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Expression and function of *AtMBD4L*, the single gene encoding the nuclear DNA glycosylase MBD4L in Arabidopsis

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ABSTRACT

DNA glycosylases recognize and excise damaged or incorrect bases from DNA initiating the base excision repair (BER) pathway. Methyl-binding domain protein 4 (MBD4) is a member of the HhH-GPD DNA glycosylase superfamily, which has been well studied in mammals but not in plants. Our knowledge on the plant enzyme is limited to the activity of the Arabidopsis recombinant protein MBD4L *in vitro*. To start evaluating MBD4L in its biological context, we here characterized the structure, expression and effects of its gene, *AtMBD4L*. Phylogenetic analysis indicated that *AtMBD4L* belongs to one of the seven families of HhH-GPD DNA glycosylase genes existing in plants, and is unique on its family. Two *AtMBD4L* transcripts coding for active enzymes were detected in leaves and flowers. Transgenic plants expressing the *AtMBD4L:GUS* gene confined GUS activity to perivascular leaf tissues (usually adjacent to hydathodes), flowers (anthers at particular stages of development), and the apex of immature siliques. MBD4L-GFP fusion proteins showed nuclear localization *in planta*. Interestingly, overexpression of the full length MBD4L, but not a truncated enzyme lacking the DNA glycosylase domain, induced the BER gene *LIG1* and enhanced tolerance to oxidative stress. These results suggest that endogenous MBD4L acts on particular tissues, is capable of activating BER, and may contribute to repair DNA damage caused by oxidative stress.

1. Introduction

Plants often face stressful conditions that damage their genomes. Chemical pollutants, UV light, ozone, ionizing radiation, and metabolic byproducts such as free radicals, can produce alterations in the DNA molecule. Genome integrity is maintained by several DNA repair mechanisms. Among them, the base excision repair (BER) pathway removes single-base lesions involving the action of DNA glycosylases [1,2]. These enzymes recognize bases altered by oxidation, alkylation, deamination, and depurination/depyrimidination, to cleave the N-glycosidic bond and generate an abasic (apurinic or apyrmidinic; AP) site. Subsequently, an AP endonuclease hydrolyzes the phosphodiester bond 5′ to the AP site, a DNA polymerase introduces the correct base, and a DNA ligase seals the nick, completing the repair process [3,4]. These BER components must act in a coordinated manner to avoid generation of AP sites or strand brakes, and their functions have been better studied in animals and microbes than in plants, where fewer enzymes have been characterized, mainly by using recombinant proteins and *in vitro* systems [1,4].

Based on structural homology, DNA glycosylases are classified into four superfamilies, uracil DNA glycosylases (UDG), 3-methylpurine glycosylases (MPG), endonuclease VIII-like glycosylases (NEIL; Nei/Fpg (MutM)), and helix-hairpin-helix DNA glycosylases (HhH-GPD) [1,4]. Each superfamily includes enzymes targeting different lesions, with most of them recognizing more than one substrate. Some DNA glycosylases, called bifunctional, possess AP lyase activity and can cleave the phosphodiester bond. The HhH-GPD superfamily is present in all kingdoms and constitutes the most diverse group. It includes mono and bifunctional enzymes that together recognize all lesions repaired by BER [4]. The hallmark of this superfamily is the presence of two helixes separated by a hairpin loop involved in DNA binding (HhH motif), followed by a Gly/Pro-rich loop and a conserved Asp required for catalytic activity (GPD motif) [3]. HhH-GPD DNA glycosylases are organized into the following families: endonuclease III-like DNA glycosylase (NTH),

Abbreviations: BER, base excision repair; DME, DEMETER; FPG, formamidopyrimidine DNA glycosylase; HhH, helix-hairpin-helix; MBD4, methyl-binding domain protein 4; MPG, 3-methyl-purine glycosylase; MutY, A/G-mismatch-specific adenine glycosylase; NEI, endonuclease VIII-like glycosylase; NTH, endonuclease III-like DNA glycosylase; OGG, 8-oxoguanine DNA glycosylase; ROS1, repressor of silencing 1; UDG, uracil DNA glycosylase.

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A/G-mismatch-specific adenine glycosylase (MutY), 8-oxoguanine DNA glycosylase 1 (OGG1), 8-oxoguanine DNA glycosylase 2 (OGG2), alkyladenine-DNA glycosylase (AlkA), N-methyl-purine-DNA glycosylase II (MpgII), and methyl-binding domain protein 4 (MBD4). An additional HhH-GPD DNA glycosylases family that is exclusive of plants is DEMETER (DME), whose members are DME, DEMETER-LIKE2 (DML2), DML3 and REPRESSOR OF SILENCING1 (ROS1) [1,3,4].

The mammalian MBD4 is a monofunctional glycosylase that excises T and U opposite G with preference for halogenated U derivatives such as 5-hydroxymethyluracil (5-hmU), a trait also reported for TDG (thymine DNA glycosylase from the UDG superfamily) [5]. This enzyme affects DNA repair, tumor progression, apoptosis and gene expression. Human carcinomas with microsatellite instability exhibit mutations on this gene [6]. Deficiency of MBD4 increases the level of C to T transitions at CpG sites, and alters tumorigenesis [7,8]. In addition, MBD4 interacts with apoptosis-associated proteins, such as MLH1 from the mismatch repair system, and the Fas-associated death domain protein FADD [9]. Moreover, MBD4 binds and represses hypermethylated gene promoters affecting transcription [10]. On the other hand, mammalian MBD4 and TDG, as well as plant DME glycosylases, are involved in active DNA demethylation associated to the BER pathway. While DME, DML2, DML3 and ROS1 remove 5-methylcytosine (5-mC) allowing its replacement by cytosine (C) [11,12], MBD4 and TDG do not efficiently excise 5-mC requiring a multistep process. In the latter case, T and 5-hmU derived from hydrolytic deamination of 5-mC or 5-hydroxymethylcytosine (5-hmC), respectively, may act as substrates of these enzymes [5,13].

Curiously, little is known about MBD4 in the plant kingdom, where other DNA glycosylases have been characterized for quite some time. UNG [14,15], Fpg [16], NTH1 and NTH2 [17], OGG1 [18,19], ROS1 and DME [11,20,21] were studied at the molecular or biochemical levels. Some of these enzymes modulate plant development, as indicated by phenotypic analysis of Arabidopsis knockout mutants [15,20,22–24]. As expected, they are also implicated in environmental stress responses. ROS1 mediates DNA repair induced by UV-B [25], and provides tolerance to methyl methanesulfonate and H_2O_2 [20]. OGG1 provides protection to osmotic and oxidative stress and enhances seed longevity [26]. In turn, DML3 affects seed germination under adverse conditions [27].

Recently, Ramiro-Merina and colleagues [28] demonstrated that the Arabidopsis *At3g07930* gene encodes an active monofunctional DNA glycosylase homologous to mammalian MBD4 (MBD4-like; MBD4L). These authors report the *in vitro* activity of the recombinant protein, its low affinity over 5-mC or 5-hmC and its capacity to remove U and T opposite G, and excise 5-halogen uracil derivatives including 5-hmU. *In vivo* synthesis of MBD4L was suggested by the finding of two derivative peptides in Arabidopsis proteomic studies (VLVICMLLNK, LGRDDDSSVMMTR; http://fgcz-pep2pro.uzh.ch/index.php; www.arabidopsis.org/cgi-bin/gbrowse/arabidopsis). However, no studies have examined so far the expression of MBD4L and its capacity to activate BER *in planta*. Arabidopsis contains three AP endonucleases, APE1L, APE2 and ARP with different incision activities and expression patterns [29], and mostly uses DNA ligase LIG1 in the last step of the repair process [30], suggesting that some of these components may accompany MBD4 in this pathway.

The current work provides a functional characterization of *At3g07930* (*AtMBD4L*), a single-copy gene encoding MBD4 in Arabidopsis. Phylogenetic and gene expression studies show features that distinguish *AtMBD4L* from other Arabidopsis DNA glycosylase genes. *AtMBD4L* generates two transcripts that co-exist in leaves and flowers, and at least one of them codes for a nuclear protein. Activation of *AtMBD4L* induces the expression of the late BER gene *AtLIG1*, and improves tolerance to oxidative damage. These are the first evidences on the action of MBD4 in a plant model.

2. Materials and methods

2.1. In silico analysis

HMM-HMM comparison (HHpred toolkit from MPI Bioinformatics) was used to select the Arabidopsis genes homologues to the *Homo sapiens MBD4* gene. DNA glycosylase families were defined by comparing all the PF00730 domains (http://pfam.sanger.ac.uk) detected in plant proteins with T-Coffee multiple sequence alignment (www.ebi.ac.uk/Tools/msa/tcoffee/). The phylogenetic tree was generated and edited with BLOSUM62 Jalview and TreeDyn v199.3 programs.

2.2. Plant material, treatments, and transgenic plants

Arabidopsis thaliana Columbia (Col-0) seeds were obtained from the Arabidopsis Biological Resource Center (ABRC; Columbus, OH). Seeds were stratified at 4 °C for 3 days, germinated on MS plates (7–10 days), and transferred to soil for growth in chambers (8 h light/16 h dark, 23 °C). The *35S:AtMBD4L3-GFP*, *35S:At*Δ*MBD4L3* and *AtMBD4L:GUS* (Col-0) transgenic plants were generated in the laboratory using pENTR/D-TOPO (Invitrogen), and pK7FWGF2, pK2GW7 or pKGWFS7 plasmids as entry and destination vectors, respectively. The *35S:AtMBD4L3-GFP* (complete coding region of *At3g07930.3* cloned in pK7FWGF2), *35S:At*Δ*MBD4L3* (initial 1065 nt of *At3g07930.3* in pK2GW7) and *AtMBD4L:GUS* (1564 bp of the *AtMBD4L* promoter fused to *GUS* in pKGWFS7) transgenes were generated using primers and conditions described in Table S1. Constructs integrity was confirmed by sequencing. Plants were transformed *via A. tumefaciens* by floral dipping. Homozygous *35S:AtMBD4L3-GFP*, *35S:At*Δ*MBD4L3* and hemi/homozygous *AtMBD4L:GUS* plants were evaluated. Leaves of 4 week-old plants were floated on 25 or 100 μM methyl viologen (MV) and maintained at 23 °C under short-day cycle, to be evaluated 30 h later.

For cell death assays, excised leaves were floated on water and treated with dots (1.5 μl) of 100 μM MV in the adaxial face (4 dots/leaf). Six hours later, leaves were stained with SYTOX Green to detect and quantify dying cells as previously described (Cecchini et al., 2011).

2.3. Gene expression and protein analysis

RT-PCR assays [31] used primers and conditions described in Table S1. Tissues analyzed by GUS histochemical assays [23] were incubated with substrate up to 72 h at 37 °C. GFP-trap agarose beads (Chromotek) were used for protein immunoprecipitation according to manufacturer's instructions. Western blots with anti-GFP antibodies (Abcam) were analyzed with Odyssey Infrared Imaging System (LI-COR Bioscience) [31]. qPCR was performed by triplicate (10 min at 95 °C; 40 cycles: 15 s at 95 °C; 15 s at 58 °C; 30 s at 72 °C; melting curve 60–95 °C). Gene expression was determined by ΔΔCt method using *UBQ5* as housekeeping gene.

2.4. Microscopy

Confocal images were collected with a FluoView FV1000 microscope and 60X/1.42 NA objective from Olympus. GFP and chlorophyll were excited at 488 nm, and DAPI at 405 nm. Fluorescence was acquired at 500–520 nm (GFP), 640–660 nm (chlorophyll) or 420–475 nm (DAPI). Linear unmixing algorithm was used to deconvolute the contribution of GFP and chlorophyll fluorescence.