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RESEARCH ARTICLE

Expression and DNA methylation of *SERK*, *BBM*, *LEC2* and *WUS* genes in in vitro cultures of *Boesenbergia rotunda* (L.) Mansf.

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Abstract The process of somatic embryogenesis and plant regeneration involve changes in gene expression and have been associated with changes in DNA methylation. Here, we report the expression and DNA methylation patterns of *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* (*SERK*), *BABY BOOM* (*BBM*), *LEAFY COTYLEDON 2* (*LEC2*) and *WUSCHEL* (*WUS*) in meristematic block of newly emerged shoots from rhizome, embryogenic and non-embryogenic calli, prolonged cell suspension culture, ex vitro leaf, and in vitro leaf of regenerated plants of *Boesenbergia rotunda*. Among all seven samples, based on qRT-PCR, the highest level of expression of *SERK, BBM* and *LEC2* was in embryogenic callus, while *WUS* was most highly expressed in meristematic block tissue followed by embryogenic callus. Relatively lower expression was observed in cell suspension culture and watery callus for *SERK, LEC2* and *WUS* and in in vitro leaf for *BBM*. For gene specific methylation determined by bisulfite sequencing data, embryogenic callus samples had the lowest levels of DNA methylation at CG, CHG and CHH contexts of *SERK*, *LEC2* and *WUS*. We observed negative correlation between DNA methylation at the CG and CHG contexts and the expression levels of *SERK*, *BBM*, *LEC2* and *WUS*. Based on our results, we suggest that relatively higher expression and lower level of DNA methylation of *SERK, BBM*, *LEC2* and *WUS* are associated with somatic embryogenesis and plant regeneration in *B. rotunda*.

Keywords Callus · Somatic embryogenesis · Plant regeneration · qRT-PCR · Bisulfite sequencing (BS-seq) · Epigenetics

Introduction

In vitro plant tissue and cell culture systems are important tools to overcome challenges in the agricultural, industrial and medicinal sectors. Multiplication of desired genotypes of plants with improved agronomic traits or with high industrial and medicinal value can be achieved by making use of cellular totipotency during in vitro culture. Induction of somatic embryogenesis is a widely used approach for rapid propagation of valuable clones, for plant regeneration, and also for genetic transformation to obtain genetically modified plants. Formation of somatic embryos during in vitro culture is a unique developmental process as somatic cells must undergo dedifferentiation, activation of cell division and reprogramming of their metabolism, and of their gene expression patterns (Elhiti et al. 2013; Fehér 2015; Yang and Zhang 2010). However, some plant species or specific desired genotypes of plants are recalcitrant to the formation of somatic embryos. The examples include; *Phaseolus vulgaris* (Hnatuszko-Konka et al. 2014); *Vigna radiata* L. Wilczek (Sagare and Mohanty 2015), cotton (Sakhanokho and Rajasekaran 2016) and *Cocos neucifera* (Bhavyashree et al. 2016). It has also been observed that

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plant cells can lose their totipotency after prolonged culture periods (Lambé et al. 1997). It has been suggested that selection pressure results in the loss of totipotent cells, either via depletion of metabolites promoting organogenesis or due to an accumulation of inhibitors of regeneration and epigenetic changes due to cultural environment (Gaspar et al. 2000). One of the environmental factors that has been associated with loss of totipotency in vitro is toxicity of 2,4-D in tomato (Patil et al. 2003) or long culture periods in media with 2,4-D in *B. rotunda* (Aziz et al. 2017), which has also been suggested as likely to be species dependent. Therefore, it is of interest to examine the underlying regulatory mechanisms of somatic embryogenesis and plant regeneration during in vitro culture.

A number of genes have been associated with the development of somatic embryos. Among the somatic embryogenesis related gene families, *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* (*SERK1, SERK2,* leucine-rich repeat receptor-like kinases), *BABY BOOM* (*BBM,* an *AP2/ERF* domain transcription factor), *LEAFY COTYLEDON* (*LEC1,* a CCAAT-box binding factor and *LEC2,* a B3 domain transcription factor), and *WUSCHEL* (*WUS*, homeodomain transcription factors), have been suggested to be markers that can distinguish individual embryo-forming cells in several plant species. Examples include *SERK* in *Solanum tuberosum* (Sharma et al. 2008), *Zea mays* (Zhang et al. 2011), *Trifolium nigrescens* (Pilarska et al. 2016) and *Brassica napus* (Ahmadi et al. 2016); *BBM* in *Arabidopsis thaliana* and *B. napus* (Boutilier et al. 2002) and *BBM* and *LEC* in *B. napus* (Malik et al. 2007). Studies on the expression levels of *SERK* in *A. thaliana* (Salaj et al. 2008), *B. napus* (Ahmadi et al. 2016) and *T. nigrescens* (Pilarska et al. 2016) showed high expression in the cells from which embryoids or embryo-like structures were produced. The expression of *BBM* was observed in almost all tissues of *A. thaliana* embryos, except distal parts of cotyledons (Kulinska-Lukaszek et al. 2012). The expression of *BBM* was much higher in embryogenic callus than non-embryogenic callus in *Coffea arabica* (Silva et al. 2014), and similarly, higher levels of *LEC2* were reported during somatic embryogenesis in *Z. mays* (Salvo et al. 2014). *WUS*, initially identified as being required to maintain a pool of pluripotent stem cells in the shoot apical meristem (SAM) in an undifferentiated state in Arabidopsis (Mayer et al. 1998), has been shown to have a role in somatic embryogenesis and shoot regeneration. Recently, it was found that *WOX2*, a member of the *WUS* family, was expressed in all stages of embryogenesis in *Larix decidua* with higher level in early stages, while a very low level of expression was observed in needle and seedlings (Rupps et al. 2016). Overexpression and ectopic expression of embryogenesis-related genes in various plant species provide supporting evidence for roles in somatic embryogenesis; for example somatic embryogenesis can be induced by ectopic expression of *BBM* in *A. thaliana* and *B. napus* (Boutilier et al. 2002) and in *Theobroma cacao* (Florez et al. 2015) and by overexpression of *LEC2* in Arabidopsis (Wójcikowska and Gaj 2015). The overexpression of *WUSCHEL* (*WUS*) in immature embryos after *Agrobacterium*-mediated transformation of *Z. mays* stimulated the growth of embryogenic tissues, which ultimately enhanced the recovery of transgenic plants in non-transformable inbred lines (Lowe et al. 2016).

Methylation of cytosine residues in DNA is one of the important epigenetic processes involved in the reprogramming of cells during in vitro culture (reviewed in Dela-Peña et al. (2015); Karim et al. (2016)). DNA methylation has been suggested to specifically regulate gene expression related to the induction of somatic embryogenesis and successful plant regeneration (Mahdavi-Darvari et al. 2015; Shibukawa et al. 2009). There is good evidence to support an influence of plant hormones on several types of epigenetic modifications, including DNA methylation (reviewed in Yamamuro et al. 2016) with auxins suggested to play a role in feedback regulation of epigenesis. DNA methylation in plants is largely located in repetitive regions of the genome and may affect adjacent gene promoters (Hollister and Gaut 2009). Increases in DNA methylation at gene promoter regions during in vitro plant culture has been reported to result in decreases in gene expression (Berdasco et al., 2008; Stroud et al. 2014) while reports on the methylation of the gene body and its effect on expression are lacking. Several studies have shown that gene body methylation may be a dynamic process that occurs in numerous plant genes, although the regulatory function remains unclear (Bewick and Schmitz 2017). The majority of reports of DNA methylation during in vitro culture of plants are based on genome-wide DNA methylation status (Grzybkowska et al. 2018; Quinga et al. 2017; Viejo et al. 2010), for example, DNA hypomethylation in *A. thaliana* (Kwiatkowska et al. 2014) and DNA hypermethylation in *Eleuterococcus senticosus* (Chakrabarty et al. 2003) were both associated with increased somatic embryogenesis. A recent study reported lower expression of somatic embryogenesis related genes coincident with increased global levels of DNA methylation in *Pinus radiata* (Bravo et al. 2017), however there are no reports comparing DNA methylation at the individual gene level between embryogenic and non-embryogenic tissue culture materials. With an aim to provide a better understanding of DNA methylation of embryogenesis-related genes during in vitro culture, here we report an analysis of the expression and DNA methylation profiles of *SERK, BBM*, *LEC2* and *WUS* in embryogenic and non-embryogenic calli/cells and tissues of *Boesenbergia rotunda*, an important

ethnomedicinal plant in South-East Asia, India and Southern China.

Materials and methods

Plant materials and establishment of in vitro samples

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 weeks after potting. 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 mg L^{-1} (5.4 µM) α-napthaleneacetic acid (NAA), 1 mg L^{-1} (5.7 µM) indole-3-acetic acid (IAA), 30 g L^{-1} sucrose and 2 g L^{-1} Gelrite® (Sigma Aldrich, Missouri, United States). The calli that formed (after around 4 weeks) were transferred to a MS medium supplemented with 30 g L^{-1} sucrose and 2 g L^{-1} Gelrite® and 2,4-dichlorophenoxy acetic acid (2,4-D) at various concentrations as follows; for watery callus (WC) (1 mg L^{-1} (4.5 µM)), for embryogenic callus (EC) (3 mg L^{-1} (13.5 µM)) and for dry callus (DC) (4 mg L^{-1} (18 µM)). The WC, EC and DC samples were collected after 4 weeks on the respective media (8 weeks 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 year in MS liquid medium supplemented with 3 mg L^{-1} 2,4-D according to Wong et al. (2013). After successful establishment, callus and cell suspension cultures were viewed under stereo microscopy 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 weeks after initial culturing from explant) and prolonged cell suspension culture (samples collected after 12 months in suspension culture or equivalent to 60 weeks after initial culturing from explant) were placed in regeneration media (MS0) using 10 plates with 9 calli per plate and were monitored daily during 8 weeks (Table S1). Leaves from plants regenerated from embryogenic calli were collected after 8 weeks (16 weeks 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 A_{260nm}/A_{280nm} ranging from 1.8 to 2.2, and an A_{260nm}/A_{230nm} 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.

Alignment and domain identification in *SERK*, *BBM*, *LEC2* and *WUS* of *B. rotunda*

The unigenes sequences of somatic embryogenesis and plant regeneration related gene for *SERK* (KY290870), *BBM* (KY290873), *LEC2* (KY290872) and *WUS* (KY290871) from *B. rotunda* transcriptome data (Md-Mustafa et al. 2014) were used to perform Basic Local Alignment Search Tool, BLASTx (v 2.6.0+) analysis to confirm the sequence similarity and identity with genes of the same function from other plant species using the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). Conserved domains of *B. rotunda* sequences were determined using the NCBI Conserved Domains Search Tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

qRT-PCR

Quantitative RT-PCR (qRT–PCR) was performed using gene specific primers designed from unigene sequences 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) contained 10 µl Power SYBR Green PCR Master Mix

Table 1 Primers for gene expression analysis using qRT-PCR

Genes name	Primer sequence	Product size (bp)
SERK	Forward: 5′-TGCAGAGTGGCAGAGCTACA	297
	Reverse: 5′-CCGACGCCAACATCTGAACC	
BBM	Forward: 5′-CAGGGGAGTGACAAGGCATC	234
	Reverse: 5′-TTCTTCATCGCCTCCAGCTC	
LEC2	Forward: 5′-TAAACGACGGATTCCCAGTC	250
	Reverse: 5′-AGAGAGATCTGCAGGCGTGT	
WUS	Forward: 5′-AGCAAGAAGCCCGACCAGG	127
	Reverse: 5′-CATCCCGCTGTCGAACAAAGC	
β-ACTIN	Forward: 5′- GCCTCACGCTCTTCTTTCGAT	100
	Reverse: 5′- AGCAGTGGTGGTGAATGA ATCTC	

(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. Reactions were run on a QuantStudio® 12 K 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 in three independent experiments with each primer pair. Meristematic block (MB) was used as the calibrator (value set as 1). A non-template control (NTC) was included to monitor the formation of non-specific products. The housekeeping gene, *β-ACTIN* was used as an internal control for each analysis. A comparative CT method ($2^{-\Delta\Delta CT}$ method) was used to perform relative quantification of gene expression (Livak and Schmittgen 2001).

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 and 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 (50 bp) 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 *SERK*, *BBM*, *LEC2* and *WUS* 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

Starting with dedifferentiation, somatic embryogenesis is a multi-step, highly regulated and complex process that occurs as part of natural plant development in vivo (e.g., apomixes) (Raghavan 2006), or is achievable in plant tissue

culture (Fehér 2005), allowing asexual reproduction and the regeneration of entire plants (Feher et al. 2003; Rocha et al. 2016). Several of the genes associated with the regulation of somatic embryogenesis and plant regeneration have been identified (Ikeuchi et al. 2016; Mahdavi-Darvari et al. 2015), however, the DNA methylation patterns of the regulatory genes during the process has not been well examined. In this study, expression patterns and DNA methylation status of four somatic embryogenesis and plant regeneration related genes (*SERK*, *BBM*, *LEC2* and *WUS*) were examined using in vitro derived cells, tissues and leaf samples. The samples used in this study were selected to represent meristematic tissue (MB) from shoots, embryogenic (EC) and non-embryogenic dry and watery callus (DC, WC), cells that originated from embryogenic callus but were non-regenerative after prolonged culture in suspension (CS), differentiated tissue (leaf) and regenerated tissue (in vitro leaf) of *B. rotunda* (Fig. S1 and Table S1). Callus produced on media containing 3 mg L^{-1} 2, 4-D (EC) was pale-yellowish, globular and friable showing clear signs of embryo structures (globular, translucent spheres) when observed under stereo microscope (Fig. S1), as previously reported in Ng et al. (2016). For the current study, these cells were used as materials for EC samples and for the production of cell suspensions. Non-embryogenic calli included DC (4 mg L^{-1} 2, 4-D) which was yellowish, friable, nodular and dry, and WC (1 mg L^{-1} 2, 4-D) which was spongy, soft and wet. Although the cell suspension (CS) sample was established from EC, these cells were non-embryogenic in nature when tested by placing on regeneration media for 8 weeks after maintenance for 12 months in suspension (Table S1).

Analysis of *B. rotunda SERK, BBM, LEC2* and *WUS* sequences

The gene sequences for *SERK* (KY290870), *BBM* (KY290873), *LEC2* (KY290872) and *WUS* (KY290871), obtained from our previous *B. rotunda* transcriptome data (Md-Mustafa et al. 2014), showed high similarity and identity with gene sequences annotated for the same functions in the NCBI nr database (Table S2). *BrSERK*, *BrBBM*, *BrLEC2* and *BrWUS* showed the highest similarity and identity scores with the sequences from *Musa acuminata* subsp. malaccensis followed by *Phoenix dactylifera* and *Elaeis guineensis* and had the expected conserved domains: BrSERK contains Leucine-rich repeat (LRR) and Protein kinase (PKc) domains; BrBBM contains two Apetala2 (AP2) domains; BrLEC2 contains B3 domain and BrWUS contains a homeobox domain (Fig. S2), and thus, supporting the validity of the gene sequences that were selected for this study from previous transcriptome data of *B. rotunda* reported in Md-Mustafa et al. (2014).

SERK, BBM, LEC2 and *WUS* are strongly expressed in EC of *B. rotunda*

From qRT-PCR results based on the gene specific primers, the expression levels of *SERK, BBM* and *LEC2* of *B. rotunda* were the highest in EC samples (Fig. 1a–c), while the expression level of *WUS* was slightly higher in MB than EC (Fig. 1d). Non-embryogenic and non-regenerable calli and cells i.e. DC, WC and CS (especially WC and CS) showed significantly lower levels of expression than EC samples for all four genes (Fig. 1a–d). *SERK*, *BBM*, *LEC2* and *WUS* have each been previously reported to be more highly expressed in embryogenic callus than non-embryogenic callus: for example *SERK* in *A. thaliana* (Singla et al. 2008) and *Passiflora edulis* (Rocha et al. 2016); *BBM* in *Nicotiana tabacum* (Srinivasan et al. 2007) and *T. cacao* (Florez et al. 2015); *LEC2* in *B. napus* (Malik et al. 2007) and *Z. mays* (Salvo et al. 2014) and *WUS* in *Coffea canephora* (Arroyo-Herrera et al. 2008) and *L. decidua* (Rupps et al. 2016). The strong expression of these genes may induce accumulation of embryo-specific proteins or the products of other key regulatory genes for embryo development during somatic embryogenesis and plant regeneration in *B. rotunda* as in other plant species. Thus, differences in the DNA methylation status of these genes are of interest to elucidate the regulatory processes of gene expression. While *WUS* was strongly expressed in EC, the relatively higher expression in the MB sample probably reflects the role of *WUS* in the maintenance of the shoot apical meristematic cells within this *B. rotunda* sample. Previously, *WUS* was shown to be essential for shoot apical meristem maintenance in Arabidopsis (Mayer et al. 1998), to promote vegetative-to-embryonic transition in Arabidopsis (Zuo et al. 2002) and to promote shoot regeneration in tobacco (Rashid et al. 2007), Arabidopsis (Chatfield et al. 2013; Li et al. 2011) and watermelon (Zhang et al. 2015). Similarly, the expression of *SERK* and *LEC2* was also relatively high in MB samples. The *ex vitro* leaf (EVL) and in vitro leaf (IVL) represented non-meristematic and differentiated tissue samples and showed intermediate levels of expression of all four embryogenesis related genes: the levels of expression of *SERK*, *LEC2*, *BBM* and *WUS* were significantly higher in EC than EVL and IVL, while *SERK*, *LEC2* and *WUS* expression levels were significantly lower in non-embryogenic callus/cell samples (DC, WC and CS). Although *BBM* gene expression in the non-embryogenic callus (DC and WC) samples was lower than that of EC, surprisingly, *BBM* expression was still significantly higher than that of EVL and IVL. However, *BBM* expression in the non-regenerable CS samples was significantly lower than that of EVL (and non-distinguishable from that of IVL). In Arabidopsis, *BBM* promoter activity was reported to be largely restricted to

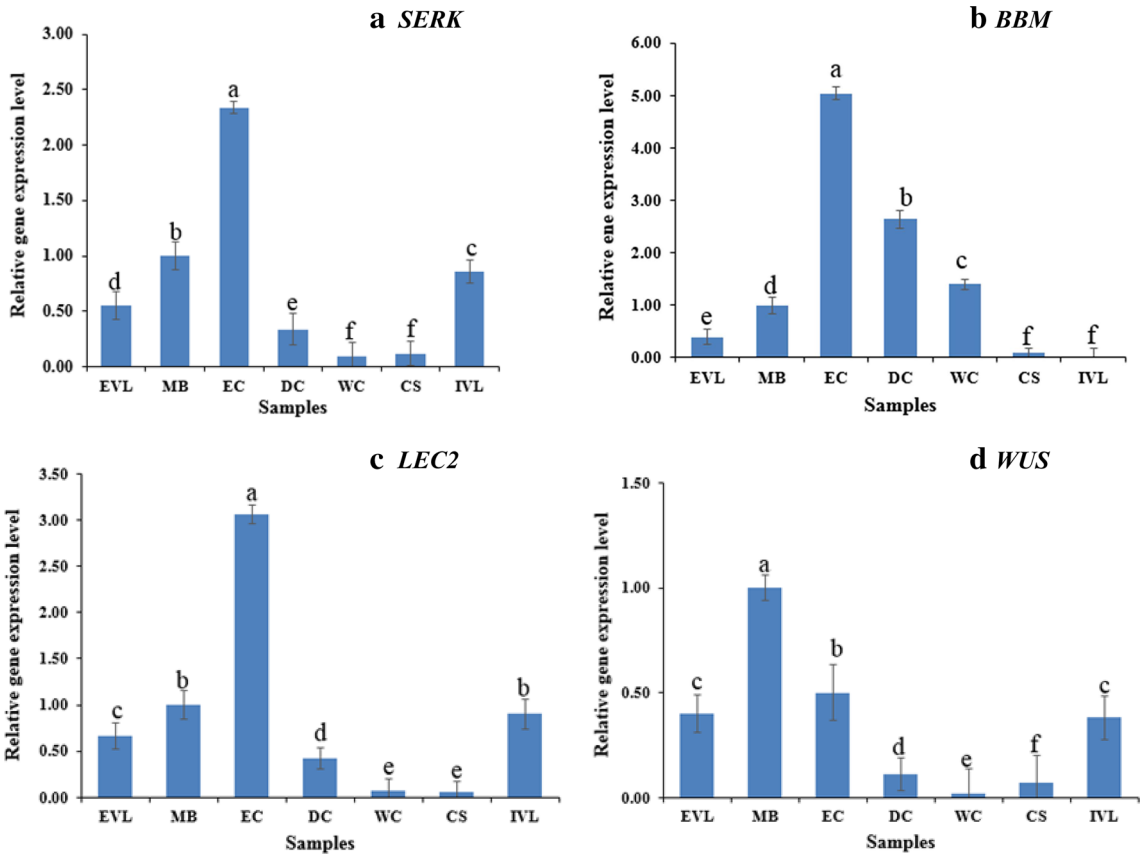

Fig. 1 Relative gene expression of somatic embryogenesis related genes in ex vitro and in vitro tissues and calli using qRT-PCR. **a** *SERK*, **b** *BBM*, **c** *LEC2* and **d** *WUS*. Relative expression levels were determined by qRT-PCR using the $2^{-\Delta\Delta CT}$ method. EVL: ex vitro leaf; *MB* meristematic block, *EC* embryogenic callus, *DC* dry callus, *WC* watery callus, *CS* cell suspension and *IVL* in vitro leaf. MB was used as the calibrator (value set as 1), and *Actin* was used as the internal control. Bars represent the standard deviation of three biological and three technical replicates. Letters indicate statistical significance, where the same letter indicates no significant difference between samples, according to Tukey's comparison test ($P > 0.05$)

dividing cells in pre-embryogenic cultures followed by expression in developing somatic embryos, shoot-like structures and callus, while there was no activity in the parts of explants not involved in morphogenesis (Kulinska-Lukaszek et al. 2012). An earlier report suggested that *BBM* may be associated with general cell proliferation and growth (Passarinho et al. 2008) as might be also found in actively dividing callus tissue and this would preclude *BBM* being a suitable marker for somatic embryogenesis in *B. rotunda*.

DNA methylation patterns of *SERK*, *LEC2*, *BBM* and *WUS* genes of *B. rotunda* and their correlation with gene expression

The expression of genes also depends on promoter or gene body methylation in plants and animals, but still its effect on gene expression and developmental regulation remain obscure (Zilberman 2017). In this study, analysis of the level of DNA methylation determined by BS-seq in each context (CG, CHG and CHH) revealed that CG methylation was higher than CHG and CHH methylation in all sample types (*SERK*, *BBM*) or a majority of the sample types (*LEC2*, *WUS*) (Fig. 2a–d). For the samples that were methylated, the average methylation ratio was much higher for *LEC2* than the other genes studied. Gene-specific DNA methylation showed wide variation of the presence of CG, CGH and CHH methylation in *SERK*, *BBM*, *LEC2* and *WUS* across the 6 sample types studied (Fig. 2). However, the levels were broadly in line with the global DNA methylation levels reported in plants i.e. a higher level of CG methylation with lower levels of CHG and CHH methylation. Most reports about DNA methylation levels in plants refer to global methylation (reviewed in Karim et al. 2016), however in the current study, we examined the DNA methylation of the *SERK*, *BBM*, *LEC2* and *WUS* coding sequences.

Correlation analysis showed a negative correlation between gene methylation and expression level of *SERK*, *LEC2* and *WUS* across the different samples (Fig. 3a–d).

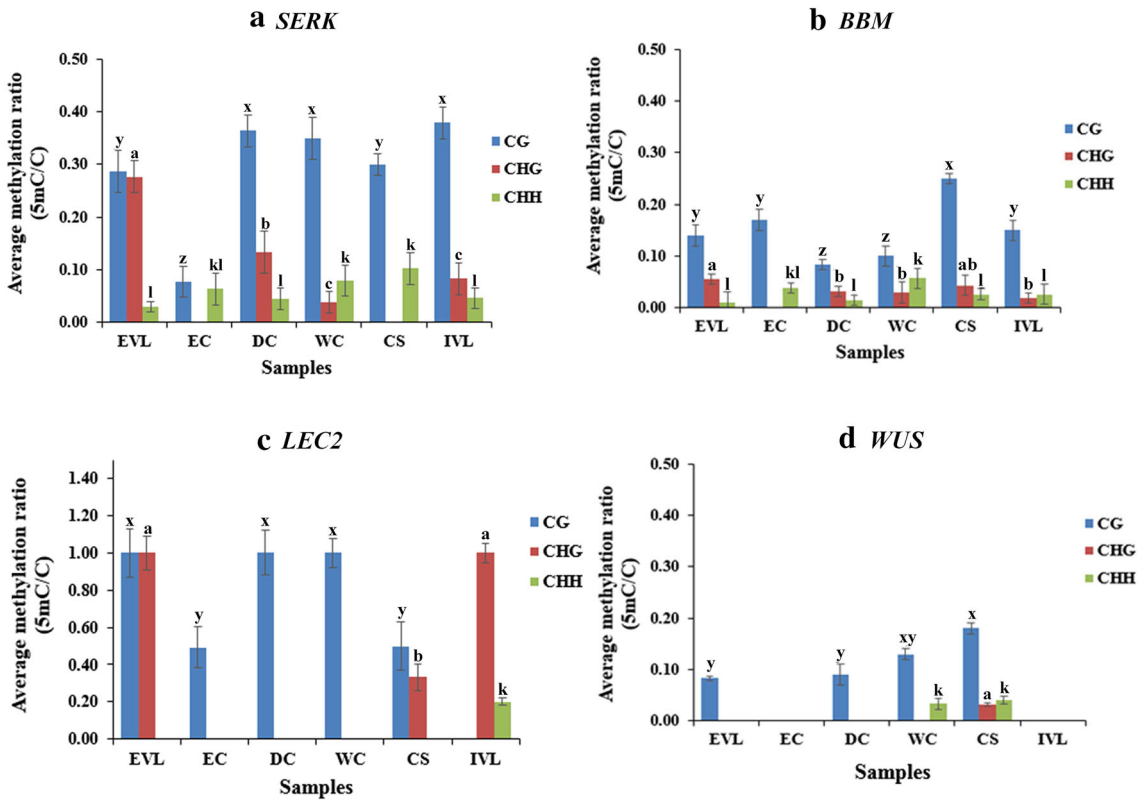

Fig. 2 DNA methylation of *SERK*, *BBM*, *LEC2* and *WUS* for *ex vitro* and in vitro calli and tissues of *B. rotunda*. **a** *SERK*, **b** *BBM*, **c** *LEC2* and **d** *WUS*. EVL: ex vitro leaf; EC: embryogenic callus; *DC* dry callus, *WC* watery callus, *CS* cell suspension and *IVL* in vitro leaf. 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 ($P > 0.05$), (*x*, *y*, *z* for CG methylation; *a*, *b*, *c* for CHG methylation; and *k, l* for CHH methylation)

However, in the case of *BBM* and *LEC2*, there was weak positive correlation of gene expression and CHH methylation (Fig. 3b, c). While authors of reports using global DNA and also histone methylation levels of plant cell cultures have hypothesized that decreased global methylation levels decrease the gene expression of embryogenesis related genes *LEC1*, *BBM1* and *WOX4* in *C. arabica* (Nic-Can et al. 2013), in contrast, increased global methylation levels coincided with decreased levels of gene expression of *WOX2*, *BBM* and *SERK1* in *P. radiata* (Bravo et al. 2017). The level of methylation of the individual genes in different conditions has not been reported. There are numerous contradictory reports suggesting that either higher [hypermethylation e.g. in *E. senticosus* (Chakrabarty et al. 2003)] or reduced [(hypomethylation e.g. in *A. thaliana* (Kwiatkowska et al. 2014)] global methylation of DNA promote somatic embryogenesis. A possible reason for the discrepancies is that DNA methylation is dynamic and might be highly sensitive to culture conditions, sampling time and also vary between species and possibly even between genotypes (Nic-Can and De la Peña 2014; Baránek et al. 2015). In addition, DNA methylation at different contexts (CG, CHG and CHH in plants) occur at different levels, with generally the highest occurrence in CG context, intermediate in CHG context and the lowest in CHH context as reported in several plant species such as in *A. thaliana* (24, 6.7 and 1.7%) (Cokus et al. 2008), *Populus trichocarpa* (41.9, 20.9 and 3.25%) (Feng et al. 2010), *Betula platyphylla* (42.64, 28.80 and 5.16%) (Su et al. 2014) and *Z. mays* (86.4, 70.9 and 1.2%) (West et al. 2014). The relatively low frequency of CHH methylation may also explain weaker correlation of methylation and gene expression for this context. If considering only CG and CHG methylation, we observed higher gene expression for *SERK*, *BBM*, *LEC*2 and *WUS* in the samples with lower DNA methylation, indicating negative correlation (Fig. 3). This is in agreement with recent reports that show relatively higher levels of global DNA methylation to be associated with decreased expression of somatic embryogenesis related genes in *P. radiata* (Bravo et al. 2017) and with loss of embryogenic potential in long term cultures of *T. cacao* (Quinga et al. 2017). Based on our data, we can suggest that the decreased level of DNA methylation in EC coinciding with enhanced expression level of somatic embryogenesis related genes permits somatic embryogenesis and regeneration, whereas

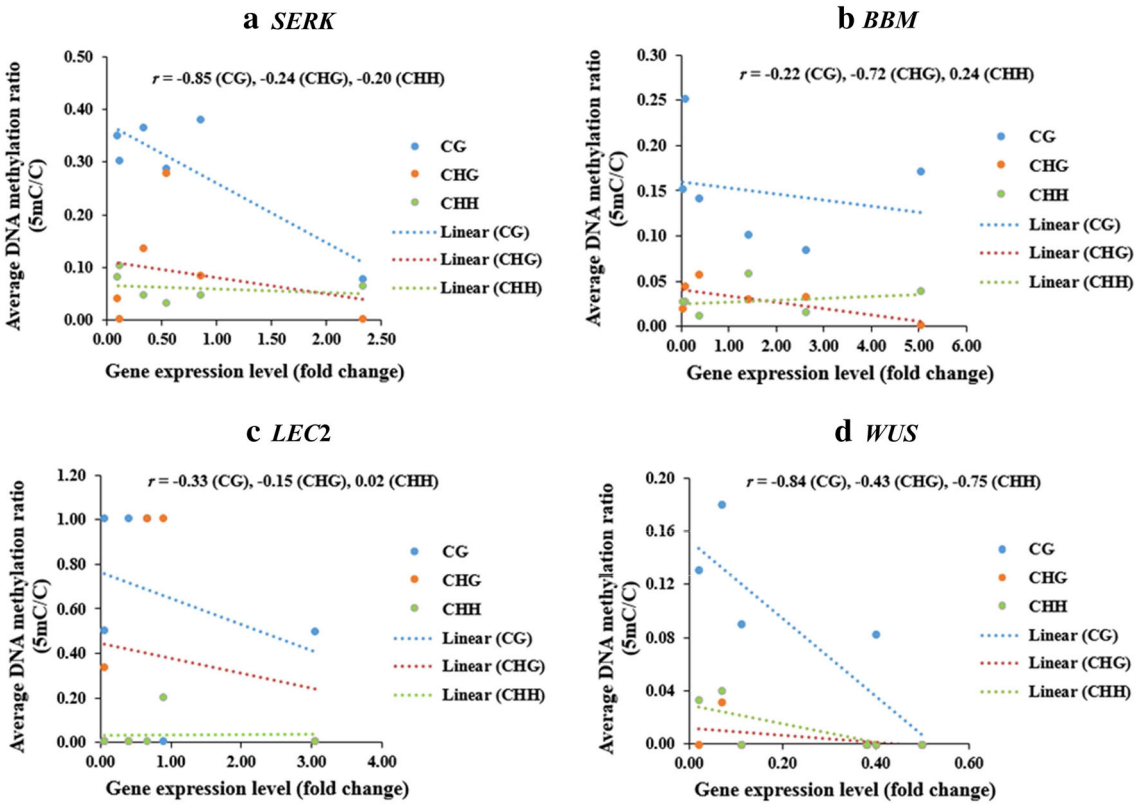

Fig. 3 Correlation between gene expression and DNA methylation status. **a** *SERK*, **b** *BBM*, **c** *LEC2* and **d** *WUS*. Analysis was performed by Pearson's correlation coefficient

the relatively increased level of DNA methylation (in DC, WC and CS) reduced the expression level of *SERK*, *BBM*, *LEC*2 and *WUS*, reducing embryogenic competency and plant regeneration in *B. rotunda*.

An emerging area of interest is the association of plant growth regulators with epigenesis, including DNA methylation, which can impact gene expression levels. Both the concentration of auxins and the level of DNA methylation, have been linked to repression of gene expression in plant cell cultures (reviewed in Yamamuro et al. 2016). The different concentrations of 2,4-D used in our study to produce WC (1 mg L^{-1}), EC (3mg L^{-1}) and DC (4 mg L^{-1}) and to maintain suspension cells (3 mg L^{-1}) thus may either independently, or via an effect on DNA methylation, effect changes in gene expression of the somatic embryogenesis related genes *SERK*, *BBM*, *LEC2* and *WUS*. In a study of EC formation in *Clitoria ternatea*, it was found that 2mg L^{-1} was optimal for SE formation while 1 mg L^{-1} or 4 mg L^{-1} produced much lower numbers of embryos (Kumar and Thomas 2012), similar to our finding with *B. rotunda*. While the EC and SC were both cultured in the presence of 3 mg L^{-1} 2,4-D, the longer exposure of SC to the auxin may well have resulted in loss of regenerability due to a build up of the hormone in the media resulting in toxicity, as suggested by Gaspar et al. (2000). However, this does not preclude an involvement of DNA methylation, as the auxin effect could be mediated via the DNA methylation changes that we also observed in those samples. Gene expression of somatic embryogenesis related gene *WUS* has been shown to be affected by concentrations of auxin and cytokinins and has also been associated with changes in epigenetic marks (Li et al. 2011, reviewed in Yamamuro et al. 2016) however, the mechanism, if any, connecting these associations remains unclear. The data obtained in the current study of the expression profiles and DNA methylation patterns of somatic embryogenesis associated genes, *SERK*, *BBM*, *LEC2* and *WUS* of *B. rotunda* show that differences in the levels of DNA methylation of these somatic embryogenesis related gene sequences are correlated with differences in gene expression and the related regenerability capacity of the callus and cell suspension samples. This information could help to design strategies to further investigate the roles of plant growth regulators and DNA methylation and their interaction towards improvement of levels of somatic embryo competence and regeneration of plants.

Conclusion

Somatic embryogenesis mediated plant regeneration during in vitro culture is a complex process involving hormone actions, transcription factors and epigenetic regulations (Yang and Zhang 2010; Yamamuro et al. 2016), and the controlling mechanisms are still unclear. In this study, we report that the expression of genes related to somatic embryogenesis and regeneration, *SERK*, *BBM*, *LEC2* and *WUS* was higher in embryogenic callus and lower in non-embryogenic calli and 12-month old suspension cells of *B. rotunda*. Lower levels of DNA methylation at the loci of *SERK*, *BBM*, *LEC2* and *WUS* in terms of CG, CHG and CHH methylation were associated with the higher expression of those genes, thus, this may promote embryogenic competence during in vitro culture of *B. rotunda*. Pearson's Correlation analyses showed that higher DNA methylation of the *SERK*, *BBM*, *LEC2* and *WUS* loci were mostly negatively correlated with the expression of these genes, especially for in vitro calli and cell suspension culture. The information provided here may also 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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