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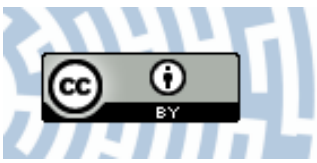

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ORIGINAL PAPER

Azacitidine (5-AzaC)-treatment and mutations in DNA methylase genes affect embryogenic response and expression of the genes that are involved in somatic embryogenesis in Arabidopsis

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Abstract
Epigenetic processes including DNA methylation play a pivotal role in regulating the genes that control plant development. In contrast to *in planta* development, the contribution of DNA methylation to the morphogenic processes that are induced in vitro are much less recognised. Hence, in the present study, we analysed the impact of DNA methylation on somatic embryogenesis (SE) that was induced in Arabidopsis. The results demonstrated a decrease in the global DNA methylation level during SE that contrasted with the up-regulation of *MET1* and *CMT3* DNA methylases and the down-regulation of DNA demethylases (*ROS1, DME* and *DML2*). Hence, the global DNA methylation level appears not to correlate with the transcriptional activity of the genes encoding DNA methylases/demethylases, thereby implying the complexity of the regulatory mechanism that controls the DNA methylation status of the SE-epigenome. Moreover, distinct changes in the expression level of the SE-regulatory genes were indicated in the 5-AzaC-treated and DNA methylase mutant cultures. Accordingly, a significant repression of the *LEC2, LEC1* and *BBM* genes was found in the 5-AzaC-treated culture that was incapable of SE induction. In contrast, the distinct up-regulation of these genes was observed in the *drm1drm2* and *drm1drm2cmt3* mutant cultures with an improved embryogenic response. The modulated expression of DNA methylase genes and the significantly modified embryogenic response of the *met1* and *drm* mutants imply that both the maintenance and the de novo pathway of DNA methylation are engaged in the regulation of SE in Arabidopsis.

Keywords 5-AzaC · DNA methylation · Somatic embryogenesis · Transcription factors

Introduction

Somatic embryogenesis (SE) is a plant-specific developmental process that involves the induction of the embryogenic programme in somatic cells, which results in the formation of somatic embryos that are capable of regenerating complete plants. The transition of already differentiated cells into embryogenic ones requires extensive changes in the somatic cell transcriptome. Accordingly, rapid changes in the gene expression patterns that accompany SE induction have been reported in various plants (Elhiti et al. 2013) including Arabidopsis (Gliwicka et al. 2013; Wickramasuriya and Dunwell 2015). The reprogramming of the cell transcriptome that results in the release of a new developmental programme is associated with extensive changes in the chromatin structure, which involves chemical modifications of DNA and histones (He et al. 2011a; De-la-Peña et al. 2015; Mozgová et al. 2017). Among the epigenetic modifications that control gene expression, the methylation of DNA is considered to play a pivotal role in plant development (Zhang et al. 2010; Turck and Coupland 2014; Victoria et al. 2018).

The methylation of plant DNA involves the addition of a methyl group to the carbon-5 of cytosine at the CpG, CpNpG and CpNpN (where N could be any nucleotide except G) sequences in DNA, which results in an increase of the content of 5-methyl cytosine (5 mC) in the genomic DNA (Law

Daria Grzybkowska and Joanna Morończyk have contributed equally to this work.

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and Jacobsen 2010). Three types of DNA methylases have been found in plants including METHYLTRANSFERASE 1 (MET1), CHROMOMETHYLASE 3 (CMT3) and DOMAINS REARRANGED METHYLTRANSFERASE (DRM1 and DRM2) (Lindroth et al. 2001; Cao and Jacobsen 2002a; Zhang et al. 2006). In *Arabidopsis thaliana*, DRMs are required for de novo methylation while MET1 and CMT3 maintain the methylation pattern during DNA replication (Zhang et al. 2010).

DNA methylases cooperate with other proteins and the recruitment of MET1 to DNA requires the activity of the variant in methylation (VIM/ORTHRUS) proteins that recognise the hemimethylated CpG sites that are generated during DNA replication (Shook and Richards 2014). The accession of DNA methyltransferases to DNA is supported by DECREASED IN DNA METHYLATION (DDM1), which is a SWI/SNF chromatin-remodeling factor (Jeddeloh et al. 1999).

In controlling genome-wide DNA methylation patterns, DNA methylation is accompanied by active DNA demethylation that is carried out by the DNA glycosylase family of DNA demethylases in plants (Penterman et al. 2007; Stroud et al. 2013). The Arabidopsis genome encodes four DNA demethylases, including DEMETER (DME), REPRESSOR OF SILENCING 1 (ROS1)/DEMETER-LIKE 1 (DML1), DML2 and DML3. ROS1 is a major DNA demethylase that is involved in the dynamic transcriptional regulation of the genome (Gong et al. 2002) that plays a role in the developmental processes and biotic and abiotic stress responses (Yamamuro et al. 2014; Schumann et al. 2017). DME is required for the expression of specific imprinted maternal alleles during seed development, while the biological function of DML2 and DML3 as yet remains mostly unknown (Bauer and Fischer 2011).

DNA methylation has been recognised as a mechanism that suppresses gene expression because the accumulation of 5 mC has been observed in the heterochromatin regions, imprinted genes, repetitive sequences and transposons (Tariq and Paszkowski 2004; Teixeira and Colot 2010). However, in a large number of expressed genes, the promoters and the transcribed regions of the genes ('gene body') are methylated (He et al. 2011b; Jones 2012). The frequent methylation of the gene body of constitutively expressed housekeeping genes suggests a homeostatic function of this type of methylation (Zilberman 2017). In addition, methylation of the gene body might also be related to the regulation of the responses of genes to internal or external cues (Aceituno et al. 2008). Thus, the function of CG methylation within the transcribed regions of genes is currently unclear (Bewick and Schmitz 2017).

During the life cycle of Arabidopsis, the global level of DNA methylation undergoes dynamic changes and the content of 5 mC decreases in gametogenesis and increases after fertilisation and during embryo development (Jullien et al. 2012; Bouyer et al. 2017). In adult plants, variations in the level and pattern of methylation have been observed between the plant organs but the overall trend is that the 5 mC content increases during the aging and maturation of a plant (Ruiz-García et al. 2005; Widman et al. 2014).

A nucleotide analogue, 5-Azacitidine (5-AzaC), of the DNA demethylation activity is commonly applied to study the role of DNA methylation in the developmental processes. 5-AzaC is randomly incorporated into a newly synthesised DNA strand instead of cytosine and, as a result, a dose- and time-dependent decrease in the DNA methyltransferase activity can be observed that is followed by genome hypomethylation at random sequences (Christman 2002; Issa and Kantarjian 2009). Treatment with 5-AzaC has been shown to induce various plant phenotypes including dwarfism, early flowering and an inhibition of vegetative growth (Kondo et al. 2006). Although 5-AzaC treatment has also been indicated to impact the morphogenic processes that are induced in vitro including the capacity of a tissue for SE (Santos and Fevereiro 2002; Yamamoto et al. 2005; Tokuji et al. 2011; Fraga et al. 2012; Teyssier et al. 2014; Solís et al. 2015), our knowledge about the role of this modification in the epigenetic regulation of the genes that control the developmental plasticity of somatic plant cells is still limited.

Hence, we were motivated to study the function of DNA methylation in regulating SE in Arabidopsis, which is a model plant that offers a rapid and efficient culture system to identify the molecular determinants that are involved in the embryogenic transition induced in somatic cells (Gaj 2001; Wójcikowska and Gaj 2016). Different analytical approaches that aimed at revealing (1) the impact of both 5-AzaC-treatment and the mutations that affect the DNA methylase genes (*drm1drm2* and *drm1drm2cmt3*) on the embryogenic capacity of a tissue and the expression profiles of the *LEC1, LEC2* and *BBM* genes that play a key role in SE induction (2) changes in the global DNA methylation level during SE and (3) the contribution of the genes encoding different DNA methylases to SE induction. The results indicated that the DNA methylation status of the explants impacts both the capacity of a culture for SE and the expression level of the SE-involved genes. The results of the DNA methylase gene profiling together with an analysis of the mutants that were defective in DNA methylases suggest that both the maintenance and the de novo pathway of DNA methylation are engaged in the regulation of SE in Arabidopsis.

Materials and methods

Plant material

Arabidopsis thaliana (L.) Heynh. plants of the Columbia (Col-0) WT ecotype and the insertional mutant lines in the Col-0 background were used in the study (Table S1). The seeds were purchased from NASC (The Nottingham Arabidopsis Stock Centre, UK; http://arabidopsis.info/). Selection of homozygous mutants was conducted following the NASC standard protocol (http://signal.salk.edu/tdnaprimers.2.html).

In vitro culture of the explants

In order to induce SE, immature zygotic embryos (IZEs) at the stage of green, fully developed cotyledons were cultured in vitro following a standard protocol (Gaj 2001). The standard medium used for SE induction (E5) contained a basal B5 medium (Gamborg et al. 1968) that was supplemented with 20 g L^{-1} sucrose, agar (8 g L^{-1}) and 5 µM of 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma-Aldrich). In the control culture, the explants were induced on an E5 medium without 2,4-D (E0), which resulted in seedling development. In some experiments, the E5 medium was supplemented with 10 µM of 5-AzaC (5-Azacitidine; Sigma-Aldrich).

Ten explants were cultured in one Petri dish and 30 explants in three replicates from each culture combination were analysed. The capacity for SE was evaluated in three-week-old cultures. Two parameters of embryogenic potential were evaluated: SE efficiency—the frequency of the explants that produced somatic embryos and SE productivity—the average number of somatic embryos that developed per embryogenic explant.

Explants that were cultured for 0 d; 3 d; 5 d; 10 d; 15 d (d = day of culture) on the E5 or E0 medium were collected to isolate RNA and DNA. A small fraction (< 10%) of the explants that were cultured on E5 failed to induce SE and developed a non-embryogenic callus that was also taken for analysis (15 days). All of the molecular analyses of the SE culture including RT-qPCR and ELISA were conducted in three biological and two technical replicates.

Plant growth and in vitro culture conditions

The plants that were used as the source of the IZE explants were grown in Jiffy-7 peat pots (Jiffy) in a 'walk-in' type phytotron under controlled conditions at 22 °C under a 16 h photoperiod of 100 µM m^{-2} s^{-1} white, fluorescent light. The plant materials that were grown in sterile conditions were kept at 23 °C under a 16 h photoperiod of 40 µM m^{-2} s^{-1} white, fluorescent light.

Total RNA isolation and RT-qPCR

An RNAqueous Kit (Ambion) was used to isolate total RNA. The concentration and purity of RNA was evaluated using an ND-1000 spectrophotometer (NanoDrop). In order to prevent DNA contamination, the RNA was treated with RQ1 RNase-free DNase I (Promega) following the manufacturer's instructions. First strand cDNA was produced in a 20 µL reaction volume using a RevertAid First Strand cDNA Synthesis Kit (Fermentas).

The product of the reverse transcription was used to evaluate the expression level of the selected genes (Table S2). RT-qPCR was carried using a LightCycler® 480 SYBR Green I Master (Roche), the appropriate mix of the master mix, cDNA and water were used for the RT-qPCR reactions (Table S3). A LightCycler 480 (Roche) real-time detection system was used under the following reaction conditions: denaturation one repeat of 10 min at 95 °C followed by 45 repeats of 10 s at 95 °C, 8 s at 55 °C, 12 s at 72 °C and 5 s at 80 °C. Denaturation for the melt curve analysis was conducted at 95 °C followed by 15 s at 65 °C and then heating to 95 °C (0.1 °C/s, with continuous fluorescence measurement).

Primary data analysis was performed using LightCycler Software 4.0 (Roche). The relative RNA levels were calculated and normalised to an internal control—the *At4g27090* gene encoded 60S ribosomal protein (Thellin et al. 1999). In all of the analysed tissue samples, the control gene displayed a constant expression pattern with $Cp = 18 \pm 1$ (Figs. 2, 3 and 4b, c) and $Cp = 23 \pm 1$ (Figs. 4a and S1).

DNA isolation and DNA methylation analysis with ELISA

The modified micro C-TAB method was used to extract genomic DNA from the explants that had been cultured on the E5 medium for 0, 3, 5, 10 and 15 days (Doyle and Doyle 1987). The global DNA methylation level was evaluated using spectrophotometric methods—a 5-mC DNA ELISA kit (ZymoResearch) following the manufacturer's protocol. The light absorbance was analysed on a Victor X5 multilabel reader system (PerkinElmer).

Statistical analyses

The Student's t test was used to calculate any significant differences (at $P = 0.05$) between the combinations that were being compared.

Results

Reduced embryogenic response of the explants treated with 5-AzaC

The embryogenic capacity of the Col-0 explants that were treated with 5-AzaC was studied. In the control culture that was induced on the E5 medium, the explants formed somatic embryos rapidly and efficiently while on the medium that had been supplemented with 10 µM of 5-AzaC, the SE response was drastically inhibited and the explants massively produced white, non-embryogenic callus tissue (Fig. 1). Treating the explants with 5-AzaC reduced both the efficiency and productivity of SE and as a result only 5% of the explants were able to undergo SE induction and the average number of somatic embryos produced by an embryogenic explant was decreased by more than half compared to the control culture. Since no signs of tissue lethality were observed in the treated cultures, we hypothesised that the inhibition of SE was not a result of the toxic effect of 5-AzaC on cell metabolism but that it resulted from DNA hypomethylation-associated effects including changes in the gene transcription.

The expression profiles of the SE-involved TF genes in response to 5-AzaC treatment

RT-qPCR analysis was used to analyse the expression of the genes encoding the transcription factors (TFs) of the documented regulatory function in SE induction in Arabidopsis including *LEC1* (*LEAFY COTYLEDON1*), *LEC2* (*LEAFY COTYLEDON2*) and *BBM* (*BBM BABY BOOM*). The gene expression profiles in response to 5-AzaC treatment of the WT (Col-0) culture were evaluated.

The results showed that 5-AzaC treatment caused significant changes in the transcription profiles of the genes that were analysed (Fig. 2). Under 5-AzaC treatment, the strongest inhibition of the transcript level was observed for *BBM* and *LEC2* whose expression was highly repressed up to 32 (*LEC2*) and 11 (*BBM*)—fold in comparison to transcript level observed in control E5 culture after the

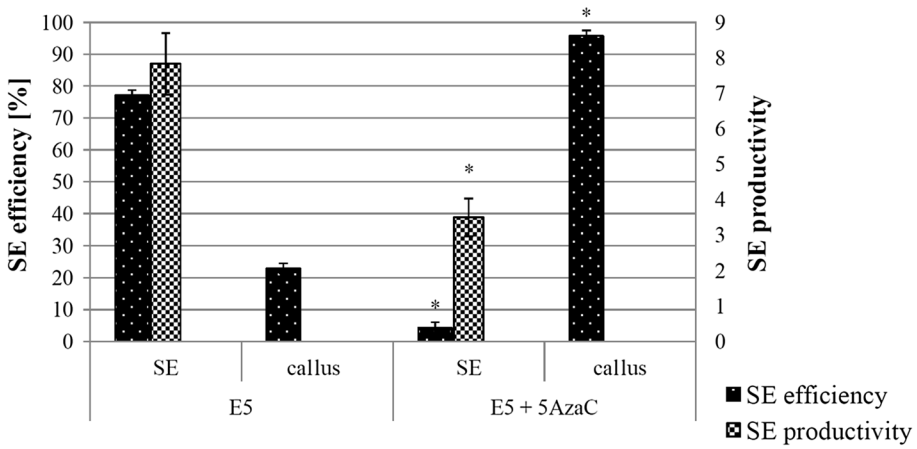

Fig. 1 Impaired SE efficiency and productivity of the Col-0 explants cultured on the E5 medium supplemented with 5-AzaC (10 µM). Values significantly different from the control culture (E5) are indicated with an asterisk (Student's t test, $P < 0.05$). Error bars indicate the standard deviation (SD)

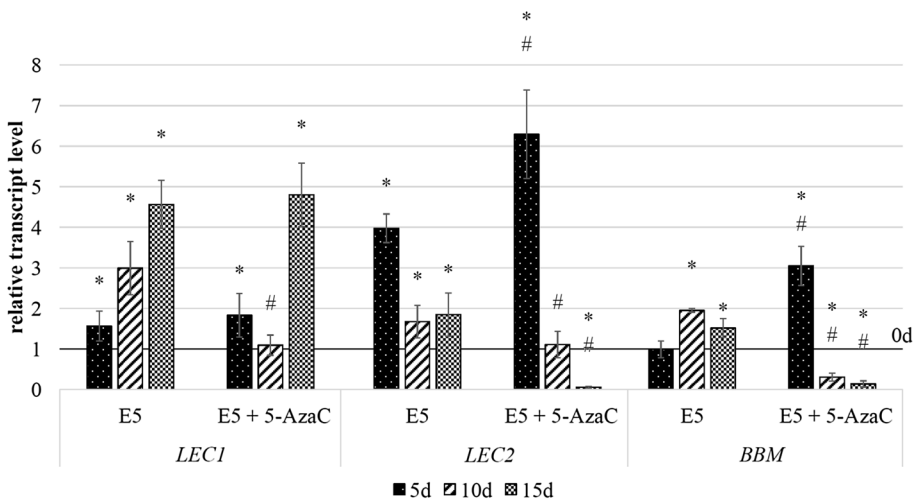

Fig. 2 Expression profiles of SE-involved *TF* genes in the Col-0 (WT) embryogenic culture induced on E5 (control) and E5 + 10 µM 5-AzaC medium. Relative transcript level was normalised to the internal control (*At4g27090*) and calibrated to the 0 day of culture. Values significantly different to 0 day are indicated with an asterisk; Values significantly different to control (E5) at corresponding day of culture are indicated with a hashtag (Student's t test, $P < 0.05$). Error bars indicate the standard deviation (SD)

transient up-regulation in the early culture (5 days). In contrast, the *LEC1* expression profile in the culture that was induced on the E5 + 5-AzaC medium was similar to the one that was observed in the control culture (E5) and an increased transcript accumulation was indicated at the early (5 days) and advanced (15 days) SE stages.

The expression profiles of the SE-involved TF genes in the DNA methylation-related mutants

To further assess the impact of DNA methylation on SE induction, we profiled the expression of the *LEC1, LEC2* and *BBM* genes in the SE-induced explants of the *drm1drm2* and *drm1drm2cmt3* mutants (henceforth referred to as *dd* and *ddc*). The results demonstrated that the expression profiles of *LEC1, LEC2* and *BBM* genes were similar in the *dd* and *ddc* mutant cultures and that the analysed genes showed a significantly increased expression in the mutant explants that were induced on the E5 medium (Fig. 3). The genes that were analysed differed in the relative level of gene transcripts and *LEC1* displayed the highest (up to 77-fold) and *BBM* the lowest (up to threefold) stimulation of gene expression in the mutant cultures. In addition, we observed that an increase in the gene transcript level was higher in the mutant (Fig. 3) than in WT (Fig. 2) culture that was induced on E5 for all of the SE-regulatory genes that were analysed.

Expression of the genes that are involved in DNA methylation and DNA demethylation during SE

To further explore the DNA methylation-related processes that are involved in the molecular mechanism that governs the embryogenic response in plant somatic cells, we evaluated the expression profiles of the genes encoding DNA methylases (*MET1, CMT3, DRM1* and *DRM2*) in the WT (Col-0) explants that had been cultured on the E5 (auxin) vs the E0 (auxin-free) medium. In contrast to the SE-promoting E5 medium, the explants that had been cultured on E0 were not able induce SE and they developed into seedlings instead.

Although we found that the DNA methylase genes were expressed in both of the culture conditions that were analysed, distinct differences in the gene expression profiles and transcript accumulation between the genes and the culture conditions were observed (Fig. 4). Accordingly, a high accumulation of *MET1* and *CMT3* in the SE culture (3–10 days) was observed that was specific to the E5 medium (Fig. 4a). Compared to *MET1* and *CMT3*, the genes that control de novo DNA methylation, *DRM1* and *DRM2*, displayed a significantly lower expression level during SE and their transcription profiles on E5 and E0 were similar. A distinct difference between the expression profiles of *DRM1* and *DRM2* genes in the advanced stage of SE culture was observed and at 10–15 days of the culture, a down-regulated *DRM1* transcription contrasted with an up-regulated expression of *DRM2*. Moreover, we found that the accumulation of the *DRM2* transcripts in the SE culture significantly exceeded (up 4000-fold) the transcript level of the *DRM1* gene (Fig. S1).

We also investigated the expression of the *DDM1* and *VIM1* genes encoding the proteins that are related to DNA methylation. We found that the transcription of *DDM1* and *VIM1* was significantly modulated during SE and that their expression patterns were similar (Fig. 4b). Accordingly, a substantial up-regulation of the *DDM1* and *VIM1* transcripts was observed during SE induction. In contrast to the genes encoding the DNA methylases, the DNA methylation-related

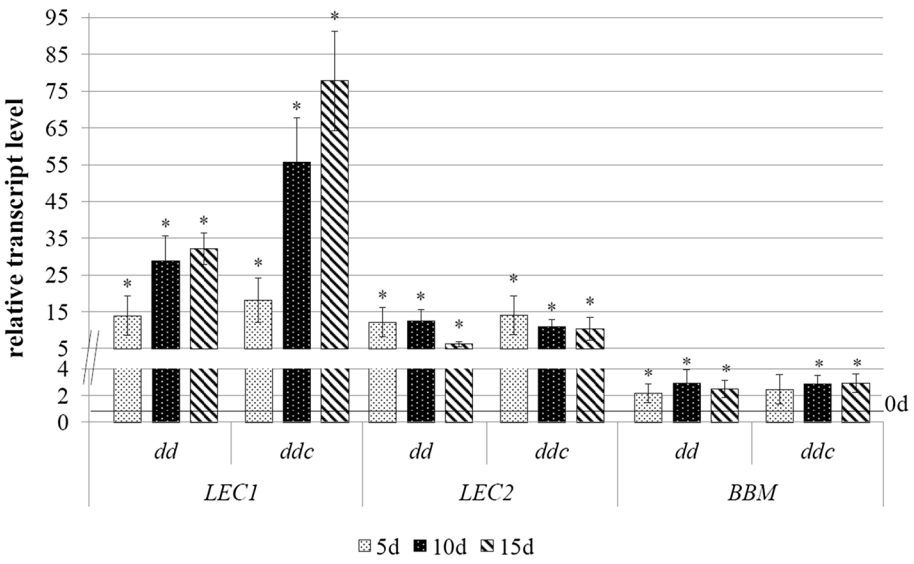

Fig. 3 Expression profiles of *LEC1, LEC2* and *BBM* genes that have a regulatory role in SE induction in the *drm1drm2 (dd)* and *drm1drm2cmt3* (*ddc*) mutant cultures induced on E5 medium. The relative transcript level was normalised to the internal control (*At4g27090*) and calibrated to the 0 day culture. The values that were significantly different to 0 day are indicated with an asterisk (Student's t test, $P < 0.05$). Error bars indicate the standard deviation (SD)

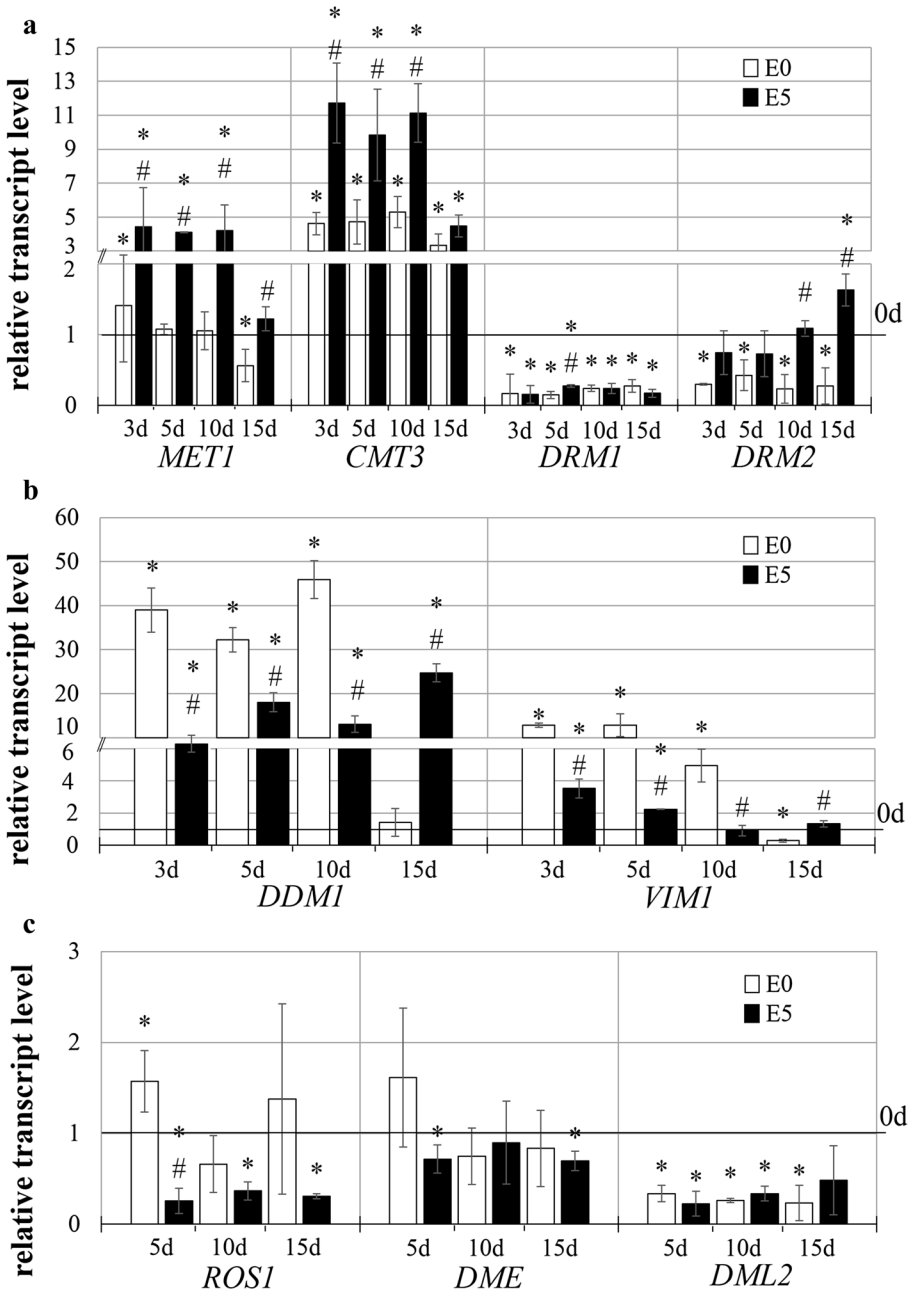

Fig. 4 Expression level of the genes encoding DNA methylases, *MET1, CMT3, DRM1, DRM2* (**a**); DNA-methylation related proteins, *DDM1, VIM1* (**b**) and the DNA demethylases, *ROS1, DME, DML2* (**c**) in the Col-0 explants cultured on the E5 and the E0 medium. Relative transcript level was normalised to the internal control (*At4g27090*) and calibrated to the 0 day of culture. Values significantly different from 0 day are indicated with an asterisk; Values significantly different from E0 are indicated with a hashtag (Student's t test, $P < 0.05$). Error bars indicate the standard deviation (SD)

genes, *DDM1* and *VIM1* displayed a down-regulation during SE (E5) and an up-regulation in the explants that developed seedlings on the E0 medium.

In addition to the genes that are involved in DNA methylation, we also analysed the expression level of three demethylase genes, including (*ROS1*) *REPRESSOR OF SILENCING* 1, (*DME*) *DEMETER* and (*DML2*) *DEMETER LIKE 2* in the explants that had been cultured on E5 and E0 (Fig. 4c). We found that in the SE culture that had been induced on the E5 medium, all of the analysed DNA demethylase genes were distinctly down-regulated up to 23–70% of the level that was found in the freshly isolated (0 day) explants. Similarly, the *DML2* expression was distinctly reduced in the seedlings that were developing on E0 while the transcription of the other two analysed genes, *DME* and *ROS1*, was not affected on E0, except for a transient up-regulation of the *ROS1* at 5 days.

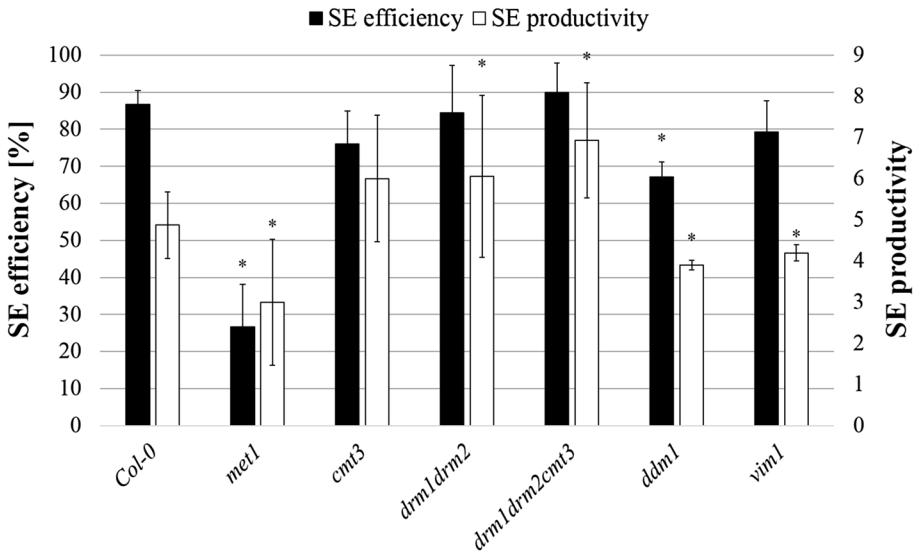

Fig. 5 Efficiency and productivity of the SE cultures derived from the mutants that were defective in the genes encoding DNA methylases (*met1, cmt3, drm1drm2, drm1drm2cmt3)* and the proteins involved in DNA methylation (*ddm1* and *vim1).* Values significantly different from the control culture (Col-0) are indicated with an asterisk (Student's t test, $P < 0.05$). Error bars indicate the standard deviation (SD)

Embryogenic capacity of the DNA methylation-related mutants

In addition to the analysis of the gene expression, we evaluated the capacity for SE of the insertional mutants that were affected in the genes encoding the DNA methylases (*met1, cmt3, dd* and *ddc*) and proteins that are involved in DNA methylation (*ddm1* and *vim1*) (Fig. 5). The results showed a distinctly decreased SE response of the *met1* and two other mutants, *ddm1* and *vim1*, with an insert in the DNA methylation-related genes. In contrast, cultures with mutations in the *DRM1* and *DRM2* methylase genes (*dd* and *ddc*) were found to display the opposite phenotype in the culture on the E5 medium and they showed an increased capacity for somatic embryo formation.

Global DNA methylation level in SE

Considering that the genes that are involved in DNA methylation displayed different expressions in the embryogenic culture, we expected that the level of DNA methylation would be modulated during SE. Therefore, we investigated the global level of 5 mC in the DNA in the explants at various time points (0, 3, 5, 10, 15 days) of the SE culture. In addition to the embryogenic explants, the non-embryogenic callus tissue that had been developed by a small fraction (less than 10%) of the E5-cultured explants was analysed.

The results indicated a significant and progressive decrease in the global level of DNA methylation in the SE-induced explants. Accordingly, the level of 5 mC was up to 30% lower (15 days) in the SE culture than in the freshly isolated 0 day explants (Fig. 6). We found that both the embryogenic (SE) and non-embryogenic (callus) tissue showed a similar level of the global DNA methylation.

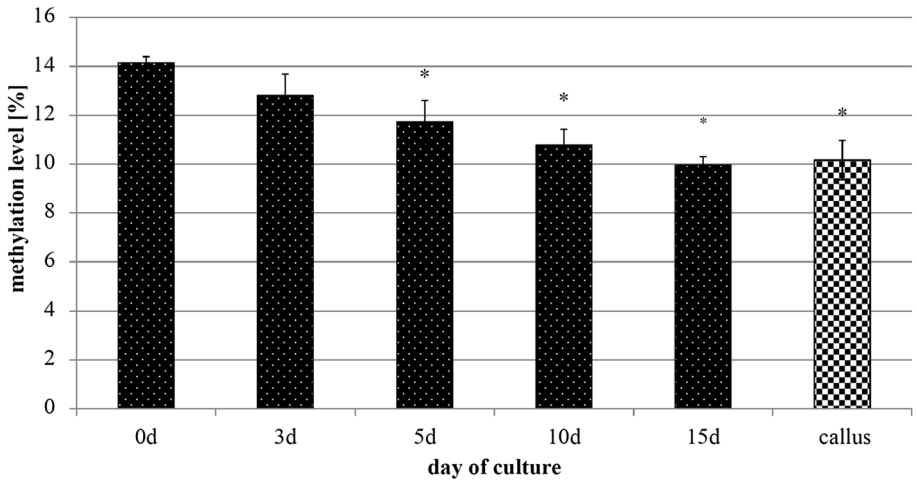

Fig. 6 Global changes in the DNA methylation level in the Col-0 explants induced towards SE on the E5 medium (0, 3, 5, 10 and 15 days) and in the non-embryogenic callus. Values significantly different from 0 day are indicated with an asterisk (Student's t test, $P < 0.05$). Error bars indicate the standard deviation (SD).4

Discussion

Recently, significant progress has been made in identifying genes and revealing their biological functions in the genetic mechanisms that control the embryogenic transition in somatic cells using the SE culture of Arabidopsis (reviewed in Nowak and Gaj 2016). In contrast, our understanding of the epigenetic processes that contribute to the regulation of SE remains very limited. Hence, the present work was focused on an analysis of the role of DNA methylation in SE induction in Arabidopsis explants that were cultured in vitro.

5-AzaC treatment and DNA methylase-related mutations affect both the expression of the SE-involved TF genes and the embryogenic response

5-AzaC treatment was indicated as distinctly changing the gene expression in the plants (Chang and Pikaard 2005; Song et al. 2017) and in vitro cultured plant cells/tissue and both the stimulation and repression of the gene transcription has been reported (Berdasco et al. 2008; Tokuji et al. 2011; Nic-Can et al. 2013). Consistent with the diverse effects of 5-AzaC on gene expression level, the hypo- and hypermethylation of DNA in response to this chemical were reported in an embryogenic culture of *Acca sellowiana*, which were dependent on the plant genotype and the supplementation of the culture medium with 2,4-D (Fraga et al. 2012).

In the present study, we demonstrated that in Arabidopsis, similar to the embryogenic cultures of other plants (Santos and Fevereiro 2002; Yamamoto et al. 2005; Teyssier et al. 2014), treating the tissue with 5-AzaC severely and negatively affected the embryogenic response of the explants. We observed that the decreased embryogenic response of the 5-AzaC-treated explants is accompanied by a deregulated expression of *LEC1, LEC2* and *BBM* that have a key regulatory role in SE induction. Similarly, 5-AzaC distinctly reduced the expression level of the shoot regeneration-related TF genes in the callus of Arabidopsis (Tokuji et al. 2011). *LEC1* and *LEC2*, which are the master regulators of zygotic embryogenesis in Arabidopsis (Braybrook and Harada 2008), were found to be essential for SE induction (Gaj et al. 2005). *LEC1* and *LEC2* contribute to SE induction via the regulation of the auxin response genes (Braybrook et al. 2006) and *LEC2* was indicated as activating auxin biosynthesis during SE (Wójcikowska et al. 2013). Recently, the *LEC1, LEC2* and *BBM* genes were indicated as functioning in the same molecular pathway in which BBM transcriptionally regulates *LEC1* and *LEC2* to induce SE (Horstman et al. 2017). Relevant to the BBM-mediated SE induction mechanism, we found the *BBM* expression to be distinctly repressed in the 5-AzaC-treated culture that was incapable of SE induction. 5-AzaC was also found to strongly inhibit the embryogenic response in coffee by decreasing the expression of *LEC1* and *BBM1* (Nic-Can et al. 2013). Given that 5-AzaC-treatment has been reported to distinctly reduce the global methylation level in the in vitro cultured tissue of different plants that have been cultured in vitro, including Arabidopsis (Tokuji et al. 2011), rape and barley (Solís et al. 2015) and coffee (Nic-Can et al. 2013), we assumed that the repression of the SE-regulatory genes that are associated with a decrease in the SE response is related to the demethylation of DNA.

In contrast to the 5-AzaC treated cultures, in the *dd* and *ddc* mutant cultures of the increased embryogenic response expression of *BBM, LEC1* and *LEC2* was significantly up-regulated (Fig. 3). Similarly, the significant up-regulation of the genes that are located in the euchromatin was reported in an *ddc* mutant (Zhang et al. 2006) and the induction of the *dd* seedling explants on the auxin medium resulted in a continuous increase of the *BBM* expression that was followed by accelerated callus production (Jiang et al. 2015). Relevant to the up-regulated expression of *LEC1, LEC2* and *BBM* in the *dd*/*ddc* mutant cultures with an increased SE response, the overexpression of these *TF*s was demonstrated to be sufficient to induce SE in Arabidopsis (Lotan et al. 1998; Stone et al. 2001; Boutilier et al. 2002) and other plants (Heidmann et al. 2011; Guo et al. 2013). Collectively, the contrasting expression level of the *LEC1, LEC2* and *BBM* genes, in particular *BBM*, a positive regulator of *LEC1* and *LEC2* during SE induction (Horstman et al. 2017), which was found in the *dd*/*ddc* versus 5-AzaC-treated cultures might account for the opposite embryogenic capacity of these cultures.

The oppose expression profiles of the SE-regulatory genes that we found in the 5-AzaC-treated and *dd*/*ddc* mutant cultures might be related to the different impact of the 5-AzaC and *dd*/*ddm* mutations on the cell methylome. By trapping the DNA methyltransferase in the replication fork, 5-AzaC causes passive DNA demethylation that results in a global and stochastic reduction of the 5metC content in the genome (Chang and Pikaard 2005; Szyf 2009). In contrast to 5-AzaC, the mutations in *CMT3* and *DRM* methylases only cause a mild decrease in the overall DNA methylation level (Zhang et al. 2006; Table S4) and the demethylation is specific to the DNA sequences (Cokus et al. 2008; Stroud et al. 2013, 2014).

In summary, these results infer a role of DNA methylation in the epigenetic control of the embryogenic response in Arabidopsis and suggest that DNA methylation might contribute to SE induction by impacting the expression of the key regulators of SE.

SE induction is associated with the decreased level of global DNA methylation

It is believed that the demethylation of DNA is characteristic of the open-chromatin state that is required to redirect the somatic cells into embryogenic development (Grafi et al. 2011; Miguel and Marum 2011). In line with this postulate, we observed a significantly decreased level of 5 mC in the embryogenic culture of Arabidopsis and the reduction in the DNA methylation level has also been associated with SE induction in other plants (Santos and Fevereiro 2002; Xu et al. 2004; Noceda et al. 2009; Rodríguez-Sanz et al. 2014; Teyssier et al. 2014). However, in some plants, an increased (Fraga et al. 2012; Rival et al. 2013), differentially modulated (Leljak-Levanić et al. 2004; Nic-Can et al. 2013) and steady (Parra et al. 2001) level of DNA methylation during SE has been reported. The diversity in the SE-associated DNA methylation levels suggests that various exo- and endogenous culture factors rather than the induction of embryonic development per se appear to contribute to the global DNA methylation level in cultured tissue (Huang et al. 2012; Rival et al. 2013; Li et al. 2014; Rathore et al. 2014). In support of this assumption, we found that the level of 5 mC in the embryogenic culture was similar to the one that was observed in the non-embryogenic callus that was formed by the explants that failed in the embryogenic transition.

In conclusion, the global content of 5 mC in the cultured tissue does not appear to be specific to the type of morphogenic development that is induced but rather reflects unspecific epigenetic status of the explant cells and their dedifferentiation in response to the in vitro culture conditions. Thus, in order to reveal the impact of DNA methylation on SE regulation, the gene-specific pattern of DNA methylation needs to be revealed.

Both the maintenance and de novo pathways of DNA methylation are involved in SE induction

The role of DNA methylation in the embryogenic response of the Arabidopsis explants was further inferred by the significantly modulated expression of the genes encoding DNA methylases during SE. We found that the genes of the methylases that are involved in the maintenance (*MET1, CMT3*) and de novo (*DRM1* and *DRM2*) DNA methylation are expressed differently during SE. We found the significant up-regulation of the *MET1* and *CMT3* genes during SE in Arabidopsis and, consistent with this result, a higher activity of these genes has been reported in zygotic embryos, in particular during the early stages of embryo formation, compared to other organs of Arabidopsis (Xiang et al. 2011; Jullien et al. 2012; Ashapkin et al. 2016). Thus, it appears that the enhanced activity of the *MET1* and *CMT3* genes is generally associated with embryonic development and might reflect a requirement for the propagation of the DNA methylation pattern in the intensively dividing pro-embryonic and embryonic cells. In support of this assumption, the period with the highest expression of the *MET1* and *CMT3* genes coincides with the time of the intensive cell divisions that are associated with the acquisition of embryonic fate by the explant cells (Kurczyńska et al. 2007).

Expression profiling of the DNA methylase genes on the E5 versus the E0 medium infers that auxin treatment activates *MET1* and *CMT3* transcription. A positive impact of auxin on the *CMT3* expression has also been reported by others (Parizot et al. 2010; Shemer et al. 2015). In support of the auxin-controlled expression of *CMT3* is the presence of the auxin-responsive motif, AuxRE, in the promoter of this gene (http://arabidopsis.med.ohio-state.edu/AtcisDB/), which implies that ARFs, the core elements of auxin signalling that are believed to play roles in SE induction in Arabidopsis (Weijers and Wagner 2016; Wójcikowska and Gaj 2017), might regulate *CMT3* in response to auxin treatment. In contrast to *CMT3*, the impact of auxin on *MET1* up-regulation in SE appears to be indirect due to the lack of auxin-responsive motifs in the gene promoter.

The impaired embryogenic capacity of the *ddm1* and *vim1* mutants (present results) suggests that similar to *in planta* development, the DNA methylation-related proteins VIM1 and DDM1 support the function of MET1 methylases during SE (Zemach et al. 2013; Shook and Richards 2014). The VIM1 protein contributes to the recruitment of MET1 to a newly replicated DNA strand (Shook and Richards 2014). Consistent with this, we found that the up-regulation of *VIM1* was associated with an increased *MET1* transcription in SE.

We observed that the explants of the *cmt3* mutant were unaffected in SE response and in contrast, the *met1* mutant was significantly defective in SE induction. The contrasting embryogenic capacity of the *cmt3* and *met1* mutants suggests that the contribution of the CMT3 and MET1 methylases to SE appears to be different. The role of *CMT3* in the callus-mediated shoot regeneration of Arabidopsis (Shemer et al. 2015) might suggest that in the SE system of Arabidopsis in which marginal callus production accompanies the direct development of somatic embryos from explant cells (Gaj 2001; Kurczyńska et al. 2007), the function of CMT3 might be not critical for SE induction. In contrast, the importance of MET1 activity for SE induction might be related with the role of the enzyme in the hormone-related processes that play a central role in SE induction (Nic-Can and Loyola-Vargas 2016). Accordingly, *MET1* has been indicated as impacting the expression of *ARF3* and *WUS*, which are involved in hormone signalling (Li et al. 2011), and the *PIN1* gene that is engaged in auxin polar transport in ZE (Xiao et al. 2006).

In the present study we found that an increased expression level of the *CMT3* and *MET1* DNA methylases contrasts with a decreased global level of DNA methylation in the embryogenic culture. Since, DNA methylation is accompanied by active DNA demethylation and the balance between these antagonistic processes is believed to control the pattern of DNA methylation in plant development including the early stages of embryogenesis (Penterman et al. 2007; Lei et al. 2015; Bouyer et al. 2017), we also analysed the expression of the genes encoding DNA demethylases during SE induction and a distinct down-regulation of *ROS1, DME* and *DML2* genes in the SE-induced explants was observed. Thus, the reduction of the global DNA methylation level that was found in the embryogenic culture (present results) appears not to directly result from the transcriptional activity of the genes encoding DNA methylases and demethylases. In support of this assumption, an analysis of the rice genome showed that the level of cytosine methylation is not directly correlated with the activity of the DNA methylases (Teerawanichpan et al. 2009). In addition, an analysis of the triple mutant that was affected in *ROS1, DML2* and *DML3* genes indicated an unchanged DNA methylation level although discrete loci were hypermethylated (Penterman et al. 2007). It was postulated that the lack of a direct correlation between the DNA methylase activity and the 5mC level might be a consequence of the complexity of the interactions that control the balance between DNA replication, the de novo maintenance DNA methylation and demethylation (Hsieh et al. 2009). The complex interplay between DNA methylation and demethylation exemplifies the fine tuning of the expression of *ROS1* to variations in the DNA methylation level in which the methylation and demethylation of the gene promoter region promote and suppress gene transcription, respectively (Lei et al. 2015). Relevant to this model, an extremely low level of *ROS1* transcripts was indicated in mutants that were impaired in DNA methylation, including *met1*, which had a significantly reduced overall level of DNA methylation (Cokus et al. 2008; Williams et al. 2015).

Our analyses indicate that in the SE of Arabidopsis de novo DNA methylation appears to mainly be controlled by DRM2 methylase due to the high expression of *DRM2* at all of the monitored time points of the culture (Fig. S1). A distinctly higher level of *DRM2* than *DRM1* transcripts was also shown to be characteristic of *in planta* development including zygotic embryo formation in Arabidopsis (Xiang et al. 2011; Jullien et al. 2012; Ashapkin et al. 2016). Moreover, we found that the auxin medium positively impacted the expression of the *DRM* genes as was reported in a callus culture induced from Arabidopsis seedlings (Jiang et al. 2015). The role of de novo DNA methylation in the control of SE induction also infers the finding that the distinct up-regulation of the SE-regulators (*LEC1, LEC2* and *BBM*) in the *dd* and *ddc* mutant cultures is associated with improved SE response (present results). The reduced content of non-CG methylated sites in DNA causing chromatin de-condensation and de-repression of genes might account for the increased *LEC1, LEC2* and *BBM* expression that was indicated in the culture of *DRM*-defected mutants (Cao and Jacobsen 2002b; Henderson and Jacobsen 2008).

Conclusions

Modification of the DNA methylation state of the cultured explants via 5-AzaC treatment and mutations in the genes encoding DNA methylases (*dd*/*ddc)* distinctly alters both the embryogenic response and the expression profiles of *LEC2, LEC1, BBM* genes, which are the master regulators of SE. Thus, DNA methylation, seems to control SE induction and expression of the *TF* genes that are essential for the embryogenic transition. Identifying the SE-specific pattern of DNA methylation within these genes will contribute to deciphering the SE-associated DNA methylome.

However, it recently became evident that DNA methylation appears to control the expression of only a small number of genes (Seymour and Becker 2017) and that the impact of DNA methylation on gene expression is distinctly influenced by other epigenetic modifications including histone modification and gene silencing by small noncoding RNA (Stroud et al. 2014; Du et al. 2015; Niederhuth and Schmidt 2017). Thus, to fully understand the function of DNA methylation in the embryogenic transition the functional outcome of DNA methylation in the context of other epigenetic changes remains to be uncovered. Moreover, it is of importance to reveal the relation of DNA methylation with the recently identified miRNA that plays a regulatory role in the SE of Arabidopsis (Wójcik and Gaj 2016; Szyrajew et al. 2017; Wójcik et al. 2017).

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Author contributions MDG conceived and designed the experiments, DG, JM and BW performed the experiments; DG, JM and MDG analysed the data and wrote the manuscript. DG and JM contributed equally to this work. All of the authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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