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Research article

Purification and characterization of rice DNA methyltransferase

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ABSTRACT

Epigenetic modification is essential for normal development and plays important roles in gene regulation in higher plants. Multiple factors interact to regulate the establishment and maintenance of DNA methylation in plant genome. We had previously cloned and characterized DNA methyltransferase (DNA MTase) gene homologues (*OsMET1*) from rice. In this present study, determination of DNA MTase activity in different cellular compartments showed that DNA MTase was enriched in nuclei and the activity was remarkably increased during imbibing dry seeds. We had optimized the purification technique for DNA MTase enzyme from shoots of 10-day-old rice seedlings using the three successive chromatographic columns. The Econo-Pac Q, the Hitrap-Heparin and the Superdex-200 columns yielded a protein fraction of a specific activity of 29, 298 and 800 purification folds, compared to the original nuclear extract, respectively. The purified protein preferred hemi-methylated DNA substrate, suggesting the maintenance activity of methylation. The native rice DNA MTase was approximately 160–170 kDa and exhibited a broad pH optimum in the range of 7.6 and 8.0. The enzyme kinetics and inhibitory effects by methyl donor analogs, base analogs, cations, and cationic amines on rice DNA MTase were examined. Global cytosine methylation status of rice genome during development and in various tissue culture systems were monitored and the results suggested that the cytosine methylation level is not directly correlated with the DNA MTase activity. The purification and characterization of rice DNA MTase enzyme are expected to enhance our understanding of this enzyme function and their possible contributions in Gramineae plant development.

1. Introduction

DNA methylation in plants plays two fundamental roles, defending against invasive DNA elements and regulating gene expression. Cytosine-5 DNA methylation is an important epigenetic modification and multiple factors interact to regulate the establishment and maintenance of DNA methylation in plant genome. It has been shown to be an epigenetic regulation of gene expression in tissue specific and developmental stage dependent manner [6,12]. Majority of cytosine-5 DNA methylation in nucleus DNA is found at CG dinucleotides for vertebrates and both at CG and CNG sequences for plants [28]. Enzymes that catalyze the methyl transfer reaction are so-called, cytosine-5 DNA methyltransferases (DNA MTase, EC 2.1.1.37). Cytosine-5 DNA methyltransferases catalyze the transfer of a methyl group from S-adenosyl-methionine (AdoMet) onto cytosine residues in specific sequences of duplex DNA, with production of 5-methylcytosine and S-adenosyl-homocysteine (AdoHcy). This reaction is irreversible. It has been shown that methylated cytosines can interact with several proteins, such as *m*ethyl-*b*inding *d*omain containing proteins (MBD) and MeCP2, resulting in transcriptional repression [22,41]. Different methylation sites require different cytosine methyltransferases, which contribute to the modification of chromatin structure and mediate epigenetics with chromatin remodeling enzymes and histone modifying factors. Although enzymatic methylation of eukaryotic DNA has been under investigation for more than 50 years, we are still far from a comprehensive understanding of the functional role of this modification of the genome.

Several *DNA MTase* genes have been cloned and sequenced. They can be classified into five classes, based on their functions and sequence homology. Dnmt1/MET1 class has predominantly a maintenance methylation activity found in both animals and plants [9,20,30]. Dnmt2 class only contains a methyltransferase domain and does not seem to have significant function based on *in vivo* and *in vitro* study [26]. Dnmt3 class, which is responsible for *de novo* methylation, has been isolated in both mammalians and

Abbreviations: AdoHcy, S-adenosyl-homocysteine; AdoMet, S-adenosyl-methionine; 5Aza, 5-azacytosine; BA, N^6-benzyladenine; CMT, chromomethylase; 2,4-D, 2,4-Dichlorophenoxyacetic acid; DNA MTase, cytosine-5 DNA methyltransferases; MBD, methyl-binding domain; DTT, dithioreitol; PMSF, phenylmethanesulphonyl fluoride.

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plants [5,25]. The chromomethylase (CMT) class, found only in plants, demonstrates the methylation activity on CNG site [2,13,27]. The last class is a chloroplast specific DNA MTase, which has been recently cloned in unicellular green algae, *Chlamydomonas reinhardii* (CrMET1). CrMET1 has an important role in maternal inheritance of chloroplast genes in *C. reinhardtii* [24].

MET1 homologues have been characterized in various plants, such as carrot, pea, tobacco, maize [3,21,28] including our work on rice [37], while CMT family members have been identified only in *Arabidopsis* and maize [2, 27]. In eukaryotes, the predicted amino acid sequence in the *Dnmt1/MET1* class comprises of two domains, a regulatory domain and a methyltransferase catalytic domain [8]. The eukaryotic methyltransferase domain of Dnmt1/MET1s contains eight out of ten conserved motifs found in prokaryotic DNA MTases and has been shown to have catalytic activity [31]. The regulatory domain of Dnmt1/MET1s contains several functional regions, including two *b*romo *a*djacent *h*omology (BAH) domains [4], a region for targeting the enzyme to DNA replication fork and a *n*uclear *l*ocalizing *s*ignal (NLS) [1]. The BAH domains are commonly found in proteins involved in gene silencing and DNA replication [4]. It has been shown that the regulatory domain of Dnmt1/MET1 can interact with histone deacetylase, resulting in transcriptional repression [10]. While *Arabidopsis* carries only one copy of the *MET1* gene [16], we reported that rice bears two *MET1* copies, *OsMET1-1* (AF462029) and *OsMET1-2* (BK001405), on chromosomes 3 and 7, respectively [37]. Based on the comparison of the rice genomic sequence with partial reverse transcription-polymerase chain reaction (RT-PCR) and 3′ rapid amplification of cDNA ends (RACE) analyses, we have assigned that *OsMET1-1* and *OsMET1-2* comprise 12 and 11 exons and encode putative proteins of 1522 and 1497 amino acids, respectively, and that the accumulation of the *OsMET1-2* mRNA is abundant in callus, young (10-day-old) root, and inflorescence, whereas only the *OsMET1-1* mRNA was slightly accumulated in young leaf, in which virtually no *OsMET1-2* transcripts were detectable.

Although several genes in *DNA MTase* family have been cloned, very little is known about the enzymatic properties of these proteins in rice. Up to now, only a few DNA MTase proteins have been purified from algae and plants [32,38,43]. Rice DNA MTase with a molecular weight of 54,000 had been previously purified from cultured rice cells with 380 purification folds [11] and showed strong preference on hemi-methylated DNA substrate. In this work, DNA MTase activity and global cytosine methylation status of rice plant during development and in various tissue culture systems were monitored. A DNA MTase with maintenance methylation activity was isolated from shoots of rice seedlings with much higher purification folds and its kinetic properties were examined in a greater detail.

2. Results

2.1. DNA MTase activity and methylation status of tissues at various stages and culture systems

The assay conditions, including pH, temperature, reaction time and DNA substrate, were initially optimized. Crude enzyme, prepared from samples harvested at different ages, was extracted and the DNA MTase activity was measured in various conditions. The data indicated that the DNA MTase activity was lowest in dry mature embryos and highest in 10-day-old shoots. The optimal condition was the reaction, carried out at pH 7.6, 25 °C and enzyme activity reached the highest level when the reaction was carried out for 1 h. These conditions were used as standard conditions for further experiments. DNA MTase activity and the amount of methylated cytosines of different tissues of rice, grown in various conditions, were investigated. DNA MTase activity was dramatically increased, during imbibing dry seeds for two days (Fig. 1A). The highest level of DNA MTase activity was observed in shoots of 10-day-old seedlings, hydroponically grown under illumination (Fig. 1A). However, the significant difference in enzyme activity between 5- and 10-day-old shoots was not observed when the seedlings were grown in the dark. On the other hand, light did not show any significant effect on DNA MTase activity in roots. Comparison of methylated cytosines in different tissues, dry embryos contained the highest amount of the methylated cytosines, which was drastically reduced three days after germination (Fig. 1B). Once shoots and roots developed from germinated embryos, shoots contained smaller amount of methylated cytosines than roots.

2.2. DNA MTase activity in different cellular compartments

Since rice shoots at 10-day-old stage contain highest DNA MTase activity, they were used as starting materials for purification of DNA MTase. Before enzyme purification, different cellular compartments, including homogenate, nuclei, plastid, and mitochondria fractions, were prepared from 10-day-old seedlings, grown either under light or dark conditions, and investigated for DNA MTase activity. To measure DNA MTase activity, the 10-day-old rice genomic DNA was used as a substrate. The data showed that DNA MTase activity was significantly enriched in the nuclei fractions of seedling, grown under the light condition (Fig. 2A). Growing in the dark inhibited DNA MTase activity in shoots, but the activity was not altered in roots. This could be observed in both homogenate and nuclei fractions. The observation that enzyme activity was undetectable in mitochondria and slightly found in plastids could be resulted from at least three possibilities: firstly, DNA MTase may not exist in these organelles; secondly, cytosine methylation level in these organelles is very low and therefore the activity of organellar DNA MTases are kept at relatively low level; or thirdly, genomic DNA used in the experiment may not be a preferred DNA substrate for organellar DNA MTases. Since the DNA MTase that functions in the chloroplast of *C. reinhardtii* has been isolated [24], we can probably exclude the first possibility.

Next, we examined global cytosine methylation status of the three organelles, nuclei, plastid and mitochondria. Results showed that cytosines were rather highly methylated in plastids of roots and etiolated shoots, but remained unmethylated in plastid DNA from photosynthetic shoots and mitochondria of all tissues investigated (Fig. 2B). We then tested the third possibility by using plastid and mitochondria DNA as a substrate for plastid and mitochondrial DNA MTase, respectively. Chloroplast DNA was prepared from light-grown shoots as it has undetectable amount of methylated cytosines. We observed 24–27 folds increase in specific activity of DNA MTase isolated from root and etiolated shoot (Fig. 2C in comparison to Fig. 2A). Nevertheless, DNA MTase isolated from the shoot of light-grown seedlings still has relatively low activity even when the plastid DNA was used as a substrate. This data revealed the presence of DNA MTase in the plastid and it preferred plastid DNA as a substrate rather than genomic DNA. In contrast to plastid, no enzyme activity was observed, although mitochondrial DNA was used as a substrate, allowing us to exclude the third possibility (Fig. 2C).

2.3. Extraction of DNA MTase

It has been reported that DNA MTase is extremely unstable [11,38,43], then preservation of DNA MTase during purification process, was essentially performed. A supplement of preservative reagents and protease inhibitors, together with storage