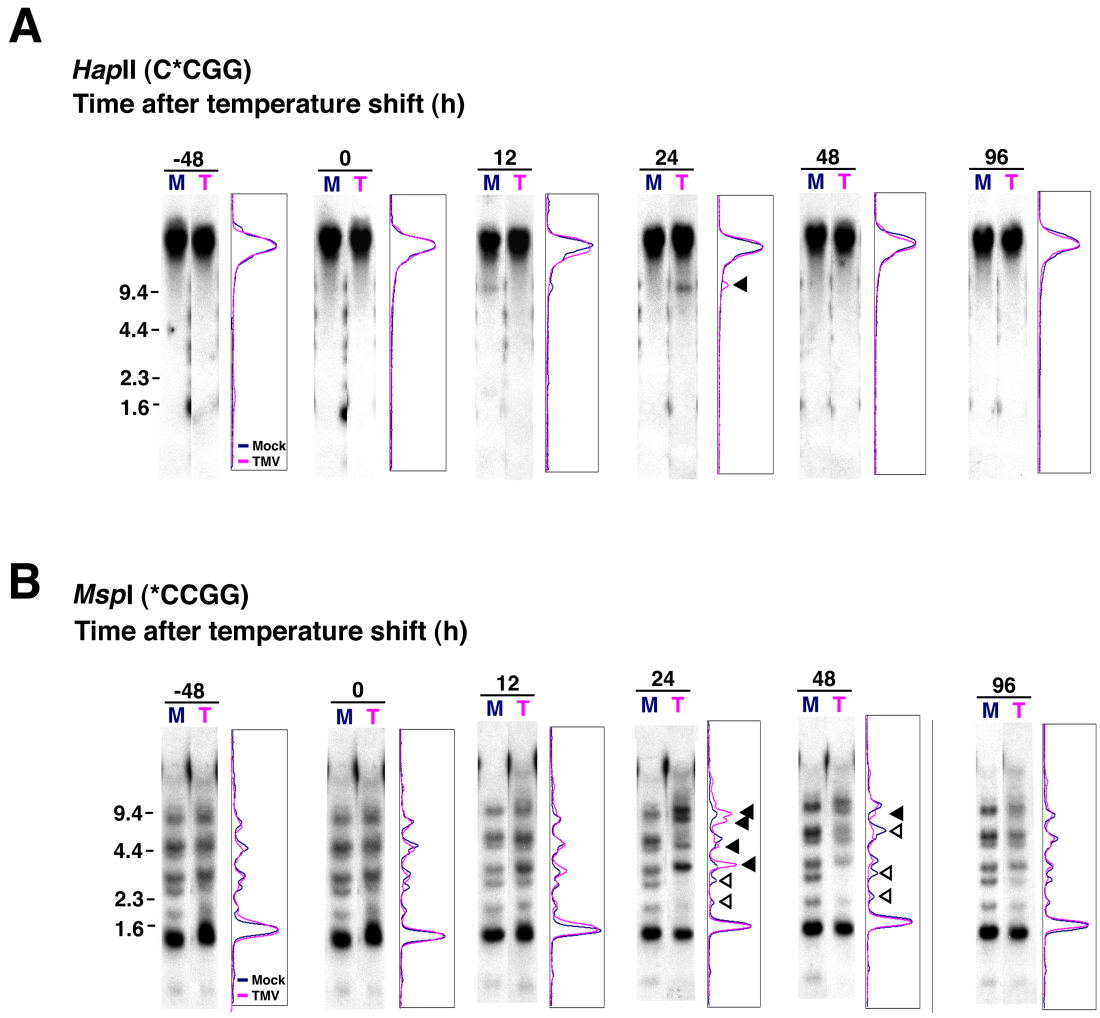

Figure 5. **Changes in methylation status of the *NtAlix1* locus during the HR.** Total DNA was isolated from leaves treated with (T) or without (M) TMV as described in the legend for Fig. 4, digested with *Hap*II (**A**) or *Msp*I (**B**) and subjected to Southern hybridization using *NtAlix1* as the probe. After exposure to x-ray film, the migration pattern was densitometrically traced and shown at the right side of each figure. Fragments increased during the HR are indicated by closed arrowheads, and those decreased by open arrowheads.

CHAPTER II

Biochemical Properties of Domains Rearranged Methyltransferase (DRM) from Tobacco Plants

Introduction

Methylation of cytosine residues in DNA is enzymatically catalyzed by DNA methyltransferases, which transfer a methyl-group from *S*-adenosyl-L-methionine (AdoMet) to the 5-position. In mammals, two types of DNA methyltransferase, which differ in DNA substrate preference, have been reported. Those belonging to the Dnmt1 group prefer cytosines in hemi-methylated CpG sites, i.e., CpG with m^5C in only one strand (Yoder et al. 1997), and are considered to be associated with the DNA replication complex *in vivo* (Chuang et al. 1997) functioning in maintenance of methylation patterns. Those belonging to the Dnmt3 group are reported to methylate cytosines in unmethylated CpG (Okano et al. 1998), and have been suggested to establish the methylation pattern during embryonic development (Okano et al. 1999).

In plants, genes encoding three types of DNA methyltransferases have so far been reported. For example, in *Arabidopsis*, MET1, CMT and DRM are distinct (Finnegan and Kovac 2000). MET1 is considered to be a maintenance DNA methyltransferase, and has been suggested to function in maintenance of global genomic methylation in plants (Finnegan et al. 1996, Ronemus et al. 1996, Nakano et al. 2000). A similar reduction of global methylation

and altered phenotypes were also observed in transgenic tobacco plants expressing an *NtMET1* gene as described in chapter I. The other two types are unique to plants; putative proteins belonging to the CMT group have a chromodomain in catalytic motifs, and are reported to be responsible for maintenance of cytosine methylation at CpNpG sites in, for example, retrotransposons (N is A, T or C) (Lindroth et al. 2001, Tompa et al. 2002). Amino acid sequence analysis indicated that the DRM type has catalytic motifs, thus resembling mammalian *de novo* enzymes such as Dnmt3 (Cao et al. 2000), although they are differ in possessing a characteristic rearrangement in catalytic motifs, between I-V and VI-X. A recent genetic analysis with mutant lines suggested that *Arabidopsis* DRMs might be responsible for the methylation of cytosines in CpNpG and asymmetric sequences of transgenes (Cao and Jacobsen 2002b). Asymmetric cytosine methylation was also seen in epigenetically silenced loci, with DRMs further suggested to function in epigenetic gene silencing (Cao et al. 2000, Cao and Jacobsen 2002b). However, no studies on biochemical properties of these proteins have so far been reported. In this article, we describe isolation of tobacco DRM. The enzyme expressed in insect cells could be shown to preferentially methylate cytosine residues in CpNpN and also CpNpG, providing concrete evidence for the predicted function of DRMs.

Materials and Methods

Plant Materials

Tobacco plants (*Nicotiana tabacum,* cv Xanthi) were grown in a greenhouse under natural

day length conditions. Tobacco BY2 cells (*N. tabacum,* cv Bright Yellow 2) were maintained in suspension culture using modified Linsmaier and Skoog medium (LS medium) (Nagata et al. 1981) at 23°C continuously in the dark. The synchronization of BY2 cells was performed as described (Nakano et al. 2000) with modification. After culture in a modified LS medium containing 5 μg ml^{-1} aphidicolin (Wako, Tokyo) for 24 h, cells were collected and washed with 3% sucrose solution, transferred to fresh medium and cultured further. They were then harvested by centrifugation and stained with 1% orcein to allow determination of the mitotic index.

Isolation of NtDRM1 cDNA

Initially, a DNA fragment encoding NtDRM1 was obtained by differential display of a cDNA population derived from wounded tobacco leaves (Hara et al. 2000). A full length 2.4 kb cDNA of NtDRM1 was isolated by colony hybridization of a tobacco (*N. tabacum*) cDNA library using the *NtDRM1* fragment as a probe and sequenced for both strands using a Big dye terminator sequencing kit (Applied Biosystems, Foster City, CA). Motif prediction was performed using the online databases PROSITE (http://tw.expasy.org/prosite/) and ISREC motif scan (http://hits.isb-sib.ch/cgi-bin/PFSCAN). Phylogenetic analysis was accomplished with CLUSTALW (http://clustalw.genome.ad.jp/).

Transformation of BY2 Cells and Histochemical Analysis

The NtDRM1 coding region (1824bp) was fused in-frame via an engineered *Nco*I site to the N-terminus of the green fluorescent protein (GFP) open reading frame. The construct was then cloned into the *Sma*I and *Eco*RI sites of pBI121 (Bevan et al. 1984) and transformed into *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) by the heat-shock method. Following a 2-day co-cultivation of the individually transformed *A. tumefaciens* with 5 ml of 4-day-old cultured tobacco BY-2 cells in the dark, transformed calli were selected using 100 µg/ml kanamycin and 250 µg/ml calbenicyrin over a 4-week period (An 1985). DNA staining was performed with an aliquot of MS medium containing 1 mg/ml 4',6-diamidino-2-phenylindole (DAPI). GFP fluorescence was observed using an AX70 fluorescence microscope equipped with UV- and B-excitation filters (Olympus, Tokyo) and standard fluorescein isothiocyanate filters. Digital images were captured with a cooled charge-coupled device camera (CoolSNAP-HQ, Photometrics, Tucson, AZ).

Expression and Purification of a GST::NtDRM1 Fusion Protein

Full length NtDRM1 was fused to the C-terminus of a glutathione-*S*-transferase (GST) and expressed in insect cells using the GATEWAY cloning system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Amplification with a set of primers (forward, 5'-AAAAAGCAGGCTTTATGGACAACAATCTTTCTGGAGAAGAC-3'; reverse, 5'-AGA AAGCTGGGTACTAATGTTTATGTCTGGACATTATGGACTT-3') was achieved and the

resulting fragment was subjected to second PCR with another set of primers (forward, 5-GGGGACAAGTTTG-TACAAAAAA-GCAGGC-3; reverse, 5'-GGGGACCACTTTGTAC AAGAAAGCTGGGT-3'). The final fragment was cloned into the pDEST20 vector plasmid and transformed into *E. coli*. together with bacumid (genome of a baculovirus) to allow *in vivo* recombination, and resulting single plaques were picked up and further propagated. Sf9 cells (6×10^9), maintained in Grace's insect medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 500 μg/ml gentamycin, were infected with the recombinant baculovirus (500 μl) and incubated at 27°C for 4 days. Cells from one dish were suspended in 1 ml of lysis buffer (20 mM Tris-HCl (pH7.5), 5 mM EDTA, 1 % Nonidet P-40, and 25 % (v/v) glycerol, 1 mM DTT, 1mM phenylmethylsulfonyl fluoride and 100 μg/ml aprotinin), and sonicated for 10 sec twice. GST-fused NtDRM1 with 77 kDa was purified through a glutathione-Sepharose column according to the manufacturer's instructions (Amersham Biosciences Piscataway, NJ). Protein concentrations were estimated by the Bradford method and immunoblot analysis was performed as described (Steward et al. 2000).

DNA Methyltransferase Assay

DNA methyltransferase activity was monitored by incorporation of the ^{3}H-labeled methyl group of AdoMet into recipient DNA substrate. For the initial assay, 2 μg of poly(dI-dC)/poly(dI-dC) (Sigma, St Lois, MI) was used as the substrate. For methylation of native DNA, samples from pGEX propagated in *E. coli* and Sf9 cells were used. 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 m^5Cs 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'-TAAGCTAGCTTAGCATATGC ATGCATAA-5' (m^5C is underlined), and those for CpNpG were 5'-ATTCAGTCAG ATCTGATCAGTACTGATT-3' and 3'-TAAGTCAGTCTAGACTAGTCATGACTAA-5'. The 100 µl reaction mixture contained 20 mM MOPS-NaOH (pH 7.0), 5 mM EDTA, 200 µg/ml BSA, 25 % (v/v) glycerol, 1 mM DTT, 100 µg/ml RNaseA. 2 µM AdoMet (methyl-^{3}H, specific activity 307.1 GBq/mmol) (New England Nuclear, Boston, MA), and appropriate amounts of substrate DNA and enzyme preparation were incubated at 37°C for the indicated time period. To the reaction mixture was added 1 mg/ml of proteinase K (Invitrogen) in 500 µl proteinase K-SDS buffer, containing 1 % SDS, 2 mM EDTA, 125 mM NaCl, and 0.5 mg/ml salmon sperm DNA. After further incubation at 50°C for 1 h, substrate DNA was extracted by phenol/chloroform treatment, precipitated with ethanol, and spotted onto DEAE-paper, which was washed with 0.5 M sodium phosphate, dried and assessed for radioactivity. Under these experimental conditions, the background level of ^{3}H-radioactivity was approximately 50 cpm per assay, indicating the quenching to be suppressed to a minimum.

Kinetic parameters were calculated from the Michaelis-Menten equation with the Anemona program (Hernandez and Ruiz 1998).

Quantitative Analysis of m^5C

Identification of m^5C by HPLC was performed using substrate DNA isolated from Sf9 cells. A 200-µl reaction mixture, containing 100 µg DNA, 2 mM AdoMet (Sigma) and appropriate amounts of NtDRM1 in the methylation buffer as described above, was incubated at 37°C for 16 h, then further with 110 ng RNaseA (Nacalai Tesque, Kyoto) at 37°C for 2 h. DNA was extracted with phenol/chloroform, precipitated with ethanol, and 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°C for 20 h. The resultant nucleotides were dephosphorylated with 20 units of calf intestine alkaline phosphatase (Takara, Otsu, Japan) at 37°C for 2 h. Samples were then fractionated by ultra filtration (Ultrafree-MC PL-10 microcentrifuge tubes, Millipore, Bedford, MA) and the permeate was injected into a Supelcosil LC-18-S column (Supelco, Bellafonte, PA). Separation was performed with a 2.5 to 20 % methanol gradient in the presence of 50 mM KH_2PO_4 (pH 4.3).

Methylation-Sensitive Restriction Enzyme Assay

A 962 bp substrate DNA was prepared and methylated as described above. An appropriate aliquot was digested with one of restriction enzymes, *Hap*II, *Pvu*II, *Bsp*12861 (methylation

sensitive) or T*aq*I (methylation insensitive), and subjected to agarose gel electrophoresis analysis. Unmethylated DNA fragments were also treated under the same condition as the control.

DNA Methylation Mapping

Substrate DNA was prepared by PCR amplifying a 962 bp fragment of pGEX-4T-1 (positions 4521-513, containing the position 1) then methylated *in vitro* in a reaction mixture containing 25 µg DNA, 2 mM AdoMet (Sigma) and 93.75 µg NtDRM1 in methylation buffer, as described above, at 37°C for 16 h. After phenol extraction and precipitation with ethanol, DNA was subjected to bisulfite modification (Raizis et al. 1995). The method was developed to identify m^5C in arbitrary sequences, based on the resistance of m^5C to bisulfite, which changes C into U. After bisulfite treatment, the modified DNA can be amplified by PCR, cloned and directly sequenced, the C and m^5C in the initial sequence being replaced with T and C, respectively. Experimentally, modified DNA was subjected to first PCR using ExTaq^{TM} enzyme (Takara) and specific forward, 5'-GTTGTGTAGTTTGAATGGTGA ATGG-3'; and reverse, 5'-CAACCACCCAACATATTATACTTATCAAC-3' primers. Both were designed for predicted sequences after modification, in which C was assumed to be converted into T in the former, and G into A in the latter. The resulting product was subjected to subsequent second PCR with the same forward primer and another reverse primer, 5'-CACAAAACCCTTAATTTTCCAATAACC-3' (G was replaced with A). Amplified 594-bp

DNA samples were ligated to the pGEM-T easy vector™ (Promega, Madison, WI), and cloned in DH5α (Stratagene, La Jolla, CA). Sequences were determined with an ABI PRISM BigDye™ Terminator DNA sequencing kit and a 3100 Genetic Analyser automated sequencer (Applied Biosystems). Fifteen clones were sequenced and the average methylation at each site was calculated.

DNA and RNA Isolation and Gel Blot Hybridization

Genomic DNA was extracted by the cetyl-trimethyl ammonium bromide method (Murray and Thompson 1980). Total RNA was isolated from indicated tissues or from BY2 cells by the acid guanidium-phenol-chloroform method (Chomczynski and Sacchi 1987) and hybridization analyses were performed as described (Yoda et al. 2002) with probes synthesized with a pair of primers specific for each gene.

Results

Identification of NtDRM1

During screening for genes whose transcripts accumulate in the early stage after wounding of tobacco leaves by the modified differential display, a particular fragment of 1.6 kb was identified. Although the clone was found later not necessarily to be specific to the wound response by northern hybridization, it was further characterized because homology searches indicated resemblance to genes for DNA methyltransferases. Subsequent screening of a

tobacco cDNA library yielded a full length of 2,540 bp cDNA, encoding a protein of 608 amino acids. Sequence analysis showed it to contain at least six highly conserved motifs found in DNA methyltransferases, but their order was unusual. In contrast to the majority of eukaryotic DNA methyltransferases, having conserved motifs that are arranged in the order of I-IV-VIII-X in the C-terminus, the present putative protein was found to possess motifs in the rearranged order of VI-VIII-IX-X-I-IV (Figure 1A). A homology search indicated high homology to domains rearranged methyltransferases (DRMs) from *Arabidopsis* (DRM1, DRM2) and maize (Zmet3) (Cao et al. 2000) (Figure 1B). Consequently, we concluded that the isolated gene encodes a DRM, and designated it as NtDRM1 (*N. tabacum* DRM1). In the N-terminal region, two domains (amino acid positions 61-97 and 166-204) were found which resembled the ubiquitin-association (UBA) domains of human p62 and yeast RAD23 (Figure 1A). UBA domains, considered to function in protein-protein interactions (Hofmann and Bucher 1996), have also been identified in DRMs of *Arabidopsis* and maize (Cao et al. 2000). A nuclear localization signal (NLS) is present at amino acid positions 234-237 (Figure 1A). A phylogenetic tree generated with the conserved catalytic motifs I to IV indicated that DRMs are more closely related to the *de novo* methyltransferases from mammals (Dnmt3a, Dnmt3b) than to other plant DNA methyltransferases (Figure 1B). A similar tree was also obtained when motifs VI to X were aligned (data not shown).

Subcellular Localization

To identify the cellular localization, a reporter gene encoding green fluorescence protein (GFP) was selected, and a plasmid expressing the NtDRM1-GFP fusion protein under the control of the cauliflower mosaic virus (CaMV) 35S promoter was constructed. As the control, a plasmid expressing only GFP was used. Each plasmid was introduced into onion epidermis cells by biolistic bombardment, and interference contrast images for whole cell structures (Figure 2A, C) and GFP fluorescence for localization of GFP proteins (Figure 2B,D) were examined. While CaMV 35S::GFP control construct showed GFP signals in both cytoplasm and nucleus (Figure 2B), with CaMV 35S:: NtDRM1-GFP they were predominantly in the nucleus (Figure 2D). To confirm the nuclear localization of NtDRM1 *in planta*, tobacco BY-2 cells were stably transformed with the same plasmids, and GFP fluorescence was detected in living cells (Figure 2E-H). As in onion cells, fluorescence of NtDRM1-GFP was observed only in the nucleus (Figure 2F, G). This pattern was identical with that of DAPI, which stains the nucleus (Figure 2H). A nuclear localization of NtDRM1 was ubiquitously observed in transformed cells (Figure 2E, F). It is evident from these results that NtDRM1 is exclusively localized in nuclei.

Purification and Enzymatic Analysis

NtDRM1 cDNA was fused in-frame to the *GST* gene and expressed using the baculovirus-mediated insect cell (Sf9) expression system, which lacks endogenous DNA

methyltransferase activity. After three cycles of propagation of infected Sf9 cells, crude proteins were recovered and further purified through a glutathione-Sepharose column. SDS-PAGE indicated the product to be almost homogeneous (Figure 3A), and this was confirmed to be the recombinant protein by immunoblot staining with anti-GST antibodies (Figure 3A). Using the crude protein preparation, methylation activity was preliminarily assayed by measuring ^{3}H-labeled methyl group transfer from AdoMet into the synthetic oligonucleotide, poly(dI-dC)/poly (dI-dC). As positive and negative controls, mammalian Dnmt3a, a *de novo* DNA methyltransferase, and bacterial β-glucuronidase (GUS), respectively, were similarly expressed and subjected to the same assay. The amounts of enzyme protein in the crude samples were estimated by densitometric quantitation of immunostaining after gel-electrophoresis, and activity was normalized. Methyl group incorporation was clear in the reaction mixture containing GST-NtDRM1 or GST-Dnmt3a proteins, whereas it was undetectable with the GST-GUS preparation (Figure 3B). The activity of NtDRM1 was 10-fold higher than that of Dnmt3a, suggesting an identity as a *de novo* DNA methyltransferase. The optimal pH for the reaction was found to be 7.0 (data not shown). To obtain kinetic information, the activity was further assayed using purified GST-NtDRM1 and varying concentrations of poly(dG-dC)/poly(dG-dC) and also poly(dI-dC)/poly(dI-dC), because the former frequently forms multi-stranded structures and even non-specific aggregation, whereas the latter does not (Figure 3C). Methylation activity increased in a substrate-dependent manner and poly(dI-dC) proved to be a better substrate for

the enzyme than poly(dG-dC), probably reflecting the structural features. The apparent K_m values for poly(dI-dC) and poly(dG-dC) were 2.58 $\pm$ 0.03 μM and 6.36 $\pm$ 0.53 μM (in methylatable cytosine mononucleotides), respectively, and the V_{max} values were calculated to be 718.9 $\pm$ 10.5 fmol/min/nmol protein and 312.0 $\pm$ 58.9 fmol/min/nmol protein, respectively (Figure 3C). The ratio of methyl-incorporation to the substrate cytosines in poly(dI-dC) was estimated to be around $1/10^5$ under the experimental conditions with low concentrations of enzyme and AdoMet and a reaction time of only 30 min. The results indicate *de novo* cytosine methylation by NtDRM1.

Sequence Specificity

In order to determine the sequence specificity for NtDRM1-mediated methylation, three synthetic oligonucleotides, with different sequences but the same base composition, were prepared. Each palindromic 24-mer contained 6 sites for CpG, CpNpG or CpNpN, respectively, where N is A or T. The highest methylation was seen with the CpNpN substrate, ^{3}H-incorporation increasing almost linearly with incubation period up to 1.5 h, whereas methyl-transfer efficiency was lower with CpNpG than CpNpN, and almost null with the CpG substrate (data not shown). The effect of substrate concentration on enzymatic activity was then examined with a fixed concentration of the enzyme and varying concentrations of DNA (Figure 4A). The best substrate was CpNpN, with a substrate-dependent hyperbolic increase in the reaction velocity (Figure 4A). The apparent K_m and V_{max} values were 45.9 $\pm$ 3.04 nM

and 224.4 ± 4.12 fmol/min/nmol protein (based on the methylatable cytosine mononucleotides), respectively. The molar ratio of methyl-group incorporated to the substrate was approximately $1/10^3$. The transfer rate of the methyl-group into CpNpG was 2/3 of that into CpNpN, and almost zero into CpG (Figure 4A). The apparent K_m and V_{max} values were 78.3 ± 5.26 nM and 192.2 ± 4.62 fmol/min/nmol protein (based on the methylatable cytosine mononucleotides), respectively. These kinetic experiments suggested NtDRM1 to preferentially methylate cytosines in non-CpG sequences.

Effects of hemi-methylation was then analyzed. A synthetic 28-mer oligonucleotide containing either CpG or CpNpG was prepared, in which all cytosines were substituted with m^5C. A non-methylated complementary strand was also generated and annealed to form a double-stranded substrate. Methyl-transfer activity was then assayed with a fixed concentration of the enzyme and varying concentrations of the substrate (Figure 4B). These kinetic analyses showed the enzyme to be active on the hemi-methylated CpNpG at a lower velocity than on the unmethylated CpNpG substrate. Taking account of the fact that the number of available cytosines in the former is only half that in the latter, the K_m and V_{max} values were calculated to be 21.2 ± 2.37 nM (based on the methylatable cytosine mononucleotides) and 90.9 ± 10.8 fmole/min/nmol protein, respectively, for the hemi-methylated substrate. The K_m and V_{max} values for the unmethylated substrate were 10.9 ± 1.50 nM (based on the methylatable cytosine mononucleotides) and 136.1 ± 13.4 fmole/min/nmol protein, respectively. These values indicate that the specificity of the enzyme

is lower toward the hemi-methylated CpNpG than toward the unmethylated CpNpG. The methylation patterns of both hemi-methylated and unmethylated CpG substrates were similar with, but lower than that of CpNpG, showing apparent Km values of 37.0 ± 8.54 nM and 27.3 ± 3.34 nM, respectively (Figure 4B). The results suggested that the enzyme does not recognize the hemi-methylated state of CpG and CpNpG, and therefore confirmed its non-maintenance, *de novo* properties.

Selective and Non-selective Methylation

Specificity of methylation sites for NtDRM1 was examined with reference to restriction patterns of methylated DNA by methylation sensitive restriction enzymes. The 962 bp fragment originated from the pGEX-4T-1 plasmid, which contained one or maximum two sites for the restriction enzymes, *Hap*II (for CpG), *Pvu*II (for CpNpG) and *Bsp*1286I (for CpNpN) was amplified by PCR (Figure 5A). The methylation was performed under various reaction conditions, in which the enzyme concentration was varied to give a different molar ratio between the substrate and enzyme (S/E). Restriction analysis of the resultant substrates showed that, when the S/E ratio was approximately 10 (mononucleotide base), methylation occurred in over 80% of CpNpN, whereas this was the case for less than 20% of CpNpG and CpG (Figure 5B, C). When the molar ratio was decreased to less than 5, all cytosines were equally methylated (Figure 5B, C). The absence of adenine methylation was confirmed by cleavage by *Taq*I, which is sensitive to m^6A (Figure 5B). These results indicated that, at low

concentration relative to the substrate DNA, NtDRM1 selectively methylates CpNpN, whereas at high concentrations, it expands its targets to include CpNpG and CpG. This property explains the kinetic result with CpG being not methylated, since, in that experiment, S/E ratio was far more than 58.

Direct Methylation Mapping

The sequence specificity was directly determined by methylation mapping. Native DNA was intensively methylated *in vitro* with excess AdoMet and the enzyme, modified by the bisulfite method, amplified with PCR, cloned and directly sequenced. The conversion efficiency of cytosine (C) into uracil (U) was directly estimated by aligning sequences, and found to be nearly complete as all cytosines in the untreated sequence were converted into thymine (T) (Figure 6A). The methylation frequencies were estimated for 15 clones, assigned to each cytosine, and expressed as percentages (Figure 6B). The enzyme showed the highest methylation of cytosines in CpNpN followed by CpNpG, and least in CpG. The percentages of methylated sites in the total in 15 clones were approximately 75% for CpNpN and 60% for CpNpG, in sharp contrast with the 10% for CpG (Table 1). When nucleotide triplets were classified, and the nearest neighbor was taken into account, the average methylation rates for CpT, CpA and CpC were 87%, 75% and 70%, respectively (Table 2). It appears that the third nucleotide, including guanine, does not appreciably influence the methylation activity, although T located at the 3' end of CpC and CpT reduced the frequency (Table 2). The

nucleotide located at the 5' end of target cytosines showed no apparent effects on site specificity (Table 3). These observations suggest that NtDRM1 essentially recognizes and methylates all cytosines, although some particular combinations, such as CpG and CpCpT, appear to be less favorable.

In vivo Methylation

To examine whether or not NtDRM1 functions *in vivo*, native DNA from Sf9 was analyzed by HPLC (Figure 7). Samples from Sf9 cells expressing NtDRM1 were isolated, digested with nuclease P1, dephosphorylated with alkaline phosphatase and assayed by HPLC. As the control, DNA from wild-type Sf9 cells was used. The positive control was prepared by methylating DNA *in vitro* by NtDRM1, and the negative control was intact Sf9 DNA. HPLC fractionation of the resulting nucleosides showed that DNA samples prepared from transformed cells yielded m^5C at 1.8% of total cytosines, showing a similar elution profile with the control *in vitro* methylated DNA containing m^5C, at 2.7% of total cytosines (Figure 7B, C). The low amount of m^5C from transformed cells may be due to the expression system, in which Sf9 cells are dying when the protein is produced. However, since intact DNA from untransformed cells did not show any m^5C (Figure 7D), the results suggest NtDRM1 to be active as a *de novo* cytosine methyltransferase *in vivo*.

Genomic Organization and Transcript Accumulation

Genomic DNA of tobacco (*N. tabacum*) was digested with appropriate restriction enzymes and hybridized with a specific probe prepared from the 3'UTR region of *NtDRM1* (positions 2064-2493). Under high-stringency conditions, the probe showed four distinct signals after digestion with *Bam*HI, *Eco*RI and *Hin*dIII, respectively (Figure 8A). Since none of these restriction sites was present in the probe sequence, and since *N. tabacum* is an amphidiploid, it is conceivable that *NtDRM1* forms a multigene family existing probably as pairs in each chromosome set originating from its ancestor lines, *N. sylvestris* and *N. tomentosiformis*. RNA hybridization analyses indicated *NtDRM1* transcripts to accumulate ubiquitously in leaves, stems, flowers, and roots (Figure 8B). Levels were also high in all floral organs except pistils (Figure 8B). During the cell cycle of synchronously cultured BY2 cells, *NtDRM1* transcripts accumulated throughout (Figure 8C), in marked contrast to those for *NtMET1* encoding a maintenance methyltransferase, expressed predominantly in the S-phase (Figure 8C).

Discussion

Methylation of DNA is characterized by two distinct features in eukaryotes: maintenance of the preexisting methylation patterns and methylation of previously unmethylated sites, the responsible enzymes being referred to as maintenance and *de novo* methyltransferases, respectively. In plants, proteins belonging to the former type have been biochemically characterized in several species (Finnegan and Kovac 2000), but examples of the latter have not been isolated so far. The presently identified NtDRM1 catalyzed methylation of both

synthetic oligonucleotides and native DNA *in vitro*. Hemi-methylated substrates were also methylated at a low efficiency. When expressed in insect cells, it methylated host DNA *in vivo*. It was thus concluded that NtDRM1 is a *de novo* methyltransferase, to our knowledge, the first clear example of this family in plants.

One of the notable features of NtDRM1 is its high specificity toward cytosines in non-CpG sites. The catalytic properties of NtDRM1 were specific. Kinetic analysis with a relatively low concentration of the enzyme and a high concentration of the substrate indicating that it predominantly methylates cytosines in asymmetric CpNpN. When the relative concentration of the enzyme to the substrate was increased, it also catalyzed methylation of cytosines in symmetrical CpNpG, followed by CpG sequences. Methylation of plant DNA occurs at cytosine residues located in symmetric CpG and CpNpG, and also in asymmetric CpNpN sequences (Finnegan and Kovac 2000). The methylation frequency for these sequences has been estimated to be over 70%, ~50% and ~8%, respectively, in maize DNA (Steward et al. 2002). This is a clear contrast to the case with mammalian DNA, which is almost exclusively methylated in symmetric CpG, at a frequency at more than 80% in many tissues (Robertson and Wolffe 2000). The methylation of CpG in mammals and plants is mainly mediated by DNA methyltransferases of the maintenance type, which, by recognizing m^5CpG in the mother strand, methylates opposite CpG in newly replicated daughter strands after semiconservative DNA replication. This essentially results in the maintenance of the same methylation pattern throughout cell division (Holliday and Pugh 1975). In contrast, the

methylation pattern at asymmetric cytosines is usually not maintained after DNA replication. For it to occur, an asymmetric *de novo* methyltransferase would be required and no clear evidence had been presented for the existence of such enzymes until the recent reports describing Dnmt3a to methylate not only CpG but also asymmetric cytosines (Ramsahoye et al. 2000), and DIM-2 of *Neurospora crassa* to be responsible for cytosine methylation at symmetrical and asymmetrical sites (Selker et al. 2002). In plants, no enzyme has so far been assigned to catalyze cytosine methylation in asymmetric sites. Reverse genetic analyses have suggested that methylation of CpNpG and CpNpN is catalyzed by CMTs and DRMs, respectively, suggesting a distinct role among enzymes (Cao and Jacobsen 2002b, Papa et al. 2001). Under certain circumstances, DRM was also shown to be responsible for CpNpG methylation at some loci, suggesting that DRM and CMT may act in a partially redundant and locus-specific manner to control methylation of CpNpG and CpNpN (Cao and Jacobsen 2002a). Our present findings are essentially consistent with this view, and further provide biochemical evidence that, at least in tobacco plants, NtDRM1 is generally responsible for non-CpG methylation.

The molecular basis for preferential methylation of non-CpG sites is worthy of consideration. Results with direct methylation mapping suggest that NtDRM1 does not necessarily favor CpNpN and its CpNpG subgroup, but rather evades CpG sequences, resulting in an apparent preference. Such rejection of a particular sequence has never been reported, but a recognition mechanism for m^5C by methyl-CpG-binding domain (MBD)

proteins is suggestive of a role of specific amino acids. For example, two guanines in the symmetric m^5CpG doublet have been shown to interact specifically with Arg (R133, R111) in MeCP2 protein (Wakefield et al. 1999). It is of interest to examine whether some amino acids in NtDRM1 repel CpG in such a way as to hinder mutual interaction. Another possibility is that the rearranged catalytic domain is responsible for non-CpG recognition. We think that this is probable, since only plants exhibit methylation in non-CpG sites and have DRMs. Identification of a cytosine-recognition region in NtDRM1 would substantiate this speculation.

The biological significance of selective methylation at asymmetric cytosines is not completely clear, but may be of particular importance for establishment of cell-specific methylation patterns (Ehrlich 2003). Asymmetric cytosine methylation would provide an opportunity to individual cells to establish independent methylation patterns in response to environmental conditions. An example is *de novo* cytosine methylation at CpNpN sites during RNA dependent DNA methylation (RdDM), which has been inferred to function in cosuppression of plant genes upon introduction of foreign DNA (Wassenegger and Pelissier 1998, Jones et al. 2001). It was recently reported that the demethylating enzyme, m^5C-specific glycosylase, regulates gene expression (Jost et al. 1995, Gong et al. 2002). In this case, methylation-mediated suppression is alleviated by demethylation, thereby reversibly controlling gene expression in the cell. A similar cycle of methylation-demethylation of genomic DNA can also be achieved through asymmetric methylation and subsequent passive

demethylation through cell division.

It must be mentioned that the putative amino acid sequences of DRMs so far identified from maize, *Arabidopsis* and soybean indicated the presence of an ubiquitin-asociated (UBA) domain (Cao et al. 2000), shown to be a interaction region for polyubiquitinated proteins (Wilkinson et al. 2001). Since NtDRM1 also possesses a UBA domain, and is constitutively expressed throughout the cell cycle and in all tissues, it is conceivable that it forms complexes with multiple proteins that contribute to chromatin structure, and thereby methylates particular regions of DNA in a *de novo* fashion.

Seq	m^5C / C+m^5C	Percentage
*CpG	66 / 645	10.1
*CpNpG	423 / 525	60.2
*CpNpN	757 / 1005	75.3

Table 1 Methylation frequency in symmetric and asymmetric sites

The sums of m^5C in the total methylatable cytosines the indicated sequences in a total of 15 clones are presented as numbers observed and as percentages (%). For example, the 645 CpG sites was calculated by multiplying 15 clones by 43 sites in the top strand of a 594-bp region of the substrate pGEX-4T-1. The numbers of CpNpG and CpNpN in the 594-bp region were 35 and 67, respectively. N indicates A, C or T. Asterisk indicates methylated cytosines.

Triplet	m^5C / C+m^5C	Percentage
*CpApA	78 / 120	65.0
*CpApC	130 / 165	78.8
*CpApG	111 / 150	74.0
*CpApT	110 / 135	81.5
Total *CpA	429 / 570	75.2
*CpCpA	90 / 120	75.0
*CpCpC	105 / 120	87.5
*CpCpG	88 / 120	73.3
*CpCpT	51 / 120	42.5
Total *CpC	334 / 480	69.6
*CpGpA	6 / 165	3.6
*CpGpC	6 / 135	4.4
*CpGpG	10 / 180	5.5
*CpGpT	43 / 165	26.1
Total *CpG	66 / 645	10.1
*CpTpA	70 / 75	93.3
*CpTpC	85 / 90	94.4
*CpTpG	224 / 255	87.8
*CpTpT	38 / 60	63.3
Total *CpT	417 / 480	86.9

Table 2 Methylation frequency in triplets

The sums of total m^5C numbers in the total methylatable cytosines for the indicated triplets in 15 clones are presented as numbers observed and as percentages (%), as described in the legend for Table 1. Asterisk indicates methylated cytosines.

Triplet	m^5C / C+m^5C	Percentage
ApAp*C	48 / 90	53.3
CpAp*C	96 / 165	58.2
GpAp*C	33 / 75	44.0
TpAp*C	84 / 120	70.0
Total Ap*C	261 / 450	58.0
ApCp*C	44 / 60	73.3
CpCp*C	89 / 120	74.2
GpCp*C	51 / 150	34.0
TpCp*C	78 / 135	57.8
Total Cp*C	262 / 465	56.3
ApGp*C	70 / 120	58.3
CpGp*C	87 / 135	64.4
GpGp*C	103 / 240	42.9
TpGp*C	105 / 180	58.3
Total Gp*C	365 / 675	54.1
ApTp*C	109 / 165	66.1
CpTp*C	54 / 90	60.0
GpTp*C	54 / 120	45.0
TpTp*C	133 / 150	88.7
Total Tp*C	350 / 525	66.7

Table 3 Methylation frequency in triplets

The sums of total m^5C numbers in the total methylatable cytosines for the indicated triplets in 15 clones are presented as numbers observed and as percentages (%), as described in the legend for Table 1. Asterisk indicates methylated cytosines.

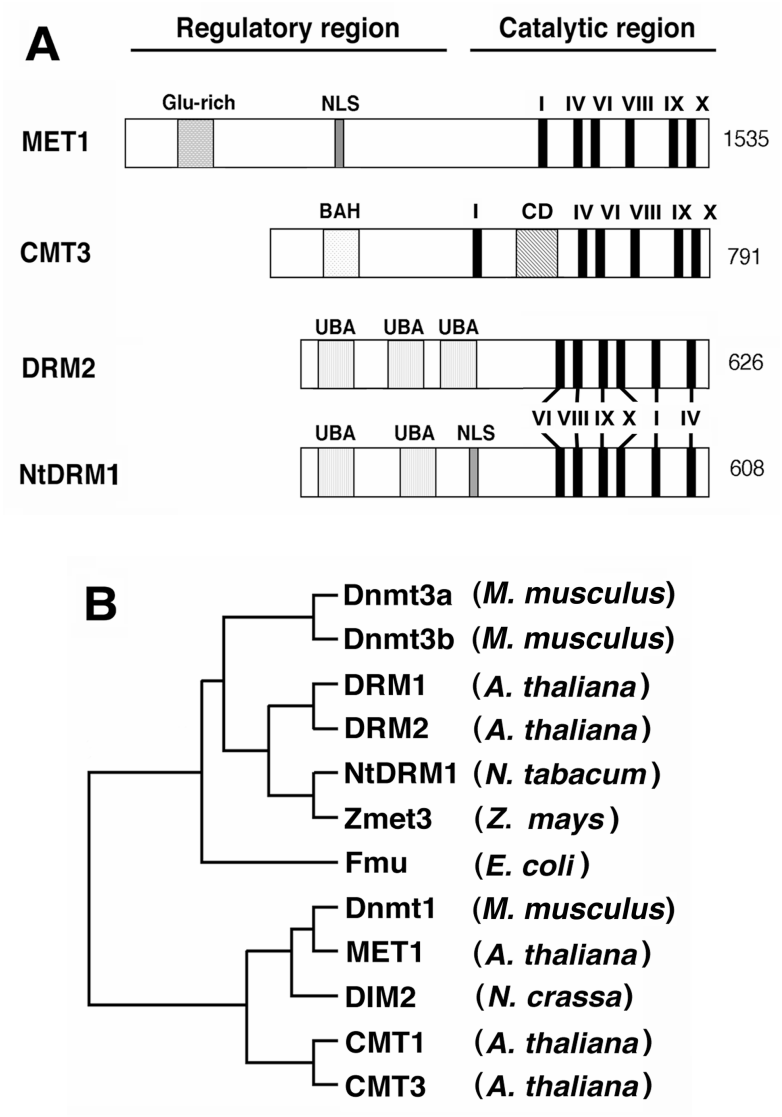

Figure 1. Comparison of plant DNA methyltransferases. **A** Schematic illustration of DNA methyltransferase structures. The size of each protein is indicated in amino acid numbers (aa), and conserved motifs in the catalytic region are indicated by closed boxes with numbers. Specific regions in the regulatory region are indicated by shaded boxes with appropriate names. Glu-rich, glutamine rich acidic region; BAH, bromo-adjacent homology domain; CD, chromodomain; NLS, nuclear localization signal; UBA, ubiquitin association domain. **B** Phylogenetic relationships among DNA methyltransferases. Sequence data were obtained from the data base; accession numbers were AF242320 (Zmet3 from *Zea mays*), ATF8M21 (DRM1 from *Arabidopsis thaliana*), AF240695 (DRM2 from *A. thaliana*), AF068625 (Dnmt3a from *Mus musculus*), AF068628 (Dnmt3b from *M. musculus*), P13864 (Dnmt1 from *M. musculus*), P23866 (Fmu from *Escherichia coli*), AF348971 (DIM-2 from *Nurospora crassa*), P34881 (MET1 from *A. thaliana*), AF039372 (CMT1 from *A. thaliana*) and AL021711 (CMT3 from *A. thaliana*). *E. coli* RNA methylase Fmu is used as an external reference.

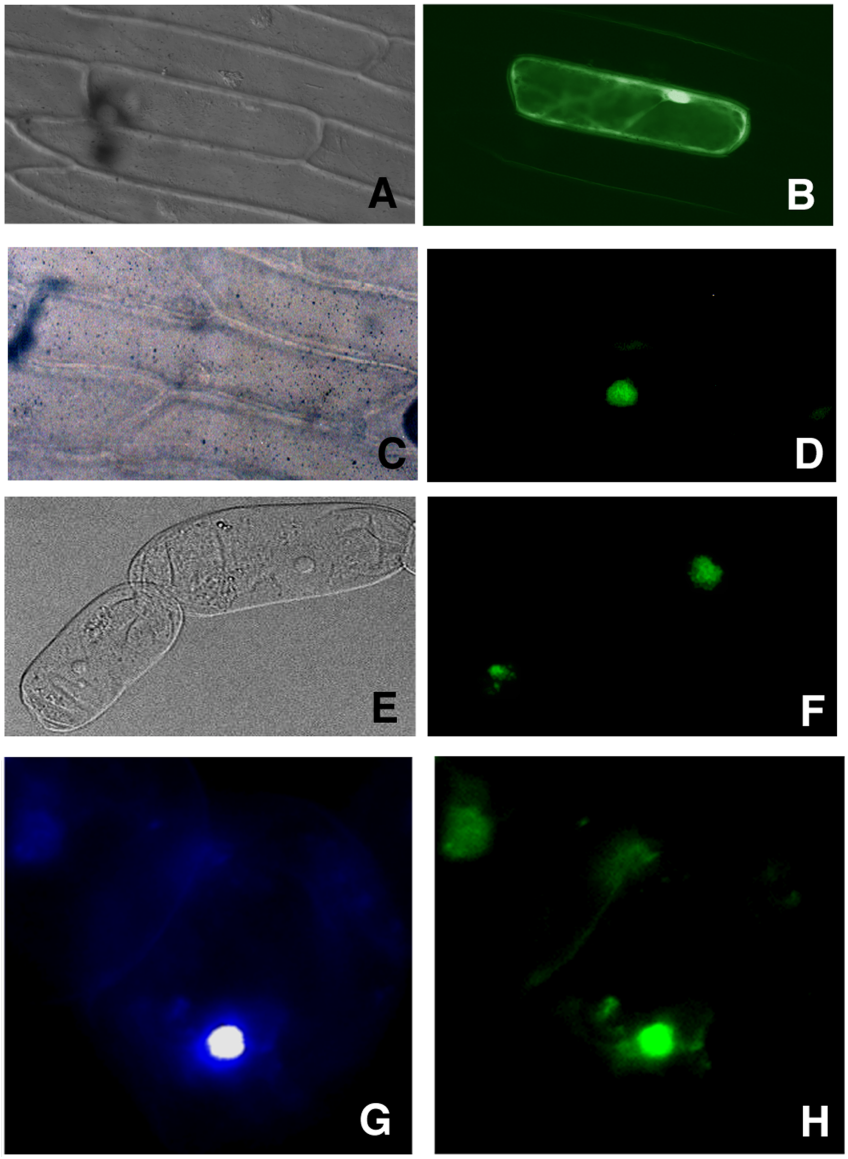

Figure 2. Subcellular localization of NtDRM1-GFP. Onion bulbs were bombarded with gold particles coated with plasmid expressing CaMV35S::GFP, or CaMV35S::NtDRM1-GFP (pNtDRM1-GFP). The proteins were transiently expressed and individual cells were observed by epifluorescence for GFP (**B**, **D**), or under interference contrast (**A**, **C**). Tobacco BY2 cultured cells were transformed with pNtDRM1-GFP and stable transformants were observed by interference contrast (**E**) or by epifluorescence for GFP (**F**). Single cells were similarly observed for GFP fluorescence (**G**) and stained with DAPI to identify the position of nuclei (**H**).

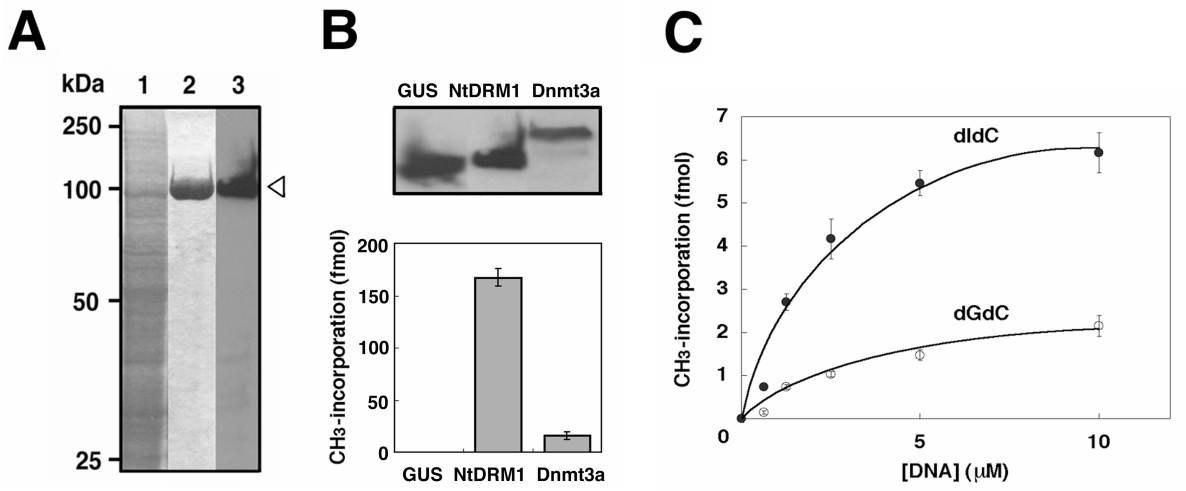

Figure 3. Production and properties of NtDRM1. **A** *In vitro* expression of NtDRM1 protein. NtDRM1 was fused to the baculo-vector, pDEST20, transformed into Sf9 cells, and amplified then crude extracts (lane 1) or purified proteins after passage through a GST-column (lane 2) were subjected to SDS-PAGE, and stained with coomasie brilliant blue (CBB). Proteins were blotted onto cellulose membrane and subjected to immuno-staining using anti-GST antibodies (lane 3). The sizes of proteins are indicated at the left side in kDa. **B** DNA methyltransferase assay. To estimate enzyme concentration, crude extracts were subjected to immunoblot staining with anti-GST antibodies, and signals were densitometrically quantified (upper panel). When the intensity for Dmnt3a was taken as 1, the relative intensities for NtDRM1 and GUS proteins were 2.37 and 2.16, respectively. Enzymatic activities were normalized based on these values. For activity assays, a reaction mixture containing 2 μM [^{3}H]AdoMet, 2 μg of poly(dI-dC)/poly(dI-dC) and 10 μl crude protein preparation as indicated was incubated for 6 h, spotted onto DEAE filter paper, washed and counted for radioactivity. A mixture containing GST-fused GUS was used as the control. Values are expressed as the means of triplicate experiments and error bars represent the standard deviations (lower panel). **C** DNA methylation activity of GST-NtDRM1 fusion protein. A reaction mixture containing the indicated concentrations of poly(dG-dC)/poly(dG-dC) (open circles) or poly(dI-dC)/poly(dI-dC) (closed circles), 2 μM [^{3}H]AdoMet and 36 ng purified GST-NtDRM1 protein was incubated at 37°C for 30 min. The amounts of transferred methyl-group were calculated based on the specific activity of [^{3}H]AdoMet (7.7 x 10^4 cpm/pmol). Experiments were repeated three times and mean values were estimated with standard deviations.

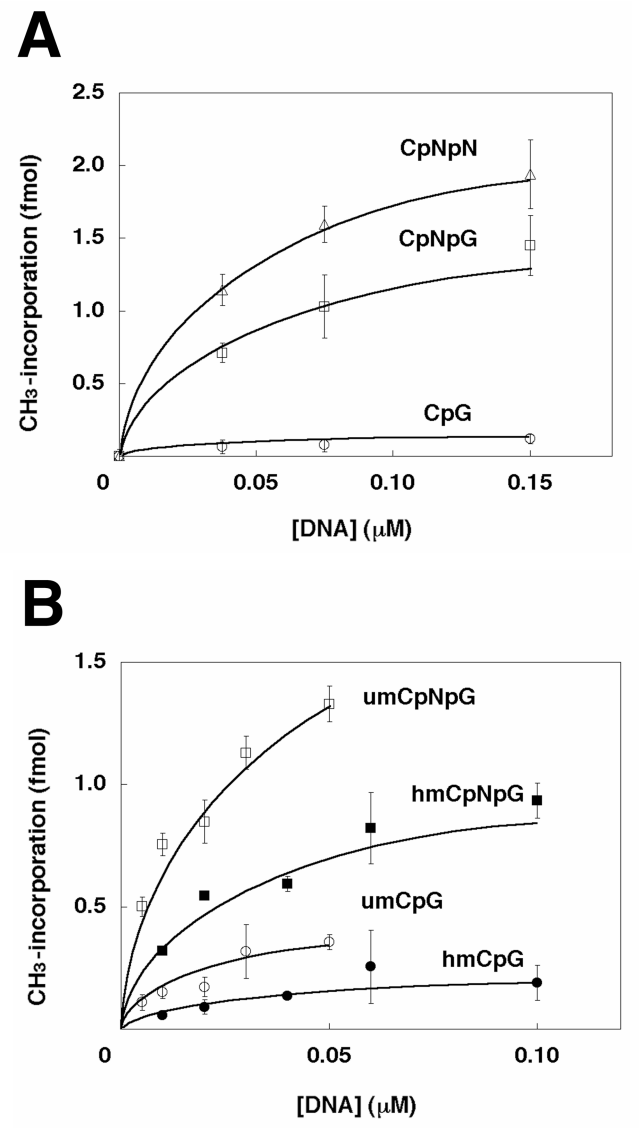

Figure 4. Sequence specificity of NtDRM1. A Sequence specificity and effects of substrate concentration. The sequences of 24-mer oligonucleotides used in this assay were 5'-ACGATCGTACGATCGTACGATCGT-3' for CpG (closed circles), 5'-ACTGCAGT ACTGCAGTACTGCAGT-3' for CpNpG (open circles) (N is A or T), and 5'-AGCATGCTAGCATGCTAGCATGCT-3' for CpNpN (open triangles). A reaction mixture containing 36 ng purified NtDRM1 protein, 2 μM [^{3}H]AdoMet and the indicated amount of synthetic oligonucleotides was incubated for 30 min, and processed as described above. Experiments were repeated three times and mean values were estimated with standard deviations. **B** Effects of hemi-methylated substrate. The hemimethylated duplex sequences of 28-mer oligonucleotides used in this assay were synthesized as described in the text. The number of methylatable cyotosines was 5 in one strand, and thus contained 5 and 10 sites in each duplex of hemi-methylated and unmethylated substrate, respectively. Substrates were unmethylated CpG (umCpG, open circle), hemi-methylated CpG (hmCpG, closed circle), unmethylated CpNpG (um CpNpG, open square) and hemi-methylated CpNpG (hmCpNpG, closed square). Assays were performed as described above.

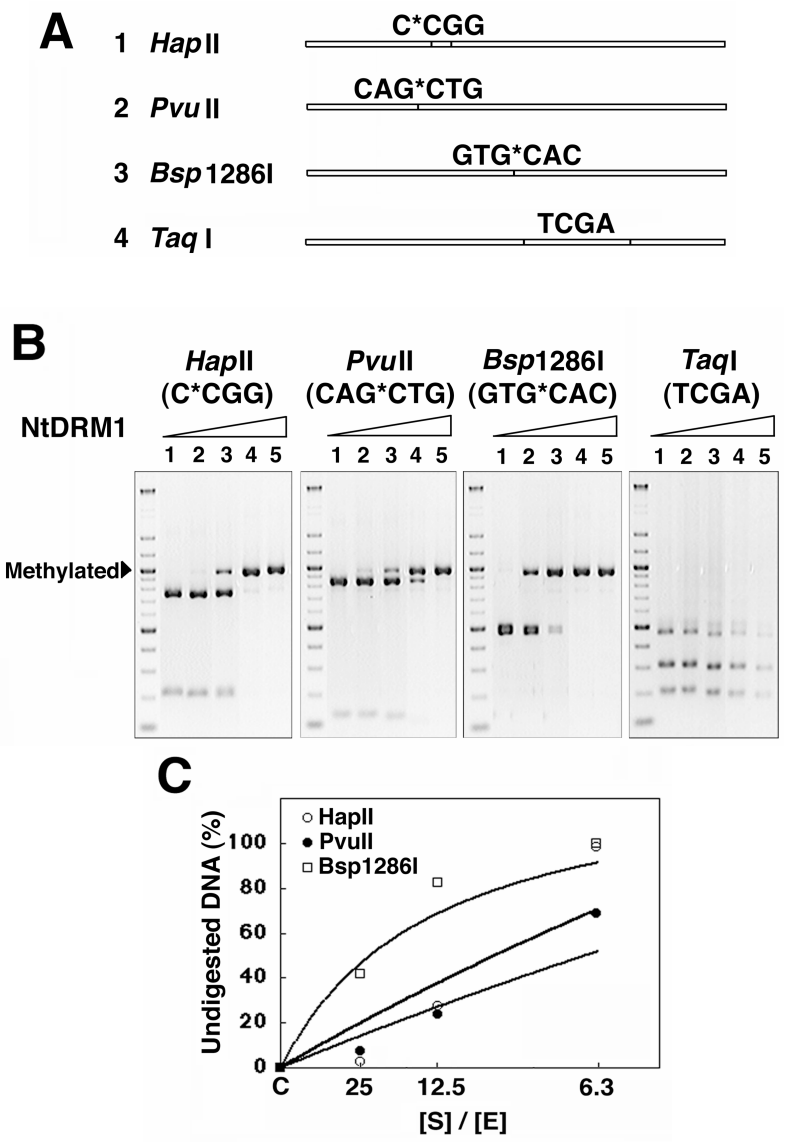

Figure 5. Restriction analysis of methylated DNA by NtDRM1. A Illustration of the restriction sites for methylation-sensitive and -insensitive restriction enzymes. A 962 bp DNA fragment between the positions 4521 and 513 of pGEX-4T-1 vector was amplified by PCR and used as the substrate in this assay. The m^5C that affects recognition is indicated by asterisk. **B** Effect of protein concentration on restriction digestion. The DNA was methylated at 37℃ for 16 h in a 2 ml reaction mixture containing a fixed amount of substrate DNA (25 μg) and varied amounts of NtDRM1 (0 ~ 0.5 mg) to give varied molar ratio between DNA (mononucleotide) and enzyme. The ratio was adjusted to 25 (lane 2), 12.5 (lane 3), 6.3 (lane 4) or 3.2 (lane 5), respectively. Unmethylated DNA is shown as the control (lane 1). A 500-ng aliquot of substrate 962 bp DNA fragment was digested with *Hap*II, *Pvu*II, *Bsp*1286I or *Taq*I as indicated. The resulting samples were fractionated on a 2% agarose gel, and visualized with ethidium bromide staining. **C** Densitometric quantitation of methylation frequency based on data presented in **B**. The vertical axis represents frequency of undigested DNA after restriction digestion in percentage (%) and the horizontal axis represents the concentration of purified GST-NtDRM1 protein.

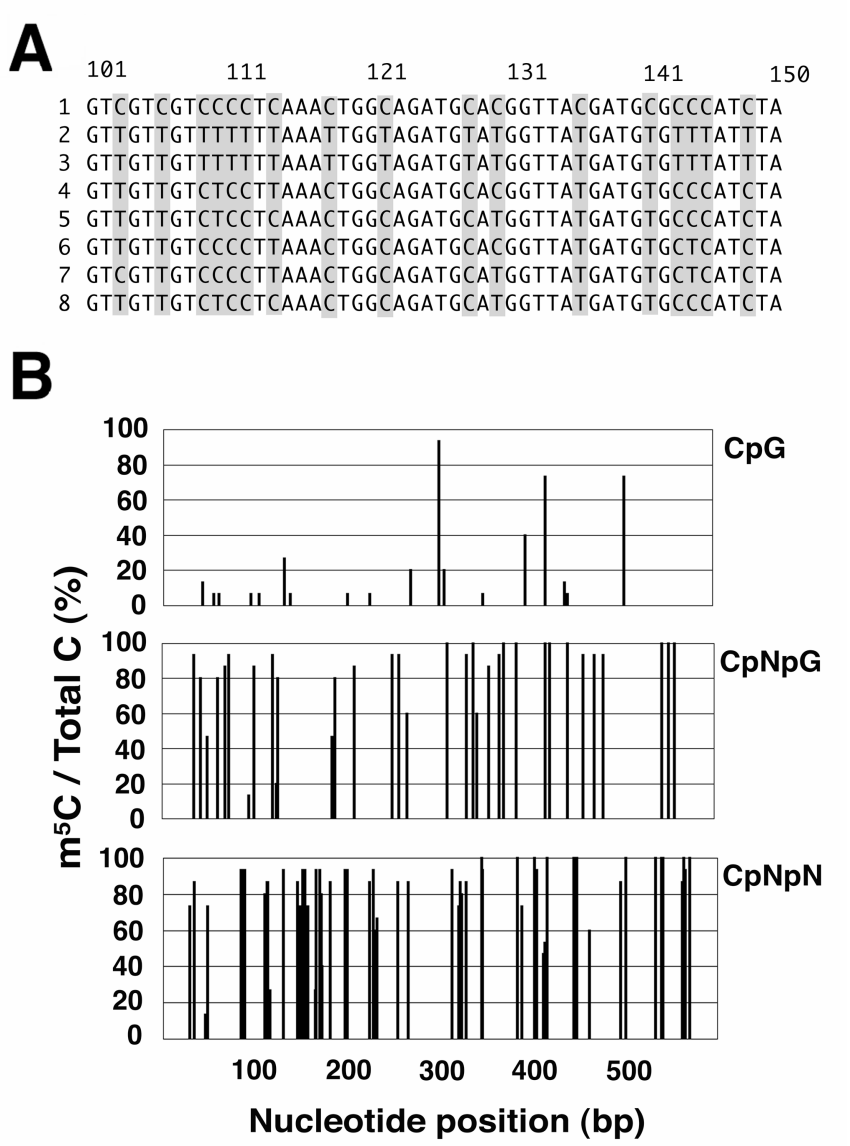

Figure 6. Direct mapping of methylated DNA. A Identification of m^5C. A 594-bp DNA fragment of pGEX-4T-1 was methylated by NtDRM *in vitro*, treated with bisulfite, cloned, and sequenced. C and m^5C in the original sequence have been converted into T and C, respectively. Nucleotide sequence at positions between 101 and 150 is illustrated. Samples were original DNA without bisulfite treatment (lane 1), unmethylated control DNA with bisulfite treatment (lane 2), and methylated DNA with bisulfite treatment (lanes 3 through 8). **B** Distribution and frequency of m^5C were estimated for 15 clones. Histograms represent the percentage of m^5C over the total cytosines at positions containing CpG (top), CpNpG (middle) and CpNpN (bottom) sequences in the top strand (N is A, C or T). The numbers of methylatable CpG, CpNpG and CpNpN sequences were 43, 35 and 67, respectively, in the 594 bp DNA fragment tested. Note that not all these sites are shown in the figure.

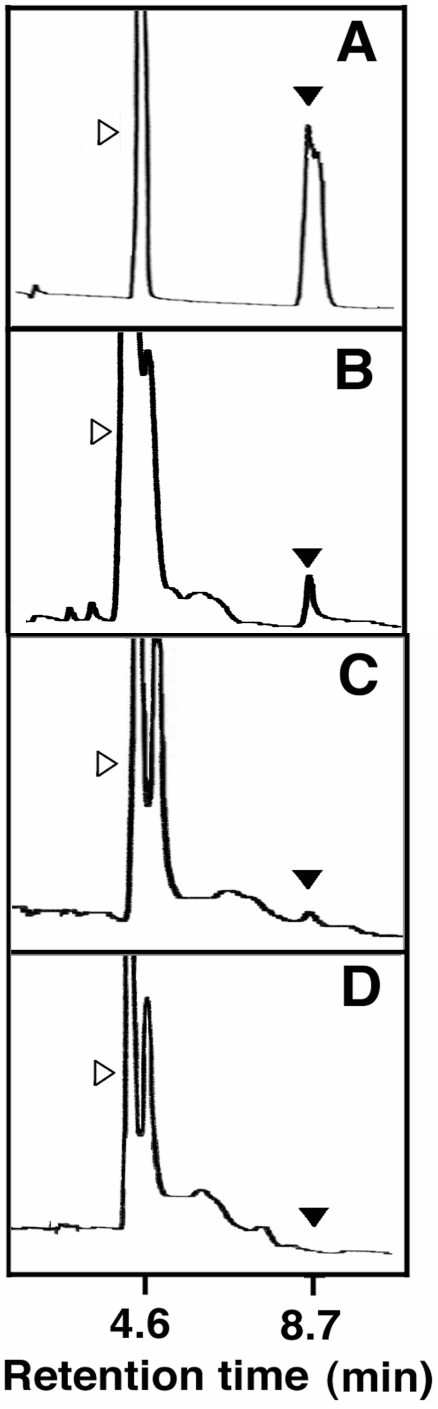

Figure 7. Identification of methylation products. DNA samples were digested to mononucleosides, dephosphorylated and subjected to HPLC analysis. **A** Elution profile of standard authentic cytosine (open arrowhead) and m^5C (closed arrowhead). The retention times of cytosine and m^5C were 4.6 min and 8.7 min, respectively. **B** Elution profile of *in vitro* methylated DNA from wild-type Sf9 cells. A 100 μg of DNA sample was methylated with NtDRM1 under the standard condition, and then digested to mononucleosides. **C** Elution profile of native DNA from NtDRM1-transformed Sf9 cells. A 100 μg of DNA sample was directly digested to mononucleosides. **D** Elution profile of native DNA from wild-type Sf9 cells. A 100 μg of DNA sample was directly digested to mononucleosides.

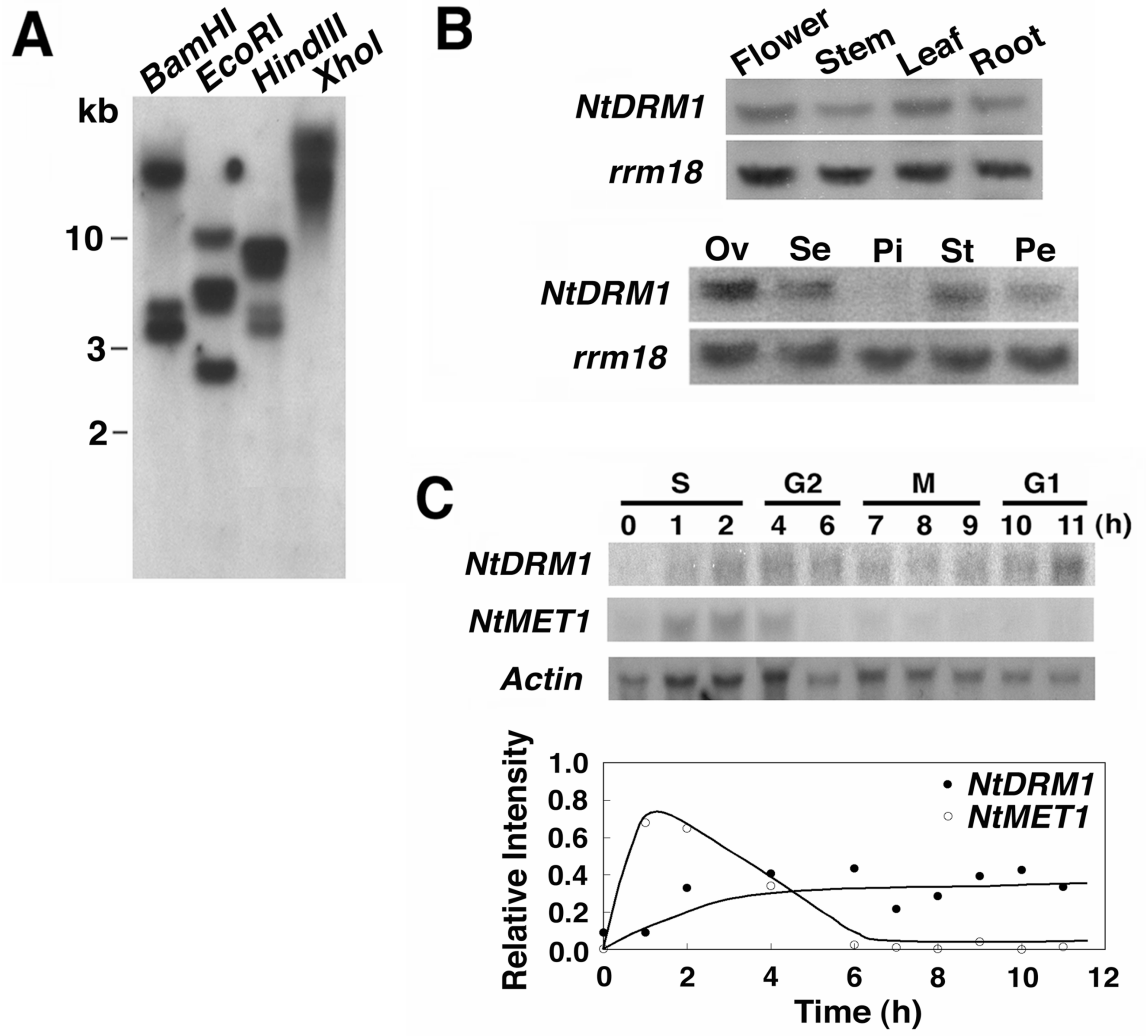

Figure 8. Genomic organization and tissue-specific expression of *NtDRM1*. **A** Southern hybridization analysis. A 10 µg aliquot of total DNA was digested with the indicated restriction enzymes and subjected to DNA hybridization with the NtDRM1 probe. **B** Tissue and organ specific transcript accumulation. Total RNA was extracted from indicated tissues or flower organs, and a 10 µg aliquot was fractionated by agarose gel electrophoresis, transferred to a nylon membrane, and subjected to hybridization with the *NtDRM1* probe. Flower organs were ovule (Ov), sepals (Se), pistils (Pi), stamen (St) and petal (Pe). As an internal standard, ribosomal protein cDNA (*rrm18*) was used (bottom panel). **C** Cell cycle-dependent expression of *NtDRM1* and *NtMET1* in synchronized tobacco BY2 cells. Membranes bearing 15 µg of total RNA per lane were successively probed with the *NtDRM1* and *NtMET1* specific probes. As the internal standard, actin cDNA (*Actin*) was used (bottom panel). The relative levels of transcripts were quantified based on densitometric measurement of each signal, normalized to actin signals. The indicated phase of the cell cycle was estimated from the change of mitotic index after aphidicolin release.

CHAPTER III

Isolation and Characterization of a Tobacco DRM1 Complex

Introduction

Methylation is a post-replicative event, catalyzed by DNA methyltransferases. However, their regulatory mechanism has not been completely understood. The most intensively investigated example is the maintenance methyltransferase Dnmt1 complex in mammalian cells. In order to recruit to Dnmt1 methylatable loci, several factors that interact with it are necessary. Dnmt1 is recruited to replication foci by interacting with proliferating cell nuclear antigen (PCNA) (Chuang et al. 1997), and forms complex with MeCP2, a m^5C targeting protein, in order to perform maintenance methylation *in vivo* (Kimura and Shiota 2003). In addition, Dnmt1 also interacts with retinoblastoma gene product (Rb) (Robertson et al. 2000) and histone deacetylase 1 (HDAC1) (Robertson et al. 2000, Fuks et al. 2000) and histone deacetylase 2 (HDAC2) (Rountree et al. 2000). A large multisubunit complex containing nucleosome remodeling factor, HDAC1, HDAC2, Rb, and Methyl-CpG-binding domain protein 3 (MBD3) (Zhang et al. 1999), is referred as NuRD, and Dnmt1 in it directs the NuRD complex to methylated DNA, providing a means of gene silencing by chromatin remodeling. NuRD can alter the chromatin structure more compact by histone deacetylase activity, thus silencing gene expressions. Another factor for silencing of methylated loci is the Dnmt1 associating protein 1 (DMAP1), a transcriptional repressor (Rountree et al. 2000).

Biochemical analysis of mammalian methyltransferase complex identified a factor that regulates methylation, and the factor for translating methylation to genes repression. Little is known, however, whether similar complexes are available in plants. In this chapter, I describe attempts of isolation of *in vivo* NtDRM1 complex and identification of the component of NtDRM1 complex.

Materials and Methods

Plant Materials

Tobacco plants (*Nicotiana tabacum,* cv Xanthi) were grown in a greenhouse under natural day length conditions. Tobacco BY2 cells (*N. tabacum,* cv Bright Yellow 2) were maintained in suspension culture using modified Linsmaier and Skoog medium (LS medium) (Nagata et al., 1981) at 23°C continuously in the dark.

Nuclear Isolation from BY-2 Cells

Nuclear isolation was performed as described (Masuda et al. 1997) with some modifications. one-week-cultured BY-2 cells were collected by centrifugation, then washed with BY-2 washing buffer [9% (w/v) sorbitol, 25 mM Mes-NaOH (pH5.6), 5 mM $CaCl_2$]. Collected cells were treated with enzyme solution containing 9% (w/v) sorbitol, 3% (w/v) Cellulase ”Onozuka” RS (Yakult Honsha, Tokyo), 1% (w/v) Macerozyme R-10 (Yakult Honsha) for 30 min under the continuous shaking. The resulting protoplasts were

homogenized in nuclear isolation buffer [25 mM Mes-NaOH (pH 5.6), 5 mM $MgCl_2$, 10 mM KCl, 0.35 M sucrose, 30% (w/v) glycerol], filtrated through nylon mesh (20 μm), and centrifuged in 48% (v/v) and 32% (v/v) percoll (Amersham Biosciences, Piscataway, NJ) gradient to isolate nuclear fraction.

Extraction of Nuclear Protein

Extraction of nuclear proteins was performed as described (Rodrigo and Franco 1990) with some modifications. Percoll-purified nuclei were pelleted by centrifugation, resuspended in digestion buffer containing 10 mM Tris-HCl (pH7.5), 1 mM $MgCl_2$, 10 mM NaCl, 0.25 M Sucrose, 5 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), then DNase I was added and incubated at 37℃ for 30 min. Chromatin fraction was obtained by centrifugation as the supernatant. Protein concentration was estimated by the Bradford method (Bradford 1976).

Immunoprecipitation

Isolated nuclei were collected by centrifugation, resuspended in nuclear lysis buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, 1 mM PMSF and 100 μg/ml aprotinin, and sonicated three times for 10 sec each on ice. The sonicated solution was centrifuged, and the supernatants were collected and diluted 10-fold IP buffer containing 16.7 mM Tris-HCl, pH 8.0, 1.2 mM EDTA, 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1 mM

PMSF and 100 μg/ml aprotinin. Chromatin solution was precleaned with preimmune serum and protein A-sepharose at 4℃ for 2 h. The precleaned supernatants were incubated with anti-NtDRM1 antibody and protein A-sepharose. After extensive washes, the protein complex was dissolved in SDS loading buffer and subjected to SDS-PAGE.

Results and Discussion

Nuclear Isolation from BY-2 Cells

Purified nuclei through percoll gradients were microscopically observed. The sample was stained with DAPI to identify nucleus, and resulting image was merged with interference contrast image. Results clearly showed that nucleus was successfully isolated (Figure 1).

Nuclear Localization of NtDRM1

Previous observation indicated that NtDRM1 protein localized in nucleus, and that its transcrips accumulated throughout cell cycle of BY-2 cultured cells. NtDRM1 localization in nucleus was confirmed by Western blot analyses using anti-NtDRM1 antibody. Signal from NtDRM1 protein was detected in both the total protein fraction of BY-2 cells and the chromatin fraction, but not in the matrix fraction (Figure 2).

Isolation of NtDRM1 Complex by Immunoprecipitation

Immunoprecipitation was performed with proteins extracted from the chromatin fraction

using rabbit anti-NtDRM1 antibody. Resulting immunoprecipitates were subjected to SDS-PAGE. Preliminary assay showed a protein with MW 150 kDa was specifically co-precipitated with NtDRM1 (Figure 3). Although further purification of these fractions was needed, this protein appeared to be one of interacting factors with NtDRM1. Further experiments by large scale purification using these materials are under progress, micro sequencing and successive gene isolation will reveal the identify of this protein.

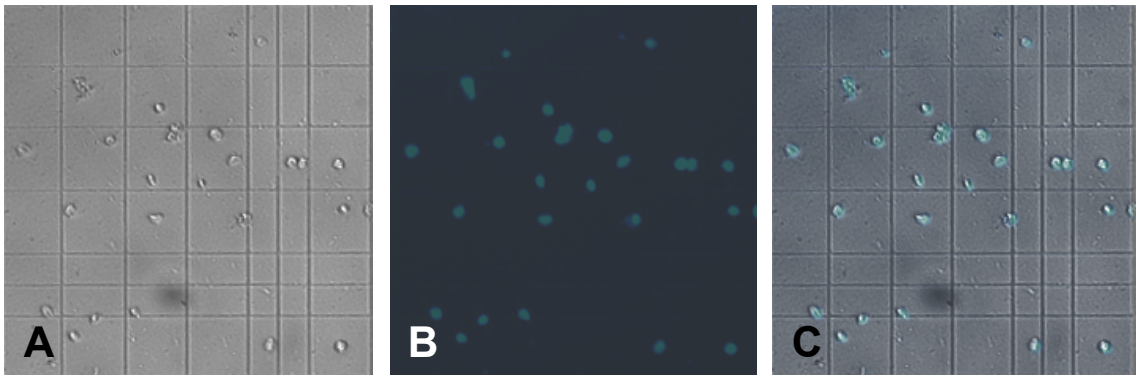

Figure 1. Isolated nuclei of BY-2 cells. Percoll purified isolated nuclei were observed under the microscopy. Interference contrast image (A), fluorescent image of DAPI staining (B) and merged (C).

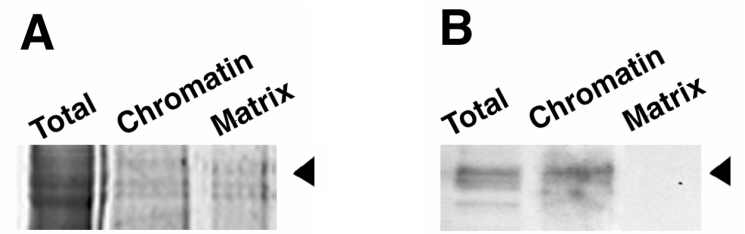

Figure 2. Presence of endogenous NtDRM1 protein in chromatin fraction. Total protein extracted from BY-2 cells (left), chromatin fraction (middle), matrix fraction (right) were resolved by SDS-PAGE and visualized by Ruby staining (A) or by western blotting probed with rabbit anti-NtDRM1 antibody (B). Expected molecular size of NtDRM1 is indicated by closed arrowheads.

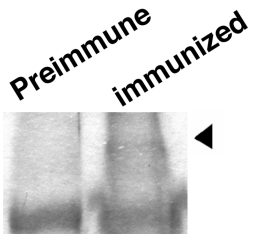

Figure 3. Erectrophoretic pattern of immunoprecipitated with endogenous NtDRM1. Chromatin fraction was incubated with preimmune sera (left) or immunized sera (right), then incubated with protein A-sepharose. Precipitates were collected by centrifugation, and subjected to SDS-PAGE. A closed arrowhead indicates interacting protein.

CONCLUDING REMARKS

In the first part of this thesis, I discussed physiological significance of maintenance of DNA methylation in tobacco plants. A dynamic change in gene expression pattern was observed in tobacco plants expressing the antisense of DNA methyltransferase, *NtMET1,* which was thought to be involved in maintenance of global DNA methylation status. The antisense plants showed a dwarf phenotype and ectopic expression of genes that are silent in wild type plants without stressing. One such gene was a pathogen responding gene (*NtAlix1).* Upon tobacco mosaic virus infection, transcripts of *NtAlix1* were found to be accumulated and the methylation status of the genomic locus was obviously changed at CCG sites. Two features are important; first, stress interferes maintenance of methylation; and second, methylation status correlates with transcriptional activity. It was conceivable that the locus was transcriptionally suppressed under the normal condition by NtMET1, and that stress-induced hypomethylation activated transcription of the gene.

These experiments indicated that NtMET1 is required for maintenance of methylation patterns, being consistent with the mechanism of hereditary of DNA methylation patterns as described by Holliday and Pugh (1975). Although the model did not refer control of gene expression, DNA methylation is now accepted to participate in many epigenetic regulation of gene expression. This role is considered to be the most important role of DNA methylation. Through the experiments, I understood that maintenance of methylation pattern is closely

related to gene expression, and consequently I was interested in the mechanism in which methylation patterns are created.

In order to address this question, I initiated biochemical analysis of enzymatic activity of tobacco DRM, which is thought to be a candidate for *de novo* DNA methyltransferase from its sequence analyses. As described in chapter II, recombinant GST-NtDRM1 fusion protein clearly showed *de novo* methyltransferase activity. An unexpected finding was specific activity of the enzyme toward both CpNpG and CpNpN sequences (N is A, C, or T.), a clear contrast to all known mammalian enzymes, that are CpG-specific. This implied that NtDRM1 was not the enzyme involved in the creation of methylation pattern, which is subsequently maintained by NtMET1. Instead, it was the enzyme that creates asymmetric methylation patterns.

In mammals, over 80% of methylated cytosines are found in symmetrical CpG dinucleotides, and all known enzymes are CpG-specific. In plants, methylation occurs at cytosine residues located in symmetric CpG and CpNpG, and also in asymmetric CpNpN sequences (Finnegan and Kovac 2000) in which methylation cannot be restored after DNA replication. This mechanistic difference between symmetric and asymmetric methylation indicates their physiological roles to differ each other. Methylation at CpG and CpNpG sites is frequently observed in transposons (Miura et al. 2001 Tompa et al. 2002), while asymmetric methylation is reported to occur during transgene silencing (Meyer and Heidmann 1994). The symmetric methylation can be maintained though DNA replication and over generations by

the action of maintenance methyltransferase. In contrast, the asymmetric methylation is not maintained, suggesting that it is involved in temporal changes in methylation status. Physiologically, RNA-directed DNA methylation was reported to be initiated by DRMs from genetic analyses using *Arabidopsis* T-DNA insertion lines (Cao and Jacobsen 2002a, Cao et al. 2003). The present study provides the first clear biochemical evidence for a catalytic specificity of the protein of this class, and for the existence of a positive mechanism of asymmetric methylations in plants. A further analysis on enzyme complex appeared to be necessary to clarify its molecular pathway.

Subsequently, I attempted to identify interacting components of NtDRM1. Genetic analyses using *Arabidopsis* mutants of DNA methylation suggested at least two methylation pathways; CpG methylation and non-CpG methylation. However, little is known on both pathways, except that a large protein complex was predicted to be involved. Although my trials to identify components of DRM is not complete, I hope that such a study will be of a help for understanding of essential framework of plant specific DNA methylation.

Overall, results of the current study, showing that DNA methylation status is interfered by environmental stresses, and created by *de novo* enzyme, will provide valuable information for physiological role of DNA methylation in plants.

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