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Effect of 5-azacytidine induced DNA demethylation on abiotic stress tolerance in *Arabidopsis thaliana*

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Abstract: The effect of 5-azacytidine (5A)-induced DNA hypomethylation on the growth and abiotic stress tolerance of *Arabidopsis thaliana* were analysed. Growth analysis revealed that aqueous solutions of 5A added to the soil did not affect the fresh and dry biomass accumulation but led to a higher percentage of flowering *A. thaliana* plants after four weeks of cultivation. The 5A treatment considerably lowered survival rates of *Arabidopsis* plants under high soil salinity, heat stress, and drought, while it did not affect the survival rates after freezing stress. 5A eliminated the stimulatory effect of the heat and drought stresses on the transcriptional levels of a number of stress-inducible genes, such as *DREB1*, *LEA*, *SOS1*, or *RD29A*. A less clear but similar trend has been detected for the effect of 5A on expression of the stress-inducible genes under salt and cold stresses. The data indicate that DNA methylation is an important mechanism regulating plant abiotic stress resistance.

Keywords: 5A; DNA methylation; freezing; drought; heat; soil salinity

Cytosine DNA methylation is an epigenetic modification that is important for maintaining genome stability and regulating gene expression in higher plants and other organisms (Zhang & Jacobsen 2006; Gehring & Henikoff 2007; Vanyushin & Ashapkin 2011). Cytosine DNA methylation of plant nuclear genomes is more extensive and involves a wider range of methylation sites than in animals (Vanyushin & Ashapkin 2011; Dubrovina & Kiselev 2016).

There are multiple studies reporting on the effect of abiotic stresses on DNA methylation levels and patterns in various plant species (Boyko *et al.* 2010; Zhong *et al.* 2010; Al-Lawati *et al.* 2016). A number of investigations demonstrated that abiotic stresses induce total genomic DNA methylation in plants (Labra *et al.* 2004; Boyko *et al.* 2010; Omidvar & Fellner 2015; Al-Lawati *et al.* 2016; Hua *et al.* 2017), while there are also reports demonstrating an opposite effect (Steward *et al.* 2002; Aina *et al.* 2004; Choi & Sano 2007). The significance of this phenomenon is not completely clear. There are few studies investigating whether inducible DNA demethylation affects plant abiotic stress resistance (Boyko *et al.* 2010; Zhong *et al.* 2010; Al-Lawati *et al.* 2016), and all these studies referred only to salt stress. 5-Azacytidine (5A), a known analogous of cytidine, is widely used as a demethylating agent in different plant and animal systems (Tyunin *et al.* 2016). Treatments with 5A have been shown to affect plant biomass accumulation, plant height, flowering time, etc. (Burn *et al.* 1993; Fieldes 1994; Kanchanaketu & Hongtrakul 2015; Li *et al.* 2015; Duan *et al.* 2016). Three studies investigated

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the effect of 5A on plant salt stress tolerance (Boyko *et al.* 2010; Zhong *et al.* 2010; Al-Lawati *et al.* 2016). However, these reports showed contradictory results. Thus, the data presented in the studies are insufficient, and further investigations are needed.

In this study, we aimed to investigate whether application of the DNA demethylation agent 5A influences abiotic stress tolerance of *Arabidopsis thaliana* (heat, cold, salt, and drought stresses) in order to find whether plant abiotic stress tolerance depends on DNA methylation. The data obtained revealed that application of 5A in the form of aqueous solutions to the soil did not affect biomass accumulation, while it markedly increased the number of flowering plants after four weeks of cultivation. Abiotic stress tolerance assays demonstrated that the addition of 5A to the soil decreased the level of heat, drought, and salinity stress resistance and prevented activation of a number of stress-inducible marker genes in the four-week-old *A. thaliana*.

MATERIAL AND METHODS

Plant materials, growth conditions, and 5A treatments. The seeds of wild-type *Arabidopsis thaliana* ecotype Columbia L. were vapour-phase sterilised for 45 min using mixed solutions of commercial bleach (100 ml) and concentrated HCl (3 ml) for further incubation at +4°C for two days on Petri dishes containing ½ Murashige and Skoog medium and 0.8% agar. Then, the Petri dishes were kept at +22°C for one week in a growth chamber (Sanyo MLR-352; Panasonic, Osaka, Japan) at 120 µmol/m^2/s over a 16-h daily light period. The one week-old *A. thaliana* seedlings were transplanted to pots (10 × 10 cm) containing 100 g of rich soil and were grown under the controlled conditions. Aqueous solutions of 5A (TCI, Zwijndrecht, Belgium) were added to soil in the pots before planting the one-week-old seedlings (5, 10, 20, 50, and 100 mg of 5A per 100 g of soil). For the subsequent experiments, 20 mg of 5A per 100 g of soil were used for plant growth.

Total RNA isolation and real-time quantitative RT-PCR. Total RNA was isolated from the four-week-old control, 5A-treated, and stressed *A. thaliana* plants using the cetyltrimethylammonium bromide-based extraction as described (Kiselev *et al.* 2012). The leaves of the control, 5A-treated, and stressed plants were collected for RNA extraction after the treatments. Complementary DNAs were synthesised using 1.5 µg of total RNA by the RNA PCR Kit (Silex-M, Moscow, Russia) as described (Kiselev *et al.* 2017). cDNAs of *ABA1*, *ABA2*, *ABF3*, *ABI1*, *ABI2*, *ABI3*, *ABI4*, *ABI5*, *CAT1*, *CBF1*, *COR15*, *COR47*, *CSD1*, *CSD2*, *Dreb1*, *Dreb2*, *Kin1*, *Lea*, *LTP3*, *NHX1*, *P5CS*, *Rab18*, *RD22*, *RD26*, *RD29A*, *RD29B*, *SOS1*, *Actin2*, and *GAPDH* genes were amplified using EvaGreen Real-time PCR dye (Biotium, Hayward, USA) and primers are listed in Supplementary Table S1 in EMS (sequences of primers). The qRT-PCRs were performed using a Real-Time PCR Kit (Syntol, Moscow, Russia) in a thermocycler supplied with Multicolor Real-Time PCR Detection System (DNA-Technology, Moscow, Russia). Expression was calculated by the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001). *AtActin2* (GenBank NM_112764) and *AtGAPDH* (GenBank NM_111283) genes were used as endogenous controls to normalise variance in the quality and the amount of cDNA of *A. thaliana* used in each real-time RT-PCR experiment (Kiselev *et al.* 2017).

DNA isolation and bisulphite sequencing. Total DNA was purified from 20 mg of dried *A. thaliana* mixed tissues (all types of rosette tissues were mixed) using the eight-week-old 5A-treated *A. thaliana* plants as described (Kiselev *et al.* 2015). The cytosine methylation status of *MEA-ISR* DNA region of *A. thaliana* was analysed using bisulphite sequencing as described in Kiselev *et al.* (2015), using primers presented in Deleris *et al.* (2010). We analysed DNA methylation status of several studied stress-inducible genes with the highest differences in gene expression levels after 5A or stress treatments (*ABA2*, *CSD2*, and *COR15* genes) using DNA bisulphite conversion followed by PCR with the treated template DNA and specific primers to the studied genes in treated and non-treated plants before and after stress treatments.

Abiotic stress tolerance assays. The plants were subjected to heat, freezing, drought, and salt stress treatments as described (Dubrovina *et al.* 2017). Briefly, the sterilised transgenic seeds of *Arabidopsis* were germinated on plates and the seven-day-old seedlings were transferred to commercially available rich well-watered soil in a controlled environmental chamber at standard conditions. Then, the plants were subjected to drought by culturing without additional irrigation for four weeks, and then re-watered. For salt stress treatments, the four-week-old plants were well-irrigated with 350 mM NaCl solution. No signs of drought were observed for plants before irrigation with the NaCl solution. One week after irrigation with NaCl, the pots were placed in water to leach

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out the salt from the soil. For cold tolerance assays, normally cultured four-week-old *A. thaliana* plants were stressed in a –10°C freezer for 0.5 h and then cultured at 8°C for 2 hours. For heat tolerance assays, normally cultured four-week-old plants were stressed at 45°C in a controlled incubator for 3 hours. The survival rates were determined as the number of visibly green plants three days after re-watering (drought), one week after heat and cold stress treatments, and one week after salt leaching (salt stress).

Statistical analysis. The physiological, DNA methylation, and transcription data are presented as mean ± standard error (SE) and were tested by Student's *t*-test. For growth analysis and stress tolerance assays, five pots of plants (five seedlings per pot) were grown for each treatment in an experiment. The experiments were repeated two times for the analysis of *A. thaliana* growth under 5A treatment and five times for the analysis of *A. thaliana* survival rates under each type of stress. The data from qRT-PCRs (expression analysis of selected stress-responsive genes) were obtained from two independent experiments with eight replicates each.

RESULTS

Growth of A. thaliana and DNA methylation analysis under 5A treatment. Before the 5A treatments, we germinated *A. thaliana* and pre-cultured the seedlings on half-strength MS medium for one week. Then, the one-week-old seedlings of *A. thaliana* were transferred into pots filled with the commercially available rich soil mixed with aqueous solutions of 5A. To select an appropriate 5A dose, aqueous solutions of 5A were added to the soil (5, 10, 20, 50, and 100 mg of 5A per 100 g of soil), and the preliminary estimates showed that 5A did not considerably affect fresh and dry biomass accumulation of *A. thaliana* at all doses but accelerated flowering after three weeks of cultivation (data not shown). For further detailed experiments and statistical analysis, we decided to use 20 mg of 5A per 100 g of soil as a moderate concentration. Several independent experiments revealed that the 5A application did not affect the quantity of leaves and biomass of the *A. thaliana* plants after three weeks of cultivation (Table 1). However, the 5A application led to a higher number of flowering plants as compared to the control conditions (Table 1).

Table 1. The effect of 5-azacytidine (5A, 20 mg per 100 g of soil) on the growth and development of four-week-old wild-type *Arabidopsis thaliana*

	Control	5A
Fresh biomass (mg/plant)	57.4 ± 6.7	53.2 ± 3.8
Dry biomass (mg/plant)	5.9 ± 0.8	7.1 ± 0.9
Leaves quantity	14.0 ± 0.3	14.3 ± 0.2
Flowering plants (%)	36.2 ± 5.7	55.3 ± 8.1*

The data were obtained from four biological replicates (n = 40); *P < 0.05; versus values in control, plants without treatment; *significantly different from *A. thaliana* growing without 5A at P < 0.05 according to the Student's t-test

As presented in the Figure 1A, 5A treatment led to a significant decrease in the DNA methylation at *MEA-ICR* locus in the treated plants. Furthermore, DNA methylation slightly decreased in the stress-treated plants. The data indicate that 5A effectively

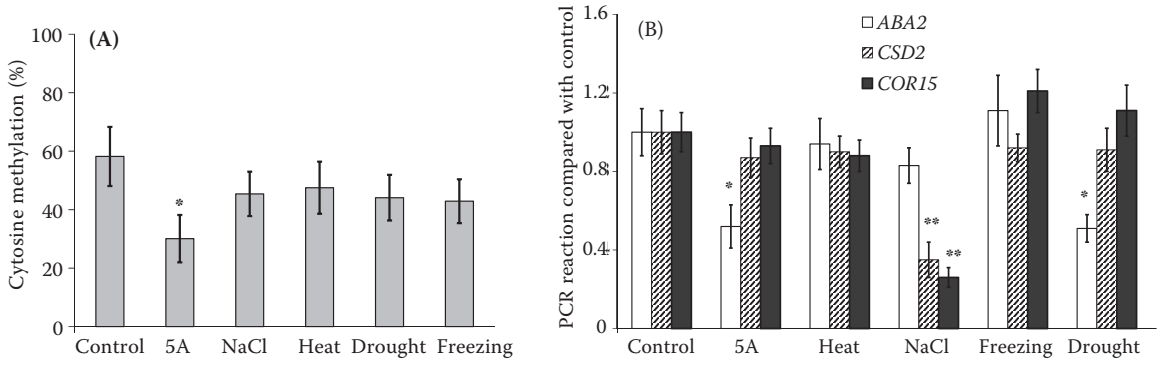

Figure 1. (**A**) DNA methylation analysis by *MEA-ISR* bisulphite sequencing of *Arabidopsis thaliana* growing under standard conditions (Control), in the presence of 5-azacytidine (5A, 20 mg per 100 g of soil), and under abiotic stress conditions (heat, high soil salinity, freezing, drought) and (**B**) efficiency of the PCR reactions with specific primers to the *ABA2*, *CSD2*, and *COR15* genes and bisulphite conversed template DNA

*P < 0.05, **P < 0.01 versus values of DNA methylation (**A**) and efficiency of PCR reactions (**B**) in the control plants

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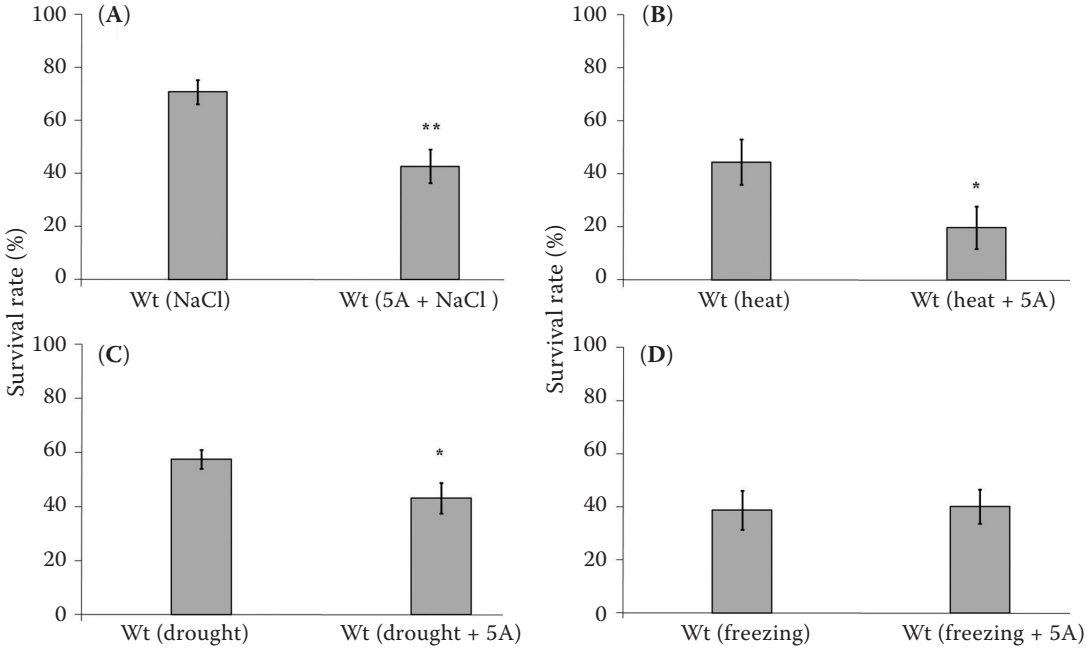

Figure 2. The effect of 5-azacytidine (5A, 20 mg per 100 g of soil) on abiotic stress tolerance of wild type (Wt) *Arabidopsis thaliana* plants: (**A**) Survival rates of *A. thaliana* growing with and without 5A under salt stress – four-week-old wild-type plants were irrigated with 350 mM NaCl solution, then free NaCl solution was removed and the plants were cultured for one week; (**B**) Survival rates of *A. thaliana* growing with and without 5A under heat stress – three-week-old plants were heat stressed at +45°C for 3 h and then transferred to normal conditions for recovery; (**C**) Survival rates of *A. thaliana* under cold stress – four-week-old plants were cold stressed at –10°C for 1 h 20 min and then transferred to normal conditions for recovery; (**D**) Survival rates of *A. thaliana* growing with and without 5A under drought stress

One-week-old plants were transplanted to soil, watered, and then cultivated for additional 4 weeks without watering to induce drought stress, and finally were re-watered. Survival rates were recorded as the number of visibly green plants after 7 days (cold, salt, heat) or 3 days (drought); the data are presented as mean ± standard error and were tested by paired Student's *t*-test; the data were obtained from four biological replicates (n = 40); *, **significantly different from *A. thaliana* growing on the same stress without 5A at $P < 0.05$ and 0.01, respectively, according to the Student's *t*-test

inhibited DNA methylation level under the selected experimental conditions.

The effect of 5A on abiotic stress tolerance and expression of stress-inducible genes in A. thaliana. To elucidate the possible impact of DNA demethylation on plant abiotic stress resistance, four-week-old *A. thaliana* plants growing with 5A and without it were exposed to high soil salinity, heat, freezing, and drought stresses. We found that addition of 5A to the soil lowered the salt, heat, and drought stress tolerance of *A. thaliana* plants in 1.3–2.2 times (Figure 2A–C). However, the 5A treatment did not affect the resistance of *A. thaliana* to cold stress (Figure 2D).

To elucidate the molecular mechanism of the 5A-induced reduction of plant stress resistance, transcription level of 27 stress-related genes of *A. thaliana* identified in different pathways was monitored by real-time PCR analysis in the control and 5A-treated *Arabidopsis* plants. We evaluated expression of the stress-responsive genes (*ABF3, CBF1*, *COR15*, *COR47*, *DREB1A*, *DREB2A*, *Kin1*, *LEA*, *LTP3*, *P5CS*, *Rab18*, *RD22*, *RD26*, *RD29A*, and *RD29B*), ion transporter genes (*NHX1* and *SOS1*), ABA biosynthesis and signalling genes (*ABA1*, *ABA2*, *ABI1*, *ABI2*, *ABI3*, *ABI4*, *ABI5*), and antioxidant genes (*CAT1*, *CSD1*, and *CSD2*) in the control and 5A-treated *Arabidopsis* plants exposed to salt, freezing, heat, and drought stress conditions (Figure 3).

We proposed that 5A could increase transcription levels of the majority of the analysed stress-responsive genes in *Arabidopsis* growing without stress treatments, since the stress-inducible genes are usually expressed at a low level in non-stress conditions and DNA methylation is a common epigenetic mechanism that is known to repress gene expression. However, expression of few genes was

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substantially affected by the 5A treatment (Figure 3). Then, we aimed to investigate whether expression of the stress-responsive genes would be altered in *Arabidopsis* when treated with 5A and under the control conditions. The results of the qRT-PCRs revealed that transcription levels of 7 genes under heat stress (*ABF3*, *CAT1*, *CSD1*, *DREB1*, *LEA*, *LTP3*, and *ABA1*), 10 genes under high soil salinity (*CAT1*, *CSD2*, *COR15*, *DREB1*, *KIN1*, *LEA*, *NHX1*, *SOS1*, *RD22*, and *RD29A*), 10 genes after freezing (*CSD1*, *CBF1*, *DREB2*, *LEA*, *LTP3*, *RD26*, *ABI1*, *3*, *4*, *5*), and 7 genes under drought stress (*LTP3*, *NHX1*, *SOS1*, *RD22*, *RD29A*, *RD29B*, *ABA2*) have been markedly up-regulated under the analysed stress conditions. Surprisingly, the heat and drought stresses combined with the 5A application had a stimulatory effect on transcription of only *ABF3* and *NHX1*, *RD22*, respectively. Thus, application of 5A removed the stimulatory effects of heat and drought stress conditions on expression of the stress-inducible genes. A similar but vague effect had been observed for salt stress and freezing. The 5A treatment removed or decreased the stimulatory effects of salt stress and freezing on transcription of 5 genes (*CSD2*, *COR15*, *LEA*, *NHX1*, *RD29A*) and 4 genes (*CSD1*, *DREB2*, *LEA*, *LTP3*), respectively. However, 5A treatment in combination with either salt or freezing stresses activated expression of some additional stress-related

	5A	Heat	5A+Heat
ABF3	1.1	2.1	2.4
CAT1	1.2	1.6	1.1
CSD1	1	2	0.4
CSD2	0.9	1.1	1
CBF1	0.8	0.4	0.7
COR15	1.8	1.5	1.3
COR47	0.8	0.4	0.3
Dreb1	1.7	1.8	0.8
Dreb2	1.7	0.7	1
Kin1	0.9	0.5	0.4
Lea	0.8	2.1	1.1
LTP3	1.2	2	1.1
NHX1	0.7	1	1
SOS1	0.4	0.8	0.6
P5CS	0.8	0.7	1.1
Rab18	0.5	0.5	0.8
RD22	1.1	0.8	1.1
RD26	0.4	1.1	1.2
RD29A	1.1	0.7	0.8
RD29B	1.4	0.7	1.4
ABA1	1.3	2.1	1.1
ABA2	0.6	0.9	0.8
ABI1	0.9	1.3	1.1
ABI2	0.8	0.6	1.1
ABI3	0.7	0.6	1.1
ABI4	1.3	0.6	0.3
ABI5	0.6	0.8	1.3

	5A	NaCl	5A+NaCl
ABF3	0.8	0.3	0.9
CAT1	0.9	1.8	2.3
CSD1	1.1	1.2	1.4
CSD2	1.4	4.8	3.5
CBF1	0.8	1.4	2
COR15	0.9	18.1	10.2
COR47	0.2	0.8	0.5
Dreb1	0.4	1.7	1.9
Dreb2	1.1	1.2	2
Kin1	0.3	3	2.9
Lea	1.8	2.4	1.9
LTP3	0.6	0.6	0.4
NHX1	1.3	1.7	1.1
SOS1	1.3	1.9	1.9
P5CS	1.1	0.6	0.5
Rab18	0.6	0.7	0.5
RD22	1.3	1.7	1.6
RD26	1.2	1	0.9
RD29A	0.5	4.3	2.3
RD29B	0.4	0.6	2.7
ABA1	1.4	0.8	0.8
ABA2	3.1	1.8	3
ABI1	1.1	0.6	1
ABI2	1.2	0.8	1.4
ABI3	0.7	0.8	0.7
ABI4	0.3	1.7	0.5
ABI5	0.5	1.2	1.1

	5A	Cold-30m	Cold-1h	5A+Cold-30m	5A+Cold-1h
ABF3	0.7	1.2	0.9	0.9	0.8
CAT1	0.8	0.8	1.1	0.8	0.8
CSD1	1.2	1.4	2.1	1.2	1
CSD2	2.5	0.9	0.8	1.2	2.4
CBF1	0.7	1.6	2.5	1.4	2.2
COR15	0.6	0.6	0.5	0.4	0.4
COR47	0.7	0.7	1.3	0.4	0.8
Dreb1	0.5	0.8	1.4	0.5	1.4
Dreb2	0.5	1	2.6	0.3	1.5
Kin1	0.4	0.5	0.6	0.5	0.5
Lea	0.6	1.2	2.7	1.2	1.1
LTP3	2.1	4.2	2.6	4.8	1.8
NHX1	1.4	1	1.6	0.5	0.8
SOS1	1.1	0.8	1	0.8	0.6
P5CS	0.8	0.5	0.7	0.3	0.8
Rab18	0.8	1.5	1.6	1.6	2.1
RD22	1.2	0.6	0.8	0.5	0.7
RD26	0.5	1.2	1.9	0.6	2.1
RD29A	0.2	0.8	1.3	0.4	0.5
RD29B	1.1	1.6	1.5	0.7	0.9
ABA1	1.4	1.6	1.9	1.9	1.7
ABA2	1.8	0.6	0.9	0.8	2
ABI1	1.2	2.3	2	1.2	2
ABI2	1.3	0.8	1.2	1.3	1.1
ABI3	0.7	1.2	3.2	1.2	3.4
ABI4	1.2	1.2	3.4	1.2	3
ABI5	0.6	1.2	1.7	0.9	1.8

	5A	Drought	5A+Drought
ABF3	0.7	1.2	0.4
CAT1	1.1	0.8	0.3
CSD1	1	0.9	0.8
CSD2	0.9	1.3	1
CBF1	0.5	0.2	0.2
COR15	0.5	0.9	0.4
COR47	0.5	0.9	0.3
Dreb1	0.9	0.2	0.3
Dreb2	0.8	0.3	0.2
Kin1	1	0.9	0.6
Lea	0.8	1	1.3
LTP3	1.3	3.1	1.5
NHX1	0.7	2.2	2.2
SOS1	1.1	1.6	0.9
P5CS	0.5	1.4	0.8
Rab18	0.6	1.2	1.3
RD22	0.5	2.2	2.4
RD26	0.4	0.4	0.4
RD29A	0.8	1.7	1.1
RD29B	1	2.5	1.3
ABA1	0.6	1.1	0.3
ABA2	3.3	2.7	1.9
ABI1	1	1.1	0.5
ABI2	0.7	0.6	0.4
ABI3	0.8	0.5	0.3
ABI4	1.2	0.7	0.5
ABI5	0.5	1.8	1.9

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Figure 3. Heat map of qRT-PCR data showing the expression fold changes of different stress-related genes between the control and treated plants

Each row of the heat map presents a gene, while each column presents different experimental conditions; 5A – *Arabidopsis* plants growing in the presence of 5-azacytidine (5A); Heat – *Arabidopsis* plants exposed to heat stress at +45°C for 40 min; 5A+Heat – *Arabidopsis* plants growing in the presence of 5A and exposed to heat stress; NaCl – *Arabidopsis* plants exposed to high soil salinity stress for 20 h (plants were well-irrigated with 350 mM NaCl solution applied at the bottom of the pots); 5A+NaCl – *Arabidopsis* plants growing in the presence of 5A and exposed to the high soil salinity conditions; Cold 30m – *Arabidopsis* plants exposed to freezing at –10°C for 30 min and then used for RNA isolation; Cold-1h – *Arabidopsis* plants exposed to freezing at –10°C for 30 min and then recovered for 1 h at normal conditions before RNA isolation; 5A+Cold 30m – plants growing in the presence of 5A and exposed to freezing at –10°C for 30 min and then recovered for 1 h at normal conditions before RNA isolation; 5A+Cold-1h – plants growing in the presence of 5A and exposed to freezing at –10°C for 1 h and then recovered for 1 h at normal conditions before RNA isolation; Drought – plants growing for 4 weeks without watering to induce drought stress; 5A+Drought – plants growing under the drought stress conditions in the presence of 5A; the colour saturation scale, shown at the bottom, represents the fold changes of gene expression relative to normal controls (normally growing *Arabidopsis* without stress and 5A treatments); red colour indicates significant increases in gene expression, while blue colour indicates significant decreases ($P < 0.05$); gray indicates the fold changes that had the $P > 0.05$

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genes (*CBF1*, *DREB2*, *RD29B*, *ABA2* under freezing and *CSD2*, *RAB18* under high soil salinity). Furthermore, we detected that 5A treatment in combination with drought stress resulted in a considerable down-regulation of four stress-inducible genes (*ABF3*, *CAT1*, *COR15*, *COR47*) and four ABA signalling genes (*ABA1*, *ABI1*, *ABI2*, *ABI4*), and the down-regulation was not detected under drought stress.

In addition, we supported the gene expression analysis by the data concerning DNA methylation status of the studied stress-related genes (Figure 1B). We used bisulphite conversion followed by PCR with template DNA and specific primers to the several studied genes. We have chosen three genes with the highest differences in gene expression levels after the 5A or stress treatments (*ABA2*, *CSD2*, and *COR15*). Treatment of denatured DNA with sodium bisulphite led to deamination of unmethylated cytosine residues to uracil, and, therefore, this process decreased the specify of the used primers and the efficiency of the PCR reaction. Thus, the highest amplification resulted in the highest methylation status. Figure 1B showed that the lowest *ABA2* amplification was detected using the DNA of the 5A treated and drought-stressed Arabidopsis plants, while the lowest *CSD2* and *COR15* amplification efficiencies were in the plants under salt stress. The data on low amplification level correlated with a considerable transcriptional upregulation of the genes under the stress conditions. Therefore, DNA demethylation of the analysed genes is probably the major cause of the gene transcriptional activation.

DISCUSSION

Epigenetic mechanisms, e.g. DNA methylation, chromatin modifications, or regulation by non-coding RNAs, are implicated in the formation of the multitude of plant responses to various environmental cues without altering DNA sequences and contribute to the plant defence reactions and proper plant development. Dynamic changes in DNA methylation is an important epigenetic phenomenon that have received considerable attention as an epigenetic mechanism regulating plant development and stress responses (Bartels *et al.* 2018; Deng *et al.* 2018). Using of 5A for studying functions of variations in DNA methylation is known to lead for global DNA genome hypomethylation revealed a substantial number of upregulated genes, with an overrepresentation of transposable element genes as revealed by transcriptome analyses (Griffin *et al.* 2016). This study indicated that 5A treatment accelerated the development of *A. thaliana* by increasing the number of flowering plants. The same acceleration of plant development by decreasing the flowering time was shown in crucifer, flax, and spinach (Burn *et al.* 1993; House 2010; Li *et al.* 2015).

There are a number of studies reporting on clear changes in the cytosine DNA methylation level of specific genes involved in various physiological processes in response to drought, salt stress, and temperature extremes (Karan *et al.* 2012; Shan *et al.* 2013). However, the methylation levels of these genes can also vary within plant species or depending on stress conditions. Using methylation-sensitive DNA fragmentation assay and bisulphite sequencing, it has previously been shown that *Arabidopsis* DNA underwent hypomethylation in the course of plant development and aging (Ogneva *et al.* 2016). The present study indicated that transcription of methyltransferase *CMT3* and *METI* genes was lowered during the development and aging of *Arabidopsis*, whereas expression of *ROS1*, *AtDME*, *AtDML2,* and *DML3* demethylase genes was up-regulated. Thus, this phenomenon of the age-related DNA hypomethylation processes was accompanied by reduction of active DNA methylation and induction of active DNA demethylation processes. While ageing of plants is generally characterised by increased stress susceptibility, abiotic stresses are, in turn, often characterised by induced plant senescence processes, which is a part of plant 'escape' strategy (Sade *et al.* 2017). The present study showed that the 5A-induced DNA-demethylation led to increased plant susceptibility to several abiotic stresses, including high soil salinity, heat stress, and drought. The results suggest that DNA methylation and demethylation processes are important for the maintenance of plant fitness and stress adaptation.

Earlier studies analysed the 5A effects on plant salt stress tolerance (Boyko *et al.* 2010; Zhong *et al.* 2010; Al-Lawati *et al.* 2016) and showed contradictory results. Treatment of the progeny of salt-stressed *A. thaliana* with 5A resulted in decreased global genomic methylation and enhanced salt stress tolerance (Boyko *et al.* 2010). Under salt stress, wheat (*Triticum aestivum*) treated with 5A had higher root dry mass, activities of superoxide dismutase, catalase, and peroxidase than the untreated plants suggesting improved salinity tolerance (Zhong *et al.* 2010). However, 5A treatment reduced seedling lengths and

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biomass of alfalfa (*Medicago* spp.) under salt stress, indicating a possible decrease in salinity tolerance (Al-Lawati *et al.* 2016). Thus, the earlier investigations focused only on the effects of 5A on salt stress tolerance and presented contradictory results. Here, we have shown that 5A decreased tolerance of *A. thaliana* to salt, heat, and drought stresses, but did not affect its cold stress tolerance. Thus, our data correlated with the results obtained by Al-Lawati *et al.* (2016). Furthermore, we found that 5A treatment suppressed transcriptional activation of some stress-inducible genes under the abiotic stress conditions.

This study indicates the importance of global DNA methylation and development-related alterations in DNA methylation for plant well-being. The mechanism of how 5A enhanced the negative effects of plant heat, drought, and salt stresses and prevented activation of stress-inducible marker genes is not clear. Thus, the data obtained support the hypothesis that plant survival, under at least some abiotic stresses, depends on alterations in DNA methylation.

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