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Functional characterization of a rice *de novo* DNA methyltransferase, *OsDRM2*, expressed in *Escherichia coli* and yeast

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ABSTRACT

DNA methylation of cytosine nucleotides is an important epigenetic modification that occurs in most eukaryotic organisms and is established and maintained by various DNA methyltransferases together with their co-factors. There are two major categories of DNA methyltransferases: *de novo* and maintenance. Here, we report the isolation and functional characterization of a *de novo* methyltransferase, named *OsDRM2*, from rice (*Oryza sativa* L.). The full-length coding region of *OsDRM2* was cloned and transformed into *Escherichia coli* and *Saccharomyces cerevisiae*. Both of these organisms expressed the *OsDRM2* protein, which exhibited stochastic *de novo* methylation activity *in vitro* at CG, CHG, and CHH di- and tri-nucleotide patterns. Two lines of evidence demonstrated the *de novo* activity of *OsDRM2*: (1) a 5′-CCGG-3′ containing DNA fragment that had been pre-treated with *OsDRM2* protein expressed in *E. coli* was protected from digestion by the CG-methylation-sensitive isoschizomer *Hpa*II; (2) methylation-sensitive amplified polymorphism (MSAP) analysis of *S. cerevisiae* genomic DNA from transformants that had been introduced with *OsDRM2* revealed CG and CHG methylation levels of 3.92–9.12%, and 2.88–6.93%, respectively, whereas the mock control *S. cerevisiae* DNA did not exhibit cytosine methylation. These results were further supported by bisulfite sequencing of the *18S rRNA* and *EAF5* genes of the transformed *S. cerevisiae*, which exhibited different DNA methylation patterns, which were observed in the genomic DNA. Our findings establish that *OsDRM2* is an active *de novo* DNA methyltransferase gene with conserved activity in both prokaryotic and eukaryotic non-host species.

1. Introduction

Covalently modified nucleotides have been discovered in almost all taxa of animals, plants, and fungi [1]. Cytosine methylation is the only known type of genomic DNA modification in eukaryotes and is one of the most common epigenetic markers [2,3]. In mammals, cytosine methylation occurs almost exclusively at the CG dinucleotide sequence [4]. In plants, cytosine nucleotides at CG, CHG, or CHH sequences can be methylated [5]. Cytosine methylation levels vary greatly between organisms: typically, 3–8% of cytosine nucleotides in vertebrates and 6–30% of cytosine nucleotides in plants are methylated [6]. The methylation of many cytosines can change throughout the life cycle and/or during ontogenic development, although both the levels and the patterns of cytosine methylation in organisms are usually faithfully maintained.

Cytosine methylation plays an essential role in maintaining the integrity and stability of the genome in all organisms that possess this epigenetic marker. Correlations between the tissue-specific expression of a number of genes and promoter hypomethylation have been observed in many plant species [7]. Two groups of enzymes are involved in the process of DNA methylation: DNA methyltransferases, which transfer methyl groups to C5 of the cytosine nucleotide, and DNA glycosylases [8–10], which remove methylated cytosines from DNA and replace them with unmethylated cytosines [11]. There are three known classes of methyltransferases in plants: MET1, a homologue of mammalian DNMT1; CMT3, a plant-specific DNA methyltransferase; and DRM2, a *de novo* methyltransferase [12,13]. MET1 is primarily responsible for the maintenance of established CG methylation [14,15]. CMT3 possesses a chromodomain catalytic motif and specifically maintains the methylation of hemimethylated CHG sites [16,17]. Both MET1 and CMT3 belong to the family of maintenance DNA methyltransferases [16,18]. DRM is a homologue of human DNMT3, it can methylate both unmethylated and hemimethylated cytosine nucleotides in any sequence context [19–21].

Cytosine methylation is often found in the rice genome [22], although information on DNA methyltransferases in this plant is limited. Recently, Moritoh et al. [23] used targeted knock-out mutation of the putative *de novo* methyltransferase gene in rice, which is homologous to the *Arabidopsis DRM2* gene and has been called *OsDRM2*, and found that the mutant (*drm2*/*drm2*) plants

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exhibited abnormal growth and development that would be consistent with altered cytosine methylation, suggesting that the *OsDRM2* gene plays a functional role in the methylation of the rice genome. However, as stated above, the three types of methyltransferases have overlapping functions; moreover, there are at least two members of the *DRM* members in the rice genome, thus complicating the functional classification of an individual gene in its endogenous host (rice).

The RNA-directed DNA methylation (RdDM) pathway is specific to plants and plays a critical role in establishing *de novo* methylation patterns [12]. The RdDM pathway requires several factors that are involved in siRNA biogenesis and metabolism [24,25]. Recent studies of the RdDM pathway and *de novo* methylation mutants have identified *drm2* mutant alleles [26]. In *Arabidopsis*, the *drm1*/*drm2*/*cmt3* triple mutant was shown to incorrectly express many genes [27] and exhibited pleiotropic developmental defects, including twisted leaves, a short stature and partial sterility [12]. In contrast, neither the *drm1*/*drm2* double mutant nor the *cmt3* single mutant displayed obvious morphological changes. The mutant phenotype of the *drm1*/*drm2*/*cmt3* triple mutant can be reverted by backcrossing the mutant plants with wild-type plants or by the transformation of a wild-type methyltransferase [28]. A loss-of-function mutation in the maize (*Zea mays*) ortholog of the *DRM2* gene was also produced; this mutant displayed severe defects in seed morphology during reproductive development, although morphological phenotypes were not observed during vegetative development [29]. This discrepancy may be attributed to the drastic changes in the methylation level of the endosperm that occurs in monocots but not dicots or to the dramatic differences in the genome size and the abundance of transposons between *Arabidopsis* and maize. In *S. cerevisiae*, both DNA methyltransferase and the proteins required to form the RISC are absent, and it is known that gene expression in this species of yeast lacks siRNA regulation [30]. This feature of *S. cerevisiae* makes it a suitable model for studying the specificity of the methyltransferase without the guiding role of siRNAs.

In this study, we isolated the *OsDRM2* gene from rice and performed a functional characterization using the alien host systems *S. cerevisiae* and *E. coli*. These two organisms are eukaryote and prokaryote, respectively, and both lack cytosine methylation. Thus, the results are not confounded by any endogenous host factors. We show that *OsDRM2* is a *bona fide* active *de novo* DNA methyltransferase gene that possesses a conserved function in unrelated foreign hosts, including both prokaryotes and eukaryotes.

2. Materials and methods

2.1. Materials

Full-length *OsDRM2* cDNA was generated by RT-PCR of total RNA extraction from immature rice leaves. The *E. coli* expression vector pET28a_*OsDRM2* and the budding yeast expression vector pB*OsDRM2* were constructed using the obtained cDNA. The *E. coli* strain BL21 (DE3) was obtained from Novagen. The yeast strain AH109 was obtained from Clontech Laboratories, Inc.

2.2. Cloning of OsDRM2

Several rice methyltransferase candidates were selected from the full-length rice cDNA databases of ChromDB and NCBI GenBank based on their sequence similarity and automated annotation. Their amino acid sequences were compared with well-characterized *Arabidopsis*, human and mouse *de novo* cytosine methyltransferase genes *AtDRM1*, *AtDRM2*, *hDNMT3a*, *hDNMT3b*, *mDNMT3a*, and *mDNMT3b* by creating alignments using ClustalW2 with the default parameters.

Total RNA was extracted from rice young leaf, reverse-transcribed and amplified, by using the following *OsDRM2* gene-specific primers with adaptors: 5′-CGAGCTCCCCGGGATGGTGG ACTGGGCTTCAGATA-3′ and 5′-CCGCTCGAGCTGCAGTCAAATGTA ATCGTAGAAGAGCGC-3′.

2.3. In vitro methylation activity of OsDRM2

The *E. coli* expression construct pET-28a_*OsDRM2* was chemically transformed into competent cells of strain BL21(DE3). The transformants were incubated with shaking until the cultures reached an OD_{600} of 0.8. The cultures were then supplemented with IPTG to a final concentration of 100 μM, and incubation with shaking was continued for an additional 2 h. Induced *E. coli* cells were collected and lysed. The lysate was loaded on a 10% SDS–PAGE gel.

E. coli strain BL21(DE3) containing the plasmid pET-28a_*OsDRM2* was induced using 200 μM IPTG for 20 h at 20 °C. The fusion *OsDRM2* protein was purified. A target DNA fragment was prepared by PCR amplification a 419-bp fragment from a cDNA clone (AK108034) which containing a *Hpa*II/*Msp*I [31] recognition site (CCGG) at its 126-bp position (the sequence is provided in Supplementary Table 1). The PCR primers sequences were as follows: sense 5′-AGCTGAAAATAACTCTACCTGATGG-3′ and anti-sense 5′-GCCCTCCCAGTCCAACCTA-3′. A total of 1 μg of column-purified PCR product was incubated with either 0.5 μl of purified *OsDRM2* protein or 0.5 μl of M. *Sss*I (a commercial CG methyltransferase, NEB #M0226S) in the supplied reaction buffer at 37 °C for 4 h. The treated PCR products were purified separately using a column; the eluted products were then digested with *Hpa*II for 2 h at 37 °C, and the digested products were run on a 1.5% agarose gel.

2.4. In vivo methylation activity of the OsDRM2 gene in S. cerevisiae

The budding yeast expression construct pB*OsDRM2* was transformed into *S. cerevisiae* strain AH109 using the LiCl method. The construct pBhDNMT3a was also transformed into *S. cerevisiae* as a positive control for the detection of methylation.

2.5. MSAP analysis of OsDRM2 methylation activity

MSAP is commonly used to assess genome-wide methylation by sampling unbiased genomic loci [32]. Five clones were chosen from each of the following groups: the untransformed *S. cerevisiae* AH109 (negative control), *S. cerevisiae* AH109 transformed with the plasmid pBhDNMT3a (positive control) and *S. cerevisiae* AH109 transformed with the plasmid pB*OsDRM2* (experimental sample). Genomic DNA was extracted from each culture. A total of 200 ng of genomic DNA from each sample was digested with either *Eco*RI + *Msp*I or *Eco*RI + *Hpa*II for 4 h. The adapters were then ligated for 4 h at 8 °C.

The MSAP procedure was performed as previously described [31,32] with modifications for silver-staining [33]. The DNA from different *S. cerevisiae* transformants were used as templates; after two rounds of amplification (the 16 pairs of PCR primers are provided in Supplementary Table 2), the PCR products were run on a 5% polyacrylamide gel and silver-stained. Bands that were differentially expressed in the various samples were recovered from the gel, and the presence or absence of the differentially expressed bands was scored as 1 or 0, respectively.

2.6. Confirmation of de novo methylated sites in S. cerevisiae by sequencing and by PCR

All of the bands that were absent in MSAP analysis of the pBO*sDRM2* transformants, but present in the untransformed negative controls, were recovered from the gel. The DNA was eluted from the gel for templates and was amplified with the same primers used in the MSAP analysis. The PCR products were ligated into the pMD18-T vector and sequenced.

After the DNA fragments from the bands were sequenced, BLAST searches were performed against the NCBI Genbank. PCR primers flanking the possible methylation sites (CCGG) were designed with one primer located inside the fragment and the other located in the adjacent genomic DNA. The same double-digested genomic DNAs that were used in both the negative control and the MSAP experiments were used as the PCR template. Methylation was confirmed by the generation of PCR products for the experimental samples and the lack/weak amplification for the negative-control samples.

2.7. Methylation pattern analysis using bisulfite sequencing

Two yeast housekeeping genes, *18S rRNA* and *EAF5*, were arbitrarily chosen to analyze their cytosine methylation pattern using bisulfite sequencing. The same batch of yeast genomic DNA samples that was used for MSAP analysis was treated with bisulfite. The treated DNA was used as the PCR template. Two fragments of each gene were analyzed (the primer sequences are provided in Supplementary Table 3). The PCR products were then ligated to the pMD18-T vector, and at least 10 colonies from each ligation reaction were sequenced.

3. Results

3.1. Identification and construction of a vector expressing the OsDRM2 gene

Based on sequence comparisons, the coding region of DMT706 (Genbank accession AK065147) was found to possess the highest level of similarity to the *Arabidopsis de novo* cytosine methyltransferase genes *AtDRM1* and *AtDRM2* (Supplementary Fig. 1). We subsequently designated DMT706 as the *OsDRM2* gene. The *E. coli* and *S. cerevisiae* expression vectors expressing *OsDRM2*, pET-28a_*OsDRM2*, pBO*sDRM2* and pBhDNMT3A were successfully constructed (Supplementary Fig. 2).

3.2. In vitro and in vivo methylation activity of a rice de novo DNA methyltransferase

The OsDRM2 protein is approximately 86 kDa when expressed in *E. coli* BL21 (DE3) cells (Supplementary Fig. 3). In the endonuclease protection assay (Fig. 1), the untreated DNA was completely digested by *Hpa*II (negative control, lane 3), and there was no evidence of *Hpa*II digestion of the CG methyltransferase (M. *Sss*I)-treated DNA (positive control, lane 4). Partially digested substrate was observed in the experimental lane, in which DNA was incubated with *OsDRM2* and then subjected to *Hpa*II digestion (lane 2), indicating that at least one of the two cytosines was methylated by *OsDRM2* in a portion of the DNA substrate.

3.3. MSAP analysis of S. cerevisiae genomic DNA from strains transformed with the OsDRM2 gene

There were 2138 clearly resolved bands on the MSAP gels; of these, 61 and 285 clear bands were lost in the pBhDNMT3A- and

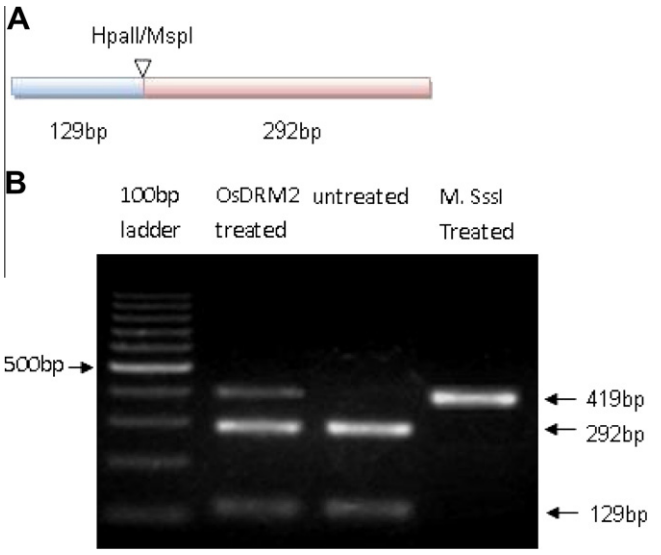

Fig. 1. Assay of *OsDRM2* methylation activity. (A) A 419-bp target DNA fragment containing a *Hpa*II/*Msp*I site. (B) *Hpa*II digestion of differently treated DNA fragments. Lane 1: 100-bp DNA ladder; lane 2: digestion of *OsDRM2* treated DNA; lane 3: negative control (untreated DNA); lane 4: positive control (digestion of M. *Sss*I treated DNA).

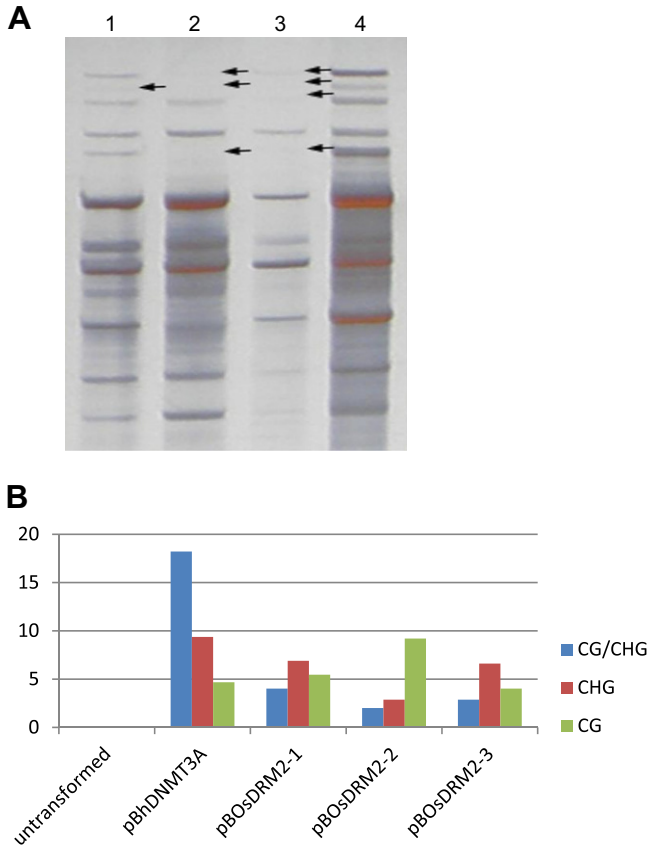

Fig. 2. MSAP analysis of genomic DNA from pBO*sDRM2*-transformed *S. cerevisiae*. (A) Different bands of transformed *S. cerevisiae* identified by the MSAP assay. Lanes 1–3: pBO*sDRM2-1*, pBO*sDRM2-2* and pBO*sDRM2-3* strains, which were transformed with the plasmid pBO*sDRM2* and digested with the endonucleases *Hpa*II and *Eco*RI. The arrows indicate bands that are absent in the experimental strain but present in the untransformed control. The missing bands correspond to CCGG sites where *de novo* methylation has occurred. Lane 4: Untransformed *S. cerevisiae* control DNA digested with *Hpa*II and *Eco*RI. (B) Frequency of cytosine methylation in *S. cerevisiae* transformants (%) as identified by MSAP analysis. All three types of cytosine methylation were identified in the genomic DNA from transformed strains.

pBO*sDRM2*-transformed samples, respectively (Fig. 2A). The positions where clear bands were visible in the untransformed control *S. cerevisiae* DNA and absent in the DNA from the strains carrying either pBhDNMT3A or pBO*sDRM2* were assumed to be methylated at the cytosine(s) of their 5′-CCGG-3′ sites by the foreign *de novo* methyltransferase. Compared to the untransformed control *S. cerevisiae* genomic DNA, there were 17.99% CG/CHG, 9.52% CHG and 4.76% CG methylation sites found in the pBhDNMT3A transformants and 2.08–3.94% CG/CHG, 2.88–6.93% CHG and 3.92–9.12% CG methylation sites found in the pBO*sDRM2* transformants (Table 1, Fig. 2B).

All of the bands that were absent in the pBO*sDRM2* transformants and some of the bands absent in the pBhDNMT3A transformants were cut from the corresponding untransformed sample gel and sequenced (Supplementary Table 4). A total of 62 and 16 bands corresponding to missing bands in the pBO*sDRM2* and pBhDNMT3A samples, respectively, were recovered from the untreated control. Eleven of the sequences identified by MSAP were chosen for validation by PCR using locus-specific primers (Supplementary Table 5). Seven of the 11 methylation sites in the pBO*sDRM2* transformants were confirmed. The PCR products that were amplified from the untransformed control were either absent or significantly weaker than those that were amplified from the pBO*sDRM2* transformants (Fig. 3), indicating that the CCGG sites within these fragments were methylated by the introduced *OsDRM2*. After performing a BLAST search against the NCBI GenBank, the functions of a portion of the methylated genes were identified (Table 2). These functional genes include transcription factors, a cell wall protein, a glutamate synthase, a phosphotyrosine-specific phosphatase protein, and uncharacterized genomic DNAs.

3.4. Bisulfite sequencing of S. cerevisiae genes in the transformants

The extent of methylation revealed by bisulfite sequencing varied for each gene. The majority of the cytosine nucleotides in the *18S rRNA* gene, regardless of whether they were part of the CG, CHG, or CHH, were highly methylated with methylation levels ranging from 89% to 96%. In contrast, virtually no methylation was detected for this gene in the untransformed control (Fig. 4A, Supplementary Fig. 4A).

The methylation levels of CG, CHG, and CHH, detected in two fragments of the *EAF5* gene were 2.20%, 5.13%, 4.08%, and 0.96%, 4.27%, 2.56%, respectively, whereas the levels in the untransformed controls were 1.54%, 6.25%, 5.00%, and 0.00%, 5.93%, 6.67%, respectively (Fig. 4A, supplementary Fig. 4B). The methylation of both the controls and experimental samples was approximately equal; because it was previously known that cytosine methylation does not occur in *S. cerevisiae* genomic DNA [30,34], we can safely spec-

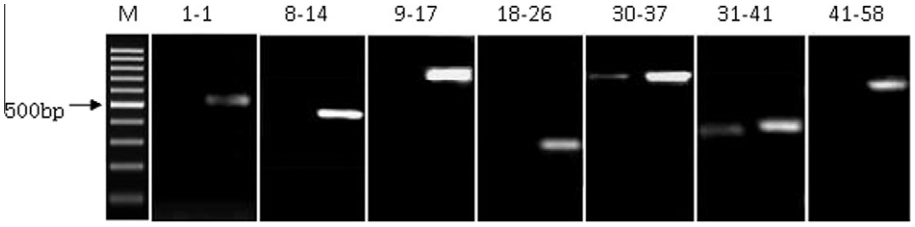

Fig. 3. Confirmation of selected methylated sites by PCR. M: 100-bp ladder; 1–1, 8–14, 30–37, 9–17, 31–41, 18–26, and 41–58 are PCR products that were amplified from MSAP templates using newly designed primers. Two lanes were used for the amplification products from each pair of primers. The left lane is the product of the primers from the negative control template; the absence of (or only very weak) bands indicate that the target CCGG site in the template was digested by *Hpa*II or *Msp*I. The right lane is the product from different pBO*sDRM2* transformants; the CCGG site inside of the fragment was fully or partially protected from *Hpa*II or *Msp*I digestion by *de novo* cytosine methylation.

Table 1
Levels of cytosine methylation in the *S. cerevisiae* transformants.

Methylation site	Untransformed	pBhDNMT3A	pBO*sDRM2-1*	pBO*sDRM2-2*	pBO*sDRM2-3*
CG/CHG	0	34	25	13	20
CHG	0	18	44	18	46
CG	0	9	35	57	27
Total changed	0	61	104	88	93
Total analyzed	0	189	635	625	689

Table 2
Identified methylation targets.

ID	BLAST result	*E*-value
1–1	NM_001182540.1 *S. cerevisiae* S288c Mcm1p (MCM1)	3e-92
8–14	NM_001179594.1 *S. cerevisiae* S288c TFIIE large subunit	5e-74
9–17	FN393077.1, *S. cerevisiae* EC1118 chromosome XI	5e-63
18–26	AB110101.1 *S. cerevisiae* K701-AWA1 gene for cell wall protein Awa1p	1e-55
30–37	NM_001180231.1 *S. cerevisiae* S288c Glt1p (GLT1)	1e-110
31–41	NM_001180290.1 *S. cerevisiae* S288c Phosphotyrosine-specific protein phosphatase	4e-80
41–58	*S. cerevisiae* S288c Sas5p (SAS5)	6e-62

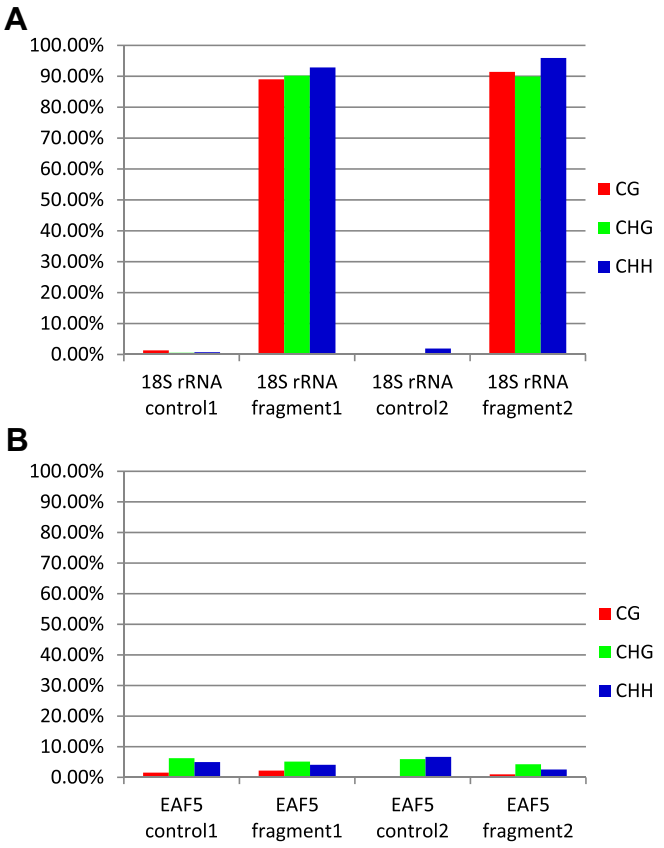

Fig. 4. Results of bisulfite sequencing of the *18S rRNA* and *EAF5* genes. (A) Methylation levels of CG, CHG and CHH nucleotide patterns in two fragments of the *18S rRNA* gene. (B) Methylation levels of CG, CHG and CHH nucleotide patterns in two fragments of the *EAF5* gene.

ulate that there is no methylation in the *EAF5* gene of pB*OsDRM2* transformants.

4. Discussion

There are approximately 10 DNA methyltransferase homologues in the rice genome. In this study, we cloned and expressed one of these homologues, *OsDRM2* (DMT706), in both *E. coli* and *S. cerevisiae*. *De novo* methylation activity was present in the transformants of both organisms. The recombinant *OsDRM* protein can methylate any type of cytosine nucleotide, including those in the patterns CG, CHG, and CHH. These results were similar to the observation of *Arabidopsis DRM2* [12,17].

In most plants, cytosine methylation is involved in the RdDM pathway, and siRNAs are needed to guide the cytosine methylation of either endogenous or exogenous DNA [12,35]. Although cytosine methylation is a common DNA modification, there are a few organisms in which DNA methylation levels are very low (*Drosophila melanogaster*) [36] or even undetectable (*S. cerevisiae*) [34]. It is likely that these organisms lost their methyltransferases and RNAi pathway during the course of evolution. The very low or undetectable levels of genomic DNA methylation of these organisms make them ideal models for studying the activity and characterizing methyltransferases. In this study, evidence of cytosine methylation was observed in the genomic DNA of *S. cerevisiae* that expressed the *OsDRM2* gene based on MSAP assays, bisulfite sequencing and a test of cytosine methylation by endonuclease digestion. This evidence demonstrated that the *OsDRM2* protein can not only methylate cytosine nucleotides *in vivo* in an RdDM pathway-free cellular environment but can also methylate cytosine nucleotides *in vitro*.

In the bisulfite sequencing experiments, two *S. cerevisiae* housekeeping genes, *18S rRNA* and *EAF5*, were used, and distinct methylation patterns were observed. The former was heavily methylated at almost every cytosine, whereas the latter exhibited virtually no detectable methylation. This difference may occur because some genes, especially in their promoter regions, are sensitive to cytosine methylation; methylation and de-methylation could inactivate and control the expression of these genes [11]. In *S. cerevisiae*, cytosine methyltransferase, Dicer or AGO necessary for the RISC are absent, and this yeast species is free of cytosine methylation and siRNA regulation [24]. As shown in the MSAP assay, the methylation levels and patterns of CG, CHG and CHH were identical for the *EAF5* gene in both the control and experimental samples. In this case, each cytosine in the genome might be equally methylated in the early generations; however, the *18S rRNA* gene promoter could be insensitive to cytosine methylation, whereas the *EAF5* gene promoter could be sensitive. In this case, although the two genes were initially methylated to the same extent, the majority of cells that possessed a methylated *EAF5* gene were unable to compete with the small number of unmethylated individuals and were eliminated from the population. In subsequent generations, the redundant maintenance methylation activity of the *OsDRM2* protein maintains the methylation pattern established in the earlier generations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.067.

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