

# The DNA dioxygenase ALKBH2 protects *Arabidopsis thaliana* against methylation damage

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## ABSTRACT

The *Escherichia coli* AlkB protein (EcAlkB) is a DNA repair enzyme which reverses methylation damage such as 1-methyladenine (1-meA) and 3-methylcytosine (3-meC). The mammalian AlkB homologues ALKBH2 and ALKBH3 display EcAlkB-like repair activity *in vitro*, but their substrate specificities are different, and ALKBH2 is the main DNA repair enzyme for 1-meA *in vivo*. The genome of the model plant *Arabidopsis thaliana* encodes several AlkB homologues, including the yet uncharacterized protein AT2G22260, which displays sequence similarity to both ALKBH2 and ALKBH3. We have here characterized protein AT2G22260, by us denoted ALKBH2, as both our functional studies and bioinformatics analysis suggest it to be an orthologue of mammalian ALKBH2. The *Arabidopsis* ALKBH2 protein displayed *in vitro* repair activities towards methyl and etheno adducts in DNA, and was able to complement corresponding repair deficiencies of the *E. coli* *alkB* mutant. Interestingly, *alkbh2* knock-out plants were sensitive to the methylating agent methylmethanesulphonate (MMS), and seedlings from these plants developed abnormally when grown in the presence of MMS. The present study establishes ALKBH2 as an important enzyme for protecting *Arabidopsis* against methylation damage in DNA, and suggests its homologues in other plants to have a similar function.

## INTRODUCTION

Living organisms are exposed to a variety of environmental DNA-damaging agents, such as chemical mutagens,

toxins from fungi and bacteria, as well as ultraviolet and ionizing radiation. In addition, various intracellular metabolites can cause DNA damage. Plants possess various mechanisms for repairing DNA, and genes encoding the components of major DNA repair pathways are found in plant genomes (1,2). However, only a few studies have focused on characterizing DNA repair enzymes from plants, and our understanding of how plants handle the deleterious effects of DNA damage remains vague.

Methylating agents introduce a number of lesions into cellular DNA and RNA. Such agents are present in the environment, e.g. as methyl halides (3,4), or they may be generated intracellularly by normal metabolism. For example, S-adenosylmethionine, which serves as a methyl donor in enzymatic methylation reactions, is also able to induce a low level of aberrant methylations (5). Several different repair mechanisms protect the genome against the harmful effects of methylating and other alkylating agents, including base excision repair (BER) initiated by alkylpurine DNA glycosylases, direct reversal by DNA alkylbase methyltransferases, and oxidative demethylation by AlkB-like dioxygenases (6). The model plant *Arabidopsis* appears to lack DNA alkylbase methyltransferases, but bioinformatics analyses indicate the existence of a large number of alkylpurine DNA glycosylases, representing all the three families of such glycosylases (7). Moreover, one of these enzymes, termed AMAG, has been shown to be an enzymatically active 3-methyladenine (3-meA) DNA glycosylase (8), and *Arabidopsis* cell extracts have been shown to contain the enzymatic machinery necessary for performing BER (9).

*Escherichia coli* AlkB (EcAlkB) is a DNA repair protein which belongs to the superfamily of iron(II) and 2-oxoglutarate dependent dioxygenases (10), enzymes which use ferrous iron as cofactor and 2-oxoglutarate as co-substrate to perform various oxidation reactions, usually hydroxylations. EcAlkB was originally shown to demethylate the

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lesions 1-meA and 3-meC in DNA by hydroxylating the methyl group, leading to the release of the resulting hydroxymethyl moiety as formaldehyde (11,12). In addition to 1-meA and 3-meC, the structurally similar, but less abundant lesions 1-methylguanine (1-meG) and 3-methylthymine (3-meT) have also been shown to be EcAlkB substrates (13–15). EcAlkB is active also on methylated RNA and on bulkier DNA lesions, such as ethyl and propyl groups, as well as on exocyclic etheno and ethano-lesions, but these activities are generally lower than on the canonical 1-meA and 3-meC lesions in DNA (16–19).

Although homologues of EcAlkB can be found in viruses, eubacteria and eukaryotes, many organisms within these groups lack AlkB homologues (20–24). However, the genomes of multicellular eukaryotes typically encode several different AlkB homologues, and mammals have eight such proteins, ALKBH1–8, as well as the somewhat more distantly related FTO (ALKBH9) protein (21,25,26). Despite extensive studies, the function of the majority of these proteins is still unknown. Also plants, such as *Arabidopsis*, possess several different ALKBH proteins (21,27).

Among the human AlkB homologues, ALKBH2 and ALKBH3 have been characterized biochemically and shown to have robust repair activities on several of the lesions that are EcAlkB substrates (17,28–31). However, these two proteins display interesting differences with respect to their substrate specificities and subcellular localization, suggesting that they perform distinct cellular functions. ALKBH2 is exclusively active on DNA substrates, whereas ALKBH3 shows comparable activities on DNA and RNA. While ALKBH2 is active both on single-stranded (ss) and double-stranded (ds) DNA substrates, with a slight preference for dsDNA, ALKBH3 displays a strong preference for single-stranded substrates (28,29). ALKBH2 is a nuclear protein which accumulates in replication foci during S phase, while ALKBH3 is found both in the nucleus and the cytosol (28). Moreover, studies of ALKBH2 and ALKBH3 knock-out mice demonstrated that ALKBH2 is the main demethylase for removal of 1-meA in DNA *in vivo* (32). ALKBH3- or EcAlkB-mediated repair leads to functional recovery of methylated tRNA and mRNA *in vitro*, and studies of viral AlkB proteins have indicated that AlkB-mediated RNA repair is indeed biologically relevant (23,33). This suggested a possible role for ALKBH3 in RNA repair, but a recent study showed that ALKBH3 is associated with the DNA helicase ASCC3, and that RNAi-mediated knock-down of ASCC3 or ALKBH3 leads to increased cellular sensitivity towards methylating agents and to accumulation of genomic 3-meC, thus indicating that DNA repair may be the only biologically relevant function for ALKBH3 (34).

Weak *in vitro* repair activities have also been reported for two other ALKBH proteins. The human FTO protein showed repair activity towards 3-meT in ssDNA and 3-methyluridine in ssRNA (25,35), and ALKBH1 was shown to demethylate 3-meC in ssDNA and ssRNA (36). However, ALKBH1 has also been implicated in transcriptional regulation, and FTO was recently shown to efficiently demethylate the RNA modification

N6-methyladenine (37–39). Indeed, by showing that mammalian and plant ALKBH8 are tRNA modification enzymes, we and others recently demonstrated that ALKBH proteins have functions other than nucleic acid repair (40–44).

We here report the functional characterization of the *Arabidopsis thaliana* protein AT2G22260, which is an AlkB homologue with comparable sequence similarities to mammalian ALKBH2 and ALKBH3. Studies of its repair activity, as well as bioinformatics analyses, established this protein as a functional ALKBH2 orthologue. Interestingly, an *Arabidopsis alkbh2* mutant displayed hypersensitivity towards the alkylating agent methylmethanesulphonate (MMS), but only showed marginally elevated levels of MMS-induced 1-meA lesions in the genome.

## MATERIALS AND METHODS

### Protein purification and plasmid construction

Constructs for expression of *Arabidopsis* ALKBH2 in pET-28a(+) (Novagen, Germany) and pJB658 (45) were generated from total cDNA of *Arabidopsis* flowers using primers 17-AtALKBH2-fwd and 18-AtALKBH2-rev (primer sequences can be found in Supplementary Table S1). The PCR product was cloned into *Nde*I and *Bam*HI sites of the vectors. His-tagged protein was expressed from the plasmid pET-28a(+) in the *E. coli* strain BL21(DE3)-CodonPlus-RIPL (Stratagene, USA) in LB medium. The recombinant protein was isolated using TALON beads as previously described (23).

### Phage reactivation experiments

The toluic acid-inducible expression plasmid pJB658 (45) expressing the *Arabidopsis* AlkB homologue ALKBH2 or EcAlkB was transfected into an F-pilus expressing, AlkB-deficient *E. coli* strain, HK82/F'. Bacterial cultures were grown at 37°C under shaking, and at  $A_{600} = 0.1$ , the expression of recombinant protein was induced by the addition of 2 mM toluic acid (Fluka/Sigma-Aldrich, USA). The bacteria were further grown after induction until they reached  $A_{600} = 0.8$ . The M13 DNA phage, was treated for 30 min at 30°C with different concentrations of MMS (Fluka/Sigma-Aldrich, USA) or chloroacetaldehyde (CAA; Fluka/Sigma-Aldrich, USA), for introduction of methyl or etheno adducts, respectively. One hundred microlitres of several dilutions of the treated phage was mixed with 300 µl of the induced bacteria and 3 ml LB top agar. The mixture was spread onto LB plates and incubated at 37°C overnight. Phage survival was scored by counting plaques. All phage dilutions and treatments were performed in M9 minimum salt medium.

### Bacterial survival experiments

Cultures of *E. coli* strain HK82/F' transformed with pJB658-derived plasmids encoding bacterial AlkB proteins were induced with toluic acid as described above and grown until  $A_{600} = 0.5$ . Then, 500 µl of

bacterial culture was pelleted by centrifugation, re-suspended in 250  $\mu$ l of M9 minimum salt medium, and then mixed with an equal volume of M9 minimum salt medium containing the appropriate MMS concentration, followed by 30 min incubation at 30°C. Appropriate (to enable colony counting) dilutions of treated bacteria were plated onto LB plates, which were incubated overnight at 37°C, and survival was assessed by colony counting.

#### Preparation of oligonucleotide substrates and *in vitro* repair reactions of site-specific lesions

DNA oligonucleotides with the sequence. TAGACATTG CCATTCTCGATAGGATCCGGTCAAACCTAGACG AATTCCG, containing either 1-meA, 3-meC, or  $\epsilon$ A at the underlined position, were synthesized by ChemGenes (Wilmington, MA; 1-meA and 3-meC) or Midland Certified Reagent Company Inc. (Midland, TX;  $\epsilon$ A). Corresponding 5'-[ $^3$ P]-labelled dsDNA substrates were generated, and repair reactions performed essentially as described previously (31,32).

#### Oxidative demethylation of [ $^3$ H]methylated oligonucleotides

The [ $^3$ H]methylated oligonucleotide (sequence: CATGAT AACC GCGACTACTGAC) was prepared by treatment with *N*-[ $^3$ H]methyl-*N*-nitrosourea, and repair assays performed, as previously described (28,29).

#### Plant growth conditions

Wild-type *Arabidopsis* (*A. thaliana* ecotype Col or *Ler*) were grown in perlite soil at 18°C under 16 h of light at 100  $\mu$ E/m<sup>2</sup>s. Seeds were surface sterilized in 7.5% hypochlorite solution containing 0.1% Tween 20, followed by treatment in 70% ethanol and rinsing in distilled water. Sterilized seeds were plated on solidified Murashige–Skoog (MS) medium (46), supplemented with 2% sucrose (MS-2).

#### RNA extraction and RT–PCR

RNA extraction was performed using Spectrum Total RNA kit (Fluka/Sigma-Aldrich), following the manufacturer's instruction. RNA concentration and purity was assessed using NanoDrop2000 (Thermo Scientific). An amount of 1  $\mu$ g RNA was processed to obtain cDNA using polyT-primer and reverse transcriptase (Superscript II, Invitrogen) following the manufacturer's instructions. The resulting cDNA was used for Reverse Transcription (RT)–PCR, using primers 1414 and 1415.

#### GUS staining

GUS staining assay of transgenic plants was performed as previously described (47). Prefixing of plant material in ice-cold 90% acetone for 10 min was followed by rinsing for 10 min in staining buffer (50 mM Na<sub>3</sub>PO<sub>4</sub>, pH 7.2; 2 mM potassium–ferrocyanide; 2 mM potassium–ferricyanide; 0.1% Triton X-100; 2 mM X-Gluc) with no substrate and incubated in staining buffer at 37°C for 3–5 h. Following a graded ethanol dehydration series to 50%

ethanol, the material was post-fixed in FAA [10:7:2:1 ethanol:distilled water:acetic acid:formaldehyde(37%)] on ice for 30 min and hydrated in an ethanol series to 50 mM Na<sub>3</sub>PO<sub>4</sub> buffer and mounted on microscope slides in chloral hydrate (8:2:1 chloral hydrate:water:glycerol). For all GUS lines, the following tissues were stained; seedlings, mature leaves, flowers and siliques. The GUS staining was observed with a Zeiss Axioplan Imaging2 microscope system equipped with Nomarski optics and cooled LCD imaging facilities.

#### Gene trap *Arabidopsis* mutant and transgenic lines

A gene trap insertion line (GT\_5\_41144, *alkbh2-1*) was obtained from The Nottingham *Arabidopsis* Stock centre. Homozygous plants were identified by PCR genotyping as previously described (48), using the primers Ds-5 and Ds-3 in combination with genomic primers 598 and 599. PCR products were cloned into the TOPO Blunt vector (Invitrogen, USA), and clones were sequenced to verify the integration site. To analyse the expression of *ALKBH2* in the *alkbh2-1* mutant, RT–PCR was performed with primers upstream of the integration site (primers 1418 and 1419), downstream of the integration site (primers 1420 and 1421), as well as spanning the integration site (primers 1414 and 1415). The p*ALKBH2::GUS* construct was generated by Gateway technology (Invitrogen, USA). The promoter fragment, which included 1044 bp of putative promoter sequence, was amplified using the primers; attB1–At*ALKBH2*–promoter and attB2–At*ALKBH2*–promoter, recombined into pDONR/Zeo (Invitrogen, USA) and further recombined into pMDC162 (49), creating the construct pMDC162 pAt*ALKBH2::GUS*. The construct was transferred to the *Agrobacterium tumefaciens* strain C58C1 pGV2260 and ecotype Col plants were transformed using the *A. tumefaciens*-mediated floral dip method (50). Transformants were selected on MS containing 20 mg/ml hygromycin.

#### Genotoxic stress treatment of plants

For assessment of sensitivity of *alkbh2-1* mutants to MMS or CAA, WT and *alkbh2-1* seeds were plated on solidified MS-2 medium supplemented with the genotoxic agents. For MMS treatment, a final concentration of 90 ppm MMS was used. For treatment with CAA 50, 100 and 250  $\mu$ M was used as previously described (51).

#### Accumulation of genomic 1-meA

For assessment of accumulation of 1-meA in the genome, 6-day-old seedlings germinated and grown on MS-2 plates were individually transferred to six-well plates containing liquid MS medium supplemented with 90 ppm MMS (Sigma-Aldrich, USA) and incubated in growth chamber at 18°C. Tissue was harvested in liquid N<sub>2</sub> and genomic DNA isolated using the Ultraprep Plant DNA kit including an RNase treatment step (AHN Biotechnologie, Germany). Before 1-meA measurements, the amount and purity of the DNA were determined by using NanoDrop 2000 (Thermo Scientific, USA), as well as assayed on gel, and all the DNA preparations were genotyped as described above. Two micrograms of DNA was

precipitated using glycogen as a carrier in ethanol supplemented with ammonium acetate, before the 1-meA level in the samples was determined by LC-MS/MS.

### LC-MS/MS analysis

Genomic DNA was enzymatically hydrolysed to nucleosides as described (52), added three volumes of methanol and centrifuged (16 000g, 30 min). The supernatants were dried and dissolved in 50  $\mu$ l 5% methanol in water (v/v) for LC-MS/MS analysis of 1-me(dA), and a portion of each sample diluted for the quantitation of the unmodified deoxynucleosides (dA, dC, dG and T). Chromatographic separation was performed on a Shimadzu Prominence HPLC system with a Zorbax SB-C18 2.1  $\times$  150 mm i.d. (3.5  $\mu$ m) column equipped with an Eclipse XDB-C8 2.1  $\times$  12.5 mm i.d. (5  $\mu$ m) guard column (Agilent Technologies). The mobile phase consisted of water and methanol (both added 0.1% formic acid), for 1-me(dA) starting with a 5-min gradient of 5–70% methanol, followed by 6.5 min re-equilibration with 5% methanol, and for unmodified nucleosides maintained isocratically with 85% methanol. Mass spectrometry detection was performed using an Applied Biosystems/MDS Sciex 5000 triple quadrupole (Applied Biosystems) operating in positive electrospray ionization mode, monitoring the mass transitions 266.2  $\rightarrow$  150.1 [1-me(dA)], 252.2  $\rightarrow$  136.1 (dA), 228.2  $\rightarrow$  112.1 (dC), 268.2  $\rightarrow$  152.1 (dG), and 243.2  $\rightarrow$  127.1 (T).

## RESULTS

### The *Arabidopsis* genome encodes a putative homologue of mammalian ALKBH2

To identify possible nucleic acid repair proteins of the AlkB family in *A. thaliana*, we performed BLAST searches using human ALKBH2 and ALKBH3 proteins as queries. These two human proteins retrieved the same *Arabidopsis* protein of 314 amino acids, encoded by the *AT2G22260* gene, as the closest hit, and this protein displayed a comparable degree of sequence similarity to human ALKBH2 and ALKBH3 (39% and 37% identity, 50% and 48% similarity, respectively). Further BLAST searches identified putative orthologues of *AT2G22260* in other plants, including rice and grapevine, indicating that this function is present in both monocots and dicots. A protein sequence alignment of ALKBH2/ALKBH3 proteins from various plants and vertebrates are shown in Figure 1. The ALKBH family of proteins are defined by a common ‘Oxygenase Core’ (OC), and, in addition, ALKBH proteins involved in DNA/RNA repair, such as ALKBH2, ALKBH3, and EcAlkB, share sequence homology in the N-terminal region shown to interact with the damaged nucleotide, referred to as the ‘Nucleotide Recognition Lid’ (NRL) (53). Interestingly, the *AT2G22260* protein and its putative plant orthologues share sequence homology with ALKBH2 and ALKBH3 both in the OC and NRL regions, indicating that these plant proteins are indeed nucleic acid repair proteins. The 3D structure of human ALKBH2 in complex with a dsDNA substrate has revealed that Phe102 is responsible for flipping the methylated base out of the dsDNA helix, while a cluster of positively charged residues, Arg241,

Lys242 and Lys243 (RKK241–243) close to the C-terminus is important for interactions with the negatively charged backbone of the complementary strand (53). Moreover, a mutational analysis of human ALKBH2 indicated that Val101, in addition to Phe102, plays an important role in base flipping (54). The *AT2G22260* protein and its plant orthologues all have a Val-Phe motif at the position corresponding to Val101–Phe102 in human ALKBH2, and they also have a positively charged four-amino-acid patch (KRAK) at the position corresponding to residues 240–243 (Figure 1). This suggests that these plant proteins have a function similar to that of mammalian ALKBH2, and the *AT2G22260* protein will in the following be referred to as ALKBH2.

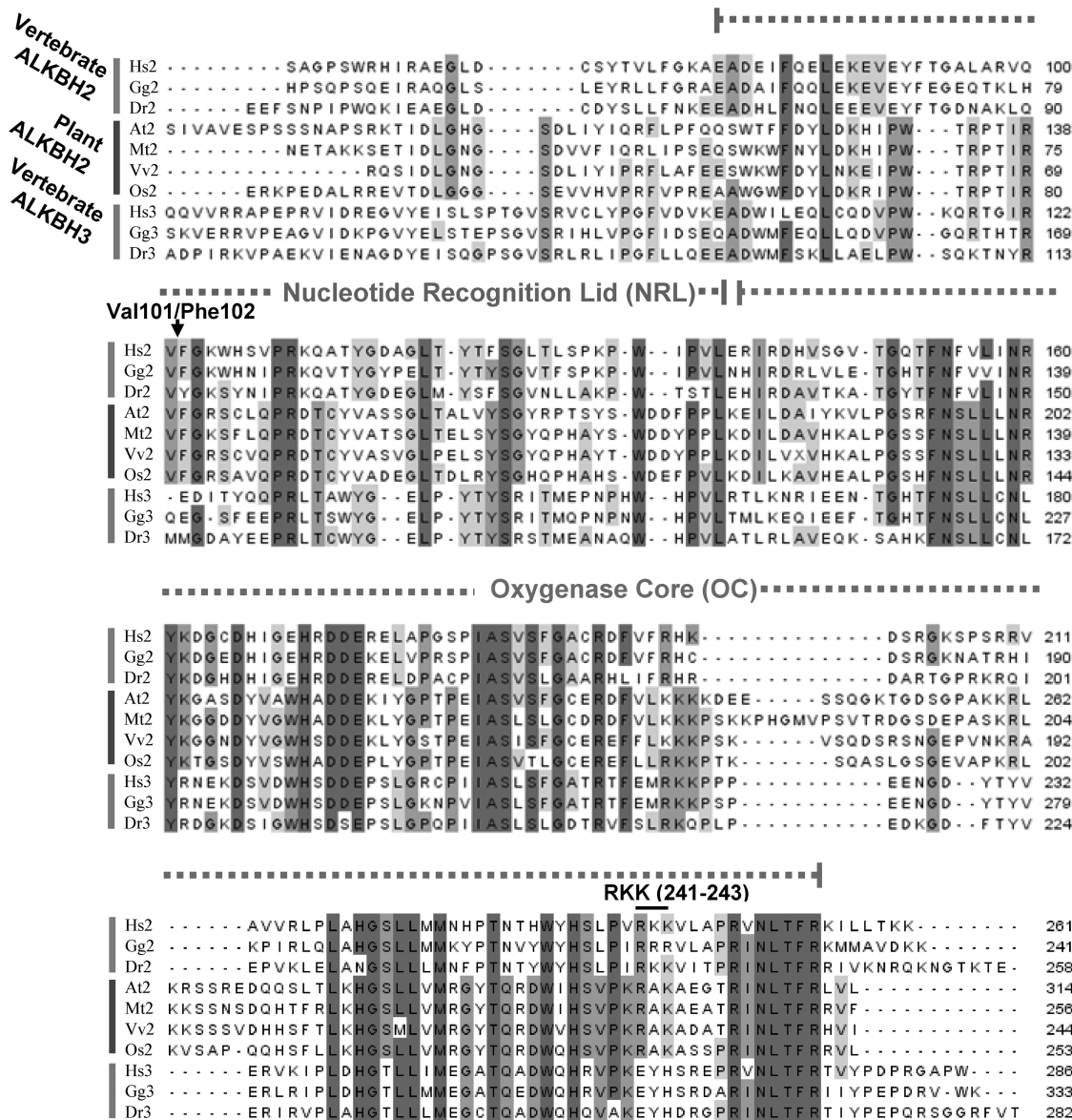
### Complementation of *alkB* mutant *E. coli* by ALKBH2

To address whether *Arabidopsis* ALKBH2 can function as an AlkB homologue in cells, we investigated its ability to complement the MMS sensitive phenotype of the *E. coli* *alkB* mutant strain. An expression construct for ALKBH2 was introduced into the *alkB* strain, and the bacteria were subjected to MMS treatment. In these experiments, ALKBH2 protected the *alkB* bacteria against MMS induced cell death to the same extent as EcAlkB (Figure 2A), indicating that *Arabidopsis* ALKBH2 is able to revert MMS-induced cytotoxic lesions that normally are repaired by EcAlkB.

The lesions that are repaired by the AlkB mechanism, such as 1-meA and 3-meC, are introduced much more efficiently by MMS treatment of ssDNA, relative to dsDNA (58). Thus, if a ssDNA phage, such as M13, is treated with MMS and used to infect *E. coli*, the production of progeny phage (‘phage survival’) is dramatically reduced in an *alkB* mutant, relative to wild-type cells (59). Accordingly, the expression of a functional AlkB homologue in the *alkB* mutant has been shown to substantially increase the survival of methylated ssDNA phage, and similar results have been obtained in the case of ssRNA phage (17,28). To investigate the ability of *Arabidopsis* ALKBH2 to reactivate ssDNA, the effect of ALKBH2 over-expression on the survival of MMS-treated M13 phage in the *alkB* mutant was investigated. Indeed, expression of ALKBH2 increased the survival of the methylated phage to an extent similar to that observed with EcAlkB (Figure 2B). When M13 phage was treated with chloroacetaldehyde (CAA), a vinyl chloride metabolite that introduces etheno adducts in DNA, ALKBH2 expression increased phage survival, albeit to a lesser extent than EcAlkB. These results indicated that *Arabidopsis* ALKBH2, like EcAlkB and mammalian ALKBH2, is capable of repairing etheno adducts (Figure 2C). The protein was unable to increase the survival of methylated ssRNA phage (data not shown). In summary, the results above indicate that *Arabidopsis* ALKBH2 is a functional EcAlkB homologue, involved in reversing methylation damage in DNA.

### *In vitro* repair activity of *Arabidopsis* ALKBH2

To further characterize the repair activity of *Arabidopsis* ALKBH2, we investigated the ability of *E. coli*-expressed

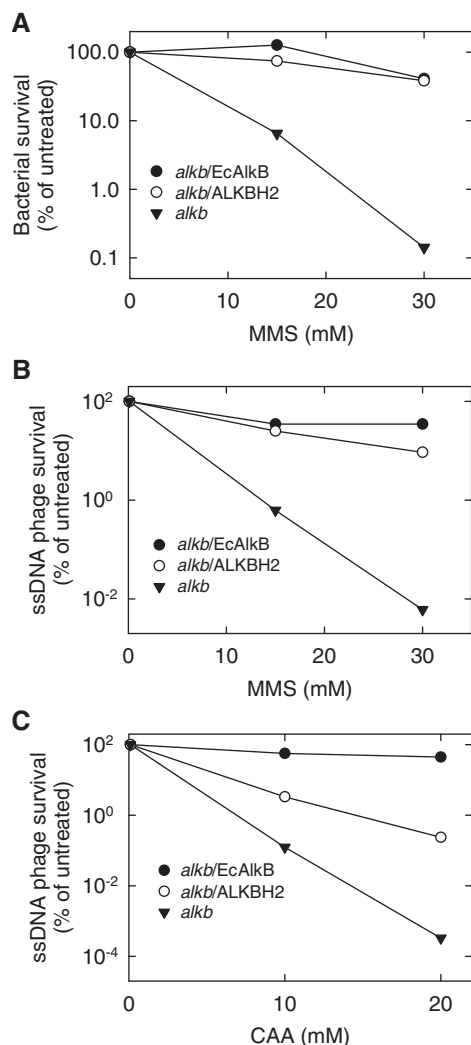


**Figure 1.** Multiple sequence alignment of putative ALKBH2 and ALKBH3 proteins from plants and vertebrates. The alignment was generated with JalView (<http://www.jalview.org/>) using the embedded MUSCLE algorithm (55,56). The 'Nucleotide Recognition Lid' and the 'Oxygenase Core', described by Yu et al. (57), are indicated, as well as residues corresponding to Val101-Phe102 (involved in base flipping) and Arg-Lys-Lys (241-243) (involved in interaction with the opposite DNA strand) of human ALKBH2. The non-conserved N-terminal part of the proteins have been omitted from the alignment. Hs, *Homo sapiens*; Gg, *Gallus gallus*; Dr, *Danio rerio*; At, *A. thaliana*; Mt, *Medicago truncatula* (barrel medic); Vv, *Vitis vinifera* (common grape vine); Os, *Oryza sativa* (rice). GI numbers of sequences: 47124096 (Hs2), 118098574 (Gg2), 68383159 (Dr2), 18399917 (At2), 87162794 (Mt2), 14777784 (Vv2), 222635406 (Os2), 21040275 (Hs3), 118091513 (Gg3), 51011105 (Dr3).

recombinant protein to repair various damaged substrates *in vitro*. Treatment of ssDNA with the radiolabelled methylating agent [<sup>3</sup>H]methylnitrosourea (MNU) generates 1-meA and 3-meC lesions containing a radiolabelled methyl group. Thus, AlkB-mediated repair can be investigated by measuring the release of ethanol-soluble radioactive formaldehyde from such a substrate, and this type of assay has previously been extensively used to study AlkB-mediated repair (11,12,17,28-30). Indeed, when a [<sup>3</sup>H]MNU treated oligonucleotide was incubated with 6×His-tagged, recombinant ALKBH2, ~40% of the total radioactivity was released from the substrate at the highest

enzyme concentrations (Figure 3A), similarly to what has previously been observed with bacterial and mammalian AlkB homologues (29). The *in vitro* activity of *Arabidopsis* ALKBH2 was also investigated on a dsDNA substrate generated by annealing the [<sup>3</sup>H]methylated ssDNA to its complementary strand, and the protein displayed a preference for dsDNA over ssDNA.

AlkB substrates generated by treatment of ssDNA with methylating agents such as MMS and MNU will contain both 1-meA and 3-meC. To investigate the activity of *Arabidopsis* ALKBH2 on individual lesions, we used [<sup>32</sup>P]-5'-end-labelled oligonucleotides where a single



**Figure 2.** *Arabidopsis* ALKBH2-mediated repair of DNA damage in the *E. coli alkB* mutant. EcAlkB-expressing plasmid and empty expression plasmid were included as positive and negative control, respectively. (A) Complementation of the MMS sensitive phenotype of *E. coli alkB* mutant bacteria by plasmid-expressed ALKBH2. The bacteria were treated with the indicated concentrations of MMS, plated out on agar plates, and the survival was assessed by colony counting. (B) Ability of ALKBH2 to increase the survival of methylated ssDNA phage. Phage M13 was treated with the indicated concentrations of MMS and mixed with *alkB E. coli* expressing the indicated proteins. Top agar was then added to the mixture, which was plated out on agar plates. Phage survival was scored by counting resulting plaques. (C) ALKBH2-mediated survival of CAA-treated ssDNA phage. Identical experiment to that in (B), except that CAA was used instead of MMS.

1-meA, 3-meC or  $\epsilon$ A lesion had been placed in the recognition sequence for the methylation sensitive restriction enzyme *DpnII* (Figure 3B). In this assay, the substrate is incubated with repair enzyme, then with *DpnII*, and the reaction mixture is analysed on an acrylamide gel, which is subsequently subjected to phosphorimaging. If the lesion is repaired, the *DpnII* site will become susceptible to cleavage, and two fragments are observed. In contrast, an unrepaired substrate will give rise to only a single fragment. In these experiments, ALKBH2 showed activity on 1-meA, 3-meC and  $\epsilon$ A, which all were

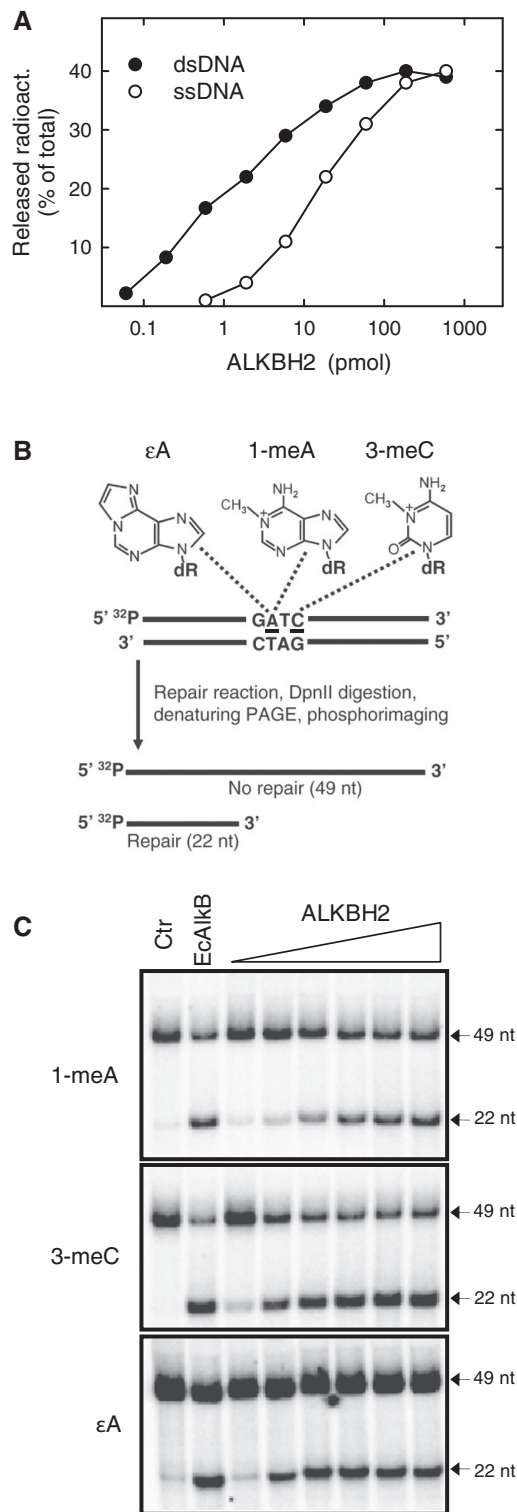
repaired to a similar extent as that observed with the EcAlkB protein (Figure 3C). Together, the complementation assays and *in vitro* experiments demonstrate that *Arabidopsis* ALKBH2, similarly to its mammalian counterpart, repairs both methyl and etheno lesions in DNA.

### Expression pattern of ALKBH2 in Arabidopsis

The expression pattern of the *ALKBH2* gene was examined in detail, utilizing an *Arabidopsis* line carrying a single copy of a transgene (*pAtALKBH2::GUS*) where the *ALKBH2* promoter was coupled to a *GUS* reporter gene. *pAtALKBH2::GUS* was shown to be active in all tissues examined (Figure 4), including seedlings, leaves and flowers. In seedlings, the expression was most notable in the shoot meristematic region, as well as in the vasculature of the hypocotyl and root (Figure 4A and B). *GUS* expression was observed in both primary and lateral root vasculature (Figure 4B). In flowers, *GUS* is expressed in sepals and stem (Figure 4C and D), as well as in carpel tissue and anther filaments (Figure 4E). *GUS* expression is also evident in true leaves (Figure 4F). *In silico* expression analysis using GENEVESTIGATOR (60), indicates that *ALKBH2* is expressed in all tissues during development (Figure 4G), in agreement with results from both the *GUS* line and RT-PCR analysis (Figure 4H). These results indicate that ALKBH2-mediated repair of alkylation damage is important in all parts of the plant.

### MMS hypersensitivity of *alkbh2-1* plants

Cells with defective alkylation repair enzymes, such as 3-methyladenine DNA glycosylases and AlkB proteins, commonly display an increased sensitivity towards methylating agents, such as MMS. This is particularly prominent in bacteria, where some alkylation repair mutants display a dramatic hypersensitivity, but there are also examples that mammalian knock-out cells lacking specific repair functions display increased sensitivity towards methylation damage. To our knowledge no studies have addressed the role of alkylation repair enzymes in protecting plants against the genotoxic effects of alkylating agents. To study the ALKBH2 function *in planta*, we took advantage of a mutant line (GT\_5\_41144, denoted *alkbh2-1*) annotated to have a Ds-transposon integrated in intron 3 (Figure 5A) and which appeared to represent a true knock-out line for the ALKBH2 function, since no mRNA for *ALKBH2* could be detected (Figure 5B). Characterization of the *alkbh2-1* line showed integration at position 257 in the *ALKBH2* gene, and that a 1-bp duplication had formed at the integration site (Figure 5C). The homozygous mutant plants were viable and did not display any visible phenotype when grown under normal conditions (data not shown). However, when the mutant seeds were germinated on MMS-containing media, the resulting plants displayed aberrant growth, while the wild-type plants were unaffected by the MMS treatment (Figure 5D and E). Since *Arabidopsis* ALKBH2 was able to reactivate phage DNA treated with CAA, which introduces etheno adducts, and since the protein showed *in vitro* activity on  $\epsilon$ A, we also investigated



**Figure 3.** *Arabidopsis* ALKBH2-mediated repair of methylated DNA *in vitro*. (A) Repair of ssDNA and dsDNA by ALKBH2. A [<sup>3</sup>H]methylated oligonucleotide, generated by treatment with [<sup>3</sup>H]MNU, was incubated with varying amounts of recombinant ALKBH2, and then precipitated with ethanol. The released ethanol soluble radioactivity (formaldehyde) was measured by scintillation counting. Open circles represent the [<sup>3</sup>H]methylated ssDNA oligonucleotide, whereas closed circles represent the corresponding dsDNA, generated by hybridization to the unlabelled complementary strand. (B) Schematic representation of the assay for repair of site-specific lesions in oligonucleotides. The substrate for repair is a 49-nt dsDNA oligonucleotide consisting of one

the effects of CAA treatment on *alkbh2-1* versus wild-type plants. We observed a negative effect of CAA on plant growth, but could not observe any differences between *alkbh2-1* and wild-type plants (data not shown). However, *Arabidopsis* has several putative DNA alkylbase glycosylases, enzymes shown capable of repairing etheno adducts (61), which may provide functional redundancy for such repair.

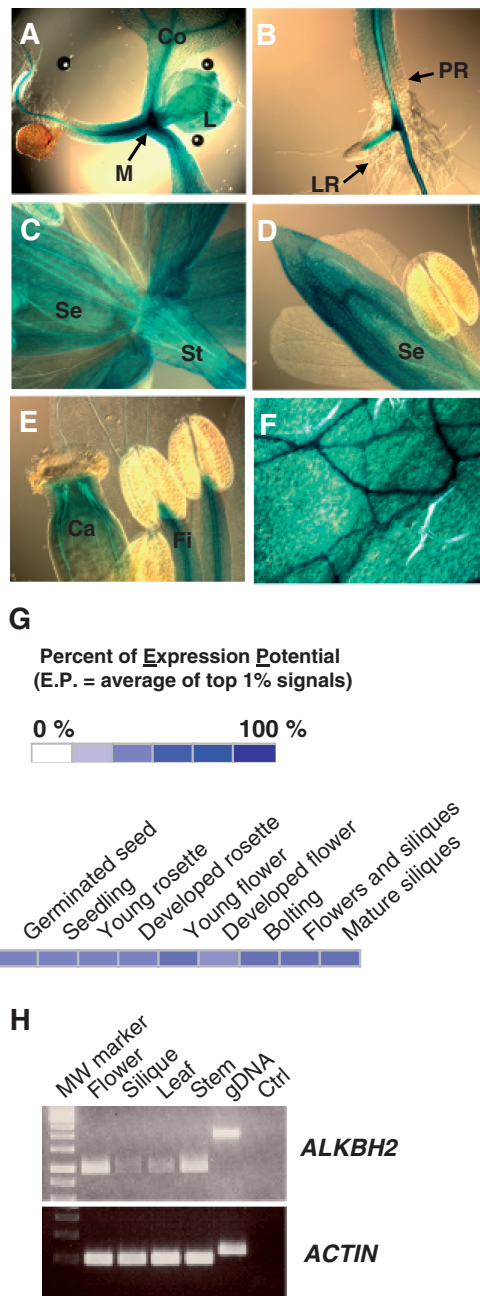
#### Accumulation of methylation damage in the genome of MMS-treated *Arabidopsis*

The most obvious explanation for the MMS sensitive phenotype of the *alkbh2-1* plants is that unrepaired, MMS-induced lesions block DNA replication and, thereby, cell growth. Thus, we investigated the levels of genomic 1-meA in wild-type and *alkbh2-1* plants after exposure to MMS. Seeds were germinated on MMS-free plates, and the resulting plants were transferred to MMS-containing liquid medium 6 days after germination, where they were incubated for up to 4 h before they were harvested, and levels of 1-meA were measured by LC-MS/MS. Clearly, growth of the plants in the presence of MMS caused a time-dependent introduction of 1-meA lesions, but the 1-meA levels were only marginally higher in the *alkbh2-1* plants than in the wild-type plants (Figure 6A). Also, when the plants were left in MMS-containing medium for a prolonged time period, the 1-meA levels were only slightly higher than in the wild-type plants (Figure 6B). These results indicate that ALKBH2-mediated repair of MMS-induced 1-meA lesions is inefficient *in planta*, possibly because the repair capacity is exceeded.

#### DISCUSSION

In this report, we describe the function of the *Arabidopsis* ALKBH2 protein, encoded by the *AT2G22260* gene, thereby establishing the existence of the AlkB mechanism for DNA repair also in plants. The *AT2G22260* protein sequence displays similarity to both mammalian ALKBH2 and ALKBH3, but our initial bioinformatics analysis revealed sequence features characteristic of ALKBH2. Moreover, the functional characterization revealed that *Arabidopsis* ALKBH2 and mammalian ALKBH2 (but not ALKBH3) share several important properties, i.e. exclusive activity on DNA (and not on RNA), preference for dsDNA

<sup>32</sup>P-end-labelled strand containing 1-meA, 3-meC or εA at a specific position, annealed to an unlabelled, lesion-free complementary strand. After incubation with recombinant repair enzyme, the substrate is digested with *Dpn* II, which is blocked by the presence of lesions in the recognition sequence. The digestion products are then analysed by gel electrophoresis and phosphorimaging, where repaired or unrepaired substrates will give rise to radiolabelled bands of 22 and 49 nt, respectively. (C) Repair activity of ALKBH2 on oligonucleotides containing site-specific lesions. The following amounts of ALKBH2 were used: 2 pmol (lane 3), 5 pmol (lane 4), 10 pmol (lane 5), 30 pmol (lane 6), 50 pmol (lane 7) and 100 pmol (lane 8). As a negative control, the oligonucleotide substrates were incubated with *Dpn* II without any previous repair reaction. Purified EcAlkB (100 pmol) was included as positive control. Note that it is commonly observed that a proportion of the DNA molecules are refractory to repair in these assays (here, this is particularly prominent for the εA containing substrate).



**Figure 4.** Expression pattern of *pALKBH2::GUS*. The expression pattern of *ALKBH2* was determined using a *pALKBH2::GUS* transgenic line. The blue GUS staining shows expression in (A) cotyledons (Co), meristematic region (M) and true leaves (L) in seedlings, (B) veins of primary (PR) and lateral roots (LR) in seedlings, (C) stem (St) and sepals (Se), (D) sepal, (E) carpels (Ca) and anther filaments (Fi), (F) Rosette leaf. (G) Expression pattern obtained from *in silico* analysis using GENEVESTIGATOR ([www.genevestigator.com](http://www.genevestigator.com)). (H) Expression pattern of *ALKBH2* using RT-PCR on cDNA from different tissues, using primers amplifying a fragment of 968 bp. Genomic DNA was used as positive control (fragment in the gDNA lane is larger due to intron sequences). The *ACTIN2-7* gene, giving a fragment of 255 bp with primers spanning intron 2, amplified at comparable levels from all tissues. Ctrl, no cDNA.

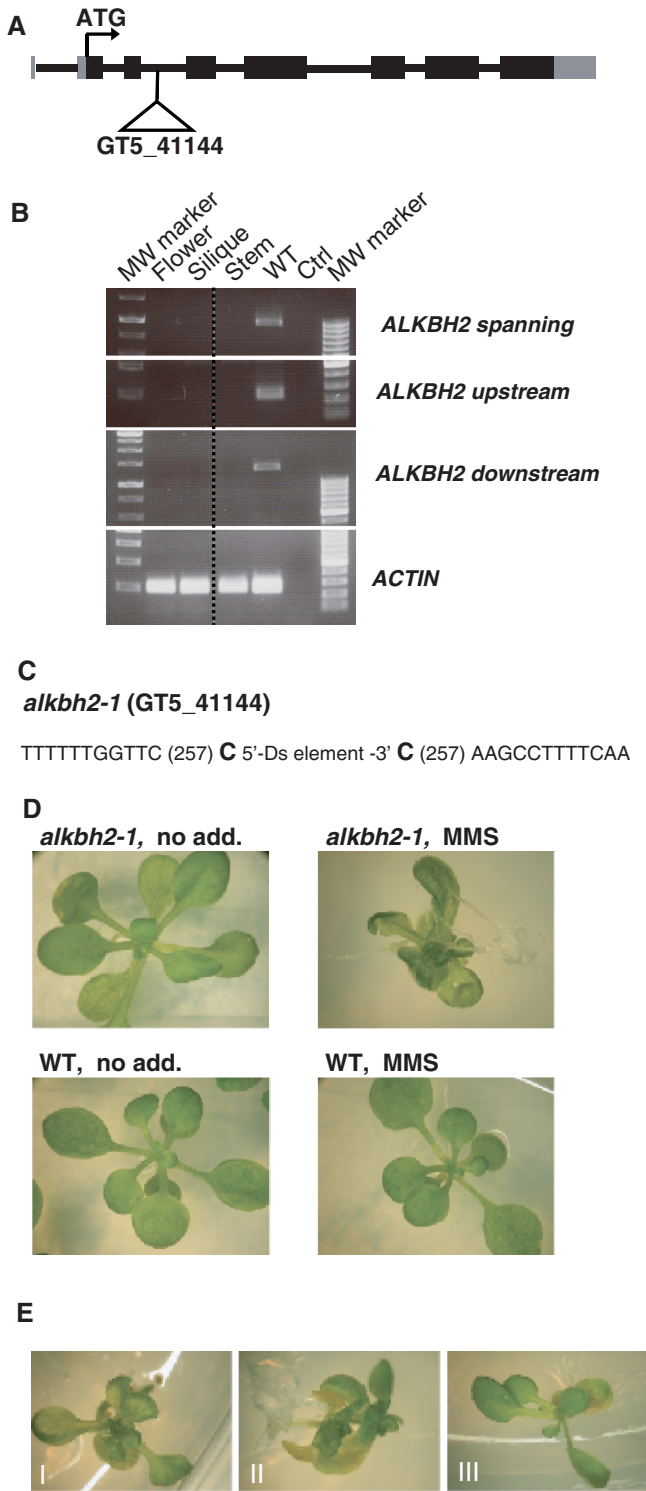
over ssDNA, and activity on etheno lesions, strongly indicating that these proteins are true orthologues. Furthermore, the observed MMS-sensitive phenotype of

*ALKBH2* defective *Arabidopsis* plants indicates that *ALKBH2* plays an important role in removing deleterious methyl lesions from the plant genome.

Based on protein sequence searches, the AlkB mechanism for DNA repair shows a rather patchy distribution across the tree of life. However, the genomes of multicellular eukaryotes generally encode several different AlkB homologues, most of which have unknown function, and are likely to have roles other than DNA repair. The mammalian ALKBH proteins represent a classic example of how function is not readily inferred from protein sequence similarity. *ALKBH1*, which displays the highest degree of sequence similarity to EcAlkB, has not been established as a DNA repair protein, whereas *ALKBH2* and *ALKBH3*, which are less similar to EcAlkB, appear to be genuine repair proteins with EcAlkB-like activities. A similar situation appear to exist in *Arabidopsis* where the proteins AT3G14160 (NP\_566479.5), and AT1G11780 (NP\_172643.1), which display sequence homology to EcAlkB and human *ALKBH1*, failed to show any repair activity in phage reactivation assays (P.Ø. Falnes and A. Bekkelund, unpublished observations) (27). Taken together with previous findings, our results indicate that nucleic acid repair by oxidative demethylation is performed by *ALKBH2/ALKBH3*-like proteins in eukaryotes, whereas *ALKBH1*-like proteins have a function other than DNA repair. Notably, many multicellular eukaryotes, including the model organisms *Drosophila melanogaster* and *Caenorhabditis elegans*, appear to lack *ALKBH2/ALKBH3*-like proteins (21). Possibly, these organisms use other enzymes, such as DNA glycosylases, for removing 1-meA lesions from the genome. Indeed, the DNA alkylbase glycosylase AlkA from the archeon *Archaeoglobus fulgidus* shows activity towards 1-meA and 3-meC lesions, as well as towards the canonical 3-meA substrate (62).

Mutant plants that lack the *ALKBH2* function developed normally and were fertile. However, these plants were hypersensitive to MMS, resulting in various developmental defects. Somewhat in contrast, we were not able to detect a significant increase in the levels of the *ALKBH2*-substrate 1-meA in plants that had been treated with MMS. Probably, the MMS-induced developmental defects displayed by *alkbh2-1* plants reflect DNA damage occurring in certain progenitor cells during development, whereas the 1-meA levels were measured as an average across the entire plant. Conceivably, the repair capacity of the expressed *ALKBH2* protein may in many tissues be too low to substantially reduce the level of MMS-induced 1-meA lesions, giving rise to a considerable background of unrepaired lesions. Alternatively, the toxic effects caused by MMS in the *alkbh2-1* mutant plants are not caused by 1-meA, but by a lesion which is more efficiently repaired. One candidate in this respect is 3-meC, which was a better *ALKBH2* substrate than 1-meA *in vitro* (Figure 3C), but which is unfortunately not detectable by our LC-MS/MS protocol (due to its identical molecular mass and similar LC elution time to the abundant DNA modification 5-methylcytosine). Similar to *alkbh2-1* plants, cells from mice with a targeted ablation of the *ALKBH2* function displayed hypersensitivity towards MMS, and they also showed a deficiency in repairing MMS-induced 1-meA lesions (32), supporting the notion that plant and mammalian *ALKBH2* have similar roles.



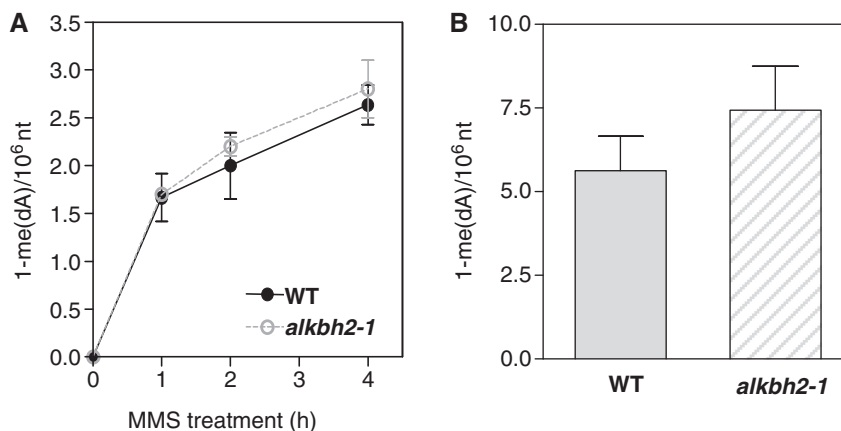


**Figure 5.** Validation and functional characterization of the *alkbh2-1* line. (A) Schematic representation of the Ds insertion in the *alkbh2-1* GT5\_41144 line. Exons are represented by boxes, while the intervening lines indicate introns. The untranslated part of exons is indicated in grey. (B) RT-PCR analysis of *ALKBH2* expression in the *alkbh2-1* line. Primer pairs upstream of, downstream of, or spanning the integration site were used. For each primer set, expression analysis was conducted on cDNA from different tissues from the *alkbh2-1* lines as indicated above, as well as on WT flowers as positive control. All samples were also tested with intron spanning *ACTIN* primers, showing presence of cDNA. Ctrl, no cDNA. (C) Determination of the DNA sequence at the site of Ds element insertion in the *alkbh2-1* (GT5\_41144) mutant.

Naturally occurring methyl halides, such as methyl chloride, methyl bromide and methyl iodide probably represent the most abundant methylating agents in the environment (6). Such compounds are emitted from plants, and they are generated during degradation of organic matter (3,4). Moreover, methyl bromide has been widely used as a fumigant to eliminate nematodes from crops (63). Methyl halides can induce various lesions in nucleic acids, including the AlkB substrates 1-meA, 3-meC and 1-meG (12,33,58). In *Arabidopsis*, the methyltransferase AtHOL1 has been shown to catalyse the formation of methyl halides through methylation of halide ions (64). Therefore, as part of the present study, we attempted to investigate whether *ALKBH2* has a role in protecting *Arabidopsis* against the potentially deleterious effects of endogenously generated methyl halides. To this end, *alkbh2-1* and wild-type seeds were germinated in the presence of iodide ions, which expectedly would cause the generation of substantial amounts of methyl iodide, a more potent methylating agent than methyl chloride or methyl bromide. The seedlings were analysed after two weeks, but we did not observe any iodide-induced 1-meA lesions in DNA, nor did we observe any difference in sensitivity towards iodide ions between wild-type and *ALKBH2*-deficient plants (data not shown). This suggested that endogenously generated methyl halides may not represent a major source of methylation damage in the *Arabidopsis* genome, at least not under the experimental conditions used here.

Mammalian *ALKBH2* is a nuclear protein associated with PCNA, which acts as a circular clamp on DNA during replication, coupling its repair function to DNA replication (28). *ALKBH2*-mediated DNA repair has also been shown to occur when DNA replication is inhibited, indicating that such repair is not strictly dependent on DNA replication, and may be important also in non-dividing tissues (32). Previous studies of the expression of DNA repair genes in plants indicate that some repair proteins are only found in proliferating tissues, whereas others, such as photoreactivation and mismatch repair enzymes, are found in non-proliferating cells as well (2). Our results show that *Arabidopsis ALKBH2* is expressed in both proliferating tissues and in leaves, which undergo little proliferation. A recent study, which investigated various AlkB homologues from *Arabidopsis*, but without detecting any repair activities, showed that *Arabidopsis ALKBH2*, like its mammalian counterpart, is a nuclear protein (27). Thus, it is reasonable to assume that the *ALKBH2* protein is involved in nuclear DNA repair in both proliferating and non-proliferating tissues in *Arabidopsis*.

The intron sequence flanking the Ds element inserted at position 257 in the *ALKBH2* gene is indicated, and the insertion of two extra nucleotides at the integration site is shown in bold. (D) MMS sensitive phenotype of *alkbh2-1* plants. Wild-type or *alkbh2-1* seeds were plated on medium with or without MMS, and seedlings resulting from 14 days of growth are shown. (E) Representative MMS-induced developmental defects of the *alkbh2-1* plants. Seedling with abnormal shape (I); seedling with rumpled leaves (II); seedling with necrosis (III).



**Figure 6.** MMS-mediated induction of 1-meA lesions in genomic DNA of wild-type and *alkbh2-1* *Arabidopsis*. (A) Time course of induction of 1-meA lesions by MMS treatment. Seedlings were treated with 90 ppm MMS at 18°C for the indicated time periods before genomic DNA was isolated and 1-meA content assessed by LC-MS/MS. (B) Induction of 1-meA lesions upon prolonged MMS treatment. Similar experiment as in (A), but the seedlings were grown in liquid media supplemented with MMS for 1 week at 18°C before 1-meA levels were assessed.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table 1.

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