N^6-Adenine DNA-methyltransferase in wheat seedlings

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Abstract The N^6-adenine DNA-methyltransferase was isolated from the vacuolar vesicle fraction of wheat coleoptiles. In the presence of *S*-adenosyl-L-methionine the enzyme de novo methylates the first adenine residue in the TGATCA sequence in the single- or double-stranded DNA substrates but it prefers single-stranded structures. Wheat adenine DNA-methyltransferase (*wadmtase*) is a Mg^{2+}- or Ca^{2+}-dependent enzyme with a maximum activity at pH 7.5–8.0. *Wadmtase* seems to be responsible for mitochondrial DNA modification that might be involved in the regulation of replication of mitochondria in plants.

Key words: Adenine DNA-methyltransferase; Aging; Apoptosis; DNA methylation; N^6-Methyladenine; Mitochondrion; Mitochondrial DNA replication; Plant; Wheat

1. Introduction

N^6-Methyladenine (m^6A) is a minor base formed as a result of methylation of the DNA adenine residues with specific adenine DNA-methyltransferases and is common in bacterial DNA [1]. This enzymatic DNA modification in bacteria is an essential element of the host modification and restriction systems and it is involved often in the regulation of replication and transcription. m^6A was also found in DNA of eukaryotes such as protozoa [2–7], slime molds [8], fungi [9], algae [10] and higher plants [11]. While the attempts to isolate this base from different animal DNA were unsuccessful [12] there is some indirect evidence of the possible presence of m^6A in mammalian DNA [13,14]. The proper plant and animal adenine DNA-methyltransferases are yet unknown; the origin of m^6A in these eukaryote DNA is still unclear and the functional role of this adenine methylation is still obscure. Nevertheless, there are some data showing that the character of transcription of many plant genes, the morphology and development of transformed plant cells and the plants are drastically changed after the introduction of genetic constructs with expressed genes of prokaryotic adenine DNA-methyltransferases. For example, the introduction and expression of the bacterial adenine DNA-methyltransferase (*dam*) gene is accompanied by methylation of the GATC sequence in DNA of transgenic tobacco plants and changes in the leaf and inflorescence morphology [15]. Moreover, *dam* methylation of promoter regions in constructs with plant genes for alcohol dehydrogenase, ubiquitin and actin results in an increase in the transcription of these genes in tobacco and wheat tissues [16]; this preliminary methylation of promoters is also important for the transcription of *PR1* and *PR2* genes in constructs introduced into tobacco protoplasts by electroporation [17]. Hence, the methylation of adenine residues in DNA may control gene expression in plants. This all means that adenine DNA methylation in plants is not an incidental or unexpected event, and it may play a significant physiological role.

We are the first to isolate N^6-adenine DNA-methyltransferase (*wadmtase*) from plant and describe some of its properties.

2. Materials and methods

2.1. Plant material

Etiolated seedlings of the wheat winter variety Mironovskaya 808 were grown at 26°C in a thermostat as described [18]. Coleoptiles of the 8-day-old seedlings were ground in a mortar with pestle at 4°C in buffer A containing 50 mM Tris–HCl, pH 7.5, 0.4 M sucrose, 5 mM EDTA and 1 mg/ml bovine serum albumin (BSA). The homogenate was filtered through two gauze layers and then centrifuged for 20 min at $600\times g$. The sediment containing nuclei was discarded. The supernatant obtained was centrifuged at $1700\times g$ for 20 min and the sediment of vesicles [18] was collected and suspended in buffer A without BSA and centrifuged again under the same conditions. The vesicle fraction obtained was then washed twice with the same buffer.

2.2. Enzyme isolation and purification

The vesicle fraction was extracted with buffer B containing 50 mM Tris–HCl, pH 7.5, 0.8 M sucrose, 5 mM EDTA and 0.35 M NaCl at 4°C with vigorous shaking for 15–20 min. The crude extract was applied onto a DEAE–cellulose column (Whatman DE-52) equilibrated with 50 mM Tris–HCl buffer, pH 7.5. Proteins were eluted with 100 ml linear NaCl gradient (0–0.5 M). Fractions with N^6-adenine DNA-methyltransferase activity eluted with 0.22–0.25 M NaCl were collected and applied to chromatography on DNA-cellulose. Proteins were eluted from a DNA-cellulose column with 50 ml linear NaCl gradient (0–0.5 M) in 50 mM Tris–HCl buffer, pH 7.5, and fractions with N^6-adenine DNA-methyltransferase activity eluted between 0.28 and 0.32 M NaCl were collected. These fractions were combined and applied to subsequent gel filtrations on Sephadex G-200 and Sephadex G-50 equilibrated with 0.05 M Tris–HCl buffer, pH 7.5. The protein fraction with adenine DNA-methyltransferase activity was collected, applied onto a C4 high-performance liquid chromatography (HPLC) column (Nucleosil) and chromatographed with a linear water–60% acetonitrile gradient in the presence of 0.1% trifluoroacetic acid.

2.3. DNA methylation assay

Methylation of DNA (2–10 μg) or oligonucleotides (10 μg) with purified enzyme (2–10 μg) was carried out in 10 μl 50 mM Tris–HCl buffer, pH 7.5 containing $MgCl_2$ and $CaCl_2$ (5 mM each) and 5–10 μM [^{3}H-*methyl*]*S*-adenosyl-L-methionine ([^{3}H-*methyl*]AdoMet; specific radioactivity 5–15 Ci/mmol, Amersham) for 2 h at 37°C. DNA and oligonucleotides isolated from the reaction mixture were

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Abbreviations: AdoMet, *S*-adenosyl-L-methionine; mtDNA, mitochondrial DNA; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; *wadmtase*, wheat N^6-adenine DNA-methyltransferase

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hydrolyzed with snake venom phosphodiesterase+alkaline phosphomonoesterase (Sigma) under standard conditions and the deoxyribonucleosides obtained were fractionated by cellulose thin-layer chromatography (TLC). N^6-Methyldeoxyadenosine was eluted with water and its radioactivity was measured.

2.4. Assay of accessibility of the in vitro methylated DNA to restriction endonucleases

The λ phage DNA (2 μg) was methylated with *wadmtase* in the presence of 10 μM non-radioactive AdoMet (Sigma) as described above, then DNA was deproteinized, the respective restriction endonuclease (15–20 U) was added and the mixture was incubated for 2 h at 37°C in the manufacturer's buffer; DNA and its hydrolysis products were then electrophoresed in 0.7% agarose gel under standard conditions. *Mbo*I, *Eco*RI, *Bcl*I and *Rsa*I were used as restriction endonucleases sensitive to adenine methylation. The commercial enzymes (Sibenzyme, Russia) used do not hydrolyze respective sequences when the adenine residue is methylated, $G^{m6}ATC$ (*Mbo*I), $G^{m6}AATTC$ (*Eco*RI), $TG^{m6}ATCA$ (*Bcl*I) and $GT^{m6}AC$ (*Rsa*I) [19,20]. To be sure that methylation but not specific binding of wheat protein to DNA is responsible for protection against DNA hydrolysis with *Bcl*I restriction endonuclease, the mixture after methylation was treated exhaustively with pronase (Sigma) until pronase was completely inactivated before endonuclease was added.

2.5. Protein electrophoresis and concentration measurement

Electrophoresis of proteins was carried out in 12.5% polyacrylamide gel (PAG) in the presence or absence of sodium dodecyl sulfate (SDS) [21]. Protein concentration was determined by Bradford's method [22].

3. Results and discussion

Relatively low adenine DNA-methyltransferase activity measured by incorporation of label from AdoMet into calf thymus DNA was detected in crude extracts from coleoptiles and leaves of etiolated wheat seedlings. In coleoptiles it increased with age by about five-fold (from 74 000 ± 500 cpm/mg protein in 3-day-old seedlings to 459 000 ± 600 cpm/mg protein in 8-day-old seedlings). High specific enzymatic activity is localized in the fraction of cellular vesicles [18] containing actively replicating mitochondria but not in nuclei or free (nonvesicular) mitochondria. The specific enzyme activity measured under standard conditions in extracts from vesicular, nuclear, and mitochondrial fractions isolated from coleoptiles of 8-day-old seedlings was about 630 000, 83 600 and 130 000 cpm/mg protein/μg DNA added to reaction mixture, respectively. Therefore, the vesicle fraction isolated from 8-day-old coleoptiles was used for enzyme isolation and purification.

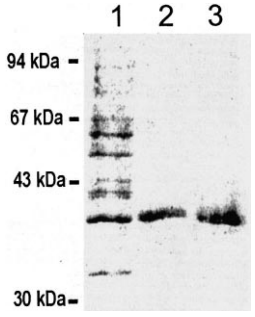

Fig. 1. SDS–PAGE of protein fractions with *wadmtase* activity isolated from vesicular fraction of 8-day-old wheat coleoptiles. 1: Extract from isolated vesicles. 2: Enzymatic protein fraction from vesicular extract purified subsequently by DEAE–cellulose chromatography, DNA-chromatography and Sephadex G-200 and G-50 filtrations. 3: Fraction 2 purified by HPLC in the 0–60% acetonitrile gradient in 0.1% trifluoroacetic acid (isolated as a single peak).

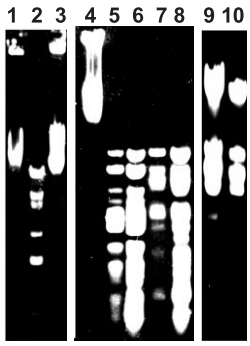

Fig. 2. Electrophoregrams in 0.7% agarose gel of λ phage DNA intact or methylated with *wadmtase* (fraction 2, Fig. 1) and the products of their hydrolysis by various restriction endonucleases. 1: λ phage DNA, 2: λ phage DNA+*Bcl*I, 3: λ phage DNA methylated with *wadmtase* in the presence of non-radioactive AdoMet+*Bcl*I, 4: λ phage DNA, 5: λ phage DNA methylated with *wadmtase*+*Mbo*I, 6: λ phage DNA+*Mbo*I, 7: λ phage DNA+*Rsa*I, 8: λ phage DNA methylated with *wadmtase*+*Rsa*I, 9: λ phage DNA+*Eco*RI, 10: λ phage DNA methylated with *wadmtase*+*Eco*RI.

The protein fraction obtained from crude extracts by gel filtration on Sephadex G-50 was purified by about 2200-fold and it possessed a high specific adenine DNA-methyltransferase activity equal to 1.4×10^6 cpm/mg protein. After HPLC on a C4 column this protein fraction was represented in SDS–PAGE as a band with an apparent molecular mass of about 39 kDa (Fig. 1).

The enzyme methylates DNA with the formation of m^6A residues: a compound with UV-light absorption data corresponding to that of N^6-methyldeoxyadenosine (λ_{max} 262 nm; λ_{min} 236 nm) was isolated by TLC from calf thymus DNA treated with *wadmtase* in the presence of non-radioactive AdoMet and hydrolyzed then to deoxyribonucleosides by snake venom phosphodiesterase and alkaline phosphatase under standard conditions.

We have investigated whether methylation with *wadmtase* may protect DNA against restriction endonucleases that are sensitive to methylation of adenine residues. The methylation with *wadmtase* entirely protects λ phage DNA against *Bcl*I restriction endonuclease but not all other nucleases used (Fig. 2). This means that *wadmtase* recognizes hexanucleotide TGATCA (but not the tetranucleotide *dam* sequence GATC) and methylates the adenine residue. The distribution patterns of fragments produced by *Mbo*I from intact and pretreated with *wadmtase* λ phage DNA are similar but not identical. Careful comparative analysis of many respective electrophoregrams always showed that after DNA methylation with *wadmtase* some lower molecular mass fragments in DNA hydrolysates are absent compared with products of intact DNA hydrolysis by *Mbo*I. This may indicate that DNA methylation with *wadmtase* seems to protect from *Mbo*I hydrolysis only the GATC sites that are constituent parts of the target TGATCA sequence recognized by *wadmtase*.

Various synthetic deoxyribooligonucleotides (oligos) were also used as substrates in the methylation carried out by *wadmtase*. The enzyme effectively catalyzes the incorporation of radioactivity from [^{3}H-*methyl*]AdoMet into separate single-stranded oligonucleotides 1, 2 and 4 with a target site TGATCA (Table 1); it is about two-fold less efficient when mixture

Table 1
Radioactivity of N^6-methyldeoxyadenosine isolated from various substrates methylated with wheat adenine DNA-methyltransferase in the presence of [^{3}H-methyl]AdoMet

Substrate	Radioactivity (cpm)
(1) ggg**tgatca**ggg	11 640 ± 300
(2) ccc**tgatca**ccc	10 070 ± 240
(3) ggg**tgatca**ggg+ccc**tgatca**ccc (annealed)	7590 ± 280
(4) gcg**tgatca**cgc	11 140 ± 320
(5) gcg**tgatca**cgc (annealed)	6070 ± 300
(6) gg**tgttca**gg	160 ± 40
(7) λ phage DNA	9980 ± 320
(8) poly-d(AT)	160 ± 40
(9) poly-d(IC)	40 ± 10
(10) poly-A	190 ± 40

3 (1:1 ratio) of the complementary oligonucleotides (1+2) or the self-complementary oligonucleotide 4 annealed at 60°C for 10 min were used. Essential radioactivity was incorporated also in intact λ phage DNA (Table 1). Thus, the enzyme de novo methylates both single- and double-stranded DNA substrates but it prefers single-stranded structures. Substitution of the first adenine residue for thymine in the recognition site TGATCA makes the substrate completely inert in the methylation (oligo 6). Thus, the first adenine residue is a target base to be modified in the recognition sequence. Methylation of the first adenine residue in the TGATCA sequence makes it resistant to *Bcl*I hydrolysis [19,20] (Fig. 2). Monotonous oligonucleotides such as poly-d(AT) or poly-d(IC) and polyribonucleotide poly-A were inactive as substrates in the methylation reaction.

Wadmtase is a Mg^{2+}- or Ca^{2+}-dependent enzyme (Fig. 3). About 2–3 mM $CaCl_2$ or $MgCl_2$ in the reaction mixture is needed for the maximal DNA methylation activity. The enzyme is strongly inhibited by EDTA. The optimal concentration of AdoMet in DNA methylation with *wadmtase* is about 10 μM (Fig. 4). The enzyme is active in a relatively wide pH interval with a maximum activity at pH 7.5–8.0 (Fig. 5) and it seems to be more stable at acid than at alkaline pH values.

It seems that in plants the methylation substrate found for N^6-adenine DNA-methyltransferase is mitochondrial but not nuclear DNA; it is known that m^6A (about 1% of DNA adenine) is present in wheat mitochondrial DNA (mtDNA) ($\rho = 1.718$ g/cm^3) but it is not found in nuclear DNA ($\rho = 1.710$ g/cm^3) [18].

By computing the GenBank data it was found that open reading frames (ORF) for putative proteins that possess

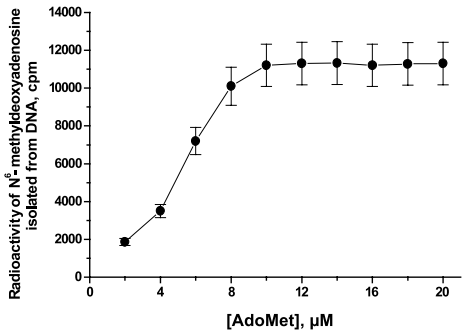

Fig. 4. Dependence of the *wadmtase* activity on the AdoMet concentration.

a high degree of homology with prokaryotic DNA-(amino)methyltransferases are present in genomes of *Arabidopsis thaliana* (GenBank, BAB02202.1), *Leishmania major* (GenBank, AAF14648), *Saccharomyces cerevisiae* (GenBank, NP_014517), *Schizosaccharomyces pombe* (GenBank, CAB38506), *Drosophila melanogaster* (GenBank, AAF52125), *Caenorhabditis elegans* (GenBank, AAF36002) and *Homo sapiens* (GenBank, CAB92726; AAG14959) [23]. Conservative motifs typical for bacterial DNA-(amino)methyltransferases are detected in the amino acid sequences of these putative proteins that are found to be encoded in nuclear DNA [23]. In mitochondrial genomes including few fully sequenced higher plant mtDNA the nucleotide sequences essentially homologous to genes of prokaryotic DNA-(amino)-methyltransferases were not detected [23]. Even though DNA-methyltransferases are very conservative [24–26], we assume that *wadmtase* encoded in the wheat nuclear DNA may be homologous to the *A. thaliana* ORF [27] (GenBank, BAB02202.1) which might be ascribed to putative adenine DNA-methyltransferases [23]. It turned out that the hypothetical protein BAB02202.1 of *A. thaliana* might have on the N-end a signal peptide for mitochondrial transportation, but it is classified by the program used as a secretory signal peptide (*RC* 3, cleavage site located after Ala21 in the sequence Glu-Ala↓Leu-Ala) [23]. Similarly, the putative protein AAF52125 of *D. melanogaster* might also have a signal peptide on the N-end (*RC* 3, the cleavage is located after Glu43 in the sequence Phe-Glu↓Leu-Tyr) [23].

It seems that m^6A detected earlier in wheat mtDNA [28] is due to modification with the enzyme *wadmtase* described. Even though this enzyme is found in vesicles with mitochon-

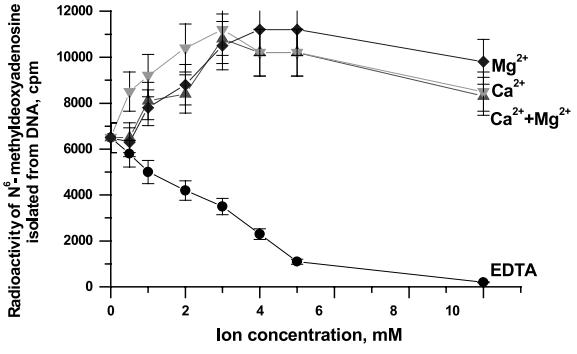

Fig. 3. Dependence of the *wadmtase* activity on concentration of divalent ions and EDTA.

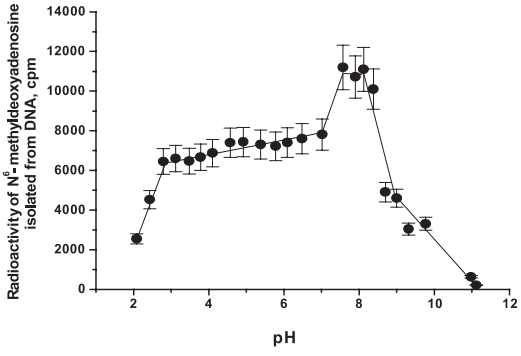

Fig. 5. The pH dependence of the *wadmtase* activity.

dria actively replicating DNA in apoptotic plant cells, its maximal activity is associated with mtDNA replication and it prefers to methylate single-stranded DNA, the *wadmtase* described seems to operate mainly with replicating mtDNA. Similar to the known *dam* enzyme controlling plasmid replication in bacteria, *wadmtase* seems to control replication of mtDNA that is represented mainly by circular molecules [29]. As mitochondria could evolutionary be of bacterial origin, the bacterial control for plasmid replication by adenine DNA methylation seems to be acquired by plant cell and it is probably used for the control of mitochondria replication.

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