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Expression and DNA Methylation of *MET1, CMT3* and *DRM2* during *In Vitro* Culture of *Boesenbergia rotunda* (L.) Mansf.

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Somatic embryogenesis and plant regeneration are important developmental processes of *in vitro* culture, in which cells must undergo dedifferentiation, activation of cell division and reprograming of their metabolism, of their physiology and of their gene expression patterns. The processes of somatic embryogenesis and plant regeneration are also associated with changes in DNA methylation. In this study, the expression of *METHYLTRANSFERASE 1, CHROMOMETHYLASE 3 and DOMAIN REARRANGED METHYLTRANSFERASE 2* was determined by Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) while the DNA methylation level was determined by Bisulfite sequencing of plant cells from meristematic block, embryogenic and non-embryogenic calli, prolonged cell suspension culture, *ex vitro* leaf and *in vitro* leaf of regenerated plants of *Boesenbergia rotunda*. We observed that the expression of DNA methyltransferase genes *METHYLTRANSFERASE 1, CHROMOMETHYLASE 3 and DOMAIN REARRANGED METHYLTRANSFERASE 2* was the highest in meristematic block followed by embryogenic callus, and the lowest was in watery callus. DNA methylation at CG, CHG and CHH sequence contexts was observed to be generally lower in embryogenic callus than in other samples. We observed relatively higher expression levels and lower levels of DNA methylation at CG, CHG and CHH sequence contexts of *MET1, CMT3* and *DRM2* associated with somatic embryogenesis and regenerability in *Boesenbergia rotunda*.

Key Words: callus, bisulfite sequencing (BS-seq), DNA methylation, gene expression, qRT-PCR, somatic embryogenesis

Abbreviations: BS – seq-Bisulfite sequencing, cDNA – complementary DNA, *CMT3 – Chromomethylase 3*, CTAB – cetyl trimethyl ammonium bromide, CS – cell suspension, DC – dry callus, DRM2 – *Domain rearranged methyltransferase 2*, EC – embryogenic callus, EVL – *ex vitro* leaf, IVL – *in vitro* leaf, MB – meristematic block, MET1 – *methyltransferase 1*, PCR – Polymerase Chain Reaction, qRT-PCR –Quantitative Reverse Transcription PCR, WC – watery callus, SPSS – Statistical Package for the Social Sciences

INTRODUCTION

DNA methylation, an important epigenetic regulatory mechanism, plays key roles in plant development (Yang et al. 2015; How-Kit et al. 2017), stress responses (Al-Lawati et al. 2016; Varriale 2017), genome stability (Law and Jacobsen 2010), gene silencing (Rodriguez-Negrete et al. 2013; Ikeda and Nishimura 2015), genomic imprinting (Vu et al. 2013; Ikeda and Nishimura 2015) and the control of transposable elements (Martienssen and Colot 2001; Wang et al. 2016). While in mammals DNA methylation is found predominantly in CG sequences (CpG-islands), in plants in addition to symmetric CG methylation, DNA methylation in symmetric CHG and asymmetric CHH sequence contexts (where H denotes A, T or C) are widely reported, though at relatively lower levels than CG methylation (Woo et al. 2008). In plants, METHYLTRANSFERASE 1 **(**MET1), CHROMOMETHYLASE 3 (CMT3) and DOMAIN REARRANGED METHYLTRANSFERASE (DRM) catalyze the methylation of 5-cytosine in DNA which is vital for epigenetic regulation and reconfiguration of genome structure (Goll and Bester 2005). DNA methylation and demethylation are dynamic processes in dividing and differentiating cells. METHYLTRANSFERASE 1 (MET1) mainly maintains

methylation in the CG context, and is a homolog of mammalian maintenance DNA Methyltransferase 1 (DNMT1) based on conserved amino acid motifs and enzyme structure (Cao et al. 2000; Meyer 2011). DOMAIN REARRANGED METHYLTRANSFERASE 2 (DRM2), a homolog of the mammalian *de novo* DNA Methyltransferase (DNMT3), maintains CHH methylation and catalyzes *de novo* methylation at all three sequence contexts *via a* small interfering RNA-directed DNA methylation (RdDM) pathway that is unique to plants (Cao and Jacobsen 2002; Zhai et al. 2015; Wendte and Pikaard 2017). Methylation of DNA cytosine in the CHG sequence context is maintained by plant-specific CHROMOMETHYLASE 3 (CMT3) (Jackson et al. 2002; Song and Cao 2017).

The expression of DNA methyltransferase genes has been reported to change in response to different factors including plant growth regulators (Taskin et al. 2015) and environmental stresses (Ahmad et al. 2014). The expression of DNA methyltransferase genes is temporal and developmental, varying with cell type, as observed for *METHYLTRANSFERASE::GUS* reporters (using *MET1, DRM2* and *CMT3*) in transgenic *Arabidopsis thaliana* (Huang et al. 2014), by genome-wide expression profiling of DNA methyltransferase genes in *Zea mays* (Qian et al. 2014) and by qRT-PCR of *MET1, CMT3*, and *DRM2* from *A. thaliana* (Ashapkin et al. 2016). Expression of DNA methyltransferases is a prerequisite for plant development as mutants *viz. met1, met1cmt3* and *drm1drm2cmt3* showed decreased seed viability and produced abnormal embryo in *A. thaliana* (Xiao et al. 2006; Zhang and Jacobsen 2006); and as full combination of mutants, i.e., *met1cmt3drm1drm2* of *A. thaliana* were also reported to have embryonic lethality (Zhang and Jacobsen 2006). The changes of expression of DNA methyltransferase genes at different stages of *in vitro* culture may affect the processes of somatic embryogenesis and plant regeneration (Karim et al. 2016).

Gene expression may be influenced by DNA methylation at a promoter region and by methylation within the gene body. However, it is apparent from various research reports that the threshold of methylation to suppress gene expression differs in each case. The presence of 5-methylcytosines in the promoter regions of genes has been generally associated with suppression of expression. DNA methylation at promoter regions is reported to occur at CG sites, maintained by *MET1* (Berdasco et al. 2008) and *DRM2* (Zhang et al. 2006). Examples of studies reporting suppression of gene expression associated with DNA methylation of gene promoters include *Oryza sativa* (Stroud et al. 2013) where hypermethylation at promoter regions was associated with inactivation of genes and hypomethylation was associated with activation of genes, *Betula platyphylla* (Su et al. 2014) where heavy methylation of promoter regions was associated with repressed gene expression; and semi-quantitative RT-PCR of RNA from heat-stressed *Nicotiana tabacum* BY-2 cell cultures indicating that hypermethylation at the promoter region of *NtEXPA5* repressed gene expression while hypomethylation at the promoter region of *CycD3-1* enhanced gene expression (Centomani et al. 2015).

While hypermethylation at promoters is associated with suppression of gene expression, this is not always the case with respect to the methylation of gene body regions. The genome-wide high resolution mapping and functional analysis of DNA methylation in *A. thaliana* found heavy methylation of the gene body of some housekeeping genes to be associated with higher levels of expression of those genes (Zhang et al. 2006). In contrast, in *B. platyphylla*, genes with moderate gene body methylation were found to be expressed at higher levels compared with genes with slight or heavy gene body methylation (Su et al. 2014) and *O. sativa*, moderate gene body methylation was associated with high levels of gene expression, whereas low or heavy gene body methylation was associated with lower levels of gene expression (Wang et al. 2013).

Boesenbergia rotunda (L.) Mansf., an important ethnomedicinal plant belonging to the ginger family (Zingiberaceae), is widely found in Southeast Asia, India and Southern China (Baker 1892; Garden and Kew 2010), and known as "Chinese key" or "Finger Root" in English, "Temu Kunchi" in Malay, and "Krachai" or "Krachai-Drag" in Thai. Regeneration of *B. rotunda* through somatic embryogenesis from callus cultures (Tan et al. 2005; Yusuf et al. 2011) and from shoot bud explants (Yusuf et al. 2013) has been reported. Plant regeneration *via* somatic embryogenesis from embryogenic cell suspension culture of *B. rotunda* was also demonstrated without any loss of capacity to produce secondary metabolites (Wong et al. 2013). However, following long-term cultivation (12 mo), *B. rotunda* suspension cells lost the ability to form somatic embryos and were unable to regenerate (Wong et al. 2013). Thus, *in vitro* cultures of *B. rotunda* provide a useful model of embryogenically competent and non-competent cells for the study of gene expression during somatic embryogenesis and plant regeneration. These processes are also associated with changes in DNA methylation. Here, we report the changes of gene expression and DNA methylation status of three DNA methylation pathway genes, *MET1, CMT3* and *DRM2* in different types of *in*

vitro and *ex vitro* calli and tissues of *B. rotunda*, as these genes maintain and mediate *de novo* DNA methylation during plant development.

MATERIALS AND METHODS

Rhizomes of *B. rotunda* (L.) Mansf. were obtained from a commercial farm in Temerloh, Pahang, Malaysia and propagated in the laboratory to generate all sample materials following methods described by Ng et al. (2016). Initially, the rhizomes were washed thoroughly under running tap water for 10 min, and then air-dried for 30 min before placing inside black polybags to initiate sprouting. Water was sprayed every day to induce sprouting or shoots. Newly emerged shoots of 1–3 cm in length were either transferred to soil in pots or were harvested for dissecting meristematic block (MB) tissue which was either used as a direct sample (MB) or as explant material for *in vitro* callus initiation (Fig. S1). The young *ex vitro* leaf (EVL) samples were collected from rhizome-derived plants at 4 wk after potting at the Department of Genetics and Molecular Biology, University of Malaya, Malaysia. Callus samples were established as described in Ng et al. (2016) by culturing MB explants on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 1 mgL^{-1} α-napthaleneacetic acid (NAA), 1 mgL^{-1} indole-3-acetic acid (IAA), 30 gL^{-1} sucrose and 2 gL^{-1} Gelrite® (Sigma Aldrich, Missouri, United States). The calli that formed (after around 4 wk) were transferred to a MS medium supplemented with 30 gL^{-1} sucrose and 2 gL^{-1} Gelrite® and 2,4-dichlorophenoxy acetic acid (2,4-D) at various concentrations as follows; for watery callus (WC) (1 mgL^{-1}), for embryogenic callus (EC) (3 mgL^{-1}) and for dry callus (DC) (4 mgL^{-1}). The WC, EC and DC samples were collected after 4 wk on the respective media (8 wk after initial culturing from explant) (Fig. S1). Embryogenic cell samples were collected by sieving clusters of embryogenic calli through a 425 μm stainless steel sieve. Cell suspension (CS) culture was established from embryogenic callus and was maintained for 1 yr in MS liquid medium supplemented with 3 mgL^{-1} 2,4-D according to Wong et al. (2013). After successful establishment, callus and cell suspension cultures were viewed under stereomicroscopy to observe the presence or absence of embryo structures (as described in Yusuf et al. (2011), Wong et al. (2013) and Ng et al. (2016)). Established embryogenic callus, watery callus, dry callus (8 wk after initial culturing from explant) and prolonged cell suspension culture (samples collected after 12 mo in suspension culture or equivalent to 60 wk after initial culturing from explant) were placed in regeneration media (MS0) using 10 plates with 9 calli per plate and were monitored daily for 8 wk (Table S1). Leaves from embryogenic calli derived regenerated plants were collected after 8 wk (16 wk after initial culturing from explant).

RNA Isolation and cDNA Synthesis

Total RNA was isolated from *ex vitro* leaf (EVL), meristematic block of newly emerged shoots (MB), embryogenic callus (EC), dry callus (DC), watery callus (WC), prolonged cell suspension cells (CS) and *in vitro* leaf of regenerated plants (IVL) using a modified cetyl trimethyl ammonium bromide (CTAB) method (Kiefer et al. 2000). Total RNA was measured spectrophotometrically using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA) and RNA integrity was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). RNA samples with absorbance ratios A260nm/A280nm ranging from 1.8 to 2.2, and an A260nm/A230nm ratio higher than 1.0 and an RNA integrity number (RIN) higher than 7.0 were used to synthesize cDNA for gene expression study using quantitative real time PCR (qRT-PCR). cDNA was synthesized for qRT-PCR analysis using a QuantiTect Reverse Transcription Kit (QIAGEN, Germany) according to the manufacturer's instructions.

Gene Expression Analysis using qRT-PCR

Quantitative RT-PCR (qRT–PCR) was performed using gene specific primers designed from unigene sequences *MET1* (KY290867), *CMT3* (KY290868) and *DRM2* (KY290869) of *B. rotunda* transcriptome (Md-Mustafa et al. 2014) using Primer BLAST and Primer3 Plus. Primers were synthesized by Integrated DNA Technologies (USA) and were as listed in Table 1. Amplification mixtures (20 μL per reaction) containing 10 μL Power SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies, Thermo Fisher Scientific, USA), 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM), 2 μL template cDNA aliquot corresponding to 20 ng of total RNA and sterile water were run on a QuantStudio® 12K Flex Real-Time PCR System (Applied Biosystems, Life Technologies, Thermo Fisher Scientific, USA). Cycling conditions were as follows: 10 min at 95 °C, 40 cycles of 95 °C – 15 s followed by 60 °C – 1 min. The reactions were performed in triplicate for each cDNA template of three independent experiments with each primer pair. A 'non template control (NTC)' was included to monitor the formation of non-specific products. Meristematic block (MB) was used as the calibrator (value set as 1). The housekeeping genes 18S rRNA were used as an internal control for normalization for each analysis. For qRT-PCR, relative

Table 1. Primers for gene expression analysis using Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR).

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Gene	Primer Sequence	Product Length (bp)	Accession No.
MET1	Forward: 5'-GCCCATGGGTAAGGTTGGAA	165	KY290867
	Reverse: 5'-TCTCCCAAAACCATTCAGTGCT		
CMT3	Forward: 5'-TCGTTGTCTTCATGGACATCGT	220	KY290868
	Reverse: 5'-TTGGGATGACTTCCCCACAG		
DRM2	Forward: 5'-ACACCGTTTGGGGATACACCT	227	KY290869
	Reverse: 5'-TGCTCCCGGTAAGATTGTTGC		
18S rRNA	Forward: 5'- CAAAAAGTGGCGGAATGCTC	226	X00794.1
	Reverse: 5'- GACAGACCAAGGGCGAACAC		

quantification of gene expression used the comparative CT method ($2^{-\Delta\Delta CT}$ method) of Livak and Schmittgen (2001). This method is based on the use of an internal control gene transcript to normalize sample variations under different experimental conditions.

DNA Methylation Analysis using Bisulfite Sequencing (BS-seq)

Total genomic DNA was extracted using a modified cetyl trimethyl ammonium bromide (CTAB) method (Doyle 1990) from all samples. The concentration and purity of DNA were determined by measuring the absorbance at 260 nm (A_{260nm}) and 280 nm (A_{280nm}) using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA) and Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, USA). Samples with an A_{260nm}/A_{280nm} ratio between 1.7–1.9 were selected for methylation analysis in the study. Genomic DNA of *B. rotunda ex vitro* leaf, embryogenic callus, dry callus, watery callus, prolonged cell suspension culture and *in vitro* leaf of regenerated plants was sequenced after being treated by sodium bisulfite. The sequencing was carried out by a commercial service provider, Sengenics Sdn. Bhd., Malaysia. A total of six samples (three biological replicates for each of six samples) were sequenced to generate paired-end reads using an Illumina HiSeq™ 2000 platform (Illumina Inc., San Diego, CA) according to the manufacturer's instructions. Reads were pre-processed by trimming low quality reads and adapters by Trim-Galore (Krueger 2015) specific for bisulfite sequencing. After trimming, the fastq reads were mapped to a *B. rotunda* transcriptome using the Bismark v 0.12.3 (Krueger and Andrews 2011), and mapping duplicates were removed using Methpipe v 3.4.2 (Song et al. 2013). Mapping of methylated and unmethylated cytosines was determined using the Methcounts program from methpipe (Song et al. 2013), where the methylation level at single base resolution was calculated based on the number of 5-methylated cytosines (5mC) in reads, divided by the sum of the C and thymines (T) in CG, CHG and CHH sequence contexts within the coding sequences of *MET1*, *CMT3* and *DRM2* from *B. rotunda*.

Statistical Analysis

Quantitative Real Time PCR (qRT–PCR) data was analyzed by ExpressionSuite Software (version 1.0.4., Life Technologies, Thermo Fisher Scientific, USA) and Microsoft Office Excel 2013. One-way ANOVA using SPSS software (version16.0, IBM, Chicago, IL, USA) was performed to assess the significant differences in the mean values of different samples obtained from qRT-PCR and BS-seq data. Comparisons between mean values were made using Tukey's comparison test ($p < 0.05$). Pearson's Correlation Analysis was performed to determine the relationship of gene expression and DNA methylation status for each gene.

RESULTS AND DISCUSSION

The field of plant genomics is just starting to generate the type of data that allow insights into the impact of DNA methylation on gene expression and developmental processes such as somatic embryogenesis and plant regeneration. DNA methylation is dynamic, involving *de novo* and maintenance activities via various DNA methyltransferases and demethylases, however, there

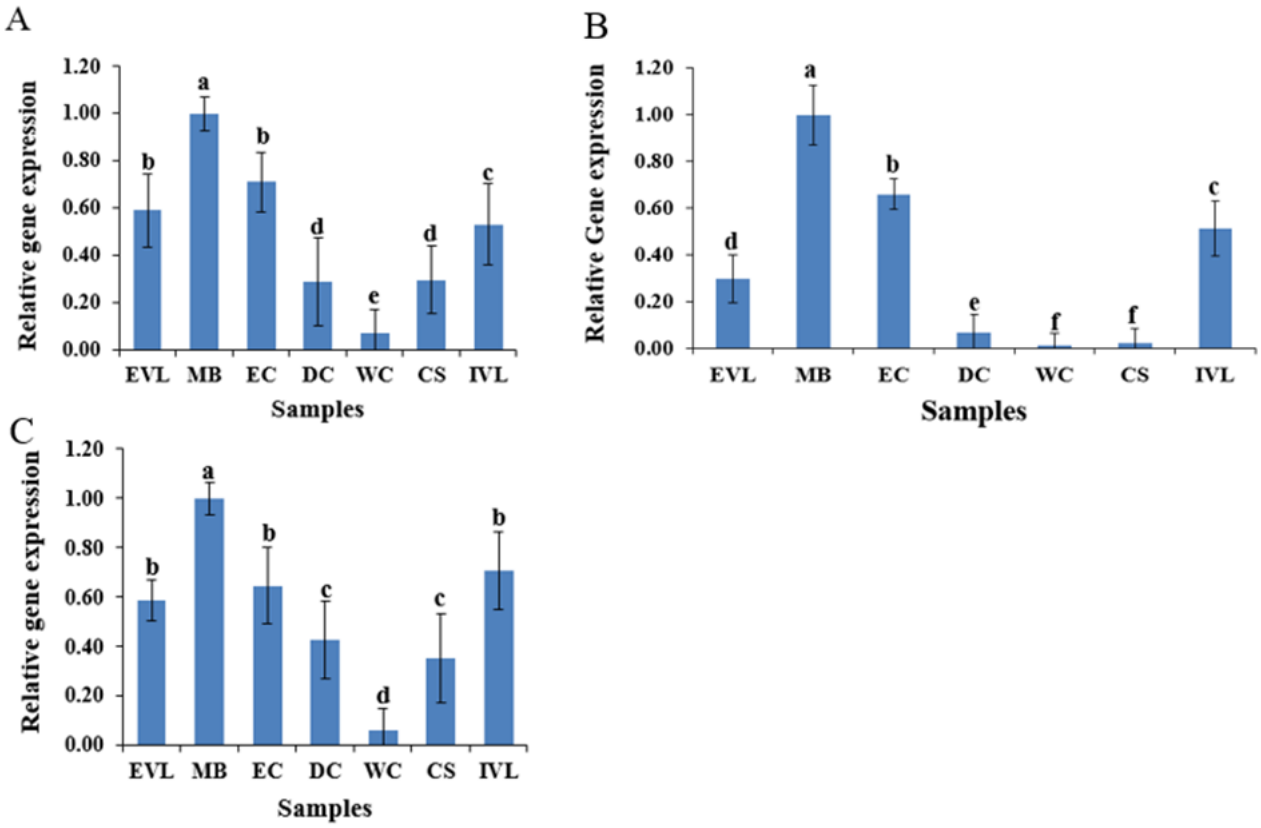

Fig. 1. Relative gene expression of DNA methyltransferases in *ex vitro* and *in vitro* tissues, and calli using Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR): *MET1* (A), *CMT3* (B) and *DRM2* (C). EVL: *ex vitro* leaf; MB: meristematic block; EC: embryogenic callus; DC: dry callus; WC: watery callus; CS: cell suspension and IVL: *in vitro* leaf. Meristematic block (MB) was used as the calibrator (value set as 1) and 18S *rRNA* was used as the internal control for qPCR. Relative quantification of gene expression was determined by using the comparative CT method ($2^{-\Delta\Delta CT}$ method) of Livak and Schmittgen (2001). Bars represent the standard deviation of three biological replicates. Letters indicate statistical significance, where the same letter indicates no significant difference between samples, according to Tukey's comparison test ($p > 0.05$).

have been limited studies of DNA methylation in relation to expression of DNA methyltransferases in plants. Enzymes encoded by *MET1*, *CMT3* and *DRM2* maintain DNA methylation at CG, CHG and CHH contexts, respectively, while *DRM2* mediates *de novo* methylation at all three sequence contexts of the genome (Cao and Jacobsen 2002; Cokus et al. 2008; Law and Jacobsen 2010; Song and Cao 2017). Here, the expression patterns of *MET1, CMT3* and *DRM2,* and their DNA methylation status at CG, CHG and CHH contexts were determined from seven sample types of *B. rotunda*, i.e., embryogenic callus (EC), representing a regenerable tissue; dry callus (DC), watery callus (WC) and prolonged cell suspension culture (CS), representing non-embryogenic tissue and cells; from initial explant materials, i.e., meristematic block of newly emerged shoots (MB); and from *ex vitro* leaf (EVL) and *in vitro* leaf of regenerated plants (IVL), representing comparable tissues prior to and following a cycle of *in vitro* regeneration.

The expression of DNA methyltransferase *MET1, CMT3* and *DRM2* was the highest in meristematic block (MB) followed by embryogenic callus (EC), *ex vitro* leaf (EVL) and *in vitro* leaf (IVL), while dry callus (DC), watery callus (WC) and prolonged cell suspension (CS) (non-embryogenic and non-regenerable) cultures showed significantly lower expression (Fig. 1). Although the CS was established from EC, after long-term culture (12 mo), cells showed a significantly decreased level of expression of all three genes compared to EC. The pattern of expression of *DRM2* between samples was similar to that for *MET1* expression (Fig. 1A, C). However, unlike *MET1* expression, the expression level of *DRM2* in IVL was not significantly different from that of EVL and EC samples.

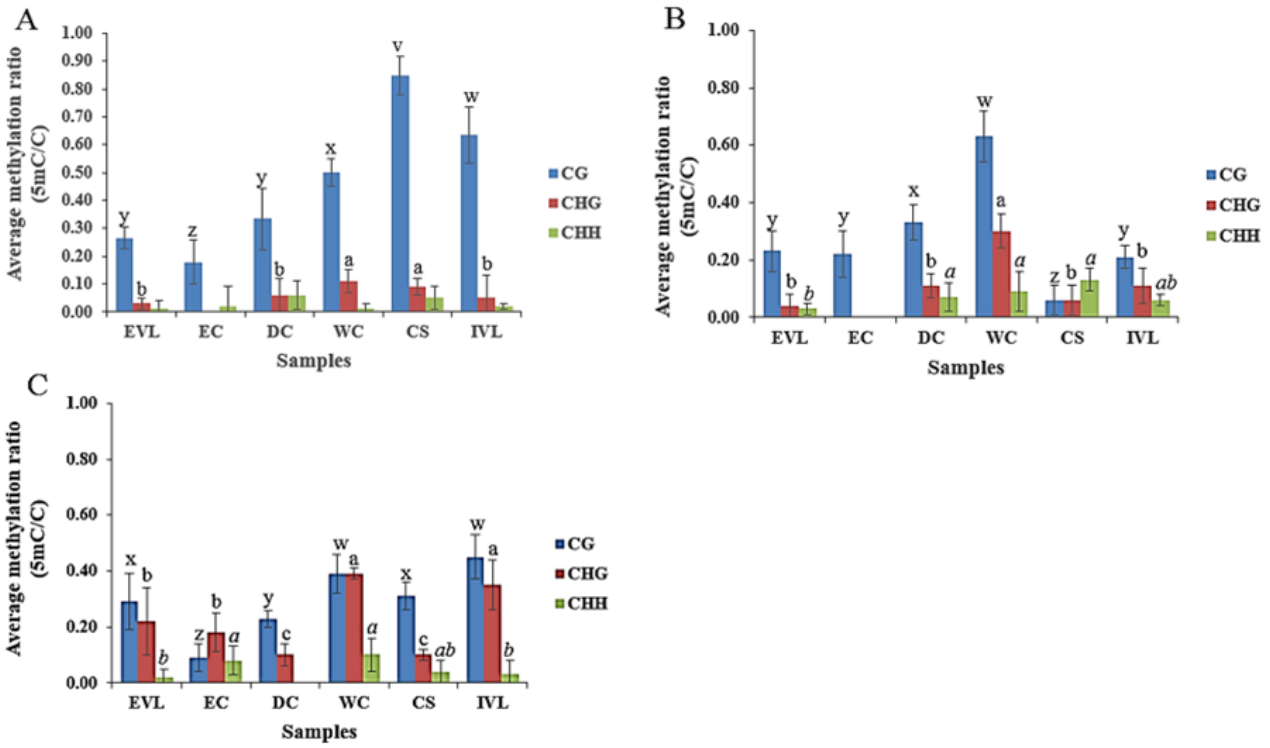

Fig. 2. DNA methylation of *MET1*, *CMT3* and *DRM2* for *ex vitro* and *in vitro* calli and tissues of *B. rotunda*. *MET1* (A), *CMT3* (B) and *DRM2* (C). EVL: *ex vitro* leaf; EC: embryogenic callus; DC: dry callus; WC: watery callus; CS: cell suspension and IVL: *in vitro* leaf. Bars represent the standard deviation of three biological replicates for each sample. Letters indicate statistical significance, where the same letter indicates no significant difference between samples, according to Tukey's comparison test ($p > 0.05$), (v, w, x, y, z for CG methylation; a, b, c for CHG methylation; and *a*, *b* for CHH methylation).

The highest expression level of *CMT3* was observed in MB, followed by EC (Fig. 1B). DC, WC and prolonged CS exhibited relatively low levels of transcripts of CMT3. Unlike *in vitro* calli and cells, EVL and IVL are organized and differentiated tissues, and thus can behave differently. However, it should be further validated considering other differentiated tissues compared with *in vitro* calli samples.

Considering only the *in vitro* calli and cell suspension samples, we observed that the levels of DNA methyltransferase gene expression were significantly higher in EC, regenerable cells, than in the non-regenerable samples DC, WC and CS for all three genes (Fig. 1). Higher expression of *MET1*, *CMT3* and *DRM2* genes was also observed in somatic embryogenic cells compared with non-embryogenic cells in *A. thaliana* (Wickramasuriya and Dunwell 2015). This suggests that DNA methylation processes are highly active in cells as they divide and multiply within embryogenic-regenerable calli. However, the actual levels of DNA methylation across the genome in these same samples would be needed to validate this.

DNA methylation level analysis at CG, CHG and CHH sequence contexts of *MET1*, *CMT3* and *DRM2* genes of *B. rotunda* revealed that CG methylation was predominant for all three genes (Fig. 2). The frequency of CHH methylation was relatively lower than that of CG and CHG methylation. Among all samples, EC showed overall lower level of methylation in all three genes. CS and WC showed the highest level of methylation in *MET1* and *CMT3*, respectively (Fig. 2A, B), while WC and IVL showed the highest level of methylation in *DRM2* (Fig. 2C). By examining the local DNA methylation of the DNA methyltransferase genes *MET1*, *CMT3* and *DRM2* in the CG, CHG and CHH contexts, it was seen that there were generally lower levels of methylation of *MET1*, *CMT3* and *DRM2* in EC compared with the other samples (Fig. 2). As DNA methylation is generally lower in regions of euchromatin to permit gene expression (Schones and Zhao 2008; Tamaru 2010; reviewed by De La Pena et al. 2015), this result fits well with the higher expression of the DNA methyltransferase genes in the EC, and likewise that the samples with lower expression of *MET1*, *CMT3* and *DRM2*, generally had higher levels of DNA

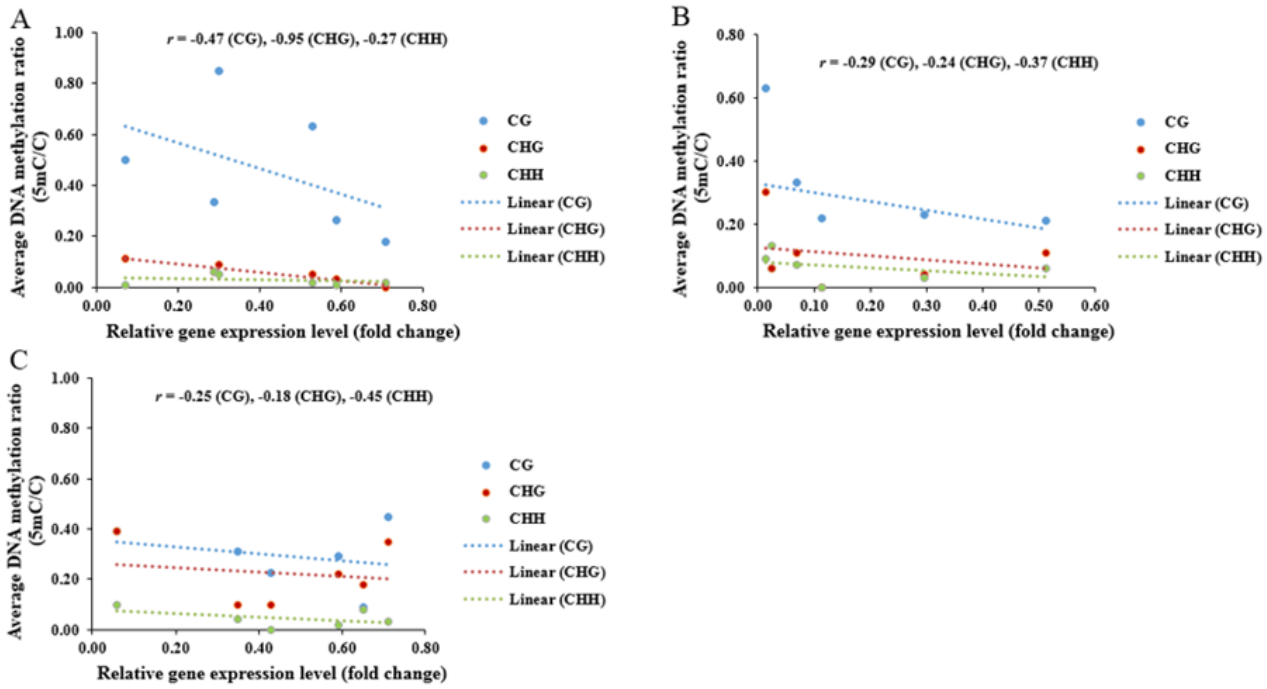

Fig. 3. Correlation between gene expression and DNA methylation level at CG, CHG and CHH contexts of *MET1* (A), *CMT3 (*B) and *DRM2* (C). Analysis was performed by Pearson's correlation coefficient.

methylation at these loci, as shown *via* correlation analysis (Fig. 3).

According to DNA methylation and gene expression data, it can be suggested that a decreased level of DNA methylation (as observed in EC) coincides with enhanced expression level which permits somatic embryogenesis and regeneration, whereas the relatively increased level of DNA methylation (in DC, WC and CS) reduced their expression level which represses the embryogenic competency and plant regeneration in *B. rotunda* (Fig. 1; Fig. 2). This hypothesis is supported by some previous observations where hypermethylation at promoter regions, e.g., in *O. sativa* (Stroud et al. 2013), in *B. platyphylla* (Su et al. 2014), and *N. tabacum* (Centomani et al. 2015), or heavy gene body methylation, e.g., in *A. thaliana* (Zhang et al. 2006), in *O. sativa* (Wang et al. 2013), and *B. platyphylla* (Su et al. 2014) is associated with suppressed gene expression.

The correlation study between gene expression and DNA methylation revealed that the methylation levels at CG, CHG and CHH contexts of each gene of three DNA methyltransferases, *MET1, CMT3* and *DRM2* showed negative correlation with their expression level (Fig. 3). Despite the increasing activities of DNA methyltransferases, hypomethylation was noticed in *Elaeis guineensis* (Rival et al. 2008) and in *Fragaria* × *ananassa* (Chang et al. 2009) similar to our results. However, as there is not yet any genome sequence available for *B. rotunda*, it is difficult to map the specific location of methylation such as in promoter or gene body or exons-introns. Nevertheless, it may be useful to determine full DNA methylation profiles of specific genes associated with plant development, or of transposable elements, when *B. rotunda* genome sequence becomes available. The information provided here may help to design strategies to enhance embryogenic capacity and regeneration, and to form the foundation for future research on genetic and epigenetic control of plant somatic embryogenesis and regeneration during *in vitro* culture.

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