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# Age-associated alterations in DNA methylation and expression of methyltransferase and demethylase genes in *Arabidopsis thaliana*

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## Abstract

Little is known about the contributions of DNA methylation/demethylation to plant aging and senescence. We used *Arabidopsis thaliana* to study how increasing age of an annual plant species influences DNA methylation. Based on methylation-sensitive DNA fragmentation assay, it could be concluded that aging *A. thaliana* was accompanied by DNA demethylation. Bisulfite sequencing reveals that cytosine methylation within the *Actin2* 3' untranslated region and internal transcribed spacer with 5.8S rRNA (*ITS1-5.8SrRNA-ITS2*) DNA regions decreased with *A. thaliana* growth and aging. We show that transcription of methyltransferase genes, chromomethyltransferase *AtCMT3* and methyltransferse *AtMETI*, significantly decreased during development and aging of the *A. thaliana* plants, whereas expression of demethylase genes – repressor of silencing *AtROS1*, demeter *AtDME*, and demeter-like *AtDML2* and *AtDML3* – increased at least at some stages of plant development. The data obtained in the present study suggest that plant DNA regions may undergo demethylation during plant aging *via* reduction of DNA methylation processes and activation of active DNA demethylation.

*Additional key words*: bisulfite sequencing, methylation-sensitive DNA fragmentation assay, plant ageing.

## Introduction

Cytosine DNA methylation is an epigenetic modification that is important for maintaining genome stability and regulating gene expression in higher plants and other organisms (Gehring and Henikoff 2007, Zhang *et al.* 2010, Vanyushin and Ashapkin 2011). Growing evidence suggest that DNA methylation is implicated in regulating gene expression across plant development and in response to environmental stresses (Zhang *et al.* 2010, Ding *et al.* 2014, Plitta *et al.* 2014, Liu *et al.* 2015). Cytosine DNA methylation of plant nuclear genomes is more extensive and involves a wider range of methylation sites than in animal nuclear genomes (Vanyushin and Ashapkin 2011), but it remains unclear how DNA methylation alters during plant ageing and whether contributes to ageing (Dubrovina and Kiselev 2016). Many studies show that DNA is methylated at a higher level in adult plant tissues than in juvenile tissues (Bitonti *et al.* 2002, Fraga *et al.* 2002, Valledor *et al.* 2010, Guo *et al.* 2011, Mankessi *et al.* 2011, Huang *et al.* 2012, Yuan *et al.* 2014, Sun *et al.* 2015), but there are also data reporting a lower level of DNA methylation in adult plant tissues than in juvenile ones (Demeulemeester *et al.* 1999, Baurens *et al.* 2004, Hasbún *et al.* 2005, Monteuuis *et al.* 2009, Meng *et al.* 2012, Michalak *et al*. 2015). Guo *et al.* (2011) and Yuan *et al.* (2014) found that the total genomic DNA methylation considerably increases with the age of bamboo plants. Taken together, the data suggest that methylation status of genomic DNA can vary during plant ageing and senescence resulting in changes in transcription of genes responsible for age-related plant deterioration.

The level and pattern of cytosine methylation are determined by both DNA methylation machinery and DNA demethylation machinery (Meyer 2011). The process of methylation is performed by methylases. Plants possess three methylase families: methyl-

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*Abbreviations*: CMT - chromomethyltransferase; DME - demeter; DML - demeter-like; DRM - domains rearranged methyltransferase; ITS - internal transcribed spacer; MET - methyltransferease; ROS 1 - repressor of silencing 1; RT - reverse transcriptase; UTR - untranslated region.
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transferases (METs), chromomethyltransferases (CMTs), and domains rearranged methyl-transferases (DRMs). The METs are responsible for maintaining methylation within symmetrical "CG" sites. In rare instances, these methylases can also affect cytosine methylation in a "CHG" context (where H = A, T or C) (Singh *et al.* 2008). The CMTs maintain cytosine methylation within partial symmetrical "CHG" sites but can also affect *de novo* methylation within asymmetrical "CHH" sites (Pavlopoulou and Kossida 2007). The DRMs maintains "CHH" methylation through persistent *de novo* methylation (Pavlopoulou and Kossida 2007). *Arabidopsis thaliana* (the ecotype Col-0) methyl-transferases are encoded by four *METs* (*AtMETI*, *AtMETIIA*, *AtMETIIB*, and *AtMETIII*), three *CMTs* (*AtCMT1*, *AtCMT2*, and *AtCMT3*), and three *DRMs* (*AtDRM1*, *AtDRM2*, and *AtDRM3*) genes, but only *AtMETI*, *AtCMT3*, *AtDRM1*, and *AtDRM2* were shown to be essential for DNA methylation (Pavlopoulou and Kossida 2007).

In contrast to DNA methylation, DNA demethylation can be passive and/or active (Ikeda and Kinoshita 2009). Whereas passive DNA demethylation may take place due to lack of maintenance of methylation during DNA replication, active demethylation occurs enzymatically by removing methylated cytosines. In plants DNA glycosylases were shown to exhibit DNA demethylation activity in combination with base excision repair process. Members of DNA glycosylase families demeter (DME) and repressor of silencing 1 (ROS1) were better studied than demeter-like 2 (DML2) and DML3 (Zhu 2009, Law and Jacobsen 2010). The four DNA glycosylases were shown to cut the N-glycosidic linkage between the DNA backbone and 5-methylcytosine. Then, the abasic site is removed by apurinic/apyrimidinic site lyase, and the resulting gap in the DNA chain is filled by DNA polymerase and ligase *in vivo* (Ikeda and Kinoshita 2009). Biochemical studies showed that AtROS1, AtDME, AtDML2, and AtDML3 are 5-methylcytosine DNA glycosylases that initiate a base excision pathway for active DNA demethylation in *Arabidopsis* (Zhu 2009).

The purpose of the present study was to elucidate whether nuclear cytosine methylation and transcription of methyltransferase/demethylase genes change during the life cycle of *A. thaliana*. We analyzed DNA methylation using methylation-sensitive DNA fragmentation assay and bisulfite sequencing. Also, we analyzed transcription of the four methyltransferase (*AtMETI*, *AtCMT3*, *AtDRM1*, and *AtDRM2*) and four demethylase (*AtROS1*, *AtDME*, *AtDML2*, and *AtDML3*) genes in *A. thaliana*.

## Materials and methods

**Plant material and growth conditions:** Plants (*Arabidopsis thaliana* L. ecotype Columbia, stored by our laboratory) were grown in pots filled with commercially available rich soil in a controlled environmental chamber (*KS-200*, *SPU*, Smolensk, Russia) at a temperature of 22 °C, an air humidity of 80 %, a 16-h photoperiod, and an irradiance of 70 μmol $m^{-2}$ $s^{-1}$ (Dubrovina *et al.* 2015). To compare DNA methylation at different stages of *A. thaliana* life cycle, two *A. thaliana* plants were collected after 1 (seedlings with two cotyledons), 4 (full rosettes with emerging flower shoots), 8 (full rosettes with developed flower shoots and developing siliques), and 12 (seed maturation and plant senescence) weeks after seed sowing (Fig. 1 Suppl.).

**DNA extraction and bisulfite sequencing:** For DNA purification, we collected shoots and the total DNA was extracted as described Kiselev *et al.* (2015b).

The cytosine methylation status of the 3' untranslated region (UTR) of the *Actin2* gene and the central part of the internal transcribed spacer with 5.8S rRNA (*ITS1-5.8SrRNA-ITS2*) DNA region of *A. thaliana* was analyzed using bisulfite sequencing as described Kiselev *et al.* (2015a). Briefly, the total of 1 μg of the genomic DNA was subjected to bisulfite modification using an *EZ* DNA methylation kit (*Zymo Research,* Irvine, CA, USA) according to the manufacturer's instructions. The DNA was converted using the following conditions: 95 °C for 5 min and 64 °C for 2 h. Cloned PCR products of the *Actin2* and *ITS* regions were used as positive controls for the bisulfite chemical reactions. The level of C to T transitions in the converted PCR products was greater than 98 %. After DNA conversion, a 361-bp *Actin2* fragment was amplified using primers 5'-AGG AATYGTTYAYAGAAAATGTT-3' and 5'-TATACA ATACTTATATTAACATTRCA-3'. Primers 5'-GAT GAAGAAYGTAGYGAAATGYGATA-3' and 5'-ACA ARARCRACCRATAAAATRTAAT-3' were used for amplification of a 281-bp product from the *ITS* DNA region. The primers were designed according to the *Zymo Research* recommendations. The PCR products were isolated from agarose gels using a *Cleanup* mini kit (*Eurogene*, Moscow, Russia) and subcloned as described Dubrovina *et al.* (2014). The clones were amplified and sequenced (8 clones for each plant and 16 clones for 2 plants for an analyzed stage) using an *ABI 3130* genetic analyzer (*Applied Biosystems*, Foster City, CA, USA) following the manufacturer's protocol and recommend-dations as described Kiselev *et al.* (2013a). There were 58 (7 CG, 4 CHG, and 47 CHH cites) and 70 (18 CG, 12 CHG, and 40 CHH cites) cytosines in the amplified regions of the *Actin* 2 and *ITS* genes, respectively. The *Basic Local Alignment Search Tool* (*BLAST*) program was used for sequence analysis. Multiple sequence alignments were performed using the *ClustalX* program (Altschul *et al.* 1990).

**Extraction of RNA and real time quantitative PCR analysis:** The total RNA was extracted as described Kiselev *et al.* (2013b) and its concentration was measured

with a spectrophotometer (*NanoPhotometer P-300*, Munich, Germany). We used RNA with the ratio of absorbances at 260 and 280 nm ($A_{260}/A_{280}$) higher than 1.8. Complementary DNAs were synthesized using 1.5 μg of the total RNA by an RNA PCR kit (*Silex M*, Moscow, Russia). The reactions were performed in 0.05 $cm^3$ aliquots of a reaction mixture, which contained reverse transcriptase (RT) buffer, 0.2 mM each of the four dNTPs, 0.2 μM oligo-$(dT)_{15}$ primer, and 200 U of Moloney murine leukemia virus polymerase at 37 °C for 1.5 h (Kiselev *et al.* 2013c). Samples of 0.5 $mm^3$ of reverse transcription products were then amplified by PCR. After cDNA synthesis, the 3'UTR of *Actin2* (GenBank accession No. NM_112764) was amplified using primers Act-S2 5'-GATTCAGATGCCCAGAA GTC-3' and Act-A2 5'-TCTGTGAACGATTCCTGGA-3' designed for the 3' end of the protein coding region. A 442-bp fragment of the *Actin* gene was amplified using DNA as template and a 356-bp fragment was amplified using cDNA as template (the difference of the 86 bp was due to the presence of an intron in the *Actin* gene). In the following real time quantitative PCRs (qPCRs), we used only those RT reactions that resulted in the 356 bp RT-PCR product for the *Actin* gene. We discarded those RT reactions that resulted in both the 356 bp RT-PCR product and the 442 bp RT-PCR product for the *Actin* gene, which indicated DNA contamination. Two biological replicates used for RNA isolation were presented by the whole one-week-old seedling and by leaves, inflorescences, and rosettes of the same whole plant 4, 8, and 12 weeks after seed sowing.

Complementary DNAs (*AtCMT3*, *AtMETI*, *AtDRM1*, *AtDRM2*, *AtDME*, *AtDML2*, *AtDML3*, *AtROS1*, *AtGAPDH*, and *AtActin*) were amplified using an *EvaGreen* real time PCR dye (*Biotium*, Hayward, USA); primers are listed in Table 1 Suppl. The real time qPCRs were performed using a real time PCR kit (*Syntol*, Moscow, Russia) in a thermocycler supplied with a multicolor real time qPCR detection system (*DNA Technology*, Moscow, Russia). An expression was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). After the calculations, value 1 was assigned to the most expressed sample in relative mRNA calculation in each qPCR reaction. Genes *AtActin2* (acc. No. NM_112764) and *AtGAPDH* (acc. No. NM_111283) were used as endogenous controls to normalize variance in the quality and amount of cDNA of *A. thaliana* used in each real time qPCR experiment. The *AtGAPDH* gene was selected in a previous study as a relevant reference gene for real time PCRs of *Arabidopsis* genes (Czechowski *et al.* 2005). We did not find significant differences in the data calculated using *AtActin2* or *AtGAPDH* as reference genes, and thus we decided to use both reference genes. Also, *Actin* genes are usually used as reference genes for plants, *e.g.*, *Actin* is one of the best reference genes for grape cells (Reid *et al.* 2006, Marum *et al.* 2012). A no-template control was included in every assay. The real time qPCR data shown were obtained from two independent experiments and are averages of eight technical replicates for each independent experiment (four qPCR reactions normalized to *Actin* and four qPCR reactions normalized to *GAPDH* expression for each independent experiment).

**Methylation-sensitive DNA fragmentation assay:** Restriction endonuclease BstHH I (50 $mm^3$, *SibEnzyme*, Novosibirsk, Russia) was used to conduct methylation-sensitive DNA fragmentation assay. A source of BstHH I is *Bacillus stearothermophilus* HH. Restriction reactions of BstHH I proceed if cytosine within its recognition site GCG↑C is not methylated. Activity of BstHH I is affected by 5'G(5mC)GC3'/3'CG(5mC)G5' and 5'G(5mC)GC3'/3'CGCG5' methylation, but it is not affected by 5'GCG(5mC)3'/3'(5mC)GCG5' and 5'GCG(5mC)3'/3'CGCG5' methylation. Restriction reactions were performed in volumes of 20 $mm^3$ containing 1 μg of DNA. The DNA samples were treated at 50 °C for 50 min and separated by electrophoresis on a 1.3 % (m/v) agarose gel.

**Statistical analysis:** Statistical analysis was carried out using the *Statistica 10.0* program. The data are presented as mean ± standard error of the mean (SE) and were tested for statistical significance by paired Student's *t*-test. $P < 0.05$ was selected as the point of minimal statistical significance of differences in all analyses.

## Results

Methylation-sensitive BstHH I restriction digestion was used to investigate DNA methylation in *A. thaliana* during plant growth, development, and ageing. The total DNA was isolated from *A. thaliana* plants 1, 4, 8, and 12 weeks after seed sowing and digested with BstHH1 (Fig. 1). Based on this assay, it could be concluded that cytosine DNA methylation gradually decreased during growth and ageing of the *A. thaliana* plants (Fig. 1).

Using bisulfite sequencing, we analyzed the total level of cytosine methylation within two control nuclear DNA regions (*Actin2* and *ITS*) of the *A. thaliana* plants grown for 1, 4, 8, and 12 weeks. The analysis reveals that the total level of cytosine methylation within the *Actin2* and *ITS* DNA regions gradually decreased with *A. thaliana* growth, maturation, and ageing (Fig. 2). The percentage of methylated cytosines at asymmetrical (CHH) sites increased with ageing the *Arabidopsis* plants in the *ITS* DNA region, whereas symmetrical and partially symmetrical (CG and CHG) methylation decreased with *A. thaliana* maturation within both the *Actin2* DNA region and the *ITS* DNA region (Table 1).

To elucidate the molecular mechanism of DNA demethylation during *Arabidopsis* growth and ageing, expression of four methyltransferase genes (*AtCMT3*,

*AtMETI*, *AtDRM1*, and *AtDRM2*) and four demethylase genes (*AtROS1*, *AtDME*, *AtDML2*, and *AtDML3*) in *A. thaliana* of different age was analyzed by real time qPCR. The analysis reveals that expression of *AtCMT3* considerably decreased during the first month of growth from one-week-old seedlings to four-week-old plants (Fig. 3*A*). Then, *AtCMT3* expression maintained essentially at the same level in leaves and rosettes of 4-, 8-, and 12-week-old plants. Transcription of *AtMETI* significantly decreased only in 12-week-old tissues ($P < 0.05$) and not in younger tissues (Fig. 3*B*). At the same time, expression of *AtDRM1* did not significantly change (Fig. 3*C*), but expression of *AtDRM2* considerably increased in the 4-, 8-, and 12-week-old leaves and 12-week-old rosettes but not in inflorescences (Fig. 3*D*).

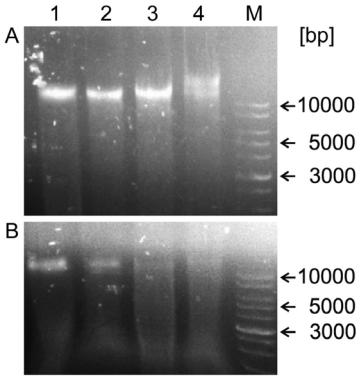


Fig. 1. An electrophoregram of total DNA samples before (*A*) and after (*B*) BstHH I treatment. The total DNA isolated from 50 *Arabidopsis* seedlings: *1* - 1-week-old, *2* - 4-week-old, *3* - 8-week-old, *4* - 12-week-old, M - molecular mass marker.

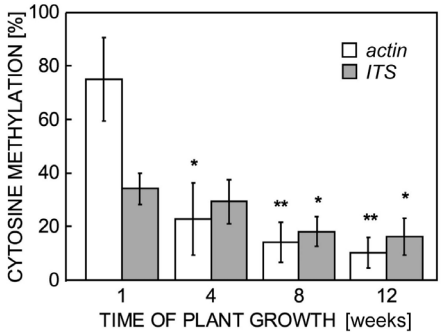


Fig. 2. Analysis of total cytosine DNA methylation within *Actin2* and internal transcribed spacer with 5.8S rRNA (*ITS*) DNA regions of *Arabidopsis thaliana*. Means ± SEs ($n$ =16) obtained from plants collected 1, 4, 8, and 12 weeks after sowing. Significant differences at * - $P < 0.05$ and ** - $P < 0.01$ *versus* values of cytosine methylation one week after seed sowing. The total number of cytosines was regarded as 100 % in each DNA region.

Table 1. Cytosine methylation in *Actin2* or internal transcribed spacer with 5.8S rRNA (*ITS*) DNA regions in different sequence contexts during growth and ageing of *A. thaliana* plants. The total number of cytosines in each DNA region and for each methylation context (CG, CHG, or CHH) was regarded as 100 %. Means ± SEs obtained from two independent experiments ($n$ = 16); significant differences at * - $P < 0.05$ and ** - $P < 0.01$ *versus* values measured in 1-week-old plants.

| | Age [week] | CG [%] | CHG [%] | CHH [%] |
|---|---|---|---|---|
| *Actin2* | 1 | 87.1 ± 13.6 | 92.4 ± 4.1 | 71.7 ± 17.4 |
| | 4 | 78.3 ± 6.3 | 96.7 ± 2.7 | 8.2 ± 1.6** |
| | 8 | 37.7 ± 5.8** | 61.2 ± 6.1* | 6.7 ± 1.7** |
| | 12 | 26.7 ± 6.6** | 42.4 ± 8.2** | 5.5 ± 1.4** |
| *ITS* | 1 | 76.7 ± 13.1 | 65.1 ± 10.6 | 6.0 ± 0.6 |
| | 4 | 42.2 ± 12.9* | 46.7 ± 14.5 | 18.1 ± 6.3** |
| | 8 | 14.8 ± 6.7** | 22.2 ± 10.0** | 18.3 ± 6.5** |
| | 12 | 18.8 ± 12.6** | 26.7 ± 14.7* | 12.1 ± 4.1* |

In contrast to *AtCMT3* and *AtMETI* expressions, expressions of the demethylase genes *AtROS1*, *AtDME*, *AtDML2*, and *AtDML3* markedly increased during growth of *A. thaliana* (Fig. 4). Expressions of the *AtROS1* gene in leaves and rosettes were 2 - 6 times higher in the 4-, 8-, and 12-week-old plants than in the 1-week-old seedlings (Fig. 4*A*). The *AtDME* gene displayed the same tendency in expression in the 4- and 8-week-old plants (Fig. 4*B*). However, its expression did not considerably increase in 12-week-old rosettes and inflorescences compared to the 1-week-old seedlings. Expressions of the *AtDML2* and *AtDML3* genes markedly increased only in the 4-week-old plants (Fig. 4*C*,*D*). Further growing the *Arabidopsis* plants led to a decrease in *AtDML2* and *AtDML3* expressions reaching expressions in the 1-week-old seedlings (Fig. 4*C*,*D*).

## Discussion

A number of studies show that ageing in humans and animals is generally characterized by genome-wide hypomethylation and site-specific hypermethylation (*e.g.*, Johansson *et al.* 2013). The available data on plants reviewed in Dubrovina and Kiselev (2015) indicate that DNA methylation vary depending on age of plant tissues and chronological age of the whole plant. However, there is lack of investigations where DNA methylation would be analyzed depending on age of the whole plant. Guo *et al.* (2011) and Yuan *et al.* (2014) found that the total genomic DNA methylation considerably increases with age of bamboo. Conversely, the data obtained in the present study show that the total genomic DNA cytosine methylation and *Actin2*- and *ITS*-specific DNA methylation decreased during growth and ageing of *A. thaliana*. Similar results were obtained by Michalak *et al.* (2015) on *Quercus robur* seeds; a decrease in seed viability during their ageing was highly correlated with a

global decline in amount of 5-methylcytosine in the genomic DNA. In the present investigation, we show that the level of asymmetrical CHH methylation increased during *A. thaliana* growth for the *ITS* DNA region, whereas the symmetrical CG and partial symmetrical CHG methylation decreased both in the *Actin2* DNA region and the *ITS* DNA region. These results are in accordance with the data published by Vaillant *et al.* (2008) that asymmetrical cytosine hypermethylation in ribosomal 5S DNA correlates with ageing of *Arabidopsis.*

There are many studies investigating methyltransferase and demethylase mutant phenotypes of *Arabidopsis* (Zhang and Jacobsen 2006, Williams *et al.* 2015). In these studies, important biochemical properties

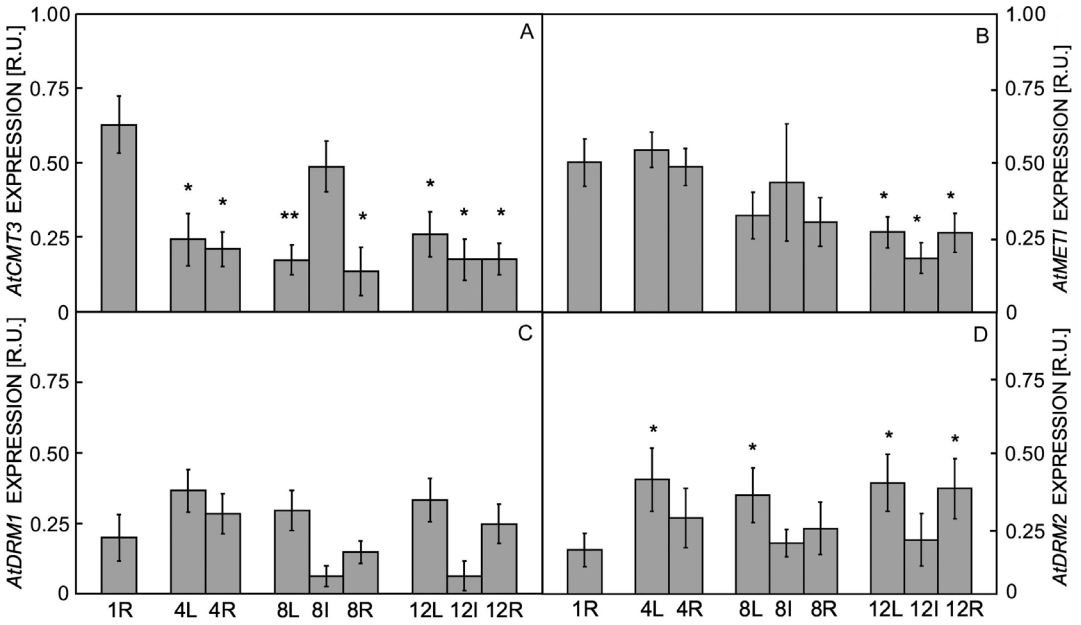


Fig. 3. Expressions of methyltransferase genes *AtCMT3* (*A*), *AtMETI* (*B*), *AtDRM1* (C), and *AtDRM2* (*D*) in *Arabidopsis thaliana*. 1R, 4R, 8R, and 12R: the total RNA extracted from 1-, 4-, 8-, and 12-week-old *Arabidopsis* seedlings and rosettes; 4L, 8L, and 12L: RNA extracted from leaves of 4-, 8-, and 12-week-old plants; 8I and 12I: RNA extracted from inflorescences of 8- and 12-week-old plants. Means ± SEs ($n$ = 16). Significant differences at * - $P < 0.05$ and ** - $P < 0.01$ *versus* values of methyltransferase gene expression in one-week-old *A. thaliana* seedlings (1R).

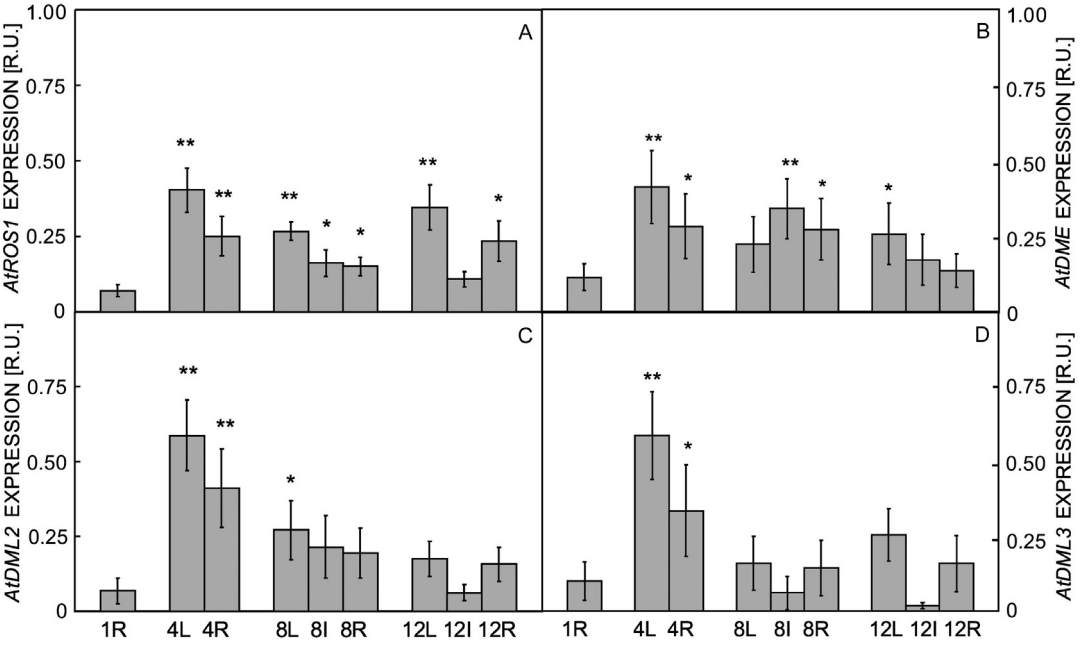


Fig. 4. Expressions of demethylase genes *AtROS1* (*A*), *AtDME* (*B*), *AtDML2* (C), and *AtDML3* (*D*) in *Arabidopsis thaliana*. For the description of the *x*-axis and statistical treatment - see Fig. 3.

of *Arabidopsis* methyltransferases and demethylases are characterized. Although there is some information about methyltransferase and demethylase gene expression in different plant organs (primary root tips, seedlings, leaves, floral organs, and siliques) and under different treatments (Huang *et al.* 2014, *AtGenExpress* database), the data were collected from different experiments and the oldest used *Arabidopsis* tissues were only 35-d-old. Thus, there was no information about methyltransferase and demethylase transcription during the whole life cycle of *A. thaliana* plants in one generation. Based on the present analysis of methyltransferase and demethylase gene transcription at different stages of *Arabidopsis* life cycle, it is clear that expressions of *CMT3* and *MET1* methyltransferases decreased with plant age, whereas expression of *ROS1* demethylase increased in the 4-, 8-, and 12-week-old plants in comparison with the 1-week-old seedlings. This suggests that plant DNA regions may undergo demethylation during plant ageing *via* reduction of DNA methylation processes and activation of active DNA demethylation. Expressions of *DME*, *DML2*, and *DML3* demethylases increased in juvenile and adult *Arabidopsis* tissues but not in senescent ones except for *DME* expression in senescent leaves. The data suggest that changes in *DME*, *DML2*, and *DML3* transcriptions could be connected with *Arabidopsis* development from juvenile to adult plants, however, it is not clear whether changes in *DME*, *DML2*, and *DML3* transcriptions contribute to *Arabidopsis* ageing and senescence.

The data obtained in the present study suggest that the plant DNA regions may also undergo hypomethylation during ageing which is likely to affect transcription and genome stability. Plants lack a reserved germ line, and their reproductive structures and gametes form late in growth cycles by differentiation of somatic meristematic cells (Buss 1983). Therefore, it is possible that plant somatic cells might pass accumulated alterations in DNA methylation patterns (epimutations) to further plant generations, which may potentially influence plant ecological and evolutionary fitness. Further investigations of occurrence and possible roles of DNA methylation/ demethylation processes in plant ageing and senescence are necessary.

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