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ddm1 plants are sensitive to methyl methane sulfonate and NaCl stresses and are deficient in DNA repair

Youli Yao · Andriy Bilichak · Andrey Golubov · Igor Kovalchuk

Abstract Plant response to stress includes changes in gene expression and chromatin structure. Our previous work showed that *Arabidopsis thaliana* Dicer-like (DCL) mutants were impaired in transgenerational response to stress that included an increase in recombination frequency, cytosine methylation and stress tolerance. It can be hypothesized that changes in chromatin structure are important for an efficient stress response. To test this hypothesis, we analyzed the stress response of *ddm1*, a mutant impaired in *DDM1*, a member of the SWI/SNF family of adenosine triphosphate-dependent chromatin remodeling genes. We exposed *Arabidopsis thaliana ddm1* mutants to methyl methane sulfonate (MMS) and NaCl and found that these plants were more sensitive. At the same time, *ddm1* plants were similar to wild-type plants in sensitivity to temperature and bleomycin stresses. Direct comparison to *met1* plants, deficient in maintenance methyltransferase *MET1*, showed higher sensitivity of *ddm1* plants to NaCl. The level of DNA strand breaks upon exposure to MMS increased in wild-type plants but decreased in *ddm1* plants. DNA methylation analysis showed that heterozygous *ddm1/DDM1* plants had lower methylation as compared to fourth generation of homozygous *ddm1/ddm1* plants. Exposure to MMS resulted in a decrease in methylation in wild-type plants and an increase in *ddm1* plants. Finally, in vitro DNA excision repair assay showed lower capacity for *ddm1* mutant. Our results provided a new example of a link between genetic genome stability and epigenetic genome stability.

Key message We demonstrate that heterozygous *ddm1/DDM1* plants are more sensitive to stress and have more severe changes in methylation than homozygous *ddm1/ddm1* plants.

Keywords Methyl methane sulfonate · Sodium chloride · *Arabidopsis thaliana* · *ddm1* mutant · *met1* mutant · Stress response · DNA repair capacity · Strand breaks · DNA methylation · Epigenetic regulation of stress response

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Y. Yao · A. Bilichak · A. Golubov · I. Kovalchuk (✉)
Department of Biological Sciences, University of Lethbridge, University Drive 4401, Lethbridge, AB T1K 3M4, Canada
e-mail: igor.kovalchuk@uleth.ca

Y. Yao
e-mail: youli.yao@uleth.ca

A. Bilichak
e-mail: a.bilichak@uleth.ca

A. Golubov
e-mail: andrey.golubov@uleth.ca

Introduction

An efficient response to stress depends on the ability of plants to express an array of genes allowing plants to weather stressful conditions. The epigenetic response to stress in *Arabidopsis* involves changes in DNA methylation as well as changes in the association of DNA with various modified histones.

In plants, DNA methylation is involved in regulating many epigenetic phenomena (Bender 2004; Chan et al. 2005; Vanyushin and Ashapkin 2011; Yaish et al. 2011). These include transcriptional silencing of transposons and transgenes, defense against pathogens, regulation of imprinting as well as the silencing of genes that control

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flowering time, floral organ identity, fertility, and leaf morphology (Finnegan et al. 1996; Jacobsen et al. 2000; Kakutani et al. 1996; Miura et al. 2001; Soppe et al. 2000).

A DECREASE IN DNA METHYLATION1 (DDM1), an ATP-dependent SWI2/SNF2 chromatin remodeling factor, is also required for normal patterns of genomic DNA methylation in Arabidopsis (Jeddeloh et al. 1999; Vongs et al. 1993). Mutations in the DDM1 gene result in a rapid loss of cytosine methylation at heterochromatic repetitive sequences and a gradual depletion of methylation at euchromatic low-copy sequences over successive generations (Kakutani et al. 1999). It is believed, however, that the mechanism by which DDM1 regulates DNA methylation is likely indirect (Verbsky and Richards 2001). Gendrel et al. (2002) showed that DDM1 might be responsible for histone H3 methylation; in ddm1 heterochromatin, DNA methylation is lost and methylation of lysine 9 is largely replaced by methylation of lysine 4 (Gendrel et al. 2002). The chromatin remodeling factor DDM1 is known to maintain 5S rDNA methylation patterns while silencing transcription through 5S rDNA intergenic spacers (IGS) (Kurihara et al. 2008). DDM1 has been shown to regulate gene imprinting, transposons, gene and transgene silencing (Hirochika et al. 2000; Vielle-Calzada et al. 1999), and possibly the occurrence of paramutations (Jeddeloh et al. 1998). DDM1 apparently stabilizes the activity of transposons; one of the ddm1-induced abnormalities was shown to be caused by insertion of CAC1, an endogenous CACTA family transposon (Miura et al. 2001).

Selfed ddm1 plants progressively accumulate morphological changes, including defects in leaf structure, flowering time and flower structure (Kakutani et al. 1996). Alterations in DDM1 induce stable epigenetic mutations such as bonsai (BNS) gene silencing, which is mediated by the activity of a long interspersed nuclear element (LINE) transposon flanking the BNS gene (Saze and Kakutani 2007). Thus, it can be hypothesized that a ddm1 mutant would be sensitive to stress. Indeed, the ddm1 mutant was shown to have sensitivity to γ-radiation and UVC (Shaked et al. 2006). Since the authors provided a very limited amount of data on stress sensitivity of the ddm1 mutant and no other data on stress response in the ddm1 mutant exist, we decided to test it.

To analyze stress sensitivity of ddm1, we used methyl methane sulfonate (MMS) and NaCl. Being a DNA-methylating agent, MMS predominantly generates 7-methylguanine (N7-MeG) and sister chromatid exchanges (Kaina et al. 2004; Wyatt and Pittman 2006); it is frequently used for the analysis of sensitivity of various mutants impaired in DNA repair and genome maintenance (Bagherieh-Najjar et al. 2005). We also tested the sensitivity to NaCl. Toxicity of NaCl is believed to be associated with homeostasis misbalance in plants, including a

sodium/potassium pump. Although there is no direct proof that NaCl can damage DNA directly, previous research suggests that exposure to NaCl results in an increase in strand breaks and homologous recombination frequency (Boyko et al. 2010a, b; Dmitrieva and Burg 2008; Puchta et al. 1995).

Our analysis showed that ddm1 plants were extremely sensitive to MMS and mildly sensitive to NaCl. The mutant also showed a disturbed methylation pattern—a lower level in plants grown under normal conditions and a higher level in plants exposed to MMS.

Materials and methods

Plant germination and exposure to methyl methane sulfonate (MMS) and NaCl stresses

The Arabidopsis ddm1 mutant [ddm1-2 mutant line; (Vongs et al. 1993); the seeds are obtained from Dr. Martienssen] was used for the experiments (Gendrel et al. 2002). As a control, wild-type Col-0 plants were used. Seeds were sterilized in 10 % commercial bleach, stratified at 4 °C for 4 days and germinated in 1/2 MS medium. One week after germination, they were transplanted to plates with different concentration of NaCl or MMS where they grew in the same Petri dish for another 2 weeks. For MMS exposure, plants were grown at 0, 80, 100 and 150 ppm MMS. The latter concentration was used for the analysis of survival rate calculated as the percentage of plants that survived (containing at least some green tissue) at 150 ppm MMS for 2 weeks after treatment. For NaCl exposure, plants were grown on 75 and 150 mM NaCl. Each experiment was repeated at least three times. Only in those plants that remained the following parameters were compared: fresh weight, root length as well as the percentage of surviving plantlets at 150 ppm MMS and on 150 mM NaCl. Twenty plants per each experimental group were used for each measurement and all experiments were performed three times.

Crosses between ddm1 mutants and wild-type plants and between met1 mutants and wild-type plants

The Arabidopsis homozygous ddm1 (ddm1-2 mutant line) and met1-3 (Saze et al. 2003) (GenBank accession no. L10692) mutants were crossed with wild-type plants (Col-0). The heterozygous progeny (ddm1/DDM1 and met1/MET1) of the cross (as determined by gene-specific and T-DNA-specific primers) was selfed, and the progeny was tested for tolerance to NaCl and MMS. Also, the homozygous ddm1/ddm1 and met1/met1 mutants were identified and propagated for four generations. Stress tolerance was compared

between wild-type plants, the population of selfed *ddm1/DDM1* and *met1/MET* plants and the fourth generation of homozygous *ddm1/ddm1* and *met1/met1* plants. For the analysis of stress tolerance, seeds were germinated in 1/2 MS medium, and 1 week after germination, they were transplanted to plates containing different concentrations of NaCl or MMS where they grew in the same Petri dish for another 1 week.

For the analysis of stress tolerance of population of selfed *ddm1/DDM1* the following arbitrary scores were given to plants: 0, non-germinated seeds; 1, germinated cotyledons without roots; 2, cotyledons with roots; 3, cotyledons with roots and two true leaves; 4, cotyledons with roots and four true leaves; 5, cotyledons with roots and more than four true leaves. The percentage of plants with scores of 0–5 was calculated and compared between wild-type plants and the population of selfed *ddm1/DDM1* plants (Fig. 3).

For comparison of stress tolerance in selfed population of *ddm1/DDM1* or *met1/MET1* and fourth generation of *ddm1/ddm1* or *met1/met1* plants, the following arbitrary scores were given: 0, non-germinated seeds; 1, plants without roots or true leaves; 2, plants with roots and true leaves. In this case, the average score was calculated according to the following formula: score = $(0 \times N + 1 \times N + 2 \times N)$/the total number of plants, where N is the number of plants in a given category (Fig. 4).

For the analysis of stress tolerance of the population of selfed *ddm1/DDM1* and *met1/MET* plants, the following arbitrary scores were given: 0, non-germinated seeds; 1, plants without roots or true leaves; 2, plants with roots; 3, plants with roots and at least two true leaves. The percentage of plants with scores of 0–3 was calculated and compared between wild-type plants and the population of selfed *ddm1/DDM1* or *met1/MET* plants (Fig. 8). In each case, the population of 20 plants per each Petri dish was analyzed. Two–three Petri dishes per each experimental group have been used and experiments were done in triplicates.

DSB measurements (the ROPS assay)

The quantification of 3′OH DNA breaks was performed as previously described (Boyko et al. 2007; Boyko and Kovalchuk 2010a). Two independent measurements from each of three independent experiments were done.

Analysis of global genome methylation

Global genome methylation was analyzed as published before (Boyko and Kovalchuk 2010b). In brief, genomic DNA was prepared from 20 three-week-old plantlets using Trizol reagent. DNA was digested for 48 h with a tenfold excess of either *Hpa*II or *Msp*I endonuclease according to the manufacturer's protocol (New England Biolabs, Beverly, MA). An additional DNA aliquot was incubated without any restriction enzyme as a background control. A single-nucleotide extension reaction was performed in 2 µg of DNA using the cytosine extension assay described previously (Boyko and Kovalchuk 2010b). The data obtained from three independent experimental groups with two measurements per each group are expressed as a percentage of dpm/µg of DNA relative to background controls.

In vitro repair assay

All reactions were done in triplicates. LITMUS29 (NEB, USA) was used in the experiments. Circular, linearized blunt-end (*Stu*I, Fermentas) non-damaged and damaged (UV light, $\lambda_{max} = 254$ nm, 450 J/m^2) as well as linearized non-sticky-end (*Bam*HI/*Kpn*I, Fermentas) non-damaged and damaged plasmids (UV light, $\lambda_{max} = 254$ nm, 450 J/m^2) were used for the assay. Each reaction (25 µl total volume) contained one form of plasmid DNA (300 ng/reaction), 2.5 µl 10× reaction buffer (Roche, Mannheim, Germany), 2.5 µl 10× dNTP/DIG-11-dUTP mix (Roche, Mannheim, Germany), and 10 µg of a partially purified plant extract (Li et al. 2002). Plant extracts were prepared from 3-week-old WT and *ddm1* plants (10 plants per each sample). Reaction mixes were incubated in the dark for 2 h at 25 °C. The reactions were stopped with 20 mM EDTA (the final concentration). DNA (120 ng) was separated by electrophoresis in 0.8 % agarose/1× TAE, visualized with ethidium bromide, photographed and transferred to a nylon membrane (Roche, Mannheim, Germany) in the 10× SSC solution using a vacuum blotter (Appligene, UK). After transfer, DNA was bound to a membrane by UV light using a Spectrolinker XL-1000 (Spectronics Corp.) and detected with the DIG Nucleic Acid Detection Kit (Roche, Mannheim, Germany) using anti-DIG-alkaline phosphatase conjugate and NBT/BCIP as a substrate according to the manufacturer's instructions (Roche, Mannheim, Germany). Membranes were scanned and quantified with ImageJ.

Statistical analysis

All experiments were repeated at least three times, and mean values ± SD were calculated. The statistical significance of the results was confirmed by performing a Student's *t* test or single factor ANOVA. Statistical analyses were performed using Microcal Origin 6.0.

Results

The *ddm1* mutant is more sensitive to MMS

We analyzed how *ddm1* plants (Mittelsten Scheid and Paszkowski 2000) respond to stress. The mutant *ddm1* is impaired in *DDM1*, a member of the SWI/SNF family of adenosine triphosphate-dependent chromatin remodeling genes (Gendrel et al. 2002). Exposure to 0, 80, 100 and 150 ppm MMS showed that *ddm1* plants were phenotypically more sensitive to MMS as compared to wild-type (WT) plants (Fig. 1a).

To obtain measurable parameters, we analyzed plant biomass (fresh weight), root length and survival rate at 21 days post-germination (dpg). First of all, it was noticed that *ddm1* plants were substantially smaller ($P < 0.01$) than control Col-0 wild-type plants (Fig. 1).

We found the fresh weight of *ddm1* plants to be smaller ($P < 0.05$) than that of WT plants grown on media containing all concentrations of MMS tested (Fig. 1b). To show the percentage of decrease in fresh weight upon exposure to MMS, we related the fresh weight of exposed plants to that of non-exposed; the analysis clearly showed that *ddm1* plants lost more fresh weight as compared to WT plants (Fig. 1S). Measurements of root length in plants exposed to MMS confirmed the aforementioned data. Significantly smaller roots ($P < 0.05$) were formed in *ddm1* plants exposed to 150 ppm MMS (Fig. 1c). Non-exposed wt and *ddm1* plants had similar root length ($P > 0.1$).

Next, we analyzed survival rates in plants exposed to MMS. The analysis showed that *ddm1* plants had lower survival rates in response to both 100 and 150 ppm MMS. This analysis confirmed the trend observed while measuring fresh weight and root length (Fig. 1d).

### Exposure to NaCl partially confirmed the trend observed for MMS exposure

To check whether similar differences can also be found for exposure to a different stress, we exposed plants to 0, 75 and 150 mM NaCl (Fig. 2a). Analysis of fresh weight showed that *ddm1* performed only slightly worse than wt plants in response to both, 75 and 150 mM of NaCl (Fig. 2b). To show the percent of decrease in fresh weight upon exposure to NaCl, we related the fresh weight of exposed plants to non-exposed; the analysis showed that *ddm1* plants lost more fresh weight than WT plants only when exposed to 150 mM NaCl (Fig. 2S).

Analysis of root length showed minor but significant ($P < 0.05$) changes; the roots of *ddm1* plants exposed to 150 mM NaCl were shorter than in WT plants (Fig. 2c).
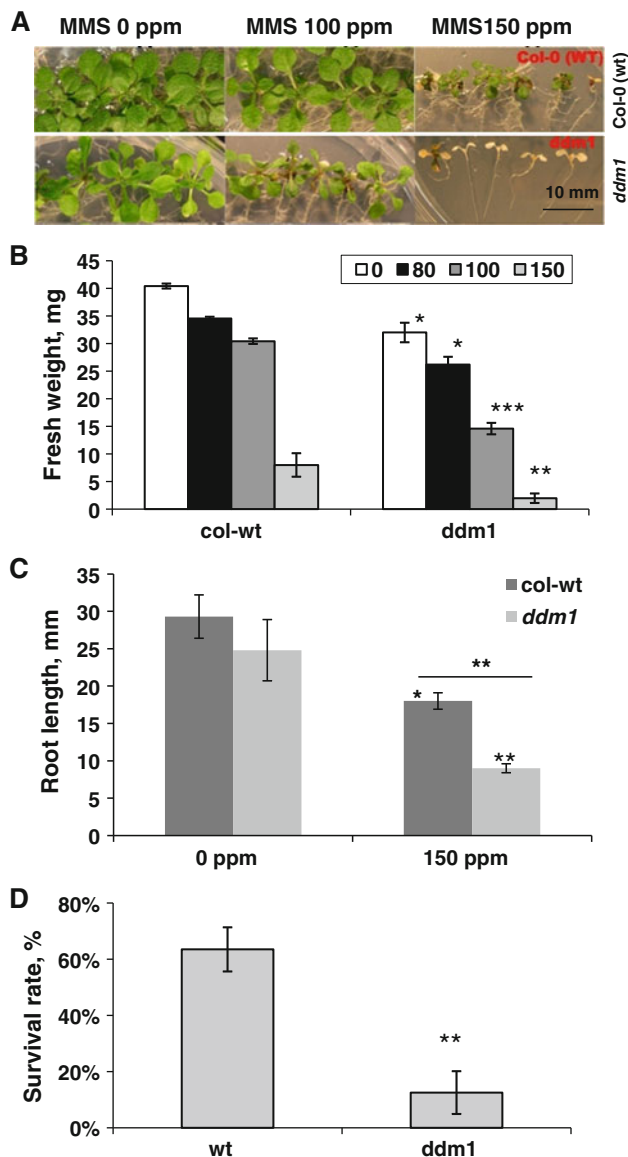
Fig. 1 *ddm1* plants are sensitive to MMS. Plants were germinated on MS media supplemented with 0, 80, 100 and 150 ppm MMS. Tissues were harvested at 3 weeks post-germination. Twenty plants per each experimental group were used for each measurement. **a** Representative images of plants taken at 3 weeks post-germination. The image of plants grown on MS media supplemented with 80 ppm MMS is not shown. The *bar* shows the size—10 mm. **b** Fresh weight (in mg) was measured in plants exposed to 80, 100 and 150 ppm MMS. The data show the average (from three independent experiments with SE). *Asterisks* show the difference between *ddm1* and wild-type plants, where *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. **c** The average length of roots (mm ± SD as calculated from three independent biological repeats) was measured in plants exposed to 0 or 150 ppm MMS. The *asterisks* indicate a significant difference between treated and non-treated and between wt and *ddm1* plants (*$P < 0.05$, **$P < 0.01$). **d** The survival rate was calculated as a percentage of plants that survived treatment with 150 ppm MMS. The data are shown as the average with SD. The *asterisk* indicates a significant difference ($P < 0.01$)
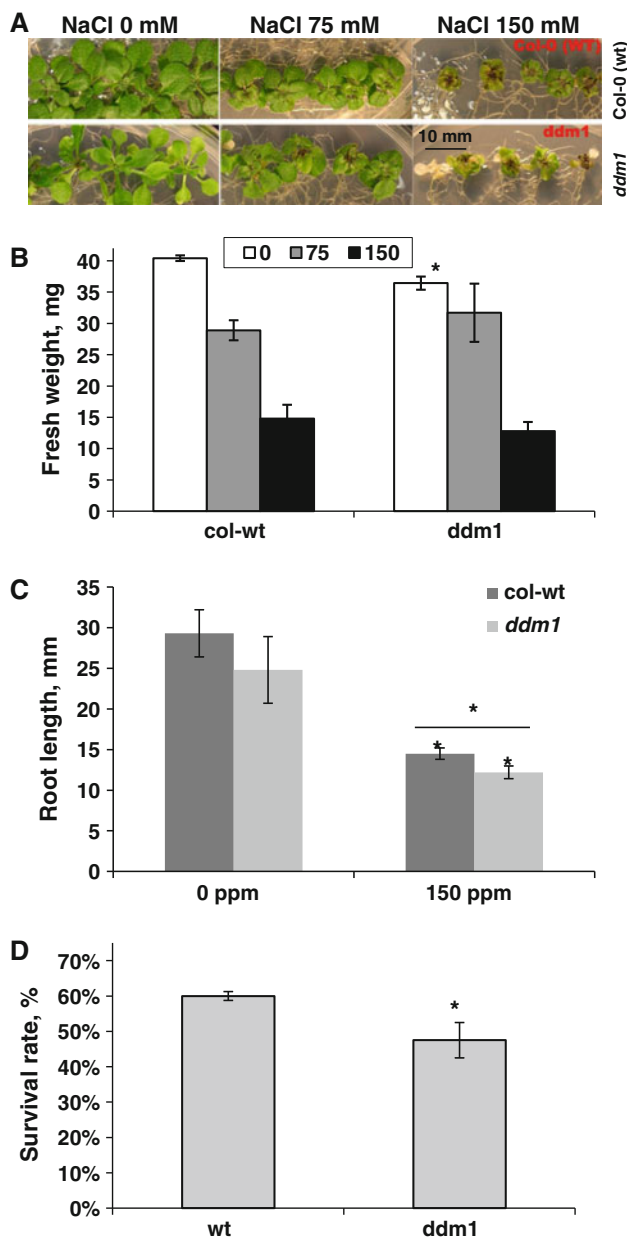
Fig. 2 *ddm1* plants are mildly sensitive to NaCl. Plants were germinated on MS media supplemented with 0, 75 and 150 mM NaCl. Tissues were harvested at 3 weeks post-germination. **a** Representative images of plants taken at 3 weeks post-germination are shown. The *bar* shows the size—10 mm. **b** Fresh weight (in mg) was measured in plants exposed to 0, 75 and 150 mM NaCl. The data show the average (from three independent experiments with SE). The *asterisk* show the difference between *ddm1* and wild-type plants (*P* < 0.05). **c** The average length of roots (mm ± SD as calculated from three independent biological repeats) was measured in plants exposed to 0 or 150 mM NaCl. The *asterisks* indicate a significant difference between treated and non-treated and between wt and *ddm1* plants (*P* < 0.05). **d** The survival rate was calculated as a percentage of plants that survived treatment with 150 mM NaCl. The data are shown as the average with SD. The *asterisk* indicates a significant difference (*P* < 0.05)

Non-exposed wt and *ddm1* plants had similar root length (*P* > 0.1). Similarly, the analysis of survival rates showed that *ddm1* plants had a significantly (*P* < 0.05) lower survival rate as compared to wild-type plants exposed to 150 mM NaCl (Fig. 2d).

The *ddm1* mutant did not differ from wild-type plants in response to temperature stress or bleomycin

Next, we checked for temperature sensitivity. We germinated plants on soil and at 7 dpg transferred them to grow at either +37 or +4 °C. Five days later, the plants were moved to normal conditions (+22 °C) and allowed to recover for 3 days. Despite some visible changes in plant response, no significant differences in fresh weight and root length between wt and *ddm1* mutant exposed to either temperature were observed (*P* > 0.1 in all cases) (Fig. 3S). We also tested exposure to bleomycin, a chemical that prevents the establishment of a proper methylation pattern, and also did not find any significant difference in plant performance (data not shown).

Heterozygous *ddm1/DDM1* plants are also sensitive to NaCl

It is known that selfed *ddm1* plants accumulate defects in leaf structure and flowering time (Kakutani et al. 1996). However, it is not known whether these plants become progressively more sensitive to stress. Unfortunately, it was not possible to obtain the original segregating populations of *DDM1/ddm1* plants. To test the hypothesis whether *ddm1* plants propagated for more generations are more sensitive to stress, we crossed *ddm1/ddm1* plants with wild-type *DDM1/DDM1* Col-0 plants. The progeny of the cross, *ddm1/DDM1* plants, were grown into the next generation and the progeny plants were tested for the resistance to NaCl by exposing plants to 75, 100 or 125 mM of salt. We hypothesized that a segregating population will show stress resistance such that 25 % of the population would be much more sensitive to stress. Our analysis showed that in fact 70–80 % of the selfed *ddm1/DDM1* plants were extremely sensitive to NaCl (Fig. 3). Non-exposed plants from both groups were similar in their performance (data are not shown). The difference in sensitivity was more apparent at higher levels of concentration of NaCl. We thus concluded that heterozygous *ddm1/DDM1* plants are also more sensitive to NaCl.

Next, we compared the sensitivity to NaCl in the population of *ddm1/DDM1* plants and the fourth generation of homozygous *ddm1/ddm1* plants. Wild-type plants were used as a control. Our analysis showed that the fourth
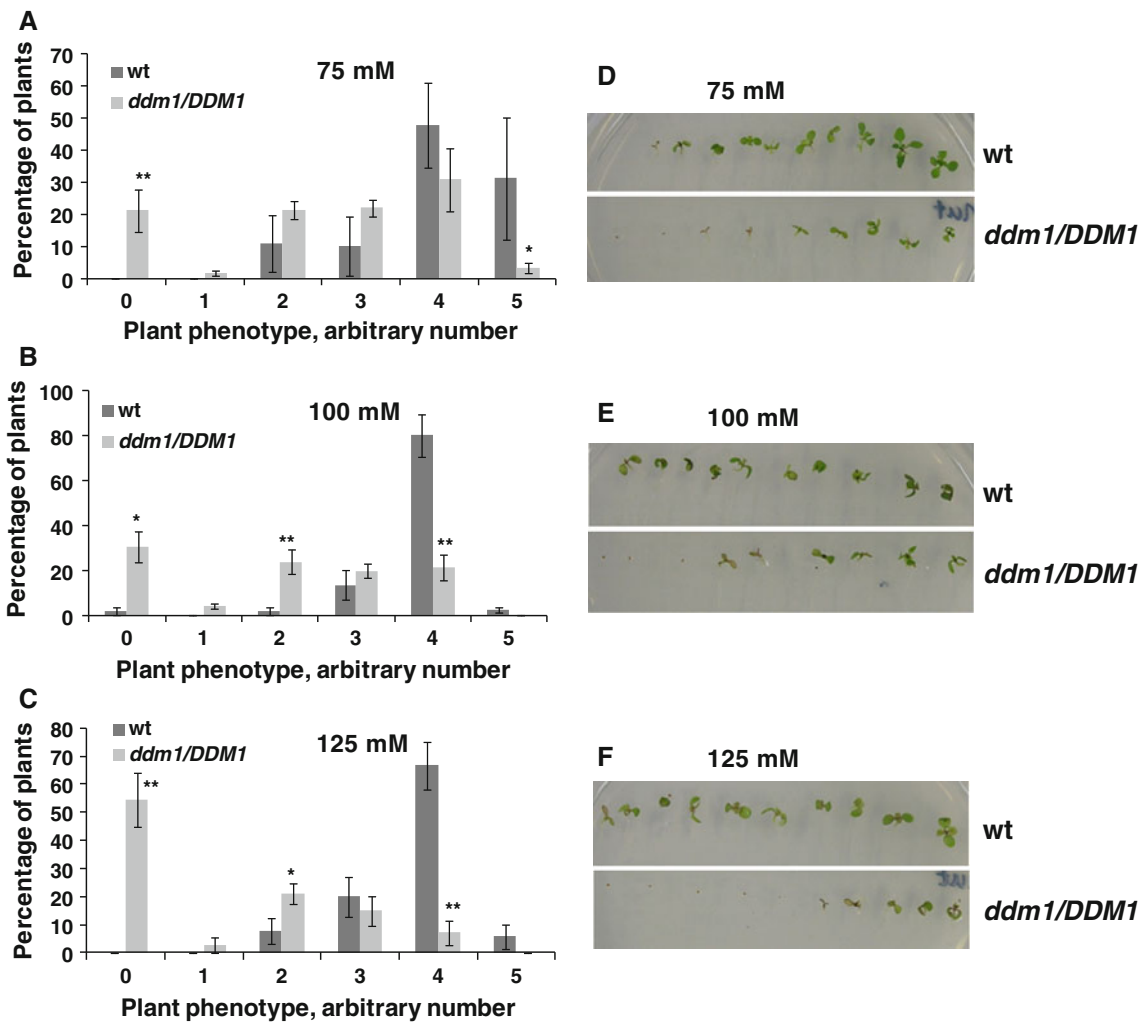
Fig. 3 Analysis of NaCl tolerance of population of selfed *ddm1/ DDM1* and wild-type plants. Wild-type plants and population of selfed *ddm1/DDM1* plants were exposed to 75 mM (**a**), 100 mM (**b**) or 125 mM (**c**) NaCl. At the age of 2 weeks phenotype of plants was analyzed and given arbitrary number: *0* non-germinated seeds, *1* germinated cotyledons without roots, *2* cotyledons with roots, *3* cotyledons with roots and two true leaves, *4* cotyledons with roots and four true leaves, *5* cotyledons with roots and four or more true leaves. *Y* axis shows the average (with SD, calculated from 3 experiments) percentage of plants with given arbitrary number. *Asterisks* show significant difference between the *ddm1/DDM1* and the wild-type groups: one asterisk—*$P < 0.05$, **$P < 0.01$. **d–f** Show representative images; plants were aligned from the weakest to the strongest in the wild type and the *ddm1/DDM1* groups

generation of *ddm1/ddm1* plants was less sensitive to salt stress (75 and 125 mM NaCl) as compared to the population of *ddm1/DDM1* (Fig. 4a, b). In contrast, exposure to 50 and 100 ppm MMS showed an opposite picture—*ddm1* plants were more sensitive to 50 ppm MMS and similarly sensitive to 100 ppm MMS as compared to *ddm1/DDM1* plants (Fig. 4c, d). Unexposed plants from all three groups were not different from each (data not shown).

The *ddm1* mutants have higher levels of strand breaks and lower levels DNA methylation

To analyze the level of DNA damage in the homozygous *ddm1* plants and WT plants, we analyzed the level of strand breaks using the ROPS assay (Boyko and Kovalchuk

2010a). The analysis showed that the spontaneous level of breaks in *ddm1* was over fivefold higher than in WT plants. To test whether growing plants on MS media supplemented with MMS would increase the number of strand breaks, we exposed 7-day-old WT and *ddm1* plants to 80 ppm MMS for 2 weeks. The analysis of strand breaks in exposed plants showed that WT plants had over 80 % increase, whereas the level of strand breaks in *ddm1* plants was decreased by over 2.5-fold.

We hypothesized that the reason *ddm1* plants had such a high spontaneous level of DNA strand breaks was due to changes in DNA methylation. Since *ddm1* plants are known to have lower levels of methylation, we decided to analyze whether a methylation pattern changes upon stress. We analyzed methylation levels using the cytosine
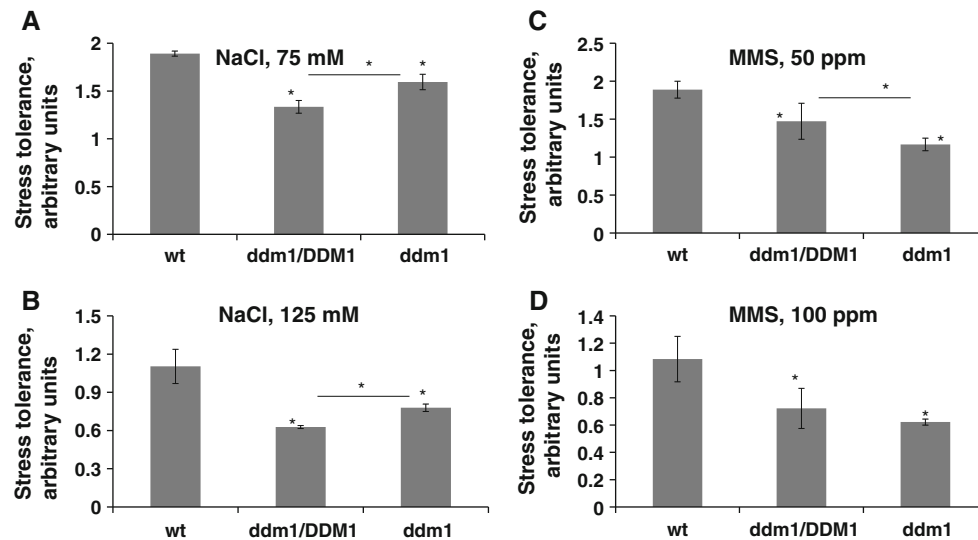
Fig. 4 Comparison of NaCl and MMS tolerance of population of selfed *ddm1/DDM1*, fourth generation of *ddm1* and wild-type plants. Wild-type plants and population of selfed *ddm1/*wt plants were exposed to 75 mM (**a**) and 125 mM (**b**) NaCl or 50 ppm (**c**) and 100 ppm (**d**) MMS. At the age of 2 weeks phenotype of plants was analyzed and given arbitrary number: *0* non-germinated seeds, *1* plants without roots or true leaves, *2* plants with roots and true leaves. *Y* axis shows the stress tolerance calculated according to the following formula: score = $(0 \times N + 1 \times N + 2 \times N)$/the total number of plants, where *N* is the number of plants in a given category. Data are shown as averages from three independent experiments (with SD). *Asterisks* show significant difference between the wild-type group and either *ddm1/DDM1* or *ddm1* groups as well as between *ddm1/DDM1* and *ddm1* groups ($P < 0.05$ in all cases)

extension assay (Boyko and Kovalchuk 2010b). The assay is based on the differential ability of *Hpa*II and *Msp*I endonucleases to cut the CCGG sequence. Both external and internal cytosines at this restriction site can be methylated. Methylation of the external cytosine in CCGG completely prevents digestion with *Msp*I and severely inhibits (~3,000-fold) digestion with *Hpa*II (McClelland et al. 1994). Methylation of the internal cytosine in CCGG does not influence digestion with *Msp*I, but it prevents digestion with *Hpa*II.

The assay showed that the level of spontaneous methylation in homozygous *ddm1* was approximately 35 % lower than that in WT plants (Fig. 5). Analysis of methylation in plants exposed to 80 ppm MMS for 2 weeks showed that methylation in WT plants was reduced by 10 % in *Hpa*II-based ROPS and by 20 % in *Msp*I-based ROPS. In contrast, the level of methylation in the exposed *ddm1* plants increased by 81 and 95 % in *Hpa*II- and *Msp*I-based ROPS, respectively. There was a strong inverse correlation between changes in DSB levels and methylation levels in WT and *ddm1* plants ($r = -0.89$ and $r = -0.72$, respectively). Thus, these experiments showed that *ddm1* plants have an abnormal response to MMS in the form of a dramatic decrease in strand breaks and an increase in methylation levels.

Curiously, when the methylation was compared between the heterozygous *ddm1/DDM1* plants (as tested by PCR) and fourth self-propagated homozygous generation of *ddm1/ddm1* plants, it was found that *ddm1/DDM1* plants

had only 40 % of methylation in wild-type plants, whereas *ddm1/ddm1* plants had 65 % (Fig. 6). This experiment suggests that self-propagation of *ddm1* plants does not result in progressive loss of methylation.

In vitro DNA repair assay

To test the possible contribution of base excision repair (BER) towards an increase in microsatellite instability, we performed BER and nucleotide excision repair (NER) assays using extracts prepared from wt and homozygous *ddm1* plants. This assay is based on the ability of partially purified protein extracts to perform the repair of non-damaged or UV-damaged circular or linear plasmid DNA molecules. Incubation of the plasmid DNA with protein extracts from wt and *ddm1* plants showed that repair efficiency of all types of DNA (circular or linear, UV-irradiated or not) molecules was dramatically lower in *ddm1* plants (Fig. 7). Lower band intensity would suggest less frequent involvement of BER/NER (Li et al. 2002) in *ddm1* plants, further confirming lower efficiency of the DNA repair pathways in this mutant.
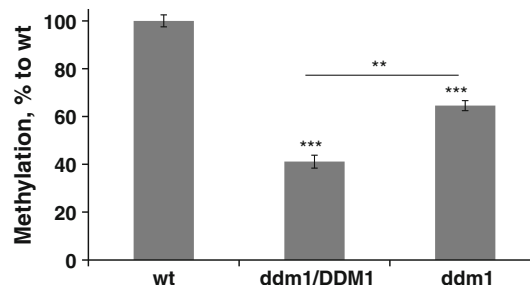
ddm1 and *met1* are differently sensitive to NaCl

A direct comparison of sensitivity of *ddm1* and *met1*, another mutant impaired in cytosine methylation (Kankel et al. 2003), showed that whereas the *ddm1* mutant was sensitive to radiation, the *met1* mutant behaved as a
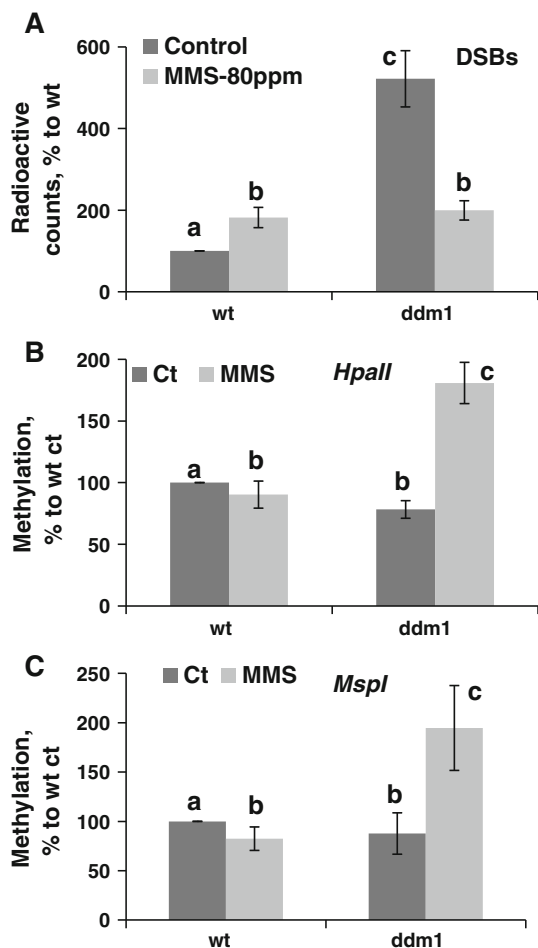
Fig. 5 Changes in strand break levels and methylation levels in wild-type and *ddm1* plants. Plants were germinated on normal MS media or MS media supplemented with 80 ppm MMS. Tissues were harvested at 3 weeks post-germination. **a** Double-strand breaks were measured via the ROPS assay. The level of strand breaks is shown as the level of radioactive counts (in % to a WT control ± SD). Data sets that are not connected with the *same letters* are statistically different. **b** Methylation levels were measured by the cytosine extension assay using *Hpa*II enzyme. Methylation levels are shown as a percentage to a WT control (with SD). Data sets that are not connected with the *same letters* are statistically different. **c** Methylation levels were measured by the cytosine extension assay using *Msp*I enzyme. Methylation levels are shown as a percentage to a WT control. Data sets that are not connected with the *same letters* are statistically different

Fig. 6 Changes in methylation in wt, *ddm1/DDM1* and fourth generation of *ddm1* plants. Plants were germinated on soil and tissues from 20 plants were harvested at 3 weeks post-germination. Heterozygous *ddm1/DDM1* plants were identified using the combination of gene-specific and T-DNA-specific primers. Methylation levels were measured by the cytosine extension assay using *Hpa*II enzyme. Methylation levels are shown as a percentage to a wild-type control (with SD). *Asterisks* show significant difference between wild-type and either *ddm1/DDM1* or fourth generation of *ddm1* plants as well as between *ddm1/DDM1* and *ddm1* groups: **$P < 0.01$, ***$P < 0.001$
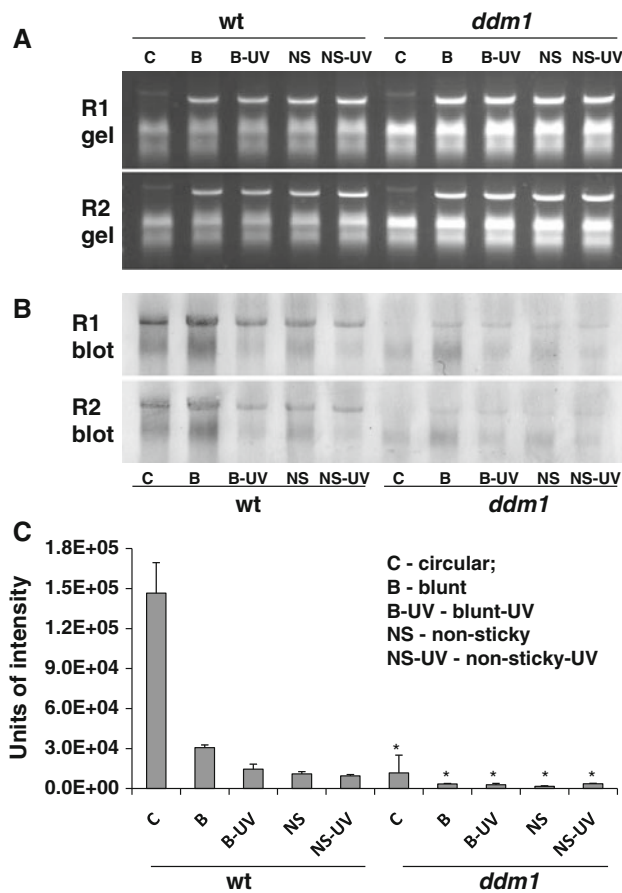
was similar to that in *ddm1/DDM1* plants and significantly smaller than that in wild-type plants (Fig. 8d). Thus, our experiments confirmed that *ddm1* plants are more sensitive to NaCl than *met1* plants. In contrast, a comparison of tolerance to NaCl in the fourth generation of *ddm1/ddm1* and *met1/met1* plants showed that these plants were equally sensitive to 75 mM NaCl as compared to wild-type plants; and *met1/met1* plants were more sensitive than *ddm1/ddm1* plants when exposed to 125 mM NaCl (Fig. 4S). It was curious to note that the fourth generation of *ddm1/ddm1* plants was more tolerant to NaCl as compared to the population of selfed *ddm1/DDM1* plants. In contrast, the fourth generation of *met1/met1* plants was more sensitive to NaCl as compared to the population of selfed *met1/MET1* plants (Fig. 4S). These experiments suggested that stress tolerance in *ddm1* plants increases, whereas, in *met1* plants, it decreases with propagation, suggesting that the changes associated with response to NaCl may accumulate in *met1* but not in *ddm1* plants.

Discussion

We demonstrated that *ddm1* plants were more sensitive to MMS and NaCl as compared to wild-type plants. Analysis of methylation and strand breaks showed that *ddm1* plants had a higher spontaneous level of strand breaks and a lower level of methylation as compared to wild-type plants. The response to MMS exhibited itself as an increase in strand breaks and a decrease in the methylation level in wild-type plants, but in *ddm1* plants the effect was opposite: there was an increase in the methylation level and a decrease in strand breaks.

MMS is a DNA-methylating agent that leads to methylation of guanines resulting in N7-MeG in over 80 % of

wild-type plant. The authors suggested that changes in chromatin structure rather than changes in methylation were more crucial for stress tolerance (Shaked et al. 2006). To test whether *ddm1* and *met1* plants are differently sensitive to NaCl, we crossed *met1* plants with wild-type Col-0 plants. A comparison of the sensitivity of populations of *ddm1/DDM1* and *met1/MET1* showed that the selfed progeny of *ddm1/DDM1* plants were much more sensitive to NaCl as compared to the selfed progeny of *met1/MET1* plants (Fig. 8). Although the progeny of *met1/MET1* plants was more tolerant to NaCl, their root length

Fig. 7 The in vitro DNA repair assay shows a lower excision repair capacity of *ddm1* plants. The DNA repair assay was performed by incubation of circular and linear non-exposed and UV-irradiated DNA with partially purified protein extracts prepared from ten WT and *ddm1* plants. **a** Loading of circular, blunt-cut, blunt-cut UV-irradiated, non-sticky end-cut and non-sticky end-cut UV-irradiated DNA after incubation with protein extracts from wild-type and mutant plants is shown. **b** Representative images of the intensity of incorporation of dig-labeled dNTPs into circular and cut DNA. **c** The average (with SD) arbitrary units of the intensity of bands from circular DNA (**b**). *Asterisks* show statistically significant differences ($P < 0.01$) between the data for mutant and wild-type plants

cases (Kaina et al. 2004; Wyatt and Pittman 2006). Base excision repair is the main mechanism involved in repair of this damage (Wyatt and Pittman 2006). Since *ddm1* plants were more sensitive to MMS, we hypothesized that they have a different capacity of BER and indeed showed that *ddm1* plants are less efficient in BER/NER pathways (Fig. 7).

The remainder of damage induced by MMS is believed to be repaired by strand break repair and mismatch repair (Wyatt and Pittman 2006). Also, strand breaks created upon BER are also repaired by strand break repair. Indeed, exposure to MMS was shown to induce recombination frequency (Kovalchuk et al. 2003; Puchta et al. 1995). It is possible that DDM1 is necessary for the repair of damaged bases and in part for the repair of strand breaks.

Alternatively, and most likely, it is possible that lack of DDM1 in the cell leads to the secondary effects that influence the outcomes of BER and HR repair pathways. Indeed, substantial changes in the transcript of *ddm1* plants were observed (Habu et al. 2006; Hudson et al. 2011). In any case, our analysis of repair capacity of *ddm1* clearly showed impairment of the BER pathway (Fig. 7). Importantly, a recent report by Hudson et al. (2011) also demonstrates that several genes involved in DNA base excision repair, such as *REPRESSOR OF SILENCING1* (*ROS1*) and *DEMETER-LIKE3* (*DML3*), are downregulated in *ddm1* plants, possibly leading to impairment of BER (Hudson et al. 2011). ROS1 and DML3 are also involved in active demethylation process, and thus it is possible that their downregulation in *ddm1* plants is a compensatory process to prevent a more severe loss of methylation. Thus, in our experiments, BER deficiency observed in *ddm1* plants may be an indirect effect of the process of hypomethylation.

ddm1 plants were shown to exhibit a reduction in cytosine methylation and a strong alteration in nuclear organization and chromatin structure that are most noticeable in the centromeric and pericentromeric regions (Probst et al. 2003). Surprisingly, little data exist on the ability of *ddm1* to withstand stress. The most comprehensive analysis of the *ddm1* plant's response to DNA-damaging stress was done by Shaked et al. (2006). The authors showed that *ddm1* plants were much more sensitive to γ-rays and UVC as compared to wild-type plants (Shaked et al. 2006). On the other hand, the *met1* mutant was not sensitive to radiation, in contrast to *ddm1* mutant (Kankel et al. 2003). Mutations in both *DDM1* and *MET1* genes coding for a nucleosome remodelling factor and a cytosine methyltransferase enzyme lead to genome demethylation. Changes in methylation in the *met1* mutant are noticeable throughout the entire genome (Kankel et al. 2003), whereas in the *ddm1* mutant, it affects mostly heterochromatin regions and partially low copy sequences (Jeddeloh et al. 1999). Both mutants have lower methylation levels, but additionally, the *ddm1* mutant has strong alterations in nuclear organization and chromatin structure (Probst et al. 2003). Thus, the authors hypothesized that the difference in response to radiation was primarily due to alterations in chromatin rather than in methylation levels. Our analysis showed that *met1* plants are sensitive to NaCl, although not to the same degree as *ddm1* plants.

Changes in stress tolerance in *ddm1* mutants could be the result of the appearance of new epigenetic states associated with hypomethylation of various loci, including transposon and transposon-related ones. Becker et al. (2011) and Schmitz et al. (2011) have recently demonstrated that spontaneous changes in DNA methylation arise fairly often and have the potential to be stabilized for many generations (Becker et al. 2011). Epimutations, and
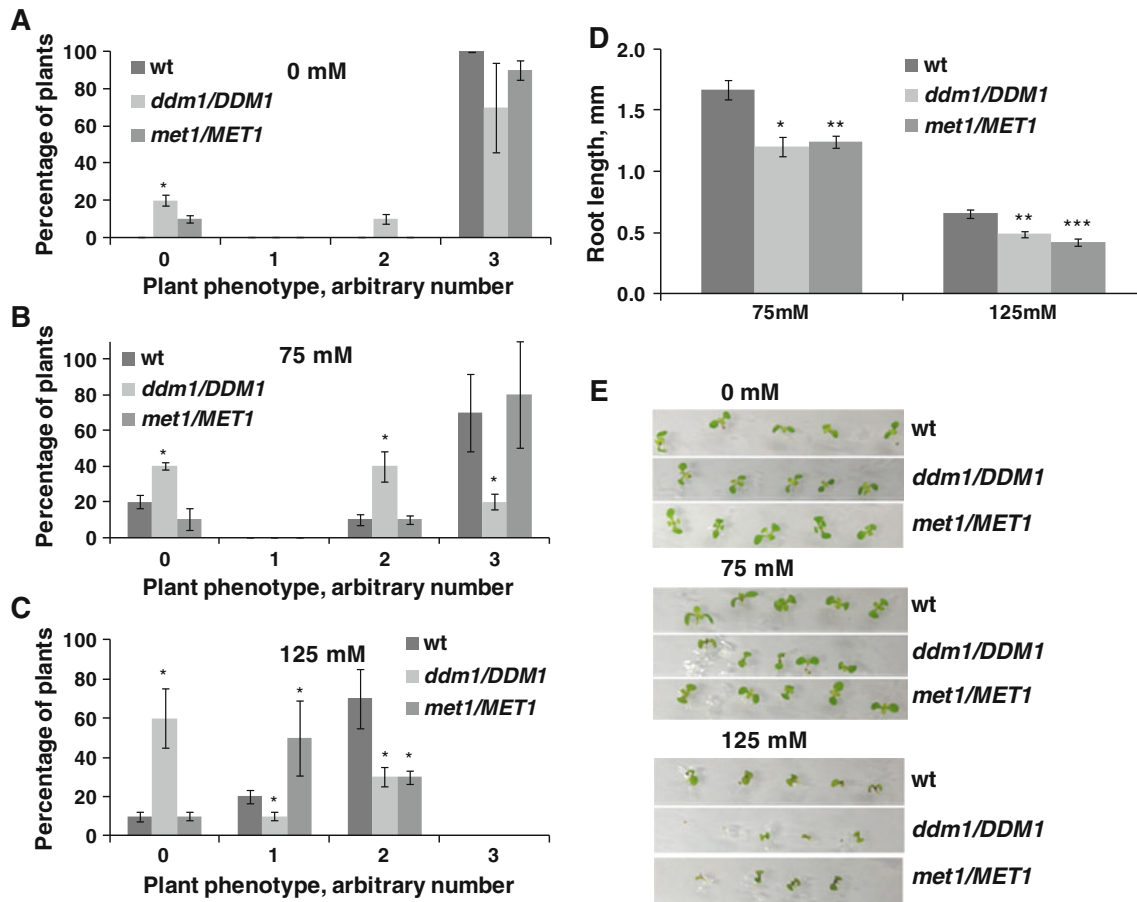
Fig. 8 Analysis of NaCl tolerance of population of selfed *ddm1/ DDM1*, *met1/MET1* and wild-type plants. Plants were exposed to 0 mM (**a**), 75 mM (**b**) or 125 mM (**c**) NaCl. At the age of 2 weeks phenotype of plants was analyzed and given arbitrary number: *0* non-germinated seeds, *1* germinated cotyledons without roots, *2* cotyledons with roots, *3* cotyledons with roots and at least two true leaves. In **a**, **b** and **c**, *Y* axis shows the average (with SD, calculated from 3 experiments with 20 plants in each experimental group) percentage of

plants with given arbitrary number. In **d**, *Y* axis shows the average (with SD) root length of plants grown in presence of NaCl. *Asterisks* show significant difference between the *ddm1/DDM1* and the wild-type groups or the *met1/MET1* and the wild-type groups: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. **e** Shows representative images of exposed plants; plants were aligned from the weakest to the strongest in the wild type, the *ddm1/DDM1* and the *met1/MET1* groups

possibly epialleles, emerge much more frequently than mutations and alleles, and according to calculations by Schmitz et al. (2011), epimutations occur 10^5 times more frequently than mutations (4.46×10^{-4} methylation polymorphism per CG per generation versus 7×10^{-9} base substitutions per site per generation). Since *ddm1* plants have a substantially hypomethylated genome, the appearance of new epimutations and epialleles may be more frequent in *ddm1* plants and thus can contribute to high stress sensitivity in mutants.

The *ddm1* mutant was shown to have a loss of up to 70 % of the methylation level (Vongs et al. 1993). More recent reports, however, suggest that there is a substantial variation in the percentage of methylation loss which probably depends on the allele used and the method employed. For example, Teixeira et al. (2009) showed about 80 % loss of methylation at CG sites of *ddm1* plants,

whereas Hudson et al. (2011) reported just 50 % (Hudson et al. 2011; Teixeira et al. 2009). Our analysis showed that heterozygous *ddm1/DDM1* plants, exhibit an over 60 % reduction in methylation levels compared to wild-type plants, whereas the fourth generation of selfed homozygous mutant plants showed only a 35 % loss of methylation, suggesting that the loss of methylation may be partially restored during the propagation of homozygous *ddm1* mutants. Fascinatingly, a similar picture was observed in *met1* plants: *met1/MET1* plants lost 52 % of methylation, whereas in the fourth generation of selfed homozygous mutant only 43 % methylation was lost (Fig. 5S). These results suggest that there might exist a certain compensation mechanism that allows restoring the level of methylation in selfed homozygous *ddm1* and *met1* mutants. It should be noted that previous publications reported the heterozygous *met1/MET1* (Kankel et al. 2003) and

ddm1/DDM1 (Teixeira et al. 2009) mutants to have intermediate levels of changes in methylation as compared to respective homozygous mutants.

Our analysis showed that *ddm1* plants also had a higher level of strand breaks. Thus, *ddm1* plants had an inverse correlation between the level of methylation and the level of strand breaks ($r = -0.82$). A curious phenomenon, however, was observed in response to MMS. In our studies, an opposite effect of MMS on the level of methylation and strand breaks in wild-type and *ddm1* plants was observed. Whereas wild-type plants responded to MMS with a decrease in methylation and an increase in levels of strand break, the *ddm1* plants responded with an increase in methylation and a decrease in strand break levels.

Metabolomic profiling of *Arabidopsis* cells exposed to salt showed a long-term reduction of the methylation cycle (Kim et al. 2007). Another study in spinach demonstrated that exposure to salt leads to high demands for metabolites involved in replenishing methylation levels (Weretilnyk et al. 2001). Wada et al. (2004) showed that infection of tobacco plants with tobacco mosaic virus resulted in a decrease in methylation of many stress-responsive genes. Another study showed that aluminium, salt and temperature stresses induce demethylation of promoter regions of several stress-associated genes (Choi and Sano 2007). A stress-induced increase in the level of strand breaks is an established phenomenon (Hays 2002). Many stresses directly cause strand breaks, but stresses like salt and temperature (Boyko et al. 2010a, b) may increase the level of strand breaks via modifications of chromatin or a decrease in methylation pattern. Thus, a decrease in the methylation level and an increase in strand breaks in response to MMS seem to be a normal response.

An increase in the methylation level in *ddm1* plants in response to MMS deserves further attention. It may indicate an abnormal response that is associated with the already lowered level of methylation and severe changes in chromatin structure. It can be suggested that the *ddm1* mutant responds to stress by increasing methylation levels at some sensitive genomic areas that have already been hypomethylated; these areas may include heterochromatic areas with repetitive elements, transposon and retrotransposon areas. Indeed, several studies showed multiple changes in gene expression in *ddm1*, with many of these genes coding for transpososable elements (Habu et al. 2006; Hudson et al. 2011). However, the remethylation of these genomic regions upon stress exposure has to be demonstrated experimentally. An increase in the methylation level in *ddm1* plants in response to MMS was paralleled by a decrease in double-strand breaks. Our experimental assay does not allow distinguishing whether these breaks are caused by direct DNA damage, or they are the result of spontaneous breaks due to changes in chromatin structure. It is possible that a decrease in strand breaks in response to MMS is associated with an increase in the methylation level followed by chromatin compaction. Again, this link needs to be established.

Our experiments suggest a link between chromatin remodelling, DNA repair and stress tolerance. A similar link between chromatin structure, gene silencing and genome maintenance has been previously proposed for the MIM gene (Hanin et al. 2000) and the BRU1 gene (Takeda et al. 2004). BRU1 is a nuclear gene that may be indirectly involved in histone chaperoning mediated by CAF-1. BRU1 possibly plays a role in chromatin replication and may also be involved in post-replicative stabilization of chromatin structure. At the same time, global genome methylation is not impaired in *bru1*. The Chromatin Assembly Factor 1 (CAF-1), a heterotrimeric complex consisting of three subunits (p150/p60/p48), is one of the replication-coupled assembly factors involved in the reconstitution of S-phase chromatin. A mutant of one of the subunits of CAF1, *fas1*, is also sensitive to stress and has an extremely increased level of homologous recombination (Endo et al. 2006; Kirik et al. 2006).

Our results have provided a new example of a link between genetic genome stability and epigenetic genome stability. In the future, it would be important to differentiate whether the sensitivity of *ddm1* plants to stress is due to their inability to establish a proper methylation pattern or it is caused by the inability to modify the chromatin structure in response to stress.

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