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

Florencia Nota, Damián A. Cambiagno, Pamela Ribone, María E. Alvarez*

Centro de Investigaciones en Química Biológica de Córdoba (CIQUIBIC, CONICET-UNC), Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Haya de la Torre y Medina Allende, Ciudad Universitaria, 5000 Córdoba, Argentina

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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.

* Corresponding author. Tel.: +54 351 5353855x3422; fax: +54 351 5353855x3422.
E-mail address: malena@mail.fcq.unc.edu.ar (M.E. Alvarez).

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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.

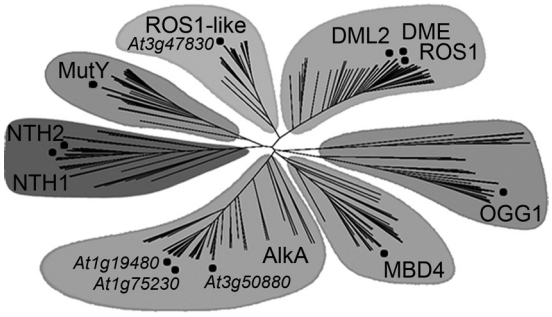

Fig. 1. Phylogenetic analysis of *AtMBD4L* (*At3g07930*). Un-rooted phylogenetic tree derived from T-Coffee alignment of the HhH-GPD domain (PF00730) present in 269 protein sequences from 32 species of the Viridiplantae kingdom. All family names are included. All Arabidopsis sequences are indicated and positioned with dots. The complete list of proteins and branch distances are shown in Fig. S1.

3. Results

3.1. At3g07930 (AtMBD4L) is the only Arabidopsis gene from the MBD4 family

Based on HMM (hidden Markov model) comparison we selected the Arabidopsis gene *At3g07930* (*AtMBD4L*) as the only ortholog of human *MBD4* gene. *In silico* analysis (Phyre2 server tool) indicated that the full length protein encoded by *AtMBD4L* contained an HhH domain (residues 399–419), followed by a GPD motif (420–429), and a conserved Asp residue (429), that were proper from active enzymes. In fact, the MBD4L protein encoded by this gene shows DNA glycosylase activity *in vitro* [28].

To position MBD4L in the context of plant DNA glycosylases we used multiple sequence alignment. First, we compared among themselves all DNA glycosylase HhH-GPD domains (PF00730) detected in annotated proteins of the Viridiplantae kingdom (269 sequences from 32 different species). The phylogenetic tree thus obtained distinguished seven plant enzyme families: DME/ROS1, OGG1, MBD4, AlkA, NTH, MutY, and ROS1-like (Fig. 1; Fig. S1). MpgII enzymes were not clustered into a family, suggesting they would be part of other HhH-GPD families or other DNA glycosylase superfamilies. After highlighting the Arabidopsis sequences in this tree, we realized that they were distributed in all enzyme families, and MBD4L was the only Arabidopsis member of this family. To begin studying the activity of *AtMBD4L* we evaluated its expression *in vivo*.

3.2. AtMBD4L generates two major splice variants in leaves and flowers

Databases inform that *AtMBD4L* can generate three alternative transcripts, *At3g07930.1*, *At3g07930.2*, and *At3g07930.3* (TAIR, http://www.arabidopsis.org) (Fig. S2A). To determine which of these transcripts were present in leaf tissues, we developed RT-PCR assays with primers described in Fig. S2A and Table S1. First, we combined one forward primer (A) with three specific reverse primers (B, C, and D), and amplified a product of similar size to *At3g07930.3* with oligonucleotides A and D (Fig. S2B). Then, we used two sets of primers complementary to all predicted cDNAs (E-F and E-G), to clone and sequence the amplified fragments (18 independent cDNAs). Once again we detected *At3g07930.3*, but not *At3g07930.1* or *At3g07930.2*.

Curiously, E-G (Fig. S2C) and E-F (Fig. S2D) primers amplified a second product smaller than *At3g07930.3*, which was named *At3g07930.4*. Both transcripts differed in an internal fragment (nt 115–462 from ATG; Fig. S2D) that was flanked by splice donor and acceptor sites (Fig. 2A), suggesting that *At3g07930.4* resulted from removal of a previously unidentified intron. *At3g07930.3* and *At3g07930.4* encode for MBD4L3 and MBD4L4 isoforms, of 445 and 329 amino acids, respectively (Fig. 2A). Both predicted proteins conserve the C-terminal DNA glycosylase domain (115 last amino acids), and at their N-terminus, MBD4L3 includes two nuclear localization signals (NLS) (residues 10–17 and 98–104), while MBD4L4 carries only one of them (Fig. 2A; Fig. S2E). Both *AtMBD4L* transcripts were detected in rosette and cauline leaves, as well as in flowers, but not in roots or stems (Fig. 2B). Hence, the results suggested that leaves and flowers may contain two MBD4L isoforms with catalytic activity.

3.3. The AtMBD4L expression is spatially and temporarily regulated

The Arabidopsis DNA glycosylase genes studied so far have either constitutive or tissue-specific expression. To study the expression pattern of *AtMBD4L* we generated the *AtMBD4L:GUS* construct carrying a 1.5 kb gene promoter fragment. Five independent T1 transgenic lines expressing this construct, and some of their T2 descendents, were analyzed by histochemistry. All plants showed low GUS activity at senescent or damaged leaves. In particular, at regions of incipient chlorosis (Fig. 3A) and perivascular tissues either from the central zone (Fig. 3C, D), or peripheral zones contacting hydathodes (Fig. 3B, C, E). GUS was also expressed in the inflorescence (three transgenic lines), in the subset of flowers whose developmental stages was defined as 9–12 (Fig. 3F) according to the classification of Smyth [32]. Within these flowers, only anthers expressed the transgene (Fig. 3G), apparently at the tapetum, but not at pollen grains (Fig. 3H). The promoter was also active at the siliques (four transgenic lines), at the developmental stage defined as 17a [33] (Fig. 3I), in a region apparently corresponding to the stigma (Fig. 3J). Curiously, GUS staining was not detected at other stages of silique development (Fig. S3). Occasionally, GUS was expressed at cauline leaves (one transgenic line), in perivascular tissues close to hydathodes, and abscission zones.

Therefore, the *AtMBD4L* promoter activity is finely controlled at the spatial and temporal levels. As discussed later, the expression pattern here described is different from that of other Arabidopsis DNA glycosylase genes studied to date.

3.4. The 35S:AtMBD4L3-GFP transgene encodes a nuclear protein

Although the activity of MBD4L on DNA has been demonstrated [28], the subcellular localization of the enzyme is unknown. To evaluate this issue, we generated transgenic plants expressing MBD4-GFP recombinant proteins. The complete *AtMBD4L3* coding region was fused to *GFP* and placed under the control of the *35S* promoter. The 1.5 kb *AtMBD4L* promoter was not used for this purpose, due to its low and tissue-specific activity. Stable *35S:AtMBD4L3-GFP* transgenic plants were analyzed by RT-PCR. As expected, A-H primers amplified a 0.7 kb fragment from transgenic, but not wild type plants, corroborating transgene expression (Fig. 4A; Table S1). Then, E-H primers were used to evaluate transgene splicing. If the *AtMBD4L* region of the transgene was processed as the endogenous gene, these primers would generate 1545 and 1197 bp products (Fig. S2). Two transcripts of the predicted sizes were amplified from *35S:AtMBD4L3-GFP* leaves, suggesting expression of MBD4L3-GFP and MBD4L4-GFP proteins in these tissues. Both transcripts accumulated at higher levels than *At3g07930.3* and *At3g07930.4* in wild type tissues (E-F primers), and the largest one, coding for MBD4L3-GFP, was the most abundant.

Next, we developed Western blot assays with anti-GFP antibodies in control (*35S:GFP*) and *35S:AtMBD4L3-GFP* plants. We did not detect MBD4L3-GFP or MBD4L4-GFP proteins (79 and 66 kDa, respectively) in total leaf extracts of *35S:AtMBD4L3-GFP* plants, but

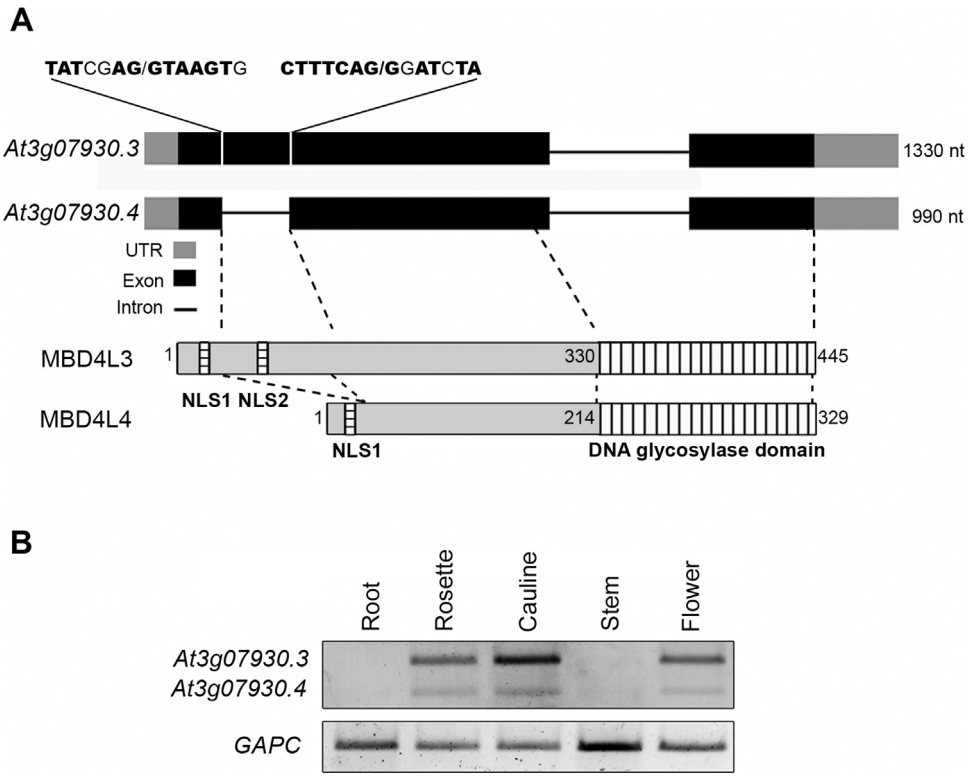

Fig. 2. *AtMBD4L* expression. (A) *At3g07930.3* and *At3g07930.4* are alternative transcripts coding for MBD4L3 and MBD4L4 proteins, respectively. Nucleotides matching the consensus for donor and acceptor Arabidopsis splicing sites [38] are shown in bold for the new gene intron. DNA glycosylase domain: Pfam PF00730. NLS: nuclear localization signal. (B) Abundance of *At3g07930.3* and *At3g07930.4* transcripts in roots, rosette and cauline leaves, stems and flowers determined by RT-PCR with E-F primers (Fig. S2A; Table S1). *GAPC* is used as internal control. Similar results were observed in four different experiments.

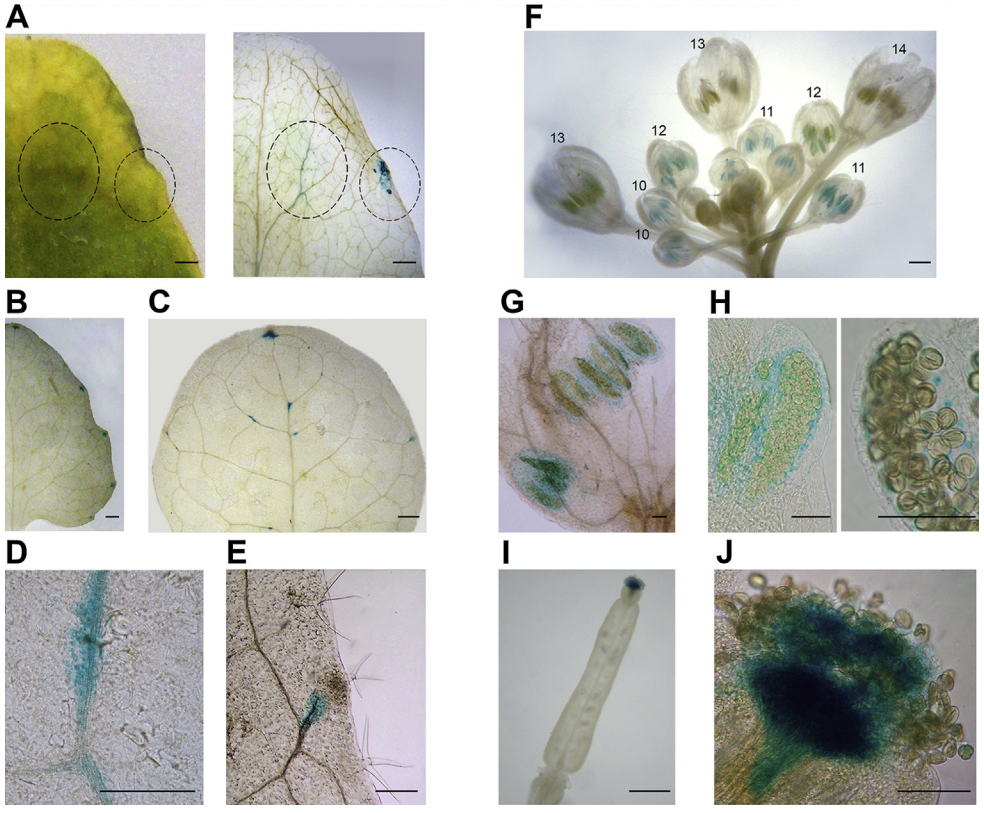

Fig. 3. Histochemical analysis of Arabidopsis *AtMBD4L:GUS* transgenic plants. (A) Circles indicate leaf areas with GUS activity. Rosette (A, C) and cauline (B) leaves with signs of senescence. Details of central (D) and peripheral (E) regions of senescent leaves. (F) Inflorescence containing flowers on different developmental stages, indicated by numbers according to Smyth and colleagues [32]. (G) Flower at stage 12 of development. (H) Anthers. (I) Silique at the 17a developmental stage [33]. (J) Magnification of the stigma. Bars, 1 mm (A, B, C, D, E, F, I); 0.1 mm (G, H, J). The number of transgenic lines analyzed in each case is described in the text.

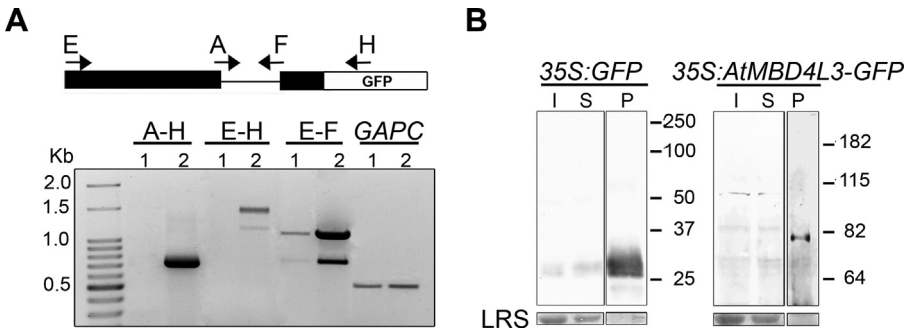

Fig. 4. Expression of the *35S:AtMBD4L3-GFP* transgene *in vivo*. (A) Leaf samples from *35S:GFP* (control) (1) or *35S:AtMBD4L3-GFP* (2) plants were analyzed by sqRT-PCR with the primers indicated in the scheme. (B) Western blot of *35S:GFP* or *35S:AtMBD4L3-GFP* samples probed with anti-GFP antibodies. Total protein extracts were subjected to pull down with GFP-trap agarose beads. Input (I; 30 μg protein), supernatant (S; 30 μg protein) and pull-down fractions (P) are shown. LRS: large Rubisco subunit. Similar results were obtained in three (A) and two (B) independent experiments.

a product of the size of MBD4L3-GFP was recovered in the pull-down fraction of extracts pre-incubated with GFP-trap agarose beads (Fig. 4B). Therefore, leaves of *35S:AtMBD4L3-GFP* plants activated the transgene, but accumulated low levels of the fusion proteins, with MBD4L3-GFP being detected by our assay.

Finally, we used confocal microscopy to examine the localization of fusion proteins in *35S:AtMBD4L3-GFP* leaves. Here again, we included *35S:GFP* leaf samples as control. As expected, GFP was detected at the nucleus and cytosol of control samples (Fig. 5). In contrast, GFP fluorescence was exclusively nuclear in epidermal, mesophyll, and guard cells from *35S:AtMBD4L3-GFP* leaf tissues. These results suggested that endogenous MBD4L resides in the nuclear compartment.

3.5. *Effects of AtMBD4L overexpression* in vivo

So far, there are no reports on the effects of MBD4L activity *in planta*. To initiate such studies, we examined two BER-associated responses in transgenic plants overexpressing either the full length *AtMBD4L* gene (*35S:AtMBD4L3-GFP* plants), or a truncated version lacking nucleotides coding for the DNA glycosylase domain (*35S:At*Δ*MBD4L3* plants) (Fig. S2). Leaves of both plants overexpressed the transgenes (Fig. 6A). Then, these leaves and leaves from *35S:GFP* plants used as control, were analyzed in parallel. These samples were initially used to monitor *AtLIG1* expression. This gene encodes an essential BER component [30], and becomes induced by overexpression of the DNA glycosylase OGG1 [26]. *AtLIG1* expression was higher in *35S:AtMBD4L3-GFP* leaves than in *35S:At*Δ*MBD4L3* or control samples (Fig. 6B). Gene activation was mild, but similar to the one observed in OGG1 overexpressing plants [26], and consistently detected in three independent experiments. In the second assay, we evaluated how leaves of *35S:AtMBD4L3-GFP*, *35S:At*Δ*MBD4L3* and *35S:GFP* plants responded to oxidative burst, a stress condition that induces BER [34]. Leaves were excised and floated in MV 25 or 100 μM, to be inspected 30 h after treatment. This herbicide generates superoxide anion in chloroplasts and affects several cellular compartments producing oxidation of DNA bases [35,36]. Most areas of *35S:GFP* and *35S:At*Δ*MBD4L3* leaves became chlorotic after treatment with 100 μM MV, with no differences between them. Interestingly, *35S:AtMBD4L3-GFP* leaves evidenced far less damage, conserving large green sectors in response to such treatment (Fig. 6C). At lower dose of MV (25 μM) the three plants showed similar phenotypes. In addition, in response to MV (100 μM), the leaves of *35S:AtMBD4L3-GFP* plants developed lower cell death levels than those of *35S:At*Δ*MBD4L3* and *35S:GFP* plants (Fig. S4). These results suggested that the DNA glycosylase activity of MBD4L generated *AtLIG1* activation, and oxidative stress tolerance in *35S:AtMBD4L3-GFP* leaves.

4. Discussion

This work describes the structure and function of the *AtMBD4L* gene, whose product is an active DNA glycosylase targeting U:G and T:G mispairs *in vitro* [28]. So far, this is the only study evaluating the *in vivo* activity of a plant gene encoding the MBD4 enzyme. *AtMBD4L* is one of the twelve HhH-GPD DNA glycosylase genes present in Arabidopsis, a plant containing all seven HhH-GPD enzyme families existing in the Viridiplantae kingdom, indicating that MBD4L can be studied in the context of all other HhH-GPD prototypes in this model.

As 20% of plant genes [37], *AtMBD4L* is subject to alternative splicing. *At3g07930.4* is a novel transcript lacking a previously unidentified intron, whereas *At3g07930.3* derives from an intron retention event, the most frequent alternative splicing form in plants [38]. *At3g07930.3* and *At3g07930.4* coexist in leaves and flowers, encoding MBD4L3 and MBD4L4, respectively. These proteins would be active enzymes since they preserve a C-terminal domain that retains catalytic activity [28]. Eventually, alternative splicing could affect the subcellular localization of MBD4L, as described for other DNA glycosylases. The human genes *MBD4* [39], *OGG1* [40] and *MUTY* [41] generate 2, 7, and 10 transcripts, respectively. The OGG1 and MUTY splice variants reach different subcellular compartments [40,41]. Similarly, the two spliced forms of the human UDG are delivered either to the nucleus or mitochondria [42]. In turn, both human MBD4 isoforms differ in the methyl binding domain, and their enzymatic capacities, with the short variant conserving U, but not T, DNA glycosylase activity [39]. In Arabidopsis, DME [24], ROS1 [20], and OGG1 [26] have only been detected in the nucleus, whereas NTH1/NTH2 were only found in chloroplast [43]. As MBD4L3 and MBD4L4 differ in their NLS motifs, they may have different abilities to localize in the nucleus. We suspect that the nuclear localization of GFP detected in the *35S:AtMBD4L3-GFP* plants corresponds to the MBD4L3-GFP protein, since it is encoded by the most abundant transcript, and is detected by Western blots. However, we cannot exclude that MBD4L4-GFP is also synthesized in these tissues at lower levels than MBD4L3-GFP.

The *AtMBD4L:GUS* plants activated GUS only in certain organs or developmental conditions, indicating that the *AtMBD4L* 1.5 kb promoter has a fairly restricted activity. This promoter includes elements sensitive to light and dehydration that may generate some of the expression patterns here described. If wild type plants control the *AtMBD4L* expression in similar way, then other DNA glycosylases should perform the same function in tissues lacking MBD4L. As mentioned before, MBD4L excises U and T opposite to G, with high activity on 5-halogen uracils (5-bromouracil, 5-fluoruracil), medium activity on U, and low effect on 5-hydroxymethyluracil, 5-hidroxyuracil, and T [28]. ROS1 has low activity on 5-halogen uracils

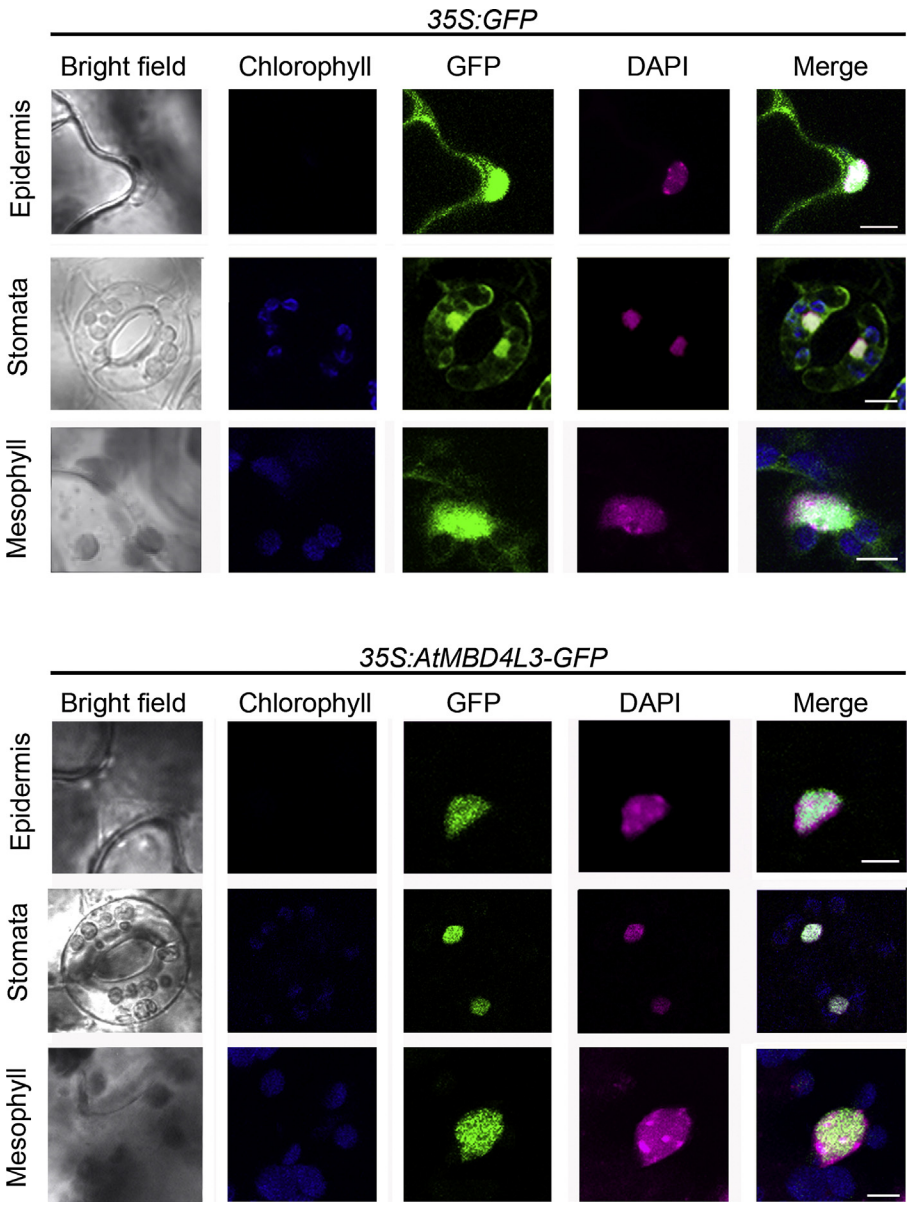

Fig. 5. Subcellular localization of MBD4L in leaf cells of *35S:AtMBD4L3-GFP* plants. Laser scanning confocal micrographs show GFP localization in *35S:GFP* (control), and *35S:AtMBD4L3-GFP* transgenic plants. Bright field and fluorescence images (chlorophyll: blue; GFP: green; DAPI: magenta) are included for each sample. Similar results were observed in four independent T2 *35S:AtMBD4L3-GFP* transgenic lines. Bars, 5 μm. Identical results were obtained with three *35S:AtMBD4L3-GFP* transgenic lines.

(5-fluoruracil, 5-bromouracil) and high activity on 5-hidroxyuracil, 5-mC, and T [44]. DME and ROS remove T:G mismatches with low efficiency, and are activated either at the central [24] and vegetative [45] cells of female and male gametophytes (DME), or at the whole plant (ROS1) [12,20]. In turn, the UNG enzyme encoded by the *At3g18630* gene [15] has not been studied at the expression level. Therefore, we currently ignore which enzymes could replace MBD4L in tissues devoid of this activity.

Proper BER function requires that AP endonucleases and DNA ligases act in concert with DNA glycosylases. Whereas at least three AP endonucleases participate in BER, LIG1 is primarily responsible for the ligation event that ends this repair process in Arabidopsis [30]. Transcriptional activation of *AtLIG1* in *35S:AtMBD4L3-GFP* plants suggested that these plants activated the complete BER pathway, and would have greater capacity to respond to DNA damaging agents. Consistently, leaves of *35S:AtMBD4L3-GFP* plants were tolerant to MV, and this response apparently derived from MBD4L DNA glycosylase activity. In mammals, MBD4 protects cells from oxidative stress and is recruited with DNMT1 at sites of oxidative DNA damage, although its effects on DNA under this condition are unknown [46]. In Arabidopsis, OGG1 overexpressing plants also increase tolerance to MV apparently by activating BER, since reduction of enzyme substrate (8-oxoguanine) was detected by immunological studies in the genome of these plants [26]. In this sense, additional work will be required to determine how *AtMBD4L* overexpression affects the plant genome, as no simple tools are currently available for quantification of its substrates.

In summary, the information provided here suggests that *AtMBD4L* generates two active enzymes that would act under specific conditions, to trigger the BER pathway that operates at the nucleus. This knowledge will contribute to study the biological role of MBD4L, the distinctive features of its isoforms, and their genomic effects, considering that its homologue in mammals leads to DNA repair, and DNA demethylation.

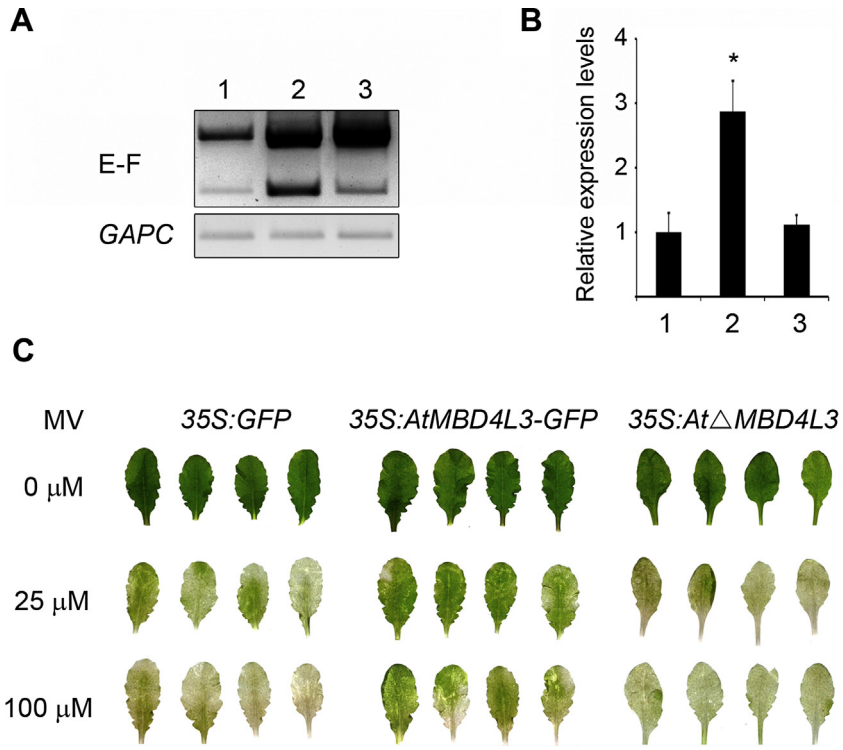

Fig. 6. *AtMBD4L* affects BER-associated responses. Leaves of *35S:GFP* (control) (1), *35S:AtMBD4L3-GFP* (2), and *35S:At△MBD4L3* (3) plants were used to determine the levels of *At3g07930.3* and *At3g07930.4* transcripts (A), the expression of *AtLIG1* gene (B), and the effect of MV (25 and 100 μM). *GAPC* and *UBQ5* were used as internal controls in sqRT-PCR (A) and qRT-PCR (B), respectively. Significant differences between *35S:GFP* and *35S:AtMBD4L3-GFP* values ($p < 0.001$, *t* test) are indicated (*) in B. Leaves treated with MV were examined at 30 h post-treatment. Similar results were obtained in three (A,B) and four (C) independent experiments. Primers are described in Fig. S2A and Table S1.

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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.plantsci.2015.03.011.

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