GENE EXPRESSION AND DNA METHYLATION DURING *IN VITRO* CULTURE AND PLANT REGENERATION IN *Boesenbergia rotunda* (L.) MANSF

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Field of Study: **GENETICS AND MOLECULAR BIOLOGY (BIOLOGY AND BIOCHEMISTRY)**

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ABSTRACT

In vitro culture and plant regeneration *via* somatic embryogenesis are important for crop improvement. These processes involve changes in gene expression and are also associated with changes in DNA methylation. Therefore, it is of interest to determine the changes in gene expression and in DNA methylation during *in vitro* culture and plant regeneration. *Boesenbergia rotunda*, an important medicinal plant in South-East Asia, India and Southern China, was chosen as a model system for this study in which the expression patterns of three DNA methylation pathway genes, *METHYLTRANSFERASE 1* (*MET1*), *CHROMOMETHYLASE 3* (*CMT3*) and *DOMAIN REARRANGED METHYLTRANSFERASE 2* (*DRM2*), and four somatic embryogenesis related genes, *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* (*SERK*), *BABY BOOM* (*BBM*), *LEAFY COTYLEDON 2* (*LEC2*) and *WUSCHEL* (*WUS*) were examined using qRT-PCR and RNA-seq in various cell samples, representing different stages of the regeneration process, from explant through somatic embryogenesis and regeneration. DNA methylation patterns and levels were determined by Methylation Sensitive Amplified Polymorphism (MSAP) and Bisulfite sequencing (BS-seq). Gene and amino acid sequences of *MET1, CMT3, DRM2, SERK, BBM, LEC2* and *WUS* from *B. rotunda* showed high similarity and conserved domains with genes of the same function from other plants in the Genbank database. Phylogenetic analysis indicated that *B. rotunda* deduced protein sequences were evolutionarily most related to those from *Musa acuminata*. There was higher expression of methyltransferase genes *MET1*, *CMT3* and *DRM2*, and hypomethylation in embryogenic calli, which may be associated with successful somatic embryogenesis and regeneration. 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 twelve-month old suspension

cells. 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 *MET1*, *CMT3*, *DRM2*, *SERK*, *BBM*, *LEC2* and *WUS* loci was mostly negatively correlated with the expression of those genes, especially for *in vitro* calli and cell suspension culture. These findings in this study may form a foundation for future research to optimize genetic and epigenetic control of plant somatic embryogenesis and regeneration during *in vitro* culture.

ABSTRAK

Kultur *in vitro* dan regenerasi semula tumbuhan melalui embriogenesis somatik adalah penting untuk penambahbaikan tanaman. Proses-proses ini melibatkan perubahan dalam gen dan juga dikaitkan dengan perubahan dalam metilasi DNA. Oleh itu, adalah menarik untuk menentukan perubahan dalam ekspresi gen dan DNA metilasi semasa kultur *in vitro* dan regenerasi semula tumbuhan. *Boesebergia rotunda*, tumbuhan yang digunakan dalam perubatandan penting di Asia Tenggara, India dan Selatan China, telah dipilih sebagai sistem model untuk kajian ini di mana corak ungkapan tiga DNA gen metilasi laluan, *METHYLTRANSFERASE 1* (*MET1*), *CHROMOMETHYLASE 3* (*CMT3*) dan *DOMAIN REARRANGED METHYLTRANSFERASE 2* (*DRM2*)), dan empat gen yang berkaitan dengan embriogenesis somatik (*SOMATIK EMBRIOGENESIS RESEPTOR-LIKE KINASE* (*SERK*), *BABY BOOM* (*BBM*), *LEAFY COTYLEDON 2* (*LEC2*) dan *WUSCHEL* (*WUS*)) telah diperiksa menggunakan qRT-PCR dan RNA-seq dalam pelbagai sampel sel, yang mewakili peringkat proses regenerasi semula, daripada eksplan melalui embriogenesis somatik dan regenerasi semula. DNA corak metilasi dan tahap ditentukan oleh Methylation Sensitive Amplified Polymorphism (MSAP) dan Bisulfite sequencing (BS-seq). Gene dan asid amino urutan MET1, CMT3, DRM2, SERK, BBM, LEC2 dan WUS dari *B. rotunda* menunjukkan persamaan tinggi dan domain dipulihara dengan gen fungsi yang sama dari tumbuh-tumbuhan lain di dalam pangkalan data GenBank itu. Analisis filogenetik menunjukkan bahawa evolusi *B. rotunda* mempunyai simpulan urutan protein paling hampir dengan *Musa acuminata*. Terdapat ungkapan yang lebih tinggi gen Methyltransferase *MET1, CMT3* dan *DRM2* dan hypomethylation dalam kalus embriogenik, yang boleh dikaitkan dengan embriogenesis somatik berjaya dan regenerasi semula. Ungkapan gen yang berkaitan dengan embriogenesis somatik dan regenerasi semula, *SERK, BBM, LEC2* dan *WUS*, adalah lebih tinggi pada kalus embriogenik dan lebih rendah dalam kalus bukan embriogenik dan dua belas bulan sel

penggantungan lama. Tahap yang lebih rendah metilasi DNA di loci of S*ERK*, *BBM*, *LEC2* dan *WUS* segi CG, CHG dan CHH metilasi dikaitkan dengan ungkapan yang lebih tinggi dari orang-orang gen, dan ini boleh menggalakkan kecekapan embriogenik semasa *in vitro* budaya *B. rotunda*. Korelasi Pearson analisis menunjukkan bahawa metilasi DNA yang lebih tinggi daripada *MET1*, *CMT3*, *DRM2*, *SERK*, *BBM*, *LEC2* dan *WUS* lokus kebanyakannya negatif dikaitkan dengan ungkapan gen, terutama *in vitro* kalus dan budaya penggantungan sel. Penemuan ini boleh membentuk asas untuk penyelidikan masa depan untuk mengoptimumkan kawalan genetik dan epigenetik loji embriogenesis somatik dan regenerasi semula kultur *in vitro*.

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LIST OF SYMBOLS AND ABBREVIATIONS

α	:	Alpha
µg	:	Microgram
µl	:	Microlitre
µM	:	Micromolar
µmol	:	Micromole
°C	:	Degree Celsius
%	:	Percentage
2,4-D	:	2,4-Dichlorophenoxyacetic acid
5mC	:	5-methylcytosine
+ve	:	Positive
-ve	:	Negative
AFLP	:	Amplified Fragment Length Polymorphism
ANOVA	:	Analysis of Variance
B. rotunda	:	*Boesenbergia rotunda*
BA	:	Benzyl adenine
BAP	:	6 – Benzylaminopurine
BBM	:	*BABY BOOM*
BLAST	:	Basic Local Alignment Search Tool
bp	:	Base pair
BS-seq	:	Bisulfite sequencing
cDNA	:	Complementary DNA
cm	:	Centimetre
CMT3	:	*CHOMOMETHYLASE 3*
CS	:	Cell suspension

CTAB	:	Cetyltrimethylammonium bromide
DC	:	Dry callus
ddH_2O	:	Double distilled water
DNA	:	Deoxyribonucleic acid
DNase	:	Deoxyribonuclease
dNTP	:	Deoxynucleotriphosphate
DRM2	:	*DOMAIN REARRANGED METHYLTRANSFERASE 2*
dsRNA	:	Double-stranded RNA
EC	:	Embryogenic callus
EDTA	:	Ethylene diamine tetra acetic acid
EVL	:	*Ex vitro* leaf
et al.	:	*Et alia*
EtOH	:	Ethanol
EtBr	:	Ethidium bromide
g	:	Gram
gL^{-1}	:	Gram per litre
HCl	:	Hydrochloride acid
IAA	:	Indole-3-acetic acid
IVL	:	*In vitro* Leaf
Kb	:	Kilo base
L	:	Litre
LEC2	:	*LEAFY COTYLEDON 2*
m	:	Metre
M	:	Molar
MB	:	Meristematic block
MEGA	:	Molecular Evolutionary Genetics Analysis

MET1	:	*METHYLTRANSFERASE 1*
mg	:	Milligram
mgL^{-1}	:	Milligram per litre
min	:	Minute
ml	:	Millilitre
mM	:	Millimolar
mRNA	:	Messenger RNA
MS	:	Murashige and Skoog
MSAP	:	Methylation Sensitive Amplified Polymorphism
NAA	:	α-napthaleneacetic acid
NaCl	:	Sodium chloride
NCBI	:	National Centre for Biotechnology Information
ng	:	Nanogram
nm	:	Nanometre
nt	:	Nucleotide
NTC	:	Non template control
OD	:	Optical density
P	:	Probability
PCR	:	Polymerase chain reaction
PGR	:	Plant growth regulator
pmol	:	Picomole
pol	:	Polymerase
PVP	:	Polyvenyl Pyrolidone
qRT-PCR	:	Quantitative Reverse Transcription PCR
RNA	:	Ribonucleic acid
RdDM	:	RNA-directed DNA methylation

RNase	:	Ribonuclease
rRNA	:	Ribosomal RNA
RIN	:	RNA integrity number
RNA-seq	:	RNA sequencing
rpm	:	Rotation per minute
s	:	Second
SDS	:	Sodium dodecyl sulfate
SE	:	Standard error
SERK	:	*SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE*
siRNA	:	Small interfering RNA
sp.	:	Species (singular)
spp.	:	Species (plural)
TBE	:	Tris boric acid EDTA
TE	:	Tris EDTA
Tm	:	Melting temperature
TPM	:	Transcript per million
TrisHCl	:	Tris hydrochloric acid
U	:	Unit
V	:	Volt
v	:	Version
WC	:	Watery callus
WUS	:	*WUSCHEL*
w/v	:	Weight per volume

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 *Boesenbergia rotunda* (L.) Mansf.

Boesenbergia rotunda (L.) Mansf., an important ethnomedicinal plant belonging to the ginger family (Zingiberaceae), is widely found in South-East Asia, India, and Southern China (Baker, 1892), and is known as "Chinese key" or "Finger Root" in English, "Temu Kunchi" in Malay, "Krachai" or "Krachai-Drag" in Thai. With the passage of time, the plant has been identified by different botanical names such as *Kaempferia cochinchinensis (*Gagnep.), *Kaempferia ovate* (Roscoe.), *Kaempferia pandurata (*Roxb.), *Curcuma rotunda* (L.), *Gastrochilus panduratus* (Roxb.) Ridl., *Gastrochilus rotundus* (L.) Alston, *Boesenbergia cochinchinensis* (Gagnep.) Loes., *Boesenbergia pandurata* (Roxb.) Schltr.; however, presently, it is known as *Boesenbergia rotunda* (L.) Mansf. (Gardens & Kew, 2010). Previously, this species was mistakenly assigned under the *Kaempferia* genus by Baker (1892), but a dendrogram obtained by unweighed pair group method algorithm (UPGMA) analysis of isozymes electrophoresis data of leaf extracts from eleven taxa of *Boesenbergia*, six taxa of *Kaempferia* and two taxa of *Scaphochlamys* from Southern Thailand, showed a low degree of similarity between *Boesenbergia* and *Kaempferia* than between *Boesenbergia* and *Scaphochlamys* (Vanijajiva et al., 2003).

Taxonomic position:

Class: Equisetopsida

Subclass: Magnolidiae

Superorder: Lilianae

Order: Zingiberales

Family: Zingiberaceae

Genus: *Boesenbergia*

Species: *Boesenbergia rotunda* (L.) Mansf.

(Source:http://www.kew.org/science-conservation/plants-fungi/boesenbergia-rotunda-fingerroot)

Boesenbergia rotunda (L.) Mansf. is a small perennial and culinary herb, with bright yellow finger-shaped rhizome, light green foliage, and generally grows to a height of 30-40 cm (Figure 1.1C). The aerial part of *B. rotunda* consists of stems, leaves and flowers, while the underground part consists of rhizome and tubers. The leaves of the plant are about 7-9 cm broad and 10-20 cm long. The flower is pink in colour with a prominent lip in darker shade pink, very delicate and short-lived, and usually blooms one at a time. The whole plant resembles the *Curcuma* species except for the inflorescence which is covered by the leaf sheath and has distichous bracts. The underground rhizome of the plant is globular in shape from which several slenders and long tubers sprout in the same direction like hand fingers. Therefore, it is called commonly finger root in English (Figure 1.1A, B).

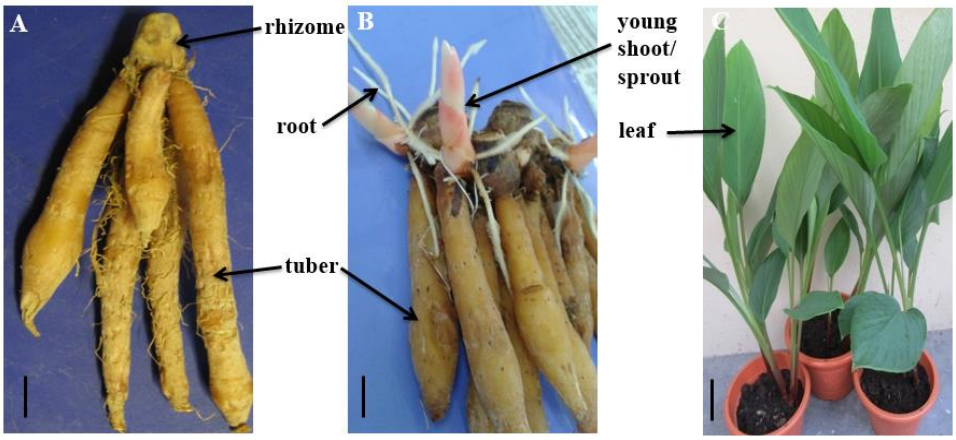


Figure 1.1: Morphology of *Boesenbergia rotunda*. A: rhizome (bar=1.0 cm); B: sprouting (bar=1.0 cm); C: whole plant (bar=10.0 cm)

In South-East Asia, the *B. rotunda* rhizome is commonly used either as a food ingredient with a pungent taste or in folk medicine. For instance, the rhizomes and roots are traditionally used to treat digestive disorders including dyspepsia, stomach discomfort, and mouth ulcers and rheumatism. It is used in the respiratory system including dry mouth and dry cough, in the reproduction system including leucorrhea, as an aphrodisiac to stimulate sexual desire. The tubers are widely used for wounds, swelling and tumors locally (Burkill, 1966; Hasnah et al., 1995; Trakoontivakorn et al., 2001; Chuakul & Boonpleng, 2003; Mahady, 2005).

Boesenbergia rotunda has pharmacological significance due to its flavonoids, flavones, essential oils and chalcones (Jaipetch et al., 1982; Trakoontivakorn et al., 2001; Tan, 2005; Kirana et al., 2007; Jing et al., 2010; Yusuf et al., 2013; Tan et al., 2015). For instance, several flavonoids were isolated from the yellow rhizome of *B. rotunda* such as boesenbergin A, panduratin A, alpinetin, boesenbergin B, cardamonin, methoxychalcone, pinostrobin, and pinocembrin. Flavonoids detected in this plant showed appreciable inhibition of Human Immunodeficiency Virus (HIV) protease (Tewtrakul et al., 2003), inhibition of Dengue-2 (DEN-2) virus NS3 protease (Kiat et al., 2006), inhibition of the

growth of MCF-7 human breast cancer and HT-29 human colon adenocarcinoma cell (Kirana et al., 2007), anti-aging activity (Shim et al., 2009), and potential antibacterial, antiviral antifungal, anti-inflammatory activities (Rukayadi et al., 2010; Wu et al., 2011).

1.2 *In vitro* culture of *B. rotunda*

Plant tissue culture offers the possibility to rapidly multiply superior genotypes with desirable characteristics such as high yield, improved content of valuable secondary metabolites, disease resistance of agronomically and commercially important crops. The ability of plant regeneration from cultured cells and tissues *via in vitro* callus and cell cultures is important for industry, agriculture and also for biodiversity conservation (Chandra et al., 2010; Deo et al., 2011; Bhojwani, 2012; De Filippis, 2014; Anis & Ahmad, 2016). Successful establishment of callus and cell suspension cultures of *B. rotunda* were performed by Tan et al. (2005), Yusuf et al. (2011) and Wong et al. (2013). However, after maintaining long-term suspension cultures (12 months) of *B. rotunda*, the cells become recalcitrant to embryogenesis and plant regeneration (Wong et al., 2013).

Somatic embryogenesis is a preferable technique for genetic improvement of plants through *in vitro* culture and genetic transformation as well, because plant regeneration from cells *via* somatic embryogenesis offers the only path for single cell based genetic transformation that shows the least degree of chimerism than direct organogenesis (Guo & Zhang, 2005; Ghosh et al., 2009; Obembe et al., 2011). In several studies, it has been reported that *in vitro* regeneration through somatic embryogenesis is a useful tool for crop improvement (Ratanasanobon & Seaton, 2010; Sivanesan et al., 2011; Baskaran & Van Staden, 2012; Cardoso et al., 2012; Nuño-Ayala et al., 2012; Chiancone & Germanà, 2013; Ozudogru & Lambardi, 2016). Plant regeneration from *B. rotunda* callus cultures through somatic embryogenesis (Tan et al., 2005; Yusuf et al., 2011) has been reported. Plant regeneration from embryogenic cell suspension culture of

B. rotunda via somatic embryogenesis was also demonstrated without losing of ability to yield secondary metabolites (Wong et al., 2013). Furthermore, callus and cell suspension cultures of *B. rotunda* were also explored as alternative sources of alpinetin, cardamonin, pinocembrin, pinostrobin and panduratin A although the production of these compounds were significantly lower than those from rhizome of *B. rotunda* (Yusuf et al., 2013). However, since long term suspension cultures (12 months) were found to be loss of embryogenic and regeneration ability, thus, this form can be a useful system for the study of the cellular and molecular changes between embryogenic-regenerable and non-embryogenic cell types of *B. rotunda*. In addition, *in vitro* cultures of *B. rotunda* can provide a useful model for the study of gene expression in relation with epigenetic modifications during somatic embryogenesis and plant regeneration.

1.3 DNA methylation during plant *in vitro* cultures

Different types of genetic changes like point mutations, new insertions of transposable elements and changes of chromosome number were observed in *in vitro* regenerated plants (Alzohairy et al., 2012; Kwasniewska et al., 2012). Other than genetic changes, epigenetic modifications during *in vitro* cultures were also widely reported (Valledor et al., 2007; Miguel & Marum, 2011; Smulders & De Klerk, 2011; Us-Camas et al., 2014; Ikeuchi et al., 2015; Mahdavi-Darvari et al., 2015; Ikeuchi et al., 2016). Epigenetics is the modification of DNA (DNA methylation) or associated proteins (histone modifications) without DNA sequence variation, which carry information content during cell division (Zhong et al., 2013; Stelpflug et al., 2014). The role of epigenetic marks has often been demonstrated and is highly important in the phenomenon of somaclonal variation (Miguel & Marum, 2011; Kooke et al., 2015; Ong-Abdullah et al., 2015). It also affects gene expression by changing the chromatin conformation. Among the different types of epigenetic modifications, DNA methylation is well

described. DNA cytosine methylation is found in most eukaryotic organisms, including plants, animals and fungi (Chan et al., 2005; Freitag & Selker, 2005; Goll & Bestor, 2005; Klose & Bird, 2006; Cokus et al., 2008; West et al., 2014; Wang et al., 2015). Although DNA methylation is predominantly found in a CG sequence context (CG-island) in animals, in plants, it is found in the CG, CHG and CHH sequence contexts, where H denotes any nucleotide other than G. In plants, the methylation in the symmetrical CG and CHG sequence context is maintained by the enzymes METHYLTRANSFERASE 1 (encoded by *MET1*) and CHROMOMETHYLASE 3 (encoded by *CMT3*), respectively, and the methylation in the asymmetric CHH sequence is maintained by CMT3 partially and *de novo* methylation that is catalyzed by DOMAIN REARRANGED METHYLTRANSFERASE 2 (encoded by *DRM2*) (shown in Figure 1.2). DRM2 also mediates *de novo* methylation at all three contexts. The activity of DRM2 is highly regulated by the RNA-directed DNA methylation (RdDM) pathway (Cao et al., 2003; Pontes et al., 2006; Matzke & Mosher, 2014; Zhai et al., 2015; Lewsey et al., 2016).

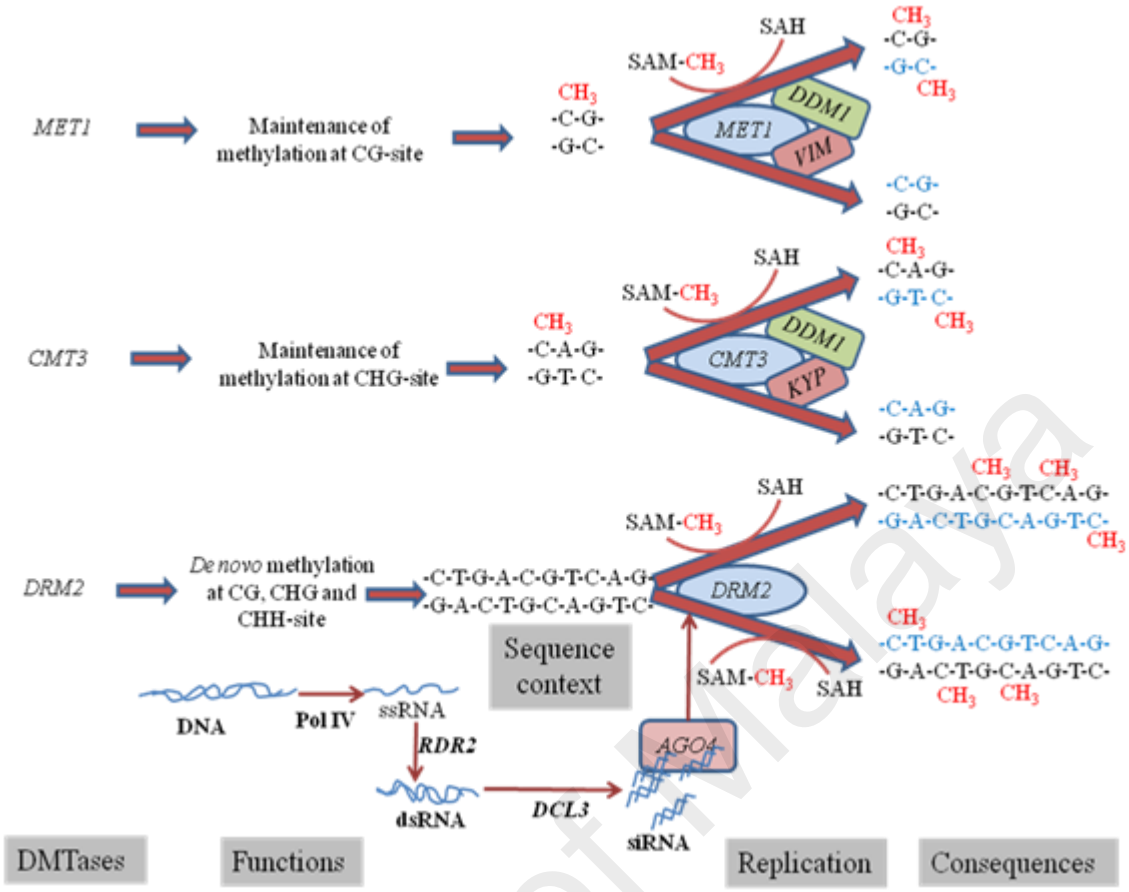


Figure 1.2: Characteristics of DNA methyltransferase genes and their functions in CG, CHG and CHH sequence where H denotes any nucleotide other than G) in plants. During replication, synthesis of new DNA strand are marked by blue colour and maintenance of methylation as well as *de novo* methylation are shown by red colour methyl group. *MET1- Methyltransferase 1, CMT3- Chromomethylase 3, DRM2- Domain rearranged methyltransferase 2, SAM- S-adenocyl methionine, SAH- S-adenocyl homocysteine, DDM1-Decrease in DNA methylation 1, VIM- Variant in methylation, KYP-Kryptonite, CMT2- Chromomethylase 2, PoIV- Polymerase IV, DCL3- Dicer like protein 3,* siRNA-small interfering RNA, *AGO4-Argonaute 4.*

In the process of somatic embryogenesis, somatic cells are induced to form totipotent embryogenic cells which are capable of regenerating into complete new plants. A series of molecular events are involved in such a developmental change stimulated by internal signals and external stimuli (Chugh & Khurana, 2002; Elhiti et al., 2013; De-la-Peña et al., 2015; Ikeuchi et al., 2015; Mahdavi-Darvari et al., 2015; Ikeuchi et al., 2016).

DNA methylation is one of the molecular changes that play key role in somatic embryogenesis and plant regeneration. DNA methylation has been found to strongly participate in dedifferentiation and redifferentiation of plant cells. In many studies, it has been observed that both DNA hypermethylation (Fraga et al., 2012; Kwiatkowska et al., 2014; Nic-Can & De la Peña, 2014) and DNA hypomethylation (Chakrabarty et al., 2003; Xu et al., 2004; Viejo et al., 2010) are associated with the somatic embryogenesis and regeneration processes. DNA methylation plays an important role for silencing of transposable elements and for regulation of endogenous genes. Alteration of methylation status of TE has been associated with creation of somaclonal variation during *in vitro* cultures (Ong-Abdullah et al., 2015). Besides transposon silencing, DNA methylation also affects plant gene regulation and development (Stroud et al., 2013a; Centomani et al., 2015). Loss of DNA methylation at promoters was associated with altered expression of particular genes (Stroud et al., 2013a).

1.4 Problem statement and research questions

It has been reported that altered patterns of DNA methylation are associated with somatic embryogenesis and plant regeneration from *in vitro* callus and cell suspension cultures, resulting in changes in expression or silencing of key genes and transposable elements. Altered DNA methylation can therefore produce epigenetically controlled phenotypes that can be meiotically or mitotically heritable and may be associated with loss of totipotency in tissue cultured plant materials. Based on previous reports, it seems that the expression levels of some important embryogenesis related genes and their DNA methylation levels in different cells and tissues is likely to contribute to their capacity to form somatic embryos and to regenerate tissue and organs; however this has not been

well studied. The current study aims to address the following research questions, using *B. rotunda* as a model plant system:

1. What are the differences in expression of DNA methylation pathway genes as well as somatic embryogenesis related genes between plant cells and tissues at different stages of *in vitro* callus, cell suspension culture and regeneration?
2. Is there any correlation between DNA methylation patterns in different cells and tissues and the capacity of those cells and tissue to undergo somatic embryogenesis or to be recalcitrant to embryogenesis and regeneration in *B rotunda*?
3. Do levels of gene expression correspond with levels of DNA methylation of these genes in the different samples used in the study?

1.5 Scope of the research and objectives

From the beginning of plant cell and tissue culture, scientists have been trying to recognize the underlying mechanisms of various morphological, physiological, biochemical and molecular changes associated with dedifferentiation, proliferation, and subsequently regeneration during *in vitro* cultures. The mechanism for these is still not completely clear, but it is likely to result from various genetic and epigenetic causes. Therefore, it would be helpful to use gene expression and DNA methylation profile during *in vitro* culture and plant regeneration to design strategies aiming to high production of elite planting materials and important secondary metabolites with pharmaceutical significance, to develop disease resistance variety, and also for biodiversity conservation of agronomically and commercially important crops.

Considering all the points, the present study was undertaken with the following **objectives**:

1. To determine the differences in expression of the DNA methylation pathway genes, *MET1*, *CMT3* and *DRM2* among *ex vitro* and *in vitro* tissues, embryogenic and non-embryogenic calli, and prolonged cell suspension culture of *B. rotunda*.
2. To determine the differences in expression of the somatic embryogenesis and plant regeneration related genes, *SERK*, *BBM*, *LEC2* and *WUS* among *ex vitro* and *in vitro* tissues, embryogenic and non-embryogenic calli, and prolonged cell suspension culture of *B. rotunda*.
3. To determine the changes in DNA methylation patterns among *ex vitro* and *in vitro* tissues, embryogenic and non-embryogenic calli, and prolonged cell suspension culture of *B. rotunda*.
4. To determine the relationship between the gene expression and DNA methylation status.

CHAPTER 2: LITERATURE REVIEW

2.1 *Boesenbergia rotunda*

Boesenbergia is a genus of the Zingiberaceae family and consists of approximately 80 species worldwide (Saensouk & Larsen, 2001). Among these, *Boesenbergia rotunda* is the most widely and commercially cultivated and its rhizomes have been used traditionally in folk medicine such as for treatment of colic disorder and as an aphrodisiac (Trakoontivakorn et al., 2001), and for culinary purposes. Several studies have demonstrated that this plant contains important metabolites including cyclohexenyl chalcone derivatives, flavones, and flavonoids such as 4-hydroxypanduratin A and panduratin A, which showed anti-inflammatory activity (Tuchinda et al., 2002); pinocembrin, pinostrobin, and cardamonin, which showed anti-HIV activity (Tewtrakul et al., 2003); isopanduratin A, which showed antimicrobial activity against the oral bacteria *Streptococcus mutans* (Hwang et al., 2004) and the spoilage bacteria *Lactobacillus plantarum* (Pattaratanawadee et al., 2006). Kiat et al. (2006) found that panduratin A and 4′-hydroxypanduratin A extracted from *B. rotunda* inhibited dengue 2 virus NS3 protease and hence is a prospective leading compound for drug design against dengue virus that is a widespread cause of disease in tropical and subtropical regions. Moreover, panduratin was also shown to have antiviral and antibacterial activity (Rukayadi et al., 2010; Wu et al., 2011). It was also shown that methanolic extract of *B. rotunda* has potential antibacterial activity against *Escherichia coli* (Lau et al., 2013).

Due to ethnomedicinal, pharmaceutical and industrial significance, in recent years, a large number of studies related to biotechnology, biochemistry and molecular biology were conducted on *B. rotunda*. For example, plantlet regeneration *via* somatic embryogenesis from callus cultures has been demonstrated (Tan et al., 2005; Wong et al., 2013). Through RNA-seq and differentially expressed genes (DEG) analysis, gene

regulation patterns in the panduratin A biosynthetic pathway was analyzed in particular with respect to the flavonoid pathway in *B. rotunda* suspension culture (Md-Mustafa et al., 2014). Expression profiles of flavonoid-related gene, 4 coumarate: coenzyme A ligase (*4CL*) and total flavonoid contents (pinostrobin, pinocembrin and panduratin A) were studied in leaves, rhizomes, and roots of *B. rotunda*, and it was found that although the highest expression of *4CL* transcript was found in roots, the highest total flavonoid contents were recorded in rhizome followed by root and leaf samples (Ata et al., 2015). The distribution patterns of flavonoids and cyclohexenyl chalcone derivatives were determined in rootlet, rhizome, shoot base, maroon stem, stalk, and leaf of *B. rotunda* using High Performance Liquid Chromatography (HPLC), and it was found that non-aerial organs had relatively higher levels of flavonoids than the aerial ones, and the most abundant flavonoid and cyclohexenyl chalcone derivative were pinostrobin and 4-hydroxypanduratin A, respectively (Tan et al., 2015). The distribution and abundance of the pharmaceutically significant flavonoids suggested that the shoot base of *B. rotunda* could be more beneficial for medicinal usages or application than other parts of the plant, and may be the location of storage or occurrence of biosynthetic enzymatic activities (Tan et al., 2015). Amino acid and secondary metabolite production in embryogenic (i.e. EC) and non-embryogenic calli (i.e. dry callus (DC), watery callus (WC)) and in shoot base of *B. rotunda* were also examined, and it was observed relatively higher levels of glutamine, arginine and lysine in embryogenic callus than in dry and watery calli, while shoot base tissue showed an intermediate level of primary metabolites. For the five secondary metabolites analyzed (i.e. panduratin, pinocembrin, pinostrobin, cardamonin and alpinetin), shoot base had the highest concentrations, followed by watery, dry and embryogenic calli (Ng et al., 2016). These studies could be helpful to get better understanding about higher production of elite planting materials and important secondary metabolites from *B. rotunda*.

2.2 Plant *in vitro* culture

In vitro plant tissue and cell culture systems are the focus of a considerable body of basic and applied research in agriculture and industry addressing large-scale micropropagation and production of superior planting materials with improved agronomic traits, higher production of important secondary metabolites (Neelakandan & Wang, 2012; De Filippis, 2014; Babu et al., 2015; Butt et al., 2015; Anis & Ahmad, 2016; Yue et al., 2016; Gupta et al., 2017). Compared to conventional crop plant propagation methods, *in vitro* methods offer a number of advantages including reduced exposure to adverse climates, pests and diseases and the capacity for high production in a small land area (Bhojwani, 2012; Hussain et al., 2012; Dunwell, 2013). Plant regeneration or cellular totipotency is a well-known characteristic which has long been exploited for plant selection, for multiplication of superior planting materials of agronomically important crops, and also for creation of new genotypes with desired characteristics (García-Gonzáles et al., 2010; Bhojwani, 2012; De Filippis, 2014; Anis & Ahmad, 2016). However, this desirable frequency is not achievable in the plant species or plant genotypes that are not amenable to culture, which has limited the application of *in vitro* cultures commercially (Withers & Alderson, 2013; Hervé et al., 2016). Due to the high relevance of plant tissue culture to agricultural production, plant breeding programs, the large scale multiplication of superior genotypes and the genetic manipulation of plants, intensive research has been conducted to overcome some of the drawbacks of these techniques (Jamsheed et al., 2013; De Filippis, 2014).

Although *in vitro* culture techniques for many plant species have been established, the techniques do not guarantee the expected outcome because of the occurrences of unexpected and potentially deleterious changes during these processes that impact negatively on crop productivity (Bairu et al., 2011; Withers & Alderson, 2013;

Cristofolini et al., 2014; Hervé et al., 2016). Moreover, some valuable species and genotypes are recalcitrant to regeneration *in vitro*, which can limit the availability of elite cultivars to industry, agriculture and medicine (Corley & Tinker, 2003; Hnatuszko-Konka et al., 2014; Sakhanokho & Rajasekaran, 2016). The molecular basis of somatic embryogenesis and plant regeneration from callus and cell suspension culture is still elusive. Besides genetic variation, epigenetic modifications such as DNA methylation affects the dedifferentiation, re-differentiation and morphogenesis during *in vitro* culture (González et al., 2011; Vining et al., 2013; Us-Camas et al., 2014; Ikeuchi et al., 2015; Mahdavi-Darvari et al., 2015; Ikeuchi et al., 2016). Therefore, better understanding concerning the cellular and molecular mechanisms that underlie the major *in vitro* plant regeneration pathways, such as *via* somatic embryogenesis may be helpful to design strategies to overcome the limitations of producing somatic embryos of agronomically and commercially important crops.

2.3 Somatic embryogenesis and plant regeneration

Somatic embryogenesis is a developmental pathway that includes cell dedifferentiation, cell division, and reprogramming of their metabolism, physiology and gene expression (Yang & Zhang, 2010; Elhiti et al., 2013; Fehér, 2015). Successful somatic embryogenesis and following plant regeneration involves various morphological, biochemical and molecular changes (Schmidt et al., 1997; Komamine et al., 2005; Jafari et al., 2015; Elhiti & Stasolla, 2016; Trontin et al., 2016). Hence, formation of somatic embryo is an important event in *in vitro* culture. Somatic embryogenesis is the most apparent reflection of totipotency in plant cells, representing that plant somatic cells contain the required genetic blueprint to complete the developmental processes to produce a new plant, and embryogenesis is not only the event of zygotic mechanism, it can occur in absence of fertilization in plants (Fehér, 2015). This system may be used to

overcome the problems facing during embryo development of zygotic embryogenesis (Smertenko & Bozhkov, 2014).

Inducible somatic embryogenesis was first demonstrated in *Daucus carota* (Steward et al., 1958), which was resulted from exposure to the synthetic auxin 2,4-D. Since the first observations of formation of somatic embryos in carrot cell suspension cultures, the potential for somatic embryogenesis has been illustrated to be characteristic of plant tissue culture systems in many other plants such as *Glycine max* (Li et al., 1985), *Coffea arabica* (Van Boxtel & Berthouly, 1996), *Gossypium hirsutum* (Zeng et al., 2006), *Capsicum annum* (Heidmann et al., 2011), *Ananas comosus* (Ma et al., 2012), *Theobroma cacao* (Florez et al., 2015), *Allium cepa* (Sivanesan et al., 2015; Wu et al., 2015); *Citrullus lanatus* (Vinoth & Ravindhran, 2016), *Pinus* spp. (Lelu-Walter et al., 2016) and *Trifolium nigrescens* (Pilarska et al., 2016), and have been propagated *via* somatic embryogenesis. However, many plant species are recalcitrant to this process. Therefore, it is of great interest to unravel the underlying regulatory mechanisms of somatic embryogenesis and plant regeneration during *in vitro* culture. In several reports, it has been identified that some genes such as *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* (*SERK*) (Ikeda et al., 2006; Savona et al., 2012; Talapatra et al., 2014; Ahmadi et al., 2016; Rocha et al., 2016), *LEAFY COTYLEDON* (*LEC*) (Ledwoń & Gaj, 2009; Guo et al., 2013; Nic-Can et al., 2013; Salvo et al., 2014; Wójcikowska & Gaj, 2015), *BABY BOOM* (*BBM*) (Heidmann et al., 2011; Nic-Can et al., 2013; Silva et al., 2014; Florez et al., 2015; Rupps et al., 2016), *WUSCHEL* (*WUS*) (Bouchabke-Coussa et al., 2013; Nic-Can et al., 2013; Zheng et al., 2014; Rupps et al., 2016) and *AGAMOUS-LIKE 15* (*AGL15*) (Harding et al., 2003; Thakare et al., 2008; Yang et al., 2014; Perry et al., 2016; Zheng et al., 2016) are specifically related to somatic embryogenesis involving transcriptional regulation and signal transduction pathways of embryogenic development. The current state of knowledge on these genes and their gene products is outlined in the next sections.

2.3.1 *Somatic embryogenesis receptor-like kinase* (*SERK*)

Among all the genes that have been found playing key roles during somatic embryogenesis process, *SERK*, encoding leucine-rich repeat receptor-like kinases (LRR-RLKs), has positively been shown to be an important marker to distinguish embryo-forming cells in many plant species, such as *Daucus carota* (Schmidt et al., 1997), *Arabidopsis thaliana* (Hecht et al., 2001; Salaj et al., 2008), *Oryza sativa* (Hu et al., 2005), *Solanum tuberosum* (Sharma et al., 2008), *Musa acuminata* (Huang et al., 2010), *Zea mays* (Zhang et al., 2011), *Triticum aestivum* (Delporte et al., 2013), *Momordica charantia* (Talapatra et al., 2014), *Brassica napus* (Ahmadi et al., 2016), *Passiflora edulis* (Rocha et al., 2015) and *Trifolium nigrescens* (Pilarska et al., 2016). At first, Schmidt et al. (1997) reported that there is a very close association between the expression of *SERK* genes and somatic embryogenesis during cell suspension culture on *Daucus carota*. Similarly, in cell culture of *Arabidopsis thaliana*, it has been observed that *SERK1* is highly expressed only in embryogenic callus, while it is not detectable in the non-embryogenic cells (Hecht et al., 2001; Salaj et al., 2008). Additionally, in the *primordia timing* (*pt*) mutant of *Arabidopsis thaliana*, it has been found that the expression of *AtSERK1::GUS* coincided with embryogenic development during *in vitro* culture (Singla et al., 2008). In Z*ea mays*, it was also found that the expression of the *ZmSERK* genes was the highest in embryogenic callus (Baudino et al., 2001; Zhang et al., 2011). Sharma et al. (2008) noticed that the expression of *StSERK1* gene was increased during embryogenic initiation in *Solanum tuberosum*. Furthermore, in *Cyclamen persicum*, RT-PCR results showed that expression of *SERK*s was high in embryogenic, moderate in organogenic, and null in recalcitrant calli. In the same study following *in situ* hybridizations, it was found that *SERK* expression started in pluripotent stem cells clumps, from which both pre-embryogenic aggregates and organ meristemoids derived, and continued in their trans-amplifying, meristem-like derivatives (Savona et al., 2012).

Thus, it was proposed that high expression of *SERK* genes in stem cells derivatives retain a pluripotent situation leading to somatic embryogenesis and subsequent totipotency. The expression analysis in the embryogenic versus non-embryogenic cultures of *Triticum aestivum* showed 20% more expression of *TaSERK* in embryogenic cells than no-embryogenic cells (Delporte et al., 2013). Talpatra et al. (2014) also noticed higher expression of *McSERK* in embryogenic callus compared to other plant organs in *Momordica charantia*, suggesting its association with somatic embryogenesis. Recently, the expressions of *SERK* were examined in *Brassica napus* (Ahmadi et al., 2016) and *Trifolium nigrescens* (Pilarska et al., 2016), and the authors found high expression in the cells from which embryoids or embryo-like structures were produced. These results suggest that *SERK* is involved in somatic embryogenesis induction, development and regeneration processes. However, the expression of *SERK* during embryogenesis was associated with the presence of exogenous auxins, for examples, *StSERK1* (Sharma et al., 2008), *ZmSERK*s (Zhang et al., 2011) and *TaSERK* (Delporte et al., 2013), indicating that its expression was influenced by the auxins.

2.3.2 *Baby boom* (*BBM*)

The regenerative and embryogenic potential of *in vitro* cells also depends on the expression of the transcription factor, *BBM*, which is a member of *AP2*/*ERF* gene family and is expressed in root meristem and in seed (Nole-Wilson et al., 2005). *BBM* was identified as a key marker to induce embryogenesis in differentiated cells during *in vitro* cultures (Boutilier et al., 2002), and also activates cell proliferation and growth related developmental pathways (Passarinho et al., 2008). In several studies, it has been reported that *BBM* gene was associated with embryogenic competency in culture, for example, *BBM* transcripts were detected in microspore-derived embryogenic cultures, but were not detected in non-embryogenic cultures in *Brassica napus* (Boutilier et al., 2002; Malik et

al., 2007). Additionally, it was observed that ectopic expression of *Brassica napus BBM* (*BnBBM*) gene induced spontaneous somatic embryos both in *Brassica napus* and in *Arabidopsis thaliana* (Boutilier et al., 2002). In *Capsicum annuum*, the ectopic expression of *BBM* has also been used to produce a large number of somatic embryos which could be easily produced seedlings *via* regeneration (Heidmann et al., 2011). Besides, the expression of *BBM* was observed in almost all tissues of *Arabidopsis thaliana* embryos, except distal parts of cotyledons (Kulinska-Lukaszek et al., 2012). By performing qRT-PCR, Florez et al. (2015) noticed that overexpression of *BBM* gene enriched somatic embryogenesis in *Theobroma cacao*. The expression of *BBM* was much higher in embryogenic callus than non-embryogenic callus in *Coffea arabica* (Silva et al., 2014), indicating that *BBM* is potential molecular marker during the *in vitro* embryogenesis process. However, although in *Arabidopsis thaliana* and in *Brassica napus, BBM* was sufficient to induce spontaneous somatic embryogenesis, in transgenic *Nicotiana tabacum*, heterologous expression of *Arabidopsis thaliana* and *Brassica napus BBM* genes was unable to produce somatic embryos on the basal medium without supplementation of cytokinin (Srinivasan et al., 2007). It was observed that *BBM* gene directly activates the *Arabidopsis thaliana* downstream genes (Passarinho et al., 2008), indicating that *BBM* stimulates a complex developmental network which is associated with cell proliferation and growth too.

2.3.3 *Leafy cotyledon* (*LEC*)

LEC1 (the CCAAT-box binding factor) and *LEC2* (the B3 domain transcription factor) are also important markers for somatic embryogenesis. The expression of *LEC* genes facilitates somatic embryogenesis process from vegetative tissues (Alemanno et al., 2008). A *lec1lec2* double mutant of *Arabidopsis thaliana* showed lack of embryo specific proteins (Meinke et al., 1994). Moreover, even in the presence of auxin (the plant

hormone which promotes somatic embryogenesis), it was found that the capacity for somatic embryogenesis was very low in *lec1lec2* double mutants, indicating that auxin mediated somatic embryo formation also need the function of *LEC* genes (Gaj, 2004). Among *LEC* genes, it was experimentally proven that *LEC2* plays a vital role for somatic embryogenesis induction during *in vitro* culture (Stone et al., 2001; Ledwoń & Gaj, 2009; Elhiti et al., 2013), perhaps by providing an environment to facilitate embryogenic competence (Ledwoń & Gaj, 2009). The overexpression of *LEC2* resulted somatic embryogenesis mediated shoot regeneration too. *LEC2* genes was expressed very early in embryogenic cells initiated by microspore culture of *Brassica napus* (Malik et al., 2007). It was reported that *LEC2* activates *YUC2* and *YUC4* (auxin biosynthesis genes) in *Arabidopsis thaliana* which promote to induce somatic embryogenesis (Stone et al., 2008). In addition, the overexpression of *LEC2* during somatic embryogenesis significantly increased the IAA content in *Arabidopsis thaliana*, indicating that *LEC2* involved in the auxin biosynthesis pathway which enhances the embryogenesis potential of somatic cells (Wójcikowska & Gaj, 2015). Lewdon and Gaj (2009) found a close link between auxin and *LEC2* activity, and observed that the expression of *LEC2* was significantly higher in embryogenic calli than non-embryogenic calli. To determine the mechanisms of *LEC2* genes in somatic embryogenesis, Guo et al. (2013) also studied global gene expression by digital gene expression profiling analysis in transgenic *Nicotiana tabacum*, and found that the expression of *LEC2* genes induced accumulation of proteins associated with embryogenesis such as seed storage proteins, fatty acid biosynthetic enzymes, late embryogenesis abundant (LEA) proteins, and the products of other key regulatory genes for embryo development. In *Zea mays*, the high level expression of *LEC2* was observed during somatic embryogenesis with increased expression of genes related stress response, such as germin-like proteins and *glutathione-S-transferases*, genes related to hormone transport, such as *PINFORMED* (Salvo et al.,

2014), suggesting coordinated expressions of genes associated with somatic embryogenesis, stress response and hormone transport.

2.3.4 *Wuschel* (*WUS*)

WUSCHEL (*WUS*) encodes a homeodomain transcription factor that is a critical regulator required for stem cell formation and maintenance of the shoot apical meristem (SAM) (Mayer et al., 1998; Bhalla & Singh, 2006). *WUS* also plays important role in somatic embryogenesis as noticed in several studies, such as, the ectopic expression of *WUS* gene induced stem cell formation in vegetative tissues that can differentiate into somatic embryos of *Arabidopsis thaliana* (Zuo et al., 2002; Bouchabke-Coussa et al., 2013), *Coffea canephora* (Arroyo-Herrera et al., 2008). A considerable correlation between higher expression of *WUS* gene and induction of somatic embryogenesis was also noticed in *Panax ginseng* (Kiselev & Tchernoded, 2009). Recently, it was found that *WOX2*, a member of the *WUS* family, was expressed in all stages of embryogenesis with higher level in early stages in *Larix decidua,* while a very low expression was observed in needle and seedlings (Rupps et al., 2016), which suggests that *WUS* family are effective marker for initiating of somatic embryogenesis. After *Agrobacterium*-mediated transformation in immature embryos of *Zea mays,* the overexpression of *WUS* gene stimulated the growth of embryogenic tissues, which ultimately enhanced the recovery of transgenic plants in non-transformable inbred lines (Lowe et al., 2016). This may also help to overcome the limitations of transformation frequencies in other monocots, particularly recalcitrant or marginally transformable varieties of rice, sorghum and sugarcane. It was also observed that in *Gossypium hirsutum,* the overexpression of *WUS* stimulates the conversion of non-embryogenic cells to embryogenic cells during somatic embryogenesis by upregulating the genes *GhLEC1*, *GhLEC2* and *GhFUS3*, suggesting that *WUS* activates the *LEC* genes that promotes cell differentiation and somatic

embryogenesis induction (Zheng et al., 2014). However, *WUS* expression is correlated with the exogenous auxin level (mainly 2,4-D) supplemented in the culture medium for somatic embryogenesis induction (Su et al., 2009; Santa-Catarina et al., 2012).

2.4 Epigenetic modifications

Epigenetic modifications are meiotically or mitotically heritable changes that modify chromatin structure without making any alteration of nucleotide sequences, and therefore contribute to flexible and reversible regulation of gene expression. Epigenetic modifications involve DNA methylation, histone modifications (histone methylation, acetylation, phosphorylation and ubiquitination), chromatin remodeling and non-coding RNAs. Each of these modifications alone, or in combination with another, controls the patterns of gene expression (Nic-Can & De la Peña, 2014; Song & Chen, 2015; Álvarez-Venegas & De-la-Peña, 2016). A number of studies showed that genetic programming can be overridden by altering epigenetic modifications in response to environmental conditions, thus contributing to flexible survival strategies of sessile plants (Kim et al., 2008; Dowen et al., 2012; González et al., 2013b). Very well-described examples of epigenetic modifications are DNA methylation and histone modifications those influence gene expression negatively or positively by altering the chromatin environment. Previous studies uncovered the vital roles of epigenetic regulation to control key developmental transitions, that is, embryogenic-to-vegetative and vegetative-to-reproductive growth phases (Chanvivattana et al., 2004; Bouyer et al., 2011; Boavida et al., 2015; Xu et al., 2015). Plant *in vitro* culture, being comprised of sequential de-differentiation (callus formation) and re-differentiation (regeneration into plants) phases (Grafi & Avivi, 2004; Grafi et al., 2011; Sugimoto, 2015) often provokes genetic and epigenetic instabilities (McClintock, 1993; Neelakandan & Wang, 2012; Ikeuchi et al., 2015; Gimenez et al., 2016). Among the different types of epigenetic mechanisms, DNA methylation, histone

methylation and histone acetylation were being well studied during plant *in vitro* culture. When the crucial roles of epigenetic modifications are well-illustrated, plant breeders may use the epigenetic information to design strategies for crop improvement based on creation of novel epialleles, selection for favourable epigenetic states, and regulation of transgene expression (Springer, 2013; Álvarez-Venegas & De-la-Peña, 2016).

2.4.1 DNA methylation and demethylation in plants

DNA methylation, an important epigenetic regulatory mechanism, plays key roles in plant development (Richards, 1997; Xing et al., 2015; Yang et al., 2015), stress responses (Al-Lawati et al., 2016; Chwialkowska et al., 2016), genome stability (Law & Jacobsen, 2010), gene silencing (Martienssen & Colot, 2001; Rodríguez-Negrete et al., 2013; Ikeda & Nishimura, 2015), genomic imprinting (Heslop-Harrison, 1990; Colot & Rossignol, 1999; Vu et al., 2013; Ikeda & Nishimura, 2015) and the control of transposable elements (Martienssen & Colot, 2001; Wang et al., 2016). Among the key and heritable epigenetic mechanisms that modify chromatin structure and thus affect gene expression and hence phenotypes, DNA methylation is one of the well-studied epigenetic mechanism. The frequency of DNA methylation are known to vary widely between organisms, such as 0-3% in insects, 2-7% in vertebrates, 10% in fish and amphibians and with remarkably high levels of DNA methylation of over 30% in some plants (Adams, 1996; Colot & Rossignol, 1999; Cokus et al., 2008; West et al., 2014; Wang et al., 2015). In plants, DNA cytosine methylation occurs at CG and the non-CG sequence contexts (CHG and CHH contexts) (where H indicates any nucleotide other than G), in which CG and CHG are symmetric and CHH is an asymmetric sequence. Unlike in animals, where CG (CG island) methylation predominates, in plants all three types of methylation occur, with the CG sequence context most highly methylated (Cokus et al., 2008; West et al., 2014). CG methylation is concentrated in the gene body of protein coding genes in both

dicots (e.g. Arabidopsis: (Watson et al., 2014)) and monocots (e.g. rice: (Hu et al., 2014; Yamauchi et al., 2014).

Since the presence of 5-methylcytosine (5mC) in DNA impacts gene regulation at different developmental stages in plants, the processes of DNA methylation and DNA demethylation are both important. DNA demethylation can be the result of either removing the methyl group from cytosine of DNA molecule (active DNA demethylation) or the inhibition of maintenance of methylation activity during DNA replication (passive DNA demethylation). It has been reported that active DNA demethylation depends on the function of the *REPRESSOR OF SILENCING 1* (*ROS1*) family genes which encode 5-methylcytosine DNA glycosylase/lyase. *ROS1* and its paralogs, *DEMETER-like 2* (*DML2*) and *DEMETER-like 3* (*DML3*), potentially function to remove the methyl group from 5-cytosine residues of the DNA molecules (Gehring et al., 2006). In several studies, it has been reported that *ROS1*, *DEMETER-like 2* (*DML2*) and *DEMETER-like 3* (*DML3*) prevent hypermethylation at thousands of genomic loci and play a crucial role in the regulation of some endogenous gene expression, and regulation of transposable elements and of transgenes (Gong et al., 2002; Penterman et al., 2007; Zhu et al., 2007; Qian et al., 2012; Yamamuro et al., 2014). On the other hand, in passive DNA demethylation, repression of DNA methyltransferase (*MET1*, *CMT3* and *DRM2*) gene expression may result in decreased DNA methylation levels. One example of such passive DNA demethylation was the repression of *MET1* expression during female gametogenesis in Arabidopsis that resulted in a decreased level of DNA methylation in some imprinted genes (Jullien et al., 2012).

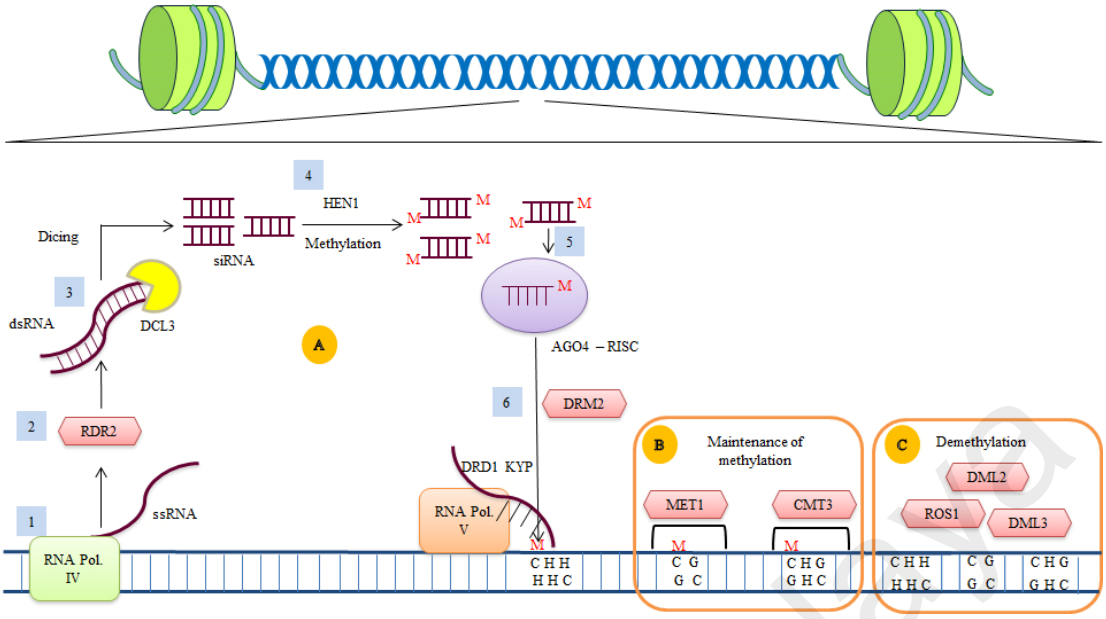


Figure 2.1: Schematic representation of methylation and demethylation in plant. (A) 1. *DRM2* catalyzes the final step of the RNA-directed DNA methylation pathway. This pathway is initiated by *RNA Polymerase IV* (*RNA Pol. IV*). 2. *RNA Pol. IV* generates small transcripts which are further converted into dsRNA by *RDR2* (*RNA DEPENDENT RNA POLYMERASE 2*). 3. These small transcripts are cleaved into 24-nucleotide small RNA (siRNA) duplexes by *DCL3* (*DICER-LIKE 3*). 4. The 24-nucleotide siRNA are methylated by *HEN1* (*Hua Enhancer 1*). 5. One of the single strands of the siRNA couples with *AGO4* (*Arogonaute 4*) and forms an RISC (RNA-Induced Silencing Complex). 6. *RNA Polymerase V* (*RNA Pol. V*) transcription is assisted by several key units such as *DRD* complex (*DRD1*), *DRM2* (*Domain Rearranged Methyltransferase 2*), *KYP* (*KRYPTONITE*) which then binds with *AGO4* leading to methylation of the DNA complex in all sequence context by *DRM2*. (B) *METHYLTRANSFERASE 1* (*MET1*) maintains methylation in the CG sequence context whereas *CHROMOMETHYLASE 3* (*CMT3*) maintains methylation in CHG sequence contexts. (C) Demethylation is catalyzed by *DME* (*DEMETER*), which belongs to a gene family including ROS1 (*REPRESSOR OF SILENCING 1*) and *DEMETER-LIKE 2* and *3* (*DML2*, *DML3*); 5-methylcytosine (5mC) is directly excised and removed.

2.4.1.1 Plant DNA methyltransferases

In plants, *METHYLTRANSFERASE 1* (*MET1*), *CHROMOMETHYLASE 3* (*CMT3*) and *DOMAIN REARRANGED METHYLTRANSFERASE* (*DRM)*, catalyze the methylation of cytosine in DNA (Figure 2.1), which is vital for epigenetic regulation and reconfiguration of genome structure (Goll & Bestor, 2005). DNA methylation and demethylation are by necessity dynamic processes in dividing and differentiating cells.

METHYLTRANSFERASE 1 (*MET1*) 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; Law & Jacobsen, 2010; Meyer, 2011; Du, 2016). *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 & Jacobsen, 2002; Matzke & Mosher, 2014; Lewsey et al., 2016). Methylation of DNA cytosine in the CHG sequence context is maintained by plant-specific *CHROMOMETHYLASE 3* (*CMT3*) (Jackson et al., 2002; Law & Jacobsen, 2010; Du, 2016).

In plants, *MET1* is regulated by *VARIANT IN METHYLATION 1* (*VIM1*), *VIM2*, and *VIM3* (Woo et al., 2008; Feng et al., 2010). In a *vim1 vim2 vim3* (*vim1/2/3*) mutant of *Arabidopsis thaliana*, CG methylation was strongly reduced resembling the *met1* mutant (Stroud et al., 2013b). Notably, *vim1*, *vim2*, and *vim3* individually did not affect CG methylation, indicating complete functional redundancy in regulating MET1 for CG methylation. The plant specific *CMT3* is regulated by H3K9 methyltransferases *KYP*, *SUVH5*, and *SUVH6* for CHG methylation (Ebbs & Bender, 2006). Stroud et al. (2013b) also noticed that loss of CHG methylation in *kyp suvh5 suvh6* (*kyp suvh5/6*) closely resembled the loss of CHG methylation mutant *cmt3*. On the other hand, Han et al. (2015) demonstrated that the DNA methylation activity of Arabidopsis *CHROMOMETHYLASE 3* (*CMT3*) is positively regulated by the *E3 SUMO ligase AtSIZ1*. The authors noticed that *CMT3* was sumoylated by the E3 ligase activity of *AtSIZ1* through a direct interaction, and that the DNA methyltransferase activity of *CMT3* was enhanced by this modification. *DRM2* activity is highly regulated by the RNA-directed DNA methylation (RdDM) pathway. *DRM2* catalyzes the final step of the RNA-directed DNA methylation pathway.

This pathway is initiated by RNA Polymerase IV (RNA Pol. IV) which generates small transcripts (ssRNA) (Wierzbicki et al., 2008), which are further converted into dsRNA by RDR2 (RNA DEPENDENT RNA POLYMERASE 2). DCL3 (DICER-LIKE 3) cleaves the small transcripts into 24-nucleotide small RNA (siRNA) which are then methylated by HEN1 (Hua Enhancer 1). One of the single strands of the siRNA couples with AGO4 (Arogonaute protein 4) and forms an RISC (RNA-Induced Silencing Complex). Then, with the help of RNA Polymerase V (RNA Pol. V), *DRM2* binds with AGO4 leading to methylation of the DNA complex in all sequence contexts (Wierzbicki et al., 2009; He et al., 2011).

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 *Arabidopsis thaliana* (Ashapkin et al., 2016). Expression of DNA methyltransferases is a prerequisite for plant development as mutations in *MET1* (alone or together with and *CMT3*) are embryonic lethal in *Arabidopsis thaliana* (Xiao et al., 2006; Zhang et al., 2006) and *drm1 drm2 cmt3* triple mutants of *Arabidopsis thaliana* were also reported to have embryonic lethality (Zhang et al., 2006). A few studies have reported the oblation of *de novo* methylation, leading to hypomethylation in mutants involving methyltransferases; these include, *drm1 drm2* Arabidopsis double mutants which lacked *de novo* methylation at CG, CHG and CHH sites (Cao & Jacobsen, 2002) and *Oryza sativa* null *met1* mutant which showed pleiotropic developmental defects, some of which were associated with activation and repression of transposable elements and altered small RNA profiles (Hu et al., 2014).

2.4.1.2 DNA methylation patterns in plant genomes

In plants, as in animals, higher levels of DNA methylation are found at the centromeric and telomeric regions, which have a low concentration of genes but are rich in repetitive DNA, including transposable elements (Chan et al., 2005; Weber & Schübeler, 2007; Meyer, 2011; Song et al., 2013a; Fultz et al., 2015; Lewsey et al., 2016). The genomes of many plants, notably including those of major crops, contain a very high content of repetitive DNA. Repetitive DNA sequences, including transposable elements are common targets of DNA hypermethylation which has been correlated with silencing of these elements (Hirochika et al., 2000; Suzuki & Bird, 2008; Sekhon & Chopra, 2009; Castel & Martienssen, 2013). Transposable elements can be reactivated and mobilized following loss of methylation (Singer et al., 2001; Neelakandan & Wang, 2012). This has been associated with stress related to *in vitro* plant culture (discussed in the following section) (Grandbastien, 2015), and also with the activation of pararetroviruses, such as *BSV* in banana (Phillips et al., 1994; Ndowora et al., 1999; Hansen et al., 2005). The activity of plant transposable elements has been shown to be repressed *via* 24-nucleotide siRNA-mediated RdDM in *Arabidopsis thaliana* (Law & Jacobsen, 2010; Matzke & Mosher, 2014). In plants and animals, the loss of DNA methylation may activate transposition of transposable elements into genic areas and lead to changes in gene expression of those genes as a result of insertions or mutation (Lisch, 2002; Naito et al., 2009; Gent et al., 2013). A recent example where methylation of a transposable element altered gene expression is the *Karma* element in *DEFICIENS 1* of *Elaeis guineensis*. Due to the presence of a splice site within *EgDEF1*, hypermethylation at this locus (termed the *Good Karma* epiallele) results in wild-type full-length transcripts of *EgDEF1* allowing normal fruit set, whereas hypomethylation of the locus (the *Bad Karma* epiallele) produces an alternative transcript with terminated transcription, resulting in the "mantled" somaclonal variation and a marked loss of fruit yield (Ong-Abdullah et al.,

2015). Previously, a positive correlation between the "mantled" somaclonal variation and DNA hypomethylation was also demonstrated in *Elaeis guineensis* (Jaligot et al., 2000; Kubis et al., 2003).

Since centromeres, telomeres and transposable elements are hypermethylated, proximal genes tend to also have higher levels of methylation that repress their expression. For example, in *Arabidopsis thaliana*, it was found that 20% (4,331out of 21,397) of *bona fide* genes (expressed and non-overlapping genes without known transposable elements) were methylated (Zilberman et al., 2007), and by grouping the genes and transposable elements annotated in RepBase into 16 1-Mb bins based on the distance from the centromeric gap, the authors noted that genes near the centromeres were much more likely to be methylated; however, this study did not mention the sequence contexts of the DNA methylation. Subsequent studies of methylated loci of *Arabidopsis thaliana* showed less methylation of CG sites in the gene body (regions comprising of introns and exons) compared to the methylation of CG sites in transposable elements, whereas methylation of CHG and CHH sequences was not notably different (Zhang et al., 2006; Cokus et al., 2008; Lister et al., 2008). However, gene body methylation may differ between species as a study on *Manihot esculenta* that examined genome-wide methylation patterns at single-base pair resolution, found that the methylation at all three sequence contexts was much higher in transposable elements than in the gene body DNA (Wang et al., 2015).

Gene expression may be influenced by 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). 5-methylcytosine (5mC) binding proteins such as the methyltransferases, KRYPTONITE (KYP) a Histone H3 lysine 9 methyltransferase, and VARIANT IN METHYLATION 1 (V1M1) bind to DNA (Woo et al., 2008), and mediate methylation at the promoter sequences, which result in repression of expression of the associated gene by blocking the movement of RNA Pol II (Chen et al., 2010). Examples of studies reporting suppression of gene expression associated with DNA methylation of gene promoters include; high-resolution DNA methylation mapping in regenerated lines of *Oryza sativa*, Stroud et al. (2013a) where hypermethylation at promoter regions was associated with inactivation of genes and hypomethylation was associated with activation of genes; genome-wide bisulfite sequencing of *Betula platyphylla*, showing that heavy methylation of promoter regions was associated with repressed gene expression (Su et al., 2014); 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 suppressed gene expression, this is not always the case with respect to the methylation of gene body regions: While genome-wide high resolution mapping and functional analysis of DNA methylation in *Arabidopsis 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, *Betula platyphylla*, gene expression was higher genes with moderate gene body methylation compared to genes with slight or heavy gene body methylation (Su et al., 2014) and in *Oryza 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). In

the latter example, the different methylation levels could also be correlated with proximal transposable elements. A confounding issue for determination of cause and effect of methylation in genes and promoter regions of plants, is the relatively poor mapping of repetitive DNA elements for many plant genome assemblies. This arises in particular for species with high repetitive element content, as is the case for several major crop plants. In view of this, changes in DNA methylation status in response to the tissue culture environment are expected to have an impact on gene expression that can influence plant development.

2.4.1.3 Changes in DNA methylation patterns during plant *in vitro* culture

Plant *in vitro* culture always involves cell multiplication and the associated maintenance of DNA methylation. Where culturing involves de-differentiation followed by embryogenesis and cell differentiation to form new organs and plants, normal maintenance of DNA methylation will occur in dividing cells as well as reshuffling of the DNA methylation patterns as part of cell reprogramming (Vining et al., 2013; Us-Camas et al., 2014; Ikeuchi et al., 2015; Ikeuchi et al., 2016). Generally, the introduction of plant cells and tissues into *in vitro* culture involves large changes in the cell environment, which may induce alterations in DNA methylation patterns (González et al., 2013a; Shan et al., 2013). The patterns of DNA methylation may be different between different explant tissues, depending on the tissue's response to *in vitro* conditions, such as different types and concentrations of plant growth regulators. Explant tissues may also respond differently over the culture period, any or all of which factors may ultimately affect developmental processes including somatic embryogenesis and plant regeneration.

Several studies have used Methylation Sensitive Amplified Polymorphism (MSAP) to assay such DNA methylation pattern changes after *in vitro* exposure of plant cells and tissues. For example, it was observed that tissue culture-derived regenerants of

Pisum sativum had increased levels of DNA methylation compared to the original plants (Cecchini et al., 1992). Similarly, in a study on *Solanum tuberosum* using MSAP, Tiwari et al. (2013) found that total alteration in DNA methylation level in the regenerants was higher (3.2 to 8.5%) than their corresponding mother plants (0.0 to 3.4%). However, from MSAP results, alteration of DNA methylation levels were noticed in *in vitro* cultured *Elaeis guineensis,* where decreased DNA methylation was exhibited in regenerants compared to mother plants (Matthes et al., 2001), and in calli derived from *Arabidopsis thaliana* leaves, where genome-wide DNA hypomethylation was noticed in the presence of kanamycin which is used as a selective agent in plant transformation (Bardini et al., 2003). Significant changes of DNA methylation patterns were noticed in calli and regenerants from pure-lines, F1 hybrids and polyploids of *Oryza sativa* which causes somaclonal variation in combination with genetic alterations (Wang et al., 2013). Differences in DNA methylation patterns between the tissues of tissue cultured plants were reported in *Brassica oleracea*, where DNA methylation levels were higher in the hypocotyl compared to the cotyledon, and that patterns at CCGG sites were different between hypocotyl and cotyledon (Li et al., 2014). Evidence that DNA methyltransferases may be associated with such patterns was provided in a study on *Boechera divaricarpa* using semi-quantitative RT-PCR, in which differential expression of DNA methyltransferase genes (*MET1*, *CMT3* and *DRM2*) in hypocotyls and seedlings was observed during *in vitro* callus culture and shoot regeneration (Taskin et al., 2015).

During plant *in vitro* culture, an important event is somatic embryogenesis that includes dedifferentiation of cells, activation of cell division, and the reprogramming of plant physiology, metabolism, and gene expression patterns. A number of studies have reported that changes in DNA methylation status are correlated with somatic embryogenesis (Leljak-Levanić et al., 2004; Fraga et al., 2012; Teyssier et al., 2014; De-la-Peña et al., 2015; Ikeuchi et al., 2015; Duarte-Aké & De-la-Peña, 2016; Ikeuchi et al.,

2016). Examples where DNA hypermethylation was associated with somatic embryogenesis include in *Acca sellowianai* (Fraga et al., 2012); in *Coffea canephora* (Nic-Can et al., 2013); in *Arabidopsis thaliana* (Kwiatkowska et al., 2014) and in *Larix* x *eurolepis* (Teyssier et al., 2014). However, DNA hypomethylation has also been associated with somatic embryogenesis in some plants (Chakrabarty et al., 2003; Xu et al., 2004; Viejo et al., 2010). For instance, in *Eleutherococcus senticosus*, High Performance Liquid Chromatography (HPLC) data showed that lower levels of methylation were associated with successful somatic embryogenesis (Chakrabarty et al., 2003), and High Performance Capillary Electrophoresis (HPCE) data in *Castanea sativa* also showed that DNA hypomethylation was associated with successful somatic embryogenesis (Viejo et al., 2010). Such differences may be a result of differences between species and genotypes, but may equally be due to differences in the tissue culture environments, such as type and level of growth factors and/or length of time in culture.

DNA methylation and somatic embryogenesis have both been associated with plant growth regulators, especially 2,4-Dichlorophenoxyacetic acid (2,4-D) or by chemical agents such as 5-azacytidine, 5-aza-2'-deoxycytidine, or Trichostatin A (Fraga et al., 2012; Huang et al., 2012; De-la-Peña et al., 2015). In many plant species, exogenous 2,4-D (auxin) has been shown to be an effective inducer of somatic embryogenesis (Michalczuk et al., 1992; Pasternak et al., 2002; Thomas & Jiménez, 2005; Kumar & Thomas, 2012; Kumar & Chandra, 2014). For instance, in *Clitoria ternatea*, optimum embryogenic callus formation (in 75 % of calli) was observed on MS medium supplemented with 2 mgL^{-1} 2,4-D (Kumar & Thomas, 2012). The effect of 2,4-D on DNA methylation has also been demonstrated for many plant species. For example, in *Cucurbita pepo*, methylation sensitive restriction digestion was used to determine the effect of 2,4-D on DNA methylation, and it was found that the highest rate of DNA methylation was exhibited in presence of 2,4-D in the early embryo stages (Leljak-

Levanic et al., 2004). This result indicates that 2,4-D may enhance DNA methylation which is essential for initiation of embryogenic cells. In a similar study, using High-Performance Liquid Chromatography Mass Spectrometry (HPLC-MS), Fraga et al. (2012) observed that the addition of 5-azacytidine (AzaC) to the cultured cells of *Acca sellowianai* resulted in less DNA methylation, while a combination of 2,4-D and AzaC increased the level of methylation, resulting in induction of somatic embryogenesis. However, in a report from callus culture of *Brassica napus*, it was found that the DNA methylation level varied with the levels of 2,4-D and 6-BA in culture media, where the DNA methylation was higher when either growth regulator was in a relatively high or relatively low concentration (Gao et al., 2014). Thus, it is suggest that the effect of 2,4-D on DNA methylation is always not functioned in a similar way, and varied depending on plant species or genotypes and culture conditions. Similarly, the actions of azaC (the demethylating agent) on DNA methylation and somatic embryogenesis are also variable, as noticed in *Pinus pinaster* somatic embryogenesis line, where the level of DNA methylation was almost same after using 5 µM or greater concentration of azaC in the culture medium (Klimaszewska et al., 2009). Somatic embryos were increased after incubation with 2.5 µM of azaC for 4 days in *Brassica napus* and *Hordeum vulgare* by decreasing DNA methylation, but due to prolonged azaC treatment, the number of embryos were diminished (Solís et al., 2015).

DNA methylation levels have been reported to change in a time-dependent fashion during *in vitro* culture, which may ultimately affect somatic embryogenesis and plant regeneration (Dubrovina & Kiselev, 2016). There are reports of both increased and decreased DNA methylation over culture time, for example, in *Solanum tuberosum* cell suspension cultures, Law and Suttle (2005) found decreased levels of DNA methylation after long term cultivation. Tanurdzic et al. (2008) observed a global reshuffling of DNA methylation in *Arabidopsis thaliana* cell suspension culture, and found that after

prolonged maintenance of culture, suspension cells showed lower levels of DNA methylation in the heterochromatic region, compared to seedlings, while in a study on *Arabidopsis thaliana* T87 cell suspension culture, Kwiatkowska et al. (2014) observed increased levels of global DNA methylation during long term cultivation. In *Taxus media* cell culture, a time-dependent increase in global DNA methylation (i.e. hypermethylation) was observed (Fu et al., 2012), and in *Elaeis guineensis* embryogenic cell suspension culture, Rival et al. (2013) noted DNA hypermethylation after prolonged cultivation and that this was associated with somatic embryogenesis, while the hypomethylated materials did not regenerate. However, it was reported that changes in DNA methylation level was low between control and somaclones obtained after long-term culture in *Coffea arabica* (Landey et al., 2015) From these reports, it appears that culture period alone cannot explain the changes in DNA methylation levels and it is likely that other genetic and environmental factors come into play.

Plants exhibit a high capacity to regenerate *via* tissue repair, *via de novo* organogenesis and *via* somatic embryogenesis. For most plants, regeneration through *de novo* organogenesis or somatic embryogenesis can be easily achieved in tissue culture by exposing explants to various combinations of auxins and/or cytokinins (Miguel & Marum, 2011; Fatima & Anis, 2012; Rocha et al., 2015; Singh et al., 2016), yet the regeneration competency varies among species, between genotypes and between tissues from the same plant (Duclercq et al., 2011; Motte et al., 2014; Carra et al., 2016). As changes in DNA methylation have been shown to occur during exposure to growth factors and over the time spent in culture, as discussed above, it seems reasonable to suggest that these new "epigenotypes" may also be a component of the variability in somatic embryogenesis and plant regeneration rates (Fraga et al., 2012; Huang et al., 2012; Rival et al., 2013; Kwiatkowska et al., 2014; Leljak-Levanić et al., 2016). These variables are likely responsible for the variation in reports on methylation in association with

regenerated plant materials, which include reports of lower levels of methylation in regenerants (*Secale cereale* (González et al., 2013a); *Oryza sativa* (Stroud et al., 2013a); triticale (Machczyńska et al., 2014)); higher levels of DNA methylation in regenerants (*Clivia miniata* (Wang et al., 2012)) and no change between regenerated plant and mother plants (*Codonopsis lanceolata* (Guo et al., 2007); *Sorghum bicolor* (Zhang et al., 2009); *Gardenia jasminoides* (Wu et al., 2012)). The alteration of DNA methylation occurring during plant regeneration through *in vitro* culture can be maintained and be stably inherited over subsequent generations in *Oryza sativa* (Stroud et al., 2013a), but in triticale, Machczyńska et al. (2014) noticed that the methylation status obtained during regeneration reversed to the initial status after the first and second successive generations. On the basis of these reports, it may be suggested that changes of DNA methylation patterns during regeneration and in the resulting regenerated plantlets are related to the types of explants, regeneration pathways and number of generations after culturing.

2.4.2 DNA methylation and environmental factors

Either *ex vitro* or *in vitro* factors may alter the DNA methylation pattern. Several studies has been demonstrated how DNA methylation is affected by environmental stresses and how these correlates with the inheritance of environmentally induced characters with some controversy (Weigel & Colot, 2012). Genome-wide methylation profiling of several plants has been shown either a decrease or an increase level of DNA methylation exposed to biotic stresses including bacterial pathogens (Dowen et al., 2012; Le et al., 2014), fungal pathogens (Sha et al., 2005), plant viral infections (Rodríguez-Negrete et al., 2013), and during tissue culture (Stroud et al., 2013a; Stelpflug et al., 2014). For example, during rice tissue culture, extensive and stochastically induced decreased level of DNA methylation was observed among individual plants, even from the same parental line (Stroud et al., 2013a). A more direct correlation between

environmental stress and DNA methylation status was noticed during fruit ripening in tomato (Zhong et al., 2013) and due to bacterial infection in Arabidopsis (Dowen et al., 2012). Zhong et al. (2013) observed that the average DNA methylation level in the 5′ ends of genes was gradually declined during fruit development of wild-type, but was remained high in the fruits of *rin* and *Cnr* mutants which are ripening-deficient. Performing DNA methylation profile of *Arabidopsis thaliana,* Dowen et al. (2012) noticed numerous stress-induced differentially methylated regions (DMR) due to exposure to salicylic acid (SA) or bacterial infection, and many of them were intimately associated with differentially expressed genes.

Abiotic stresses such as cold, drought, high salinity and UV radiation may also cause of DNA methylation changes which can be associated with gene regulation (Lang-Mladek et al., 2010; Pecinka et al., 2010; Tittel-Elmer et al., 2010; Wang et al., 2016). Although stress-induced transcriptional activation is temporary and silencing is promptly re-established upon exposure to optimal growth conditions in most cases, many reports revealed extensive and potentially stable DNA methylation changes induced by different environmental conditions. In Arabidopsis, high level of epimutation was found in response to salt stress (Jiang et al., 2014). In this study, the authors proposed that level and patterns of DNA methylation may significantly alter because of environmental factors, and can be inherited to next generations contributing to plant evolution. Changes in DNA methylation have also been reported for plants exposed to osmotic stress such as increased salinity (Ahmad et al., 2014; Al-Lawati et al., 2016) and water deficiency (Chwialkowska et al., 2016). Therefore, it would be of interest to determine how DNA methylation changes occur in response to environmental factors and how long it persists, and also to identify the mechanisms for maintenance and reversal of DNA methylation in these cases, to potentiate the application of this type of epimutation for crop improvement.

2.4.3 DNA methylation of somatic embryogenesis and plant regeneration related genes

Gene markers associated with somatic embryogenesis and plant regeneration (shown in Table 2.1) offer the opportunity to determine the potential of embryogenic competence of somatic cells, and to provide an insight of molecular mechanism on somatic embryogenesis and plant regeneration (Rocha & Dornelas, 2013; Mahdavi-Darvari et al., 2015; Rupps et al., 2016). Conversion of somatic cells to embryogenic stages involves induction of embryogenesis and expression of the embryogenic program, and in these developmental stages, the genes those are involved for cell differentiation should be repressed, and the genes those are associated with somatic embryogenesis should be stimulated. Better understanding about the fundamental molecular mechanisms that trigger somatic embryogenesis and subsequently plant regeneration could help to improve the propagation protocols, especially for recalcitrant plants during *in vitro* culturing.

In recent years, it has been reported that somatic embryogenesis and plant regeneration which results the production of a whole plant, is influenced by epigenetic mechanisms *via* alteration of gene expression (Miguel & Marum, 2011; Nic-Can & De la Peña, 2014). DNA methylation has been identified to regulate somatic embryogenesis induction and subsequent successful regeneration by modulating gene expression (Soppe et al., 2000; Shibukawa et al., 2009; Vanyushin & Ashapkin, 2011; Duarte-Aké & De-la-Peña, 2016; Trontin et al., 2016). While a majority of studies are based on estimated genome-wide DNA methylation status, there are very few reports that have focused on the methylation of specific genes related with somatic embryogenesis and plant regeneration (Us-Camas et al., 2014; De-la-Peña et al., 2015). An example of correlation of a single copy gene (*LEC1*) expression with its methylation status is a study in *Daucus*

carota embryogenic calli, where Shibukawa et al. (2009) found that DNA methylation in the 5′-upstream region of *LEC1* was negatively correlated to its expression. Similar finding of *LEC1* was noticed in *Coffea canephora* (Nic-Can et al., 2013). In the same study, the authors also found that the decreased DNA methylation levels of *SERK* and *BBM* during somatic embryogenesis process was correlated to its increased expression, while an increase of DNA methylation was correlated with its low expression. In *Arabidopsis thaliana*, the expression of *WUS*, an important marker for somatic embryogenesis and plant regeneration, was regulated by DNA methylation (Li et al., 2011), where the authors noted that due to *met1* mutant, *WUS* expression was higher, resulting early shoot primordial initiation. The decreased level of DNA methylation status of *SERK*, *BBM*, *LEC* and *WUS* promotes somatic embryogenesis and plant regeneration (De-la-Peña et al., 2015; Mahdavi-Darvari et al., 2015). However, the expressions of specific genes associated with somatic embryogenesis and plant regeneration in relation with DNA methylation status have not been fully addressed or are poorly demonstrated. Therefore, it is of interest to unravel the relationship between the expression profile of specific genes associated with somatic embryogenesis as well as plant regeneration and their DNA methylation status in different embryogenic and non-embryogenic tissues/cells.

Table 2.1: List of some gene markers associated with somatic embryogenesis and plant regenration that could be affected by cytosine DNA methylation

Gene name	Properties and mechanism of gene product	Species (References)
SERK (*Somatic embryogenesis receptor like kinase*)	encoding leucine-rich repeat receptor-like kinases (LRR-RLKs), and associated with somatic embryogenesis induction, somatic embryo development and plant regeneration during *in vitro* culture	*Arabidopsis thaliana* (Hecht et al., 2001), *Oryza sativa* (Hu et al., 2005), *Momordica charantia* (Talapatra et al., 2014), *Brassica napus* (Ahmadi et al., 2016)
WUS (*Wuschel*)	Homeo domain transcription factor. A critical regulator associated with stem cell formation and maintenance, induction of somatic embryogenesis and *de novo* shoot regeneration during *in vitro* culture	*Arabidopsis thaliana* (Li et al., 2011), *Larix decidua* (Rupps et al., 2016)
LEC1 (*Leafy Cotyledon 1*)	CCAAT-box binding factor. Over-expression at postembryonic stage stimulated to form embryo-like structure	*Arabidopsis thaliana* (Lotan et al., 1998; Ledwoń & Gaj, 2009)
LEC2 (*Leafy Cotyledon 2*)	*B3* domain transcription factor. Over-expression facilitates to stimulate embryogenic induction and to form embryo-like structure during *in vitro* culture	*Arabidopsis thaliana* (Stone et al., 2001; Ledwoń & Gaj, 2009; Wójcikowska & Gaj, 2015), *Nicotiana tabacum* (Guo et al., 2013)
FUS3 (*Fusca 3*)	DNA binding / transcription activator/ transcription factor. Significantly up-regulated in the *35S::TIR1* and down-regulated in *tir1-1* mutant	*Arabidopsis thaliana* (Ledwoń & Gaj, 2009; Qiao et al., 2012)

Table 2.1, continued

BBM (*Baby Boom*)	*AP2* domain transcription factor. Promotes callus and embryo formation due to inducible overexpression	*Zea mays* (Boutilier et al., 2002), *Theobroma cacao* (Florez et al., 2015), *Coffea arabica* (Silva et al., 2015)
AGL15 (*Agamous-like 15*)	*MADS* box transcription factor. Constitutive overexpression stimulated somatic embryogenesis.	*Glycine max* (Thakare et al., 2008), *Gossypium hirsutum* (Yang et al., 2014)
WIND1 (*Wound-induced dedifferentiation 1*)	*AP2/ERF* family transcription factor. Inducible and constitutive over-expression facilitate induction and maintenance of dedifferentiation of adult cells in a hormone-independent manner.	*Arabidopsis thaliana* (Iwase et al., 2011)

2.5 Techniques of gene expression analysis

The characteristics (phenotype) of an organism are determined by gene expression, environment and interaction between these. Basically, in prokaryotes and eukaryotes, gene expression regulates all the functions and adaptability of living cells. However, the ordered and timely expression of genes represents the complexity equally important to the definition and to the biology of the organism. Several techniques were reported to study and to quantify the level of gene expression (shown in Figure 2.2). Some techniques are well established and widely used to determine gene expression. Some important approaches those are commonly used to determine the level of mRNA expression or differential mRNA expression are: (1) Northern blotting, (2) RNase Protection Assay (RPA), (3) Fluorescent In Situ Hybridization (FISH), (4) Reverse Transcription (RT)-PCR, (5) Real-time PCR (qPCR) or Quantitative Reverse Transcription-PCR (qRT-PCR), (6) DNA Microarrays, and (7) Next Generation Sequencing (NGS) or RNA sequencing (RNA-seq).

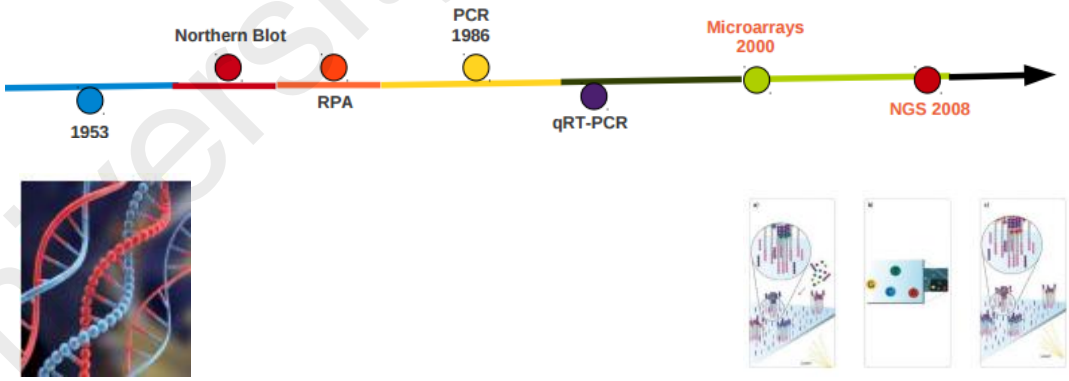


Figure 2.2: Schematic representation of different methods of gene expression with the passage of time.
Source:http://biochemie.unigoettingen.de/fileadmin/user_upload/tal/student_courses/2012/Vortrag_TAL_Microarray_Introduction2012.pdf)

2.5.1 Quantitative Reverse Transcription-PCR (qRT-PCR)

Quantitative Reverse Transcription-PCR (qRT-PCR) is a popular and widely used method to examine gene expression level (Heid et al., 1996; Winer et al., 1999). In this approach, the $2^{-\Delta\Delta CT}$ method is applied to analyze the relative changes in gene expression (Livak & Schmittgen, 2001). In qRT-PCR, very small amount of sample can be used to quantify the expression level of mRNA. Being a very powerful technology for gene expression analysis, numerous studies were conducted using qRT-PCR. In plants, some studies on expression analysis of genes related to the current thesis are: *SERK* in *Momordica charantia* (Talapatra et al., 2014), *SERK* in *Brassica napus* (Ahmadi et al., 2016); *BBM* in *Theobroma cacao* (Florez et al., 2015); *SERK* and *BBM* in *Coffea Arabica* (Torres et al., 2015), *LEC2* in *Arabidopsis thaliana* (Ledwoń & Gaj, 2009); *WUSCHEL* in *Gossypium hirsutum* (Zheng et al., 2014), *MET1*, *CMT3* and *DRM2* in *Arabidopsis thaliana* (Ashapkin et al., 2016).

2.5.2 RNA sequencing (RNA-seq)

RNA-seq used the cDNA molecules synthesized from RNA *via* reverse transcription technique to sequence RNA molecules so that the primary sequence and abundance nucleotide of each RNA molecule can be known. RNA-seq is rapidly outcompeting microarrays as the technology of choice is for whole-transcriptome studies (Van Verk et al., 2013). However, the bioinformatic knowledge and skills required for RNA-seq data analysis often create difficulty for many biologists. Despite this difficulty, researchers are performing a large number of studies for gene expression analysis using this sophisticated technique. Some examples where the expression levels of genes related this study were determined using this technique are: *LEC2* in *Nicotiana tabacum* (Guo et al., 2013), *BBM, LEC* and *SERK* in *Zea mays* (Salvo et al., 2014), *SERK, BBM, LEC, WUS, MET1, CMT3* and *DRM2 in Arabidopsis thaliana* (Wickramasuriya & Dunwell,

2015), *SERK* and *WUS* in *Cocos nucifera* (Rajesh et al., 2016), *SERK* and *LEC* in *Raphanus sativus* (Zhai et al., 2016).

2.6 Techniques of DNA methylation analysis

DNA methylation has profound impact on gene expression, genome stability as well as in developmental processes in many eukaryotic organisms (Jaenisch & Bird, 2003; Smith & Meissner, 2013; Ikeda & Nishimura, 2015; Yang et al., 2015). Over the previous decades, various strategies including established biochemical methodologies and advanced genomic approaches were applied for detection, measurement, and mapping of DNA methylation inside the genome. Basically, DNA methylation analysis techniques vary depending on their sensitivity, coverage, and on the desired level of application. Potential results may be to determine the global 5mC content, to determine the level of methylation status at genome-wide level or at specific loci. The earliest methods of examining DNA methylation were based on the separation of unmethylated and methylated cytosines using chromatography (Wagner & Capesius, 1981; Fraga et al., 2000). The following years, some molecular techniques were used to determine DNA methylation status at both a genome-wide and gene specific level, such as selective digestion with methylation sensitive restriction endonucleases, and immunoprecipitation *via* anti 5-methylcytosine (Hatada et al., 1991; Leljak-Levanić et al., 2004; Parle-Mcdermott & Harrison, 2011). A major advance to analyze DNA methylation was the development of sodium bisulfite conversion of DNA molecules that converts unmethylated cytosine to uracil, while methylated cytosines remain unchanged (Clark et al., 2006; Parle-Mcdermott & Harrison, 2011; Su et al., 2014). Recently, these three approaches have been used combined with more sophisticated methods such as DNA microarrays and next-generation sequencing platforms. Use of more advanced methods to analyze DNA methylation has brought us closer for better understanding

about the whole methylome (Umer & Herceg, 2013). Among the earliest methods, High Performance Liquid Chromatography (HPLC), a conventional approach to measure global DNA methylation in plants and animals, was used (Ehrlich et al., 1982; Johnson et al., 2007; Machczyńska et al., 2014). However, this technique requires a large amount of high-quality genomic DNA, and is not appropriate for high-throughput analyses. In plants, the most frequently used techniques to enable analysis of DNA methylation are either (1) methylation-sensitive restriction enzymes followed by PCR, (2) bisulfite conversion followed by either microarray or high-throughput sequencing (Do Kim et al., 2014; Adusumalli et al., 2015; Harris & Lonardi, 2016).

2.6.1 Methylation sensitive restriction enzymes

Methylation sensitive restriction endonuclease enzymes were commonly used to analyze DNA methylation both locus specific (Bird & Southern, 1978) and genome-wide (Hatada et al., 1991) by a method known as Methylation Sensitive Amplified Polymorphism (MSAP). MSAP is a slightly modified method of Amplified Fragment Length Polymorphism (AFLP), where the restriction isoschizomer pair *Hpa*II and *Msp*I were used to cut at the sequence 5′-CCGG-3′ based on the methylation status of the internal or external cytosine residues. *Hpa*II cleaves the hemimethylated sequence (in which only one of the DNA strands is methylated) of external cytosine, and is inactive if one or both cytosine are fully methylated or any internal cytosine is methylated, whereas *Msp*I cuts both hemi or fully methylated internal cytosine, but is inactive if any external cytosine is methylated (Cedar et al., 1979; McClelland et al., 1994; Xiong et al., 1999; Leljak-Levanić et al., 2004; Gimenez et al., 2016). MSAP was first established to detect DNA methylation patterns in dimorphic fungi (Hatada et al., 1991) and later was adjusted for the detection of DNA cytosine methylation in many plants, such as *Oryza sativa*

(Xiong et al., 1999), *Cucurbita pepo* (Leljak-Levanic et al., 2004), *Zea mays* (Shan et al., 2013), *Allium sativum* (Gimenez et al., 2016).

Another methylation sensitive endonuclease is McrBC which has also been used to determine DNA methylation by specifically cleaving methylated DNA (Sutherland et al., 1992). These enzymes enrich either methylated or unmethylated DNA in a sample, and according to methylation status, the subsequent patterns of diversely digested DNA fragments are detected by array hybridization (McrBC assay (Lippman et al., 2005), *Hpa*II tiny fragment Enrichment by Ligation-mediated PCR (HELP) (Khulan et al., 2006), Comprehensive High-Throughput Arrays for Relative Methylation **(**CHARM) (Irizarry et al., 2008)) or high throughput sequencing (Methyl-seq (Brunner et al., 2009), HELP-seq (Oda et al., 2009)). These restriction enzyme-dependant techniques were used to analyze DNA methylation at the early stages of methylation studies in plants (Lippman et al., 2005; Tran et al., 2005; Vaughn et al., 2007), however, these techniques have decreased in popularity in recent years due to several limitations, such as (i) low resolution of the data, (ii) false positives due to sequence polymorphisms in the recognition site, (iii) false positives due to incomplete digestion, (iv) bias due to the uneven distribution of recognition sites and (v) difficulties to detect non-CG DNA methylation. Thus, restriction enzyme-based techniques are inferior approach to detect DNA methylation in plant genomes, where non-CG DNA methylation is common. Decreasing sequencing cost day by day is also attracting to perform advanced next generation sequencing method like bisulfite sequencing (BS-seq) to analyze DNA methylation.

2.6.2 Bisulfite conversion

Bisulfite conversion followed by sequencing, a novel approach for the detection of DNA methylation offers a qualitative, quantitative measurement of 5mC at single base-

pair resolution. Bisulfite conversion can be combined with microarrays (Golden-Gate methylation assay (Bibikova et al., 2006), Infinium methylation assays (Bibikova et al., 2009)) as well as with high-throughput sequencing (BS-seq (Lewis et al., 1992), MethylC-seq (Lister et al., 2008), RRBS (Smith et al., 2009; Gu et al., 2011)). In the case of BS-seq, during sodium bisulfite ($NaHSO_3$) treatment, unmethylated cytosines of DNA molecule are converted into uracil, and are recognized as thymine in subsequent sequencing after PCR amplification, but, 5mC are not converted into uracil residues, and are remained as cytosines allowing 5mC to be distinguished from unmethylated cytosines. This method was first introduced to analyze DNA methylation in human by Frommer et al. (1992), and in recent years, was applied in many plants, such as *Betula platyphylla* (Su et al., 2014), *Manihot esculenta* (Wang et al., 2015), *Zea mays* (Xu et al., 2016), *Brassica napus* (Li et al., 2016a). The major constraint of restriction enzyme-based techniques to analyze DNA methylation in plants is the incapability to differentiate among DNA methylation at CG, CHG and CHH sequence contexts, and these give only average methylation profile over a region. Bisulfite treatment overcomes this limitation by providing methylation profile at single base-pair resolution (Frommer et al., 1992; Clark et al., 2006; Henderson et al., 2010; Harris & Lonardi, 2016). BS-seq permits one to sensitively quantity DNA methylation at genome-wide scale and at locus-specific sequence contexts.

Microarray platforms have been broadly applied to assess DNA methylation using bisulfite-conversion in mammals, but, studies are limited in plants using these methods. In recent years, MethylC-seq (also known as whole genome bisulfite sequencing (WGBS)) has become a popular technique to analyze DNA methylation in plants (Lister et al., 2008; Zemach et al., 2010; Schmitz & Zhang, 2011; Calarco et al., 2012), because the methods allow quantitative analysis of 5mC abundances in all three sequence contexts (Lister & Ecker, 2009; Schmitz & Zhang, 2011). Although this technique is the best way

to detect DNA methylation in plants and are widely used, there are some limitations such as over-representation of 5mC due to partial conversion, low quality sequencing and poor mapping to reference genome (Laird, 2010; Harris & Lonardi, 2016; Shafiq & Khan, 2016).

2.7 Importance of gene expression and DNA methylation during *in vitro* culture

During somatic embryogenesis, somatic cells gain their embryogenic competence as a result of various physical and chemical stimuli, which have underlying genetic components. Embryo formation and the subsequent regeneration of plant organs and whole plants, require the reprogramming of gene expression patterns comprising cascades of genetic and epigenetic signals that regulate expression of different groups of genes (Feher et al., 2003; Yang & Zhang, 2010; Neelakandan & Wang, 2012; Elhiti et al., 2013; Mahdavi-Darvari et al., 2015; Yakovlev et al., 2016). In addition to the genetic components of somatic embryogenesis, DNA methylation is one of the best-studied epigenetic mechanisms that has been correlated with embryogenesis and plant regeneration. A relationship between DNA hypermethylation / DNA hypomethylation and somatic embryogenic cell formation has been shown in several plant species such as *Daucus carota* (LoSchiavo et al., 1989), *Pinus nigra* (Noceda et al., 2009) and *Arabidopsis thaliana* (Xiao et al., 2006; Kwiatkowska et al., 2014), *Araucaria angustifolia* (Fraga et al., 2016), *Cucurbita pepo* (Leljak-Levanić et al., 2016). However, knowledge is limited on the DNA methylation of genes related to somatic embryogenesis and regeneration during these processes. Therefore, it is important to establish the role of DNA methylation in somatic embryogenesis and regeneration by the study of DNA methylation status during *in vitro* culture and plant regeneration. New techniques in whole genome DNA methylation analysis at the nucleotide sequence level, together with complete genome sequences now make this approach feasible for recalcitrant species.

B. rotunda is widely distributed in South-East Asia, India and China, and is an important source of active compounds for medicinal treatment. Due to the properties of medicinal and other uses like culinary purposes, *B. rotunda* has gained attention for the studies of various molecular and biochemical aspects. In this study, gene expression and DNA methylation profiles were generated during *in vitro* culture and plant regeneration in *B. rotunda* that could provide useful data for future studies on *B. rotunda* epigenetics, and that could be a model system for other plant species.

CHAPTER 3: MATERIALS AND METHODS

3.1 Plant materials

Rhizomes of *Boesenbergia 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. When newly emerged shoots were formed 1-3 cm in length, those 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 (shown in Figure 3.1). The young *ex vitro* leaf (EVL) samples were collected from rhizome derived the plants at 4 weeks after potting at the Department of Genetics and Molecular Biology, University of Malaya, Malaysia.

Figure 3.1: Initial plant materials used in this study. A: Rhizomes; B: Newly emerged shoot buds or sprouts; C: *Ex vitro* plant (4 weeks old); D: Meristematic block tissues dissected from newly emerged shoot buds on MS media for initial callus establishment.

3.2 Establishment of *in vitro* culture and plant regeneration

Callus samples were established as described in Ng et al. (2016) by culturing MB explants on Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) (Appendix A) 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 weeks) 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 weeks on the respective media (8 weeks after initial culturing from explant). Propagation of embryogenic cells from embryogenic callus was performed 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 one year 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 stereo microscopy to observe the presence or absence of embryo structures, and under light microscopy for histological studies with Schiff's reagent (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 for 8 weeks. Leaves from plants regenerated from embryogenic calli were collected after 8 weeks (16 weeks after initial culturing from explant). Plants regenerated from embryogenic calli were multiplied and maintained on MS0. The experimental flow for the samples is shown in Figure 3.2.

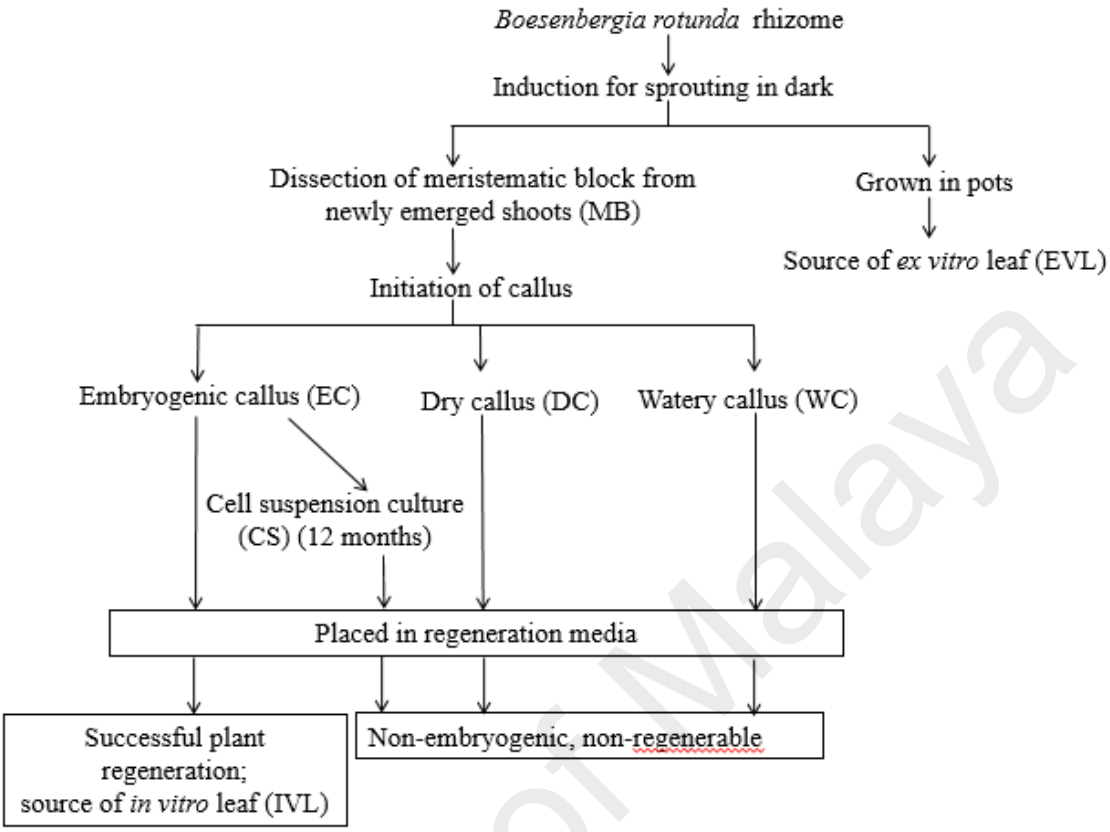


Figure 3.2: Schematic diagram for generation of embryogenic and non-embryogenic calli and their plant regeneration ability from *B. rotunda*.

3.3 Isolation of RNA and synthesis of cDNA

Total RNA was extracted from tissues and calli according to modified cetyl trimethyl ammonium bromide (CTAB) method (Kiefer et al., 2000). A slight modification was made, in which an additional extraction step using phenol-chloroform-isoamylalcohol (25:24:1) was performed prior to extraction using chloroform-isoamylalcohol (24:1). Briefly, different types of calli and tissues of *B. rotunda* were ground in liquid nitrogen using mortar and pestle making into fine powder properly before transferring to homogenization buffer containing 100 mM TrisHCl (pH 8.0), 2% (w/v) CTAB, 2% (w/v) PVP-40, 25 mM EDTA (pH8.0), 2 M NaCl and 2-Mercaptoethanol

($HOCH_2CH_2SH$) (Sigma-Aldrich®, St. Louis, MO, USA), and were mixed well by inversion repeatedly. About 0.2-0.3 g of calli and tissue was put into 850 µl of homogenization buffer (CTAB buffer) contained in a 2 mL microcentrifuge tube. An equal volume of phenol- chloroform-isoamyl alcohol (25:24:1) (from Sigma-Aldrich®, St. Louis, MO, USA; Fisher Scientific International Inc., Hampton, NH, USA; and Amresco LLC, Solon, OH, USA respectively) was added into the mixture of tissue and homogenization buffer, and mixed well by continuous inversion for 5 min. The mixtures were spun at 10,000 *g* for 10 min. This step was repeated once, followed by an extraction step using chloroform-isoamyl alcohol (24:1) (from Fisher Scientific International Inc., Hampton, NH, USA; and Amresco LLC, Solon, OH, USA, respectively). The nucleic acids were then precipitated overnight in an equal volume of isopropanol (Sigma-Aldrich®, St. Louis, MO, USA) at -80 °C. Overnight-precipitated RNA was spun at 10,000*g* for 30 min at 4 °C. The resulting RNA pellet was properly air-dried and was dissolved in RNase free water and was stored at -80 °C.

3.3.1 Determination of RNA quantity, purity and integrity

The presence of RNA was determined on a 0.5 µg/mL ethidium bromide-stained 1% (w/v) agarose gel. RNA concentration was determined based on spectrophotometry at 260 nm and 280 nm using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA) and RNA integrity was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). Only RNA samples with absorbance ratios A_{260nm}/A_{280nm} ranging from 1.8 to 2.2, A_{260nm}/A_{230nm} ratios higher than 1.0 and an RNA integrity number (RIN) higher than 7.0 were used to synthesize complementary DNA (cDNA) for gene expression study using quantitative Reverse Transcription-PCR (qRT-PCR), and for library construction followed by sequencing using Illumina sequencing platforms (Illumina Inc., San Diego, CA, USA).

3.3.2 Synthesis of complementary DNA (cDNA)

The first strand of cDNA was synthesized from 2 µg of RNA in a 40 µl reaction volume using QuantiTect® Reverse Transcription Kit (QIAGEN, Germany). The QuantiTect Reverse Transcription method takes only 20 min to synthesize cDNA from mRNA molecule, and involves 2 main steps: genomic DNA elimination and reverse transcription (Figure 3.3). Firstly, the purified RNA sample (up to 2 µg) was incubated on ice in total 28 µl of reaction mixture containing Genomic DNA (gDNA) Wipeout Buffer (4 µl) and RNase free water (volume as required to top up 28 µl) at 42 °C for 2 min to eliminate gDNA contamination effectively. After elimination of gDNA, the reaction mixture (in total of 40 µl) containing Quantiscript Reverse Transcriptase (2 µl), Quantiscript RT (Reverse Transcriptase) Buffer (8 µl), RT Primer Mix (2 µl) and entire genomic DNA elimination reaction (28 µl) was incubated at 42 °C for 15 min for reverse transcription, and was then placed immediately on ice. Finally, the mixture was incubated at 95 °C for 3 min to inactivate Quantiscript Reverse Transcriptase. Then, the synthesized cDNA was stored at -20 °C for long-term storage.

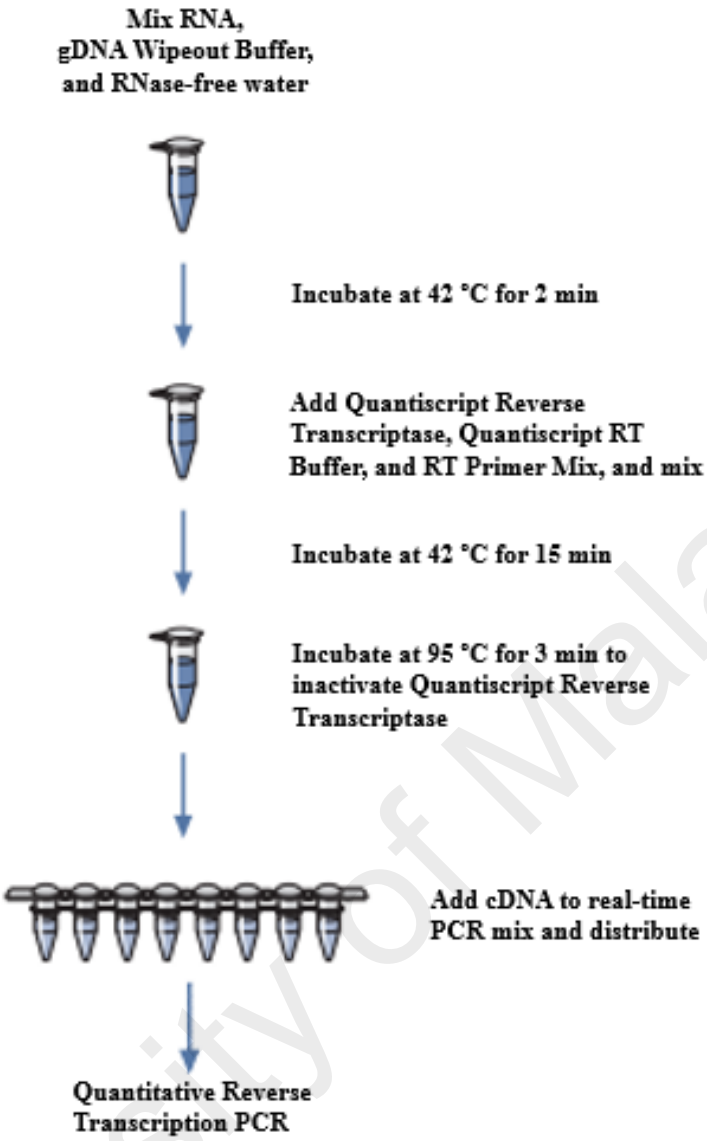


Figure 3.3: Procedure of cDNA synthesis using Quantitect Reverse Transcription. (Source: QuantiTect® Reverse Transcription Handbook, QIAGEN, Germany).

3.3.3 Determination of yield and purity of cDNA

The concentrations and purity of synthesized cDNA from different calli and tissues of *B. rotunda* were determined based on spectrophotometry at 260 nm and 280 nm using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA). The ratio of $A_{260nm}/A_{280nm,}$ and samples with values within the range of 1.7-1.9 were used for subsequent experiments.

3.4 Primer design

Primers for amplification of transcripts from DNA methyltransferase genes (*MET1*, *CMT3* and *DRM2*) and somatic embryogenesis and plant regeneration related genes (*SERK*, *BBM*, *LEC2* and *WUS*) were designed from unigene sequences from previous transcriptome studies of *B. rotunda* (Md-Mustafa et al., 2014) using Primer BLAST and Primer3 Plus. Primers were obtained from a commercial service of Integrated DNA Technologies (IDT) (USA). The primers are listed in Table 3.1.

Table 3.1: Primers for gene expression analysis using Quantitative Reverse Transcription PCR (qRT-PCR)

Gene name	Primer sequences	Expected product size (bp)
MET1	Forward: 5'-GCCCATGGGTAAGGTTGGAA	165
	Reverse: 5'-TCTCCCAAAACCATTCAGTGCT	
CMT3	Forward: 5'-TCGTTGTCTTCATGGACATCGT	220
	Reverse: 5'-TTGGGATGACTTCCCCACAG	
DRM2	Forward: 5'-ACACCGTTTGGGGATACACCT	227
	Reverse: 5'-TGCTCCCGGTAAGATTGTTGC	
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	
18S rRNA	Forward: 5'- CAAAAAGTGGCGGAATGCTC	226
	Reverse: 5'- GACAGACCAAGGGCGAACAC	
Actin	Forward: 5'- GCCTCACGCTCTTCTTTCGAT	100
	Reverse: 5'- AGCAGTGGTGGTGAATGAATCTC	

3.5 Homology searching and phylogenetic analysis

The unigene nucleotide sequences of DNA methyltransferase gene transcripts for *MET1* (KY290867), *CMT3* (KY290868) and *DRM2* (KY290869) and of somatic embryogenesis and plant regeneration related gene transcripts 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+) 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).

Nucleotide sequences of *MET1*, *CMT3*, *DRM2*, *SERK*, *BBM*, *LEC2* and *WUS* from *B. rotunda* were translated to amino acid sequence using all 6 possible reading frames through ExPASy translate tool (http://web.expasy.org/translate/) and were aligned with the 15 best scoring respective amino acid sequences obtained from NCBI Non-redundant protein sequences (nr) (https://www.ncbi.nlm.nih.gov/protein) through BLASTx(v2.6.0+)(https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome), with the help of Clustal Omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/). A phylogenetic tree was constructed using the aligned sequences of each gene by Molecular Evolutionary Genetics Analysis (MEGA) 6.0 using neighbor joining method with 500 bootstrap replicates. The reliability of each node was established based on bootstrap calculation using MEGA 6.0 software (Tamura et al., 2013).

3.6 Gene expression analysis

Levels of gene expression were determined by quantitative Reverse Transcription PCR (qRT-PCR) and from analysis of transcriptome sequencing data.

3.6.1 Gene expression analysis using Quantitative Reverse Transcription-PCR (qRT-PCR)

Quantitative Reverse Transcription-PCR (qRT-PCR) was performed using gene specific primers designed from the *B. rotunda* transcriptome sequence for each gene. 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* and *Actin* were used as an internal control for normalization for each analysis. The primers used for these analyses are listed in Table 3.1. For qRT-PCR, relative quantification of gene expression used the comparative CT method ($2^{-\Delta\Delta CT}$ method) of Livak & Schmittgen (2001). This method is based on the use of an internal control gene transcript to normalize sample variations under different experimental conditions.

3.6.2 Gene expression analysis using transcriptome sequencing data

The Illumina RNA sequencing (RNA-Seq) service was provided by a commercial service (Sengenics Sdn. Bhd., Malaysia). Whole transcriptome sequencing of six samples (three biological replicates for each of six samples) from *B. rotunda ex vitro* leaf, embryogenic callus, dry callus, watery callus, prolonged cell suspension culture and *in vitro* leaf of regenerated plants was carried out by using an Illumina HiSeq™ 2000 platform (Illumina Inc., San Diego, CA) according to the manufacturer's instructions. Detailed methodology and analyses on transcriptome study are described in another thesis paper headed by a fellow group member and PhD Candidate Mr. Yew Seong Tan (in preparation). The data provided by Mr Tan was the level of transcripts of *MET1*, *CMT3*, *DRM2*, *SERK*, *BBM*, *LEC2* and *WUS* genes, from the RNA-seq data sets, each in three biological replicates, for the six samples following data set normalization using a Transcript Per Million (TPM) method.

3.7 Isolation of DNA

Total genomic DNA was isolated using a modified cetyl trimethyl amonium bromide (CTAB) method (Doyle, 1990) from all samples. In brief, samples were ground in liquid nitrogen with mortar and pestle making fine powder, and 200-300 mg sample were collected in a 2 ml Eppendorf tube. Then, 850 µl homogenization buffer containing 100 mM Tris-HCl (PH 8.0), 2 % w/v CTAB, 20 mM EDTA, 1.42 M NaCl, 2 % w/v PVP-40 and 2-Mercaptoethanol ($HOCH_2CH_2SH$) (Sigma-Aldrich®, St. Louis, MO, USA) was added. RNase A (4 µl) was also to remove the contamination of RNA. The mixture was then gently vortexed and incubated at 65 $^{\circ}$C for 30-45 min (tubes were inverted every 15 min). Then, an equal volume of extraction solution (850 µl) containing chloroform:isoamyl alcohol (24:1) (from Fisher Scientific International Inc., Hampton, NH, USA, and Amresco LLC, Solon, OH, USA, respectively) was added to the tubes and

was mixed by inverting gently for 10 min. Next, the mixtures were centrifuged at 13000 g for 10 minutes. The top aqueous layer of around 500 µl was pipetted into a new 1.5 ml Eppendorf tube and 1 ml of 70% cold ethanol (double the sample volume) was added. The mixture was then gently inverted several times to precipitate DNA. The mixtures were kept at 4 °C for 2 hours to increase the DNA yield, and were centrifuged at 13000g for 15 min at room temperature. Then, the supernatant was decanted and the pellet air-dried for 20 min. Finally, the pellet was re-suspended in 50-100 µl ddH_2O and was stored in -20 °C for subsequent analysis.

3.7.1 Determination of DNA quantity and purity

The presence of DNA was detected on a 0.5 µg/ml ethidium bromide-stained 1% (w/v) agarose gel by running for 30 min with 120 volt in 1× TBE buffer. 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). Polysaccharides and other metabolites may interfere with OD readings of DNA extracted from plant tissues. Samples with an A_{260nm}/A_{280nm} ratio between 1.7-1.9 were selected and stored at -20 °C for subsequent analysis.

3.8 DNA methylation analysis

3.8.1 DNA methylation analysis using Methylation Sensitive Amplified Polymorphism (MSAP)

MSAP is a modified AFLP method where two methylation sensitive restriction enzymes (*Hpa*II and *Msp*I) are used to detect DNA methylation changes at CCGG sequence context (CG islands). *Hpa*II cuts unmethylated (CCGG) and only when the outer (5′) cytosine of a single strand is methylated (hemimethylation) i.e. ^{m}CCGG. *Msp*I cuts unmethylated (CCGG) and only when the inner cytosine is methylated

(hemimethylation or fully methylation) i.e. $C^{m}CGG$. MSAP was performed to detect DNA methylation changes at CCGG sequence context among different tissues and samples of *B. rotunda* based on a method described by Xiong et al. (1999) with some modifications. The MSAP method is comprised of three main parts: digestion and ligation reaction; pre-amplification and selective amplification reactions, and detection reactions. The ligation adapters, pre-amplification and selective amplification primers for *Eco*RI and *Hpa*II-*Msp*I were obtained from Integrated DNA Technologies (IDT) (USA) and are listed in Table 3.2.

Table 3.2: Adapters and primers for MSAP analysis

Adapters / Primers	Sequences (5′-3′)
*Eco*RI adapter 1 (+)[a]	5′-CTCGTAGACTGCGTACC-3′
*Eco*RI adapter 2 (-)[b]	5′-AATTGGTACGCAGTC-3′
*Hpa*II/*Msp*I adapter 1 (+)[a]	5′-GACGATGAGTCCTGAG-3′
*Hpa*II/*Msp*I adapter 2 (-)[b]	5′-CGCTCAGGACTCAT-3′
*Eco*RI pre-amplification primer (E00)	5′-GACTGCGTACCAATTC-3′
*Eco*RI selective amplification primers (E00+3)	5′-GACTGCGTACCAATTC+AAC (E1)
	5′-GACTGCGTACCAATTC+AAG (E2)
	5′-GACTGCGTACCAATTC+ACC (E3)
*Hpa*II/*Msp*I pre amplification primer (HM00)	5′-GATGAGTCCTGAGCGGC-3′
*Hpa*II/*Msp*I selective amplification primers (HM00+3)	5′-GATGAGTCCTGAGCGGC+CAA (HM1)
	5′-GATGAGTCCTGAGCGGC+CAC(HM2)
	5′-GATGAGTCCTGAGCGGC+CAG(HM3)

[a]'+' denotes positive or sense DNA strand; [b]'-' denotes negative or anti-sense DNA strand.

3.8.1.1 Digestion and ligation reactions

Genomic DNA (500 ng) samples isolated from different calli and tissues of *B. rotunda* were digested with *Eco*RI+*Hpa*II in one set of reactions and with and *Eco*RI+*Msp*1 in a separate set of reactions (New England Biolabs, USA). To reduce discrepancy caused by experimental errors, digestion and ligation was done in single reactions simultaneously. The digestion and ligation reaction was conducted in a volume of 50 µl containing 5 µl CutSmart Buffer, 500 ng DNA template, 10 U *Eco*RI, 20 U *Hpa*II/*Msp*I, 3 U T4 DNA ligase (New England Biolabs, USA), 5 pmol *Eco*RI adapters, 50 pmol *Hpa*II/*Msp*I adapters (Table 3.2) (Integrated DNA Technologies, USA) by incubating at 37 °C for 6 hours. Reactions were stopped by incubating at 65 °C for 10 min, and then samples were stored at -20 °C.

3.8.1.2 Pre-amplification and selective amplification reactions

Digested and ligated DNA was diluted 10 times by using 0.1× TE buffer. Then pre-amplification PCR was conducted with the diluted product using pre-amplification primers (E00_HM00) in a DNA thermal cycler (Eppendorf, Germany). The PCR reaction mixture was in a final volume of 20 µl containing 5 µl diluted digested and ligated DNA mixture, 1 µl *Eco*RI pre-amplification primer, 1 µl *Hpa*II/*Msp*I pre amplification primer (Table 3.2) and 10 µl GoTaq PCR Master Mix (Promega, USA). The cycling conditions were as follows: 94 °C for 60s, 25 cycles at 94 °C for 30s, at 55 °C for 30s and at 72 °C for 1 min, and final extension at 72 °C for 10 min. Then, the pre amplified PCR products were checked by running into ethidium bromide stained 1% agarose gel. Next, the pre amplified PCR product was diluted 10 times with 0.1× TE buffer, and selective amplification was performed with selective amplification primers which had three extra selective nucleotides compared to the pre amplification primers (Table 3.2). In total nine combinations (E00+3_HM00+3) were performed. The PCR reactions were in a final

volume of 20 µl containing 5 µl diluted pre-amplified PCR product, 1 µl *Eco*RI selective amplification primers, 1 µl *Hpa*II/*Msp*I selective amplification primer (Table 3.2) and 10 µl GoTaq PCR Master Mix (Promega, USA). The cycling conditions were as follows: 94 °C for 60s, 13 cycles at 94 °C for 30s, at 65 °C for 30s (reduced by 0.7 °C each cycle) and at 72 °C for 1 min; 23 cycles at 94 °C for 30s, at 56 °C for 30s and at 72 °C for 1 min, and final extension at 72 °C for 10 min.

3.8.1.3 Detection reactions

The selective amplified products were denatured by heating for 3 min at 94 °C, and were then chilled on ice quickly. Then, the denatured products were run at ethidium bromide stained 2% (w/v) agarose gel with constant power of 65 V for 2 hours. Reproducible and clear bands were scored based on presence or absence of band at specific site for analysis. Methylation percentage was determined using the formula = number of methylated bands × 100/total number of bands. Percentage of methylation polymorphism was measured as number of polymorphic methylated bands × 100/total number of methylated bands.

3.8.2 DNA methylation analysis using Bisulfite Sequencing (BS-seq)

3.8.2.1 Library construction and sequencing

Genomic DNA of *B. rotunda ex vitro* leaf, embryogenic callus, dry callus, watery callus, prolonged cell suspension culture and leaf of regenerated plants was sequenced after treated by sodium bisulfite. The sequencing was carried out by a commercial service provider, Sengenics Sdn. Bhd., Malaysia. A total of six samples (3 biological replicates for each of six samples) were sequenced by using an Illumina HiSeq™ 2000 platform (Illumina Inc., San Diego, CA) according to the manufacturer's instructions. Briefly, after bisulfite treatment, genomic DNA was fragmented to a mean size of approximately 200-

300 bp, using a Diagenome sonicator followed by blunting of ends, addition of dA (Adenine deoxyribonucleotides) to the 3′-end, and ligation of adaptors according to the manufacturer's instructions (Illumina, San Diego, CA, USA). Paired-end reads were generated. Reads were preprocessed by trimming low quality reads and adapters by Trim-Galore (Krueger, 2015) specific for bisulfite sequencing.

3.8.2.2 Mapping of BS-seq data and DNA methylation level

After trimming, the fastq reads were mapped to *B. rotunda* transcriptome using the Bismark v 0.12.3 (Krueger & Andrews, 2011), and mapping duplicates were removed using Methpipe v 3.4.2 (Song et al., 2013b). Then, overall mapping results of methylated and unmethylated cytosines were determined by "methcounts" program from methpipe (Song et al., 2013b), where methylation level at single base resolution was calculated based on number of 5-methylated cytosines (5mC) in reads at position corresponding to the site, divided by the sum of the C and thymine (T) mapping to that position in CG, CHG and CHH contexts. Percentage of global methylation at CG, CHG and CHH contexts across genome-wide was calculated by number of 5-methylated cytosine (5mC) / Total number of cytosines × 100 in triplicates of each sample. Global DNA methylation levels at genome-wide, and for four somatic embryogenesis and plant regeneration related genes (*SERK*, *BBM, LEC2* and *WUS*) and three housekeeping genes (*Actin*, *Beta-tubulin 1* and *Elongation Factor 1-alpha* (*EF1-α*)) were calculated by number of reads methylated at particular region by total number of observation from reads in the region at CG, CHG and CHH methylation contexts.

3.9 Correlation analysis between gene expression and DNA methylation

The association between the expression levels of genes used in this study (from qRT-PCR data) and the methylation level in terms of CG, CHG and CHH sequence contexts (BS-seq data) were compared using Pearson's Correlation Analysis.

3.10 Data 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 Analysis of Variance (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 by qRT-PCR, RNA-seq and BS-seq. Comparisons between mean values of different samples were made according to Tukey's comparison test ($p < 0.05$).

CHAPTER 4: RESULTS

The present investigation was carried out to determine the gene expression patterns and DNA methylation status during *in vitro* culture and plant regeneration of *Boesenbergia rotunda.* To achieve the objectives of this study, firstly, the expression patterns of three DNA methylation pathway genes (*METHYLTRANSFERASE 1* (*MET1*), *CHROMOMETHYLASE 3* (*CMT3*) and *DOMAIN REARRANGED METHYLTRANSFERASE 2* (*DRM2*)), and four somatic embryogenesis related genes (*SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* (*SERK*), *BABY BOOM* (*BBM*), *LEAFY COTYLEDON 2* (*LEC2*) and *WUSCHEL* (*WUS*)) were examined in meristematic block, embryogenic and non-embryogenic calli, prolonged cell suspension culture, *ex vitro* leaf, and in *in vitro* leaf of regenerated plants of *B. rotunda*. Subsequently, the overall genome-wide DNA methylation in the same samples was examined, and the gene specific DNA methylation of three methylation pathway genes (*MET1*, *CMT3* and *DRM2*) and four somatic embryogenesis and plant regeneration related genes (*SERK*, *BBM*, *LEC2* and *WUS*), and of three housekeeping genes (*Actin, Beta-tubulin 1* and *Elongation Factor 1-alpha*) was also examined. This allowed an analysis to determine the correlation between the expression of specific genes and their DNA methylation status. To produce regenerable ((embryogenic callus (EC)) and non-regenerable calli or cells (dry callus (DC), watery callus (WC) and cell suspension (CS)), the auxin-supplementation method of Yusuf et al. (2011) and Wong et al. (2013) was followed, and these samples were compared to *ex vitro* leaf (EVL), meristematic block of newly emerged shoots (MB) and *in vitro* leaf of regenerated plants (IVL) of *B. rotunda* to determine gene expression and DNA methylation status. The results of this study are described in this chapter.

4.1 Morphology and confirmation of embryogenic and regenerable competence of callus and cell suspension culture of *B. rotunda*

For the documentation of the morphology of callus types and cell suspension culture, microscopy analysis was carried out. It was observed that morphologically embryogenic callus i.e. EC was white-yellowish, globular and friable, whereas non-embryogenic calli i.e. DC was brown, nodular and dry, and WC was spongy, soft and wet. Under stereo microscope, embryo structures were observed in EC (Figure 4.1C, D), while DC and WC did not show any embryo structure (Figure 4.1E, F; 4.1G, H, respectively). DC became hard callus clumps that resist growth upon sub-culture while cells in EC were observed as globular, translucent spheres which differentiate and develop into somatic embryos for germination. Although the cell suspension i.e. CS was established from EC, after maintenance for 12 months, these cells were non-embryogenic in nature (Figure 4.1I, J). Histological section revealed that embryogenic callus showed dense cytoplasm with prominent nuclei and laden with protein giving rise to blue-black stained cells, whereas non-embryogenic cells showed vacuolated and did not comprise of embryogenic cells which showed blue coloration (previously described in other papers from our group by Yusuf et al., 2011; Wong et al., 2013; Ng et al., 2016; Appendix B). After incubation on MS0 regeneration media, all the EC produced shoots, while the non-embryogenic calli and cells suspension i.e. DC, WC and CS were unable to regenerate shoots (Table 4.1 and Figure 4.1K; Appendix B).

Table 4.1: Regenerability of embryogenic callus, non-embryogenic callus and long-term cell suspension cultures

Types of *in vitro* samples	Level of 2,4-D added to media (mg/L)	No. of calli incubated for regeneration	Calli with regenerated shoots (%)	No. of shoots/ callus (±SE)	Nature of calli/ regeneration ability
Watery Callus (WC)	1	90	0	0	[1]Non-embryogenic/ [2]Non-regenerable
Embryogenic Callus (EC)	3	90	100	4.4±0.3	[1]Embryogenic/ [2]Regenerable
Prolonged Cell Suspension (established from embryogenic callus and maintained for 12 months (CS)	3	90	0	0	[1]Non-embryogenic/ [2]Non-regenerable
Dry Callus (DC)	4	90	0	0	[1]Non-embryogenic/ [2]Non-regenerable

[1]Embryogenic nature of calli determined by presence / absence of embryo-like structures under sterio microscopy at 4 weeks after culture as described in Yusuf et al. (2011), Wong et al. (2013) and Ng et al. (2016). [2]The regeneration capacity was recorded after 8 weeks of culture on regeneration media.

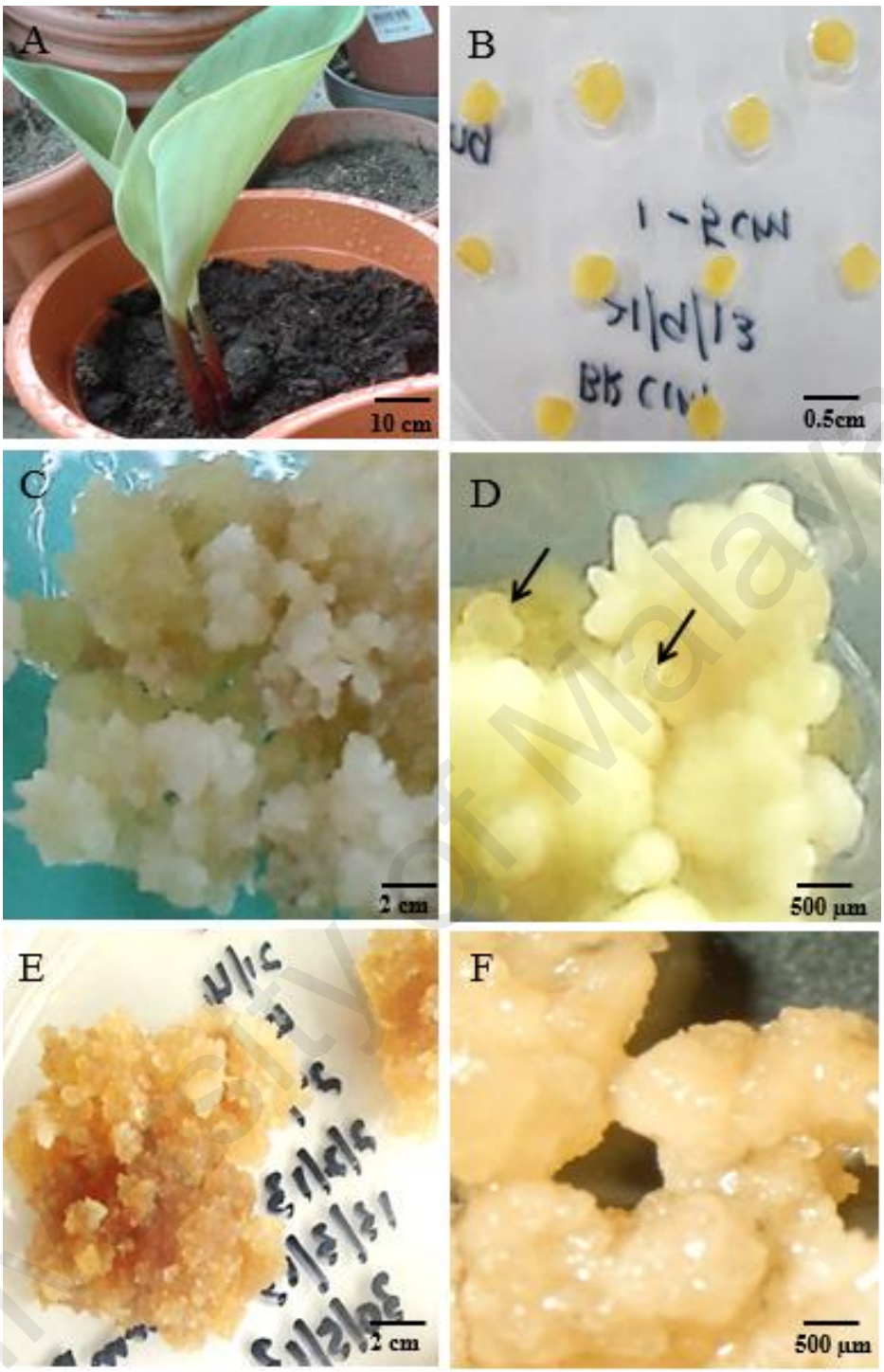


Figure 4.1: Types of samples and morphology of callus and cell suspension used in this study. A: *B. rotunda* plant which was the source for *ex vitro* leaf (EVL); B: Meristematic block (MB) slices placed on callus initiation medium; C, D: Embryogenic callus (EC); E, F: Dry callus (DC); G, H: Watery callus (WC); I, J: Cell suspension (CS); K: *In vitro* plants regenerated from embryogenic callus. Black arrow indicates embryo structure in embryogenic callus.

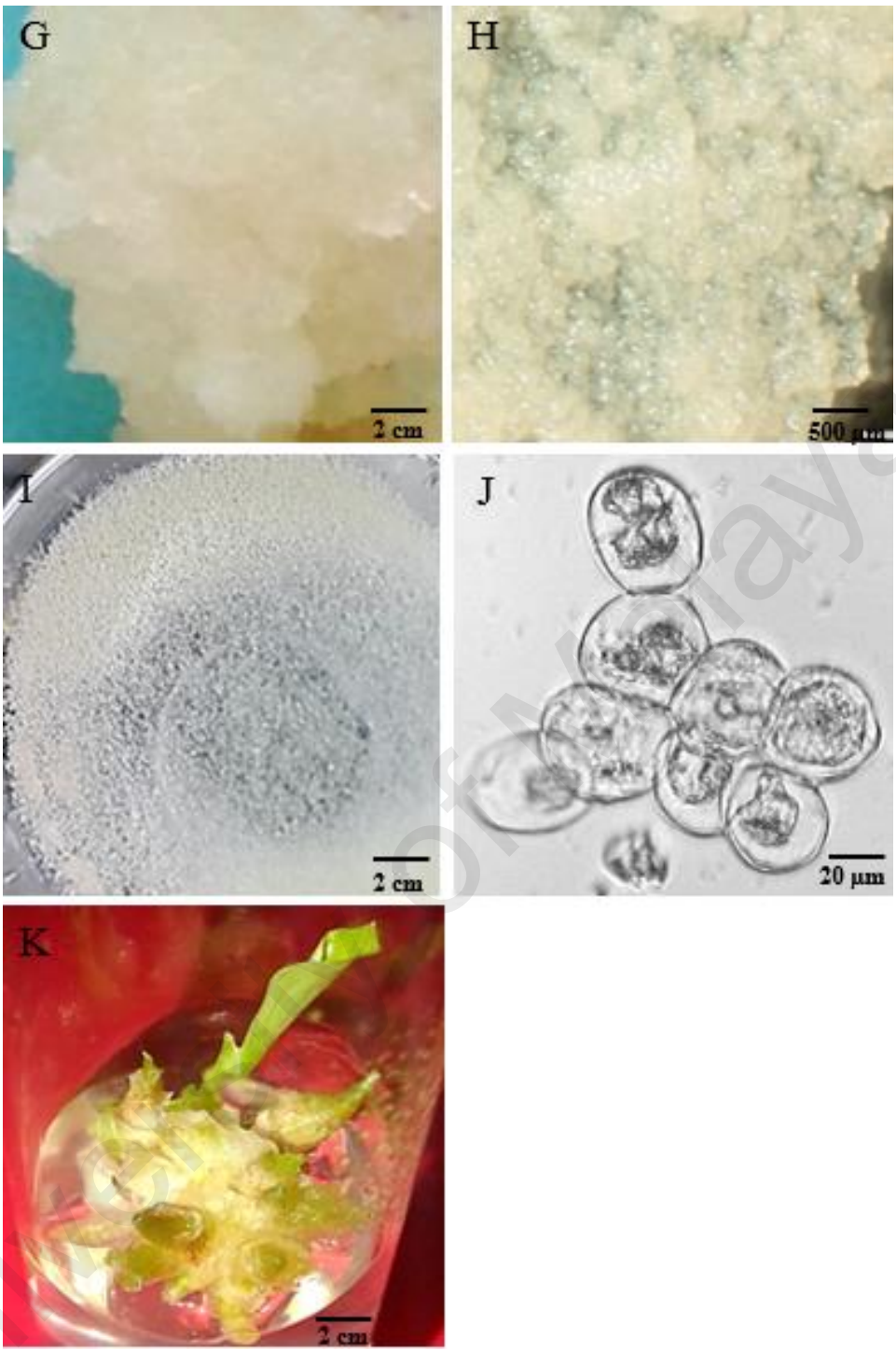


Figure 4.1, continued

4.2 RNA yield and quality

A modified cetyl trimethyl amonium bromide (CTAB)-based method for total RNA isolation described by Kiefer et al. (2000) was suitable to yield intact total RNA from *ex vitro* and *in vitro* calli and tissues of *B. rotunda* as 25S rRNA showed more band intensity than that of the 18S rRNA (Figure 4.2). All seven samples (EVL, MB, EC, DC, WC, CS and IVL) used for gene expression analysis by qRT-PCR and RNA-seq exhibited the A_{260nm}/A_{280nm} ratio about 2.0 and an A_{260nm}/A_{230nm} ratio greater than 1.0 (Table 4.2). The ratio of 25S and18S rRNA of the isolated RNA as determined by spectrophotometry was greater than 1.0, and RNA Integrity Number (RIN) was higher than 7.0 for all seven samples (Table 4.3 and Appendix C).

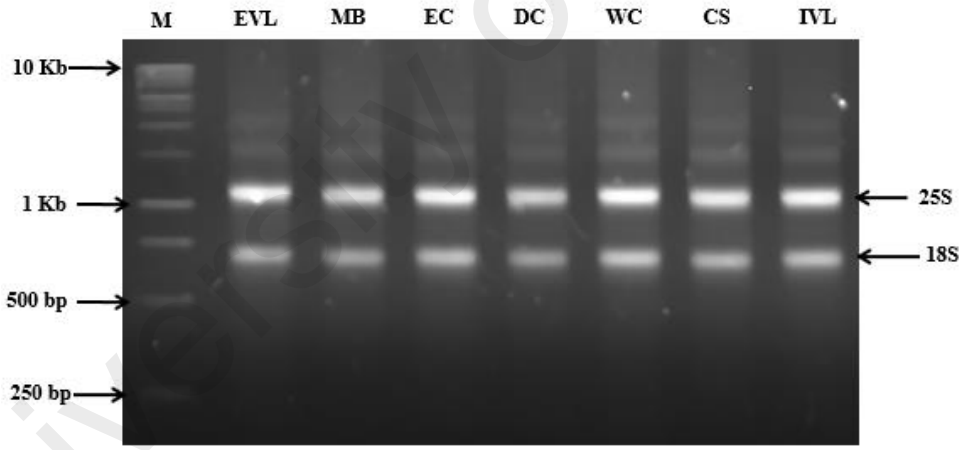


Figure 4.2: Agarose gel electrophoresis image for RNA samples of different types of calli and tissues of *B. rotunda*. EVL: *ex vitro* leaf; MB: meristematic block; EC: embryogenic callus; DC: dry callus; WC: watery callus; CS: cell suspension and IVL: *in vitro* leaf; M: 1 kb ladder (Promega, USA).

Table 4.2: Optical density readings of RNA samples extracted from different types of calli and tissues of *B. rotunda* determined by NanoDrop 2000 Spectrophotometer

Sample	A_{260nm}/A_{280nm}±SE	A_{260nm}/A_{230nm}±SE
EVL	2.10±0.06	1.96±0.04
MB	2.11±0.02	1.80±0.03
EC	2.07±0.04	1.90±0.01
DC	2.13±0.03	1.79±0.02
WC	2.14±0.03	1.91±0.05
CS	2.12±0.05	1.79±0.04
IVL	2.07±0.06	1.61±0.02

Table 4.3: Output of Agilent's Bioanalyzer 2100 assay for RNA samples extracted from different types of calli and tissues of *B. rotunda*

Sample	Concentrations (ng/µl)±SE	25S:18S±SE	RIN±SE
EVL	173.3±5	1.2±0.01	7.6±0.03
MB	545.4±3	1.1±0.01	7.4±0.02
EC	558.2±4	1.4±0.02	7.2±0.01
DC	556.1±5	1.8±0.01	7.7±0.03
WC	393.2±2	1.8±0.02	7.6±0.02
CS	132.4±3	1.3±0.03	7.7±0.04
IVL	391.5±4	1.3±0.02	7.5±0.02

4.3 DNA yield and quality

A CTAB-based method for DNA isolation (modified from Doyle (1990)) was able to extract DNA from all seven samples (EVL, MB, EC, DC, WC, CS and IVL) of *B. rotunda* used in this study as shown in Figure 4.3. Neither protein contamination nor genomic DNA degradation were observed on the gel. The A_{260nm}/A_{280nm} ratios were between 1.7-1.9 and the A_{260nm}/A_{230nm} greater than 1 (Table 4.4).

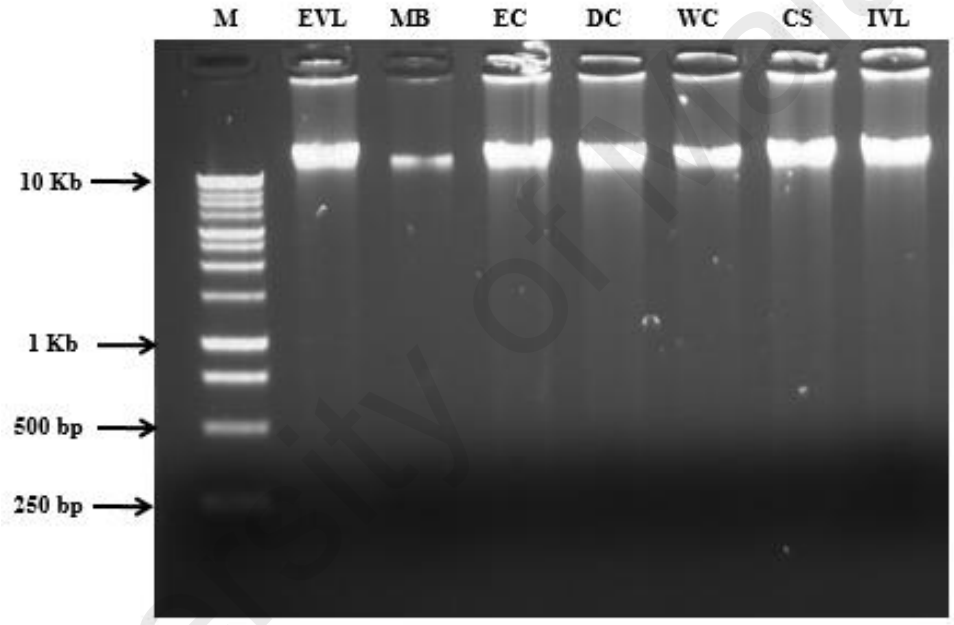


Figure 4.3: Agarose gel electrophoresis image for DNA samples from different types of calli and tissues of *B. rotunda*. EVL: *ex vitro* leaf; MB: meristematic block; EC: embryogenic callus; DC: dry callus; WC: watery callus; CS: cell suspension and IVL: *in vitro* leaf; M: 1 kb ladder (Promega, USA).

Table 4.4: Concentrations and optical density readings of DNA samples extracted from different types of calli and tissues of *B. rotunda* determined by NanoDrop 2000 Spectrophotometer

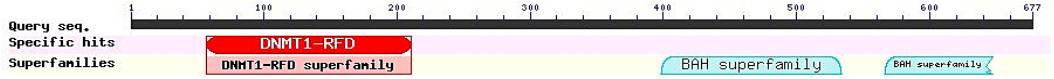
Samples	Concentrations (ng/µl) ±SE	A_{260nm}/A_{280nm}±SE	A_{260nm}/A_{230nm}±SE
EVL	890.2±6	1.85±0.03	1.41±0.06
MB	668.2±4	1.77±0.02	1.24±0.11
EC	1111.2±5	1.87±0.03	1.28±0.09
DC	1045.8±4	1.84±0.01	1.42±0.05
WC	945.5±3	1.86±0.04	1.35±0.06
CS	829.6±5	1.88±0.03	1.32±0.05
IVL	817.7±2	1.75±0.02	1.15±0.08

4.4 Homology searching and phylogenetic analysis of *B. rotunda MET1, CMT3, DRM2, SERK, BBM, LEC2* and *WUS* sequences

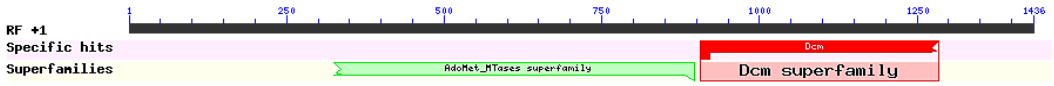
To validate the similarity and identity of the sequences used in this study (*MET1* (KY290867), *CMT3* (KY290868), *DRM2* (KY290869), *SERK* (KY290870), *BBM* (KY290873), *LEC2* (KY290872) and *WUS* (KY290871)) obtained from our previous *B. rotunda* transcriptome data (Md-Mustatfa et al., 2014), the FASTA format of these unigene sequences was subjected to BLAST using National Center for Biotechnology Information (NCBI) database (v 2.6.0+) through http://www.ncbi.nlm.nih.gov/. BLAST results revealed that all seven gene sequences of *B. rotunda* showed high similarity with gene sequences annotated for the same functions in the Genbank database. The similarity score was >200 for *MET1*, *CMT3*, *DRM2*, *SERK*, *BBM* and *LEC2*, and 80-200 for *WUS* (Appendix D). Considerable sequence identity was also observed; such as the identity with the best scoring 15 sequences of 58-72% for *MET1*, 59-75% for *CMT3*, 67-79% for *DRM2*, 74-89% for *SERK*, 53-59% for *BBM*, 49-77% for *LEC2* and 46-50% for *WUS* (Appendix B). All *B. rotunda* genes showed the highest similarity and identity scores with the sequences from *Musa acuminata* subsp. malaccensis followed by *Phoenix dactylifera* and *Elaies guineensis*.

Conserved domains analysis of *B. rotunda* MET1, CMT3, DRM2, SERK, BBM, LEC2 and WUS proteins obtained from NCBI Conserved Domains Search Tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) revealed that BrMET1 contains domains from the BAH and DNMT1-RFD superfamily (Figure 4.4A); BrCMT3 and BrDRM2 contains domains from the Dcm superfamily (Figure 4.4B, C); BrSERK contains Leucine-rich repeat (LRR) and Protein kinase (PKc) domains (Figure 4.4D);

A



B



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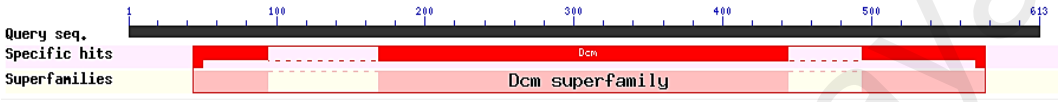

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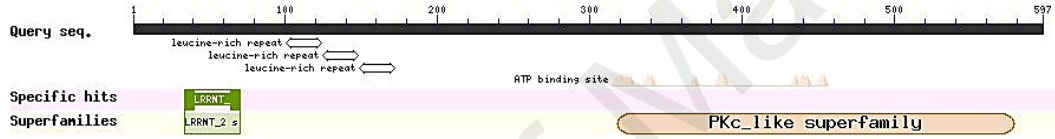

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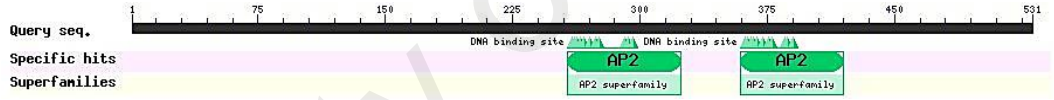


F

G

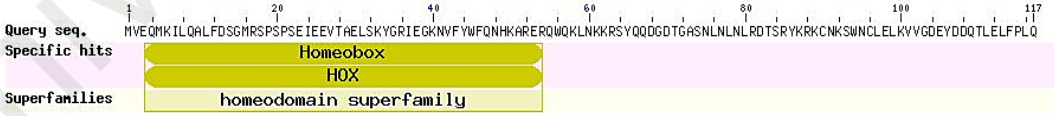


Figure 4.4: Conserved domains of *B. rotunda* proteins. A: MET1, B: CMT3, C: DRM2, D: SERK, E: BBM, F: LEC2 and G: WUS.

BrBBM contains two Apetala2 (AP2) domains (Figure 4.4E); BrLEC2 contains B3 domain (Figure 4.4F) and BrWUS contains a homeobox domain (Figure 4.4G).

The alignment results of translated amino acid sequences of *B. rotunda* also showed the similarity with their homolog proteins reported in the databases (Appendix E). Phylogenetic analysis (using the 15 best scoring respective amino acid sequences obtained from protein databases (nr) through BLASTx (v 2.6.0+) indicated that MET1, CMT3, DRM2, SERK, BBM and LEC2 *B. rotunda* sequences clustered with those of *Musa acuminata*, *Elaies guineensis* and *Phoenix dactylifera* (Figure 4.5A-F), whereas *B. rotunda* WUS clustered with *M. acuminata, Cynara cardunculus* and *Morus notabilis* (Figure 4.5G). BrMET1, BrCMT3, BrDRM2, BrSERK, BrBBM, BrLEC2 and BrWUS (where Br indicates *B. rotunda*) displayed very close relationship with *M. acuminata* MET1 (XP_009398381.1, XP_009398383.1) (Figure 4.5A), *M. acuminata* CMT3 (XP_009392766.1) (Figure 4.5B), *M. acuminata* DRM2 (XP_009393227.1) (Figure 4.5C), *M. acuminata* SERK (XP_009390528.1) (Figure 4.5D), *M. acuminata* BBM (XP_009413817.1) (Figure 4.5E), *M. acuminata* LEC2 (XP_009413626.1) (Figure 4.5F) and *M. acuminata* WUS (XP_009417388.1) (Figure 4.5G), respectively.

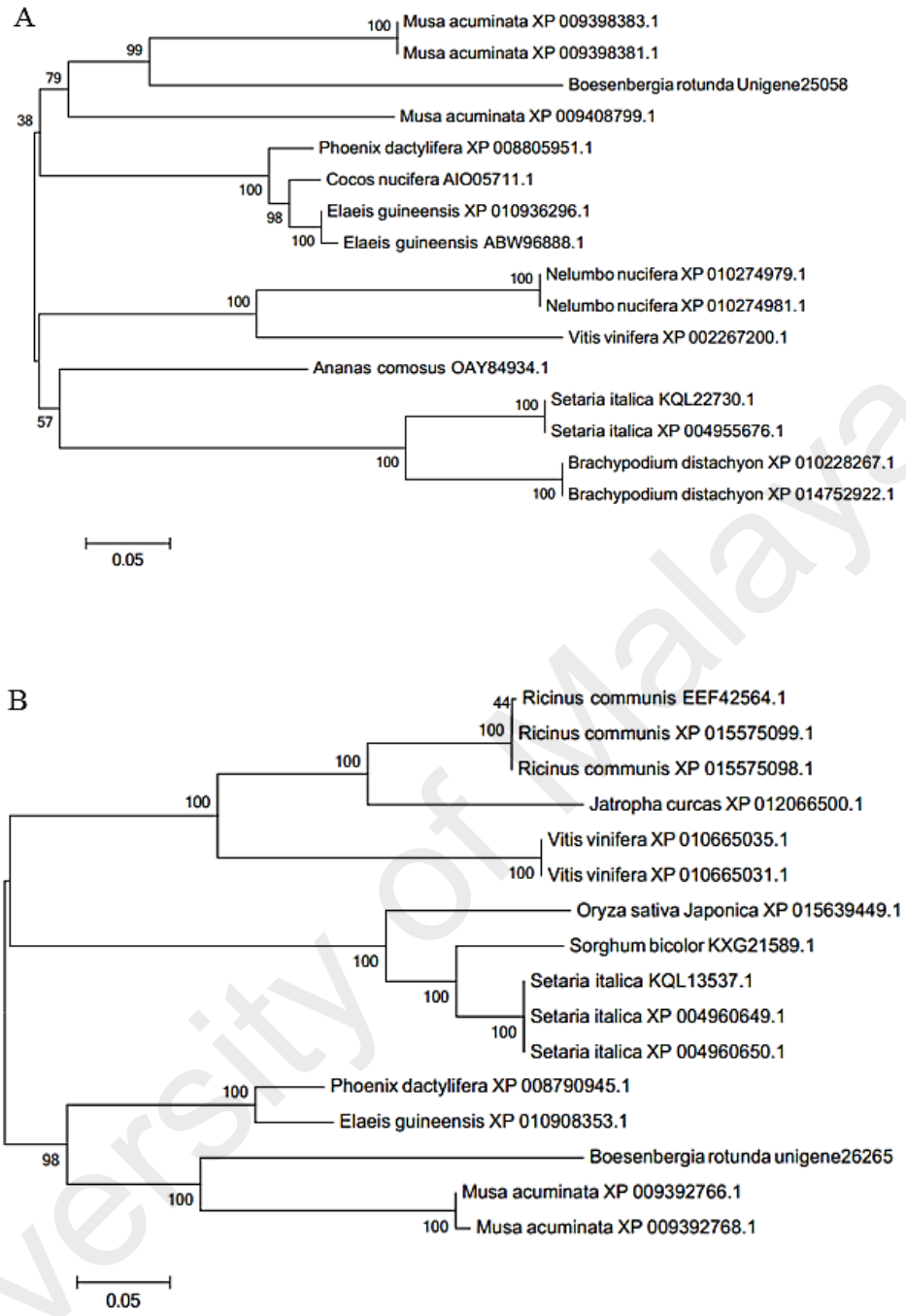
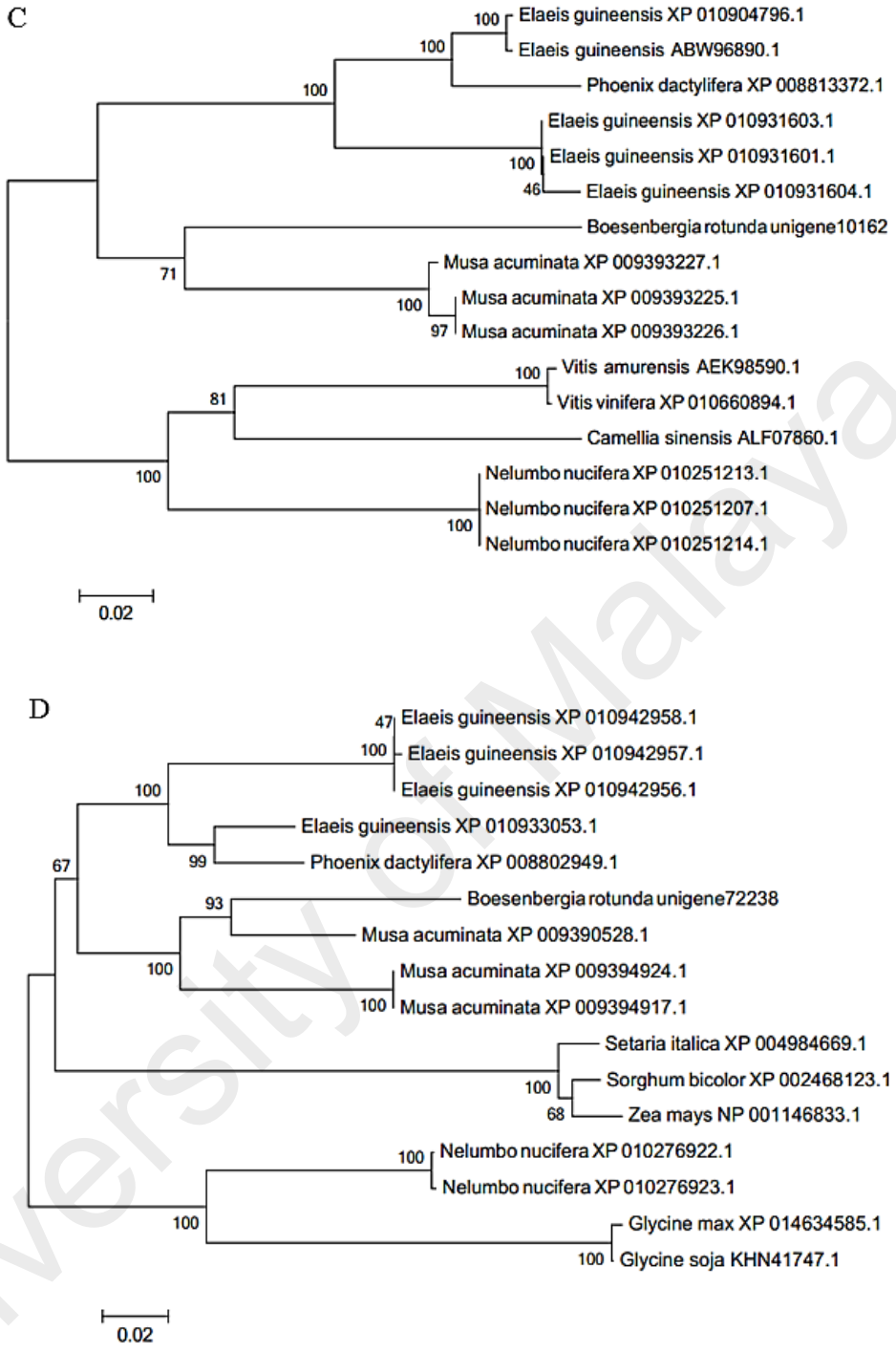


Figure 4.5: Phylogenetic analysis of *B. rotunda* proteins. A: MET1; B: CMT3; C: DRM2; D: SERK; E: BBM; F: LEC2 and G: WUS. The phylogenetic tree was constructed with most likely 15 proteins with *B. rotunda* protein using neighbor-joining method using MEGA 6.0 software.



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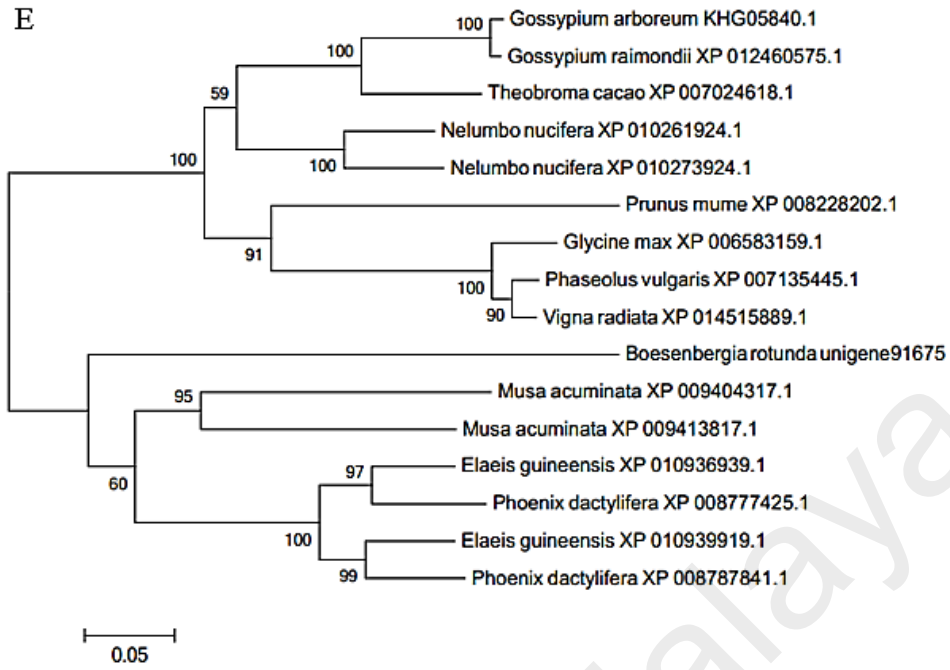

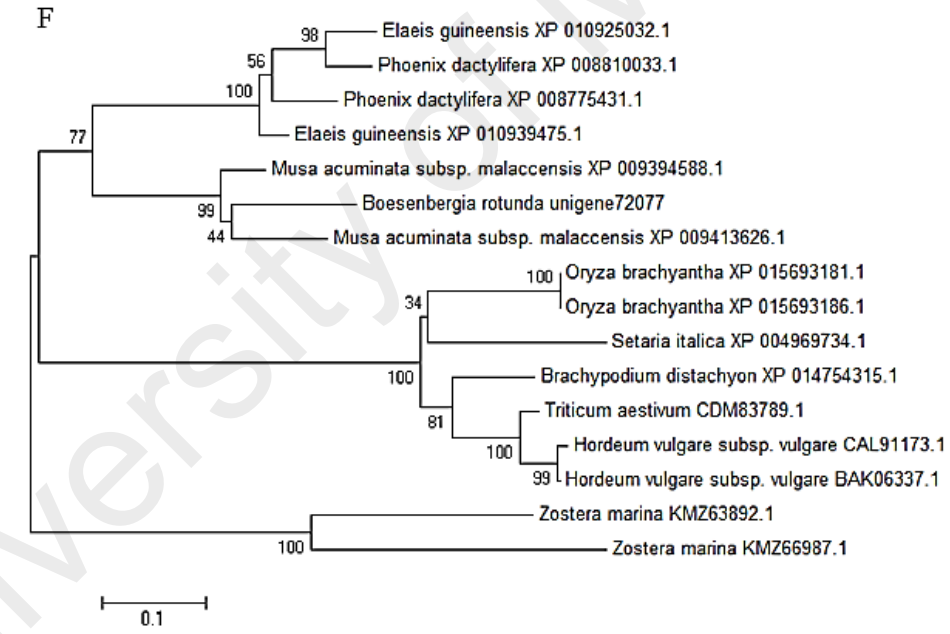

Figure 4.5, continued

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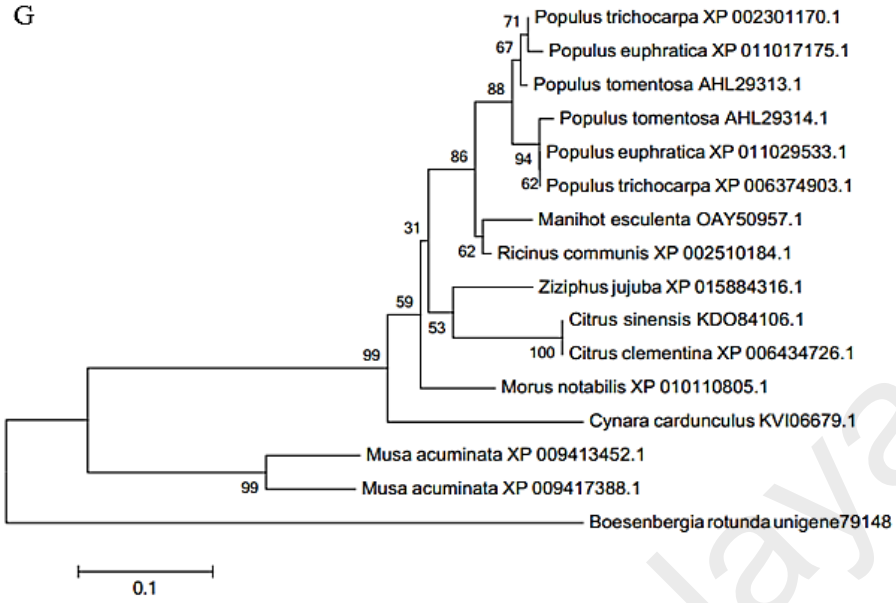

Figure 4.5, continued

4.5 Gene expression in different types of calli / tissues

4.5.1 Expression profile of DNA methyltransferase genes *MET1*, *CMT3* and *DRM2*

The expression levels of *METHYLTRANSFERASE 1* (*MET1*), *CHROMOMETHYLASE 3* (*CMT3*) and *DOMAIN REARRANGED METHYLTRANSFERASE 2* (*DRM2*) were determined by Quantitative Reverse Transcription-PCR (qRT-PCR) and by transcriptome sequencing (RNA-seq). *18S rRNA* was used as an internal control for qRT-PCR to observe differences in expression of DNA methylation pathway genes among *ex vitro* and *in vitro* tissues, embryogenic and non-embryogenic calli, and prolonged cell suspension culture of *B. rotunda*.

qRT-PCR results revealed that the expression of DNA methyltransferase *MET1* 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 (Figure 4.6A). Although the CS was established from EC, after long-term maintenance, the cells showed a significantly decreased level of *MET1* expression compared to EC. After being normalized to transcript per million (TPM) of RNA-seq data, it was observed that the expression of *MET1* was the highest in EC, followed by EVL and IVL (RNA-seq of MB was not performed), which coincide with the qRT-PCR results (Figure 4.6A). Among *in vitro* calli and cells, RNA-seq results showed that the expression of *MET1* was the highest in EC followed by DC and WC (i.e. EC>DC>WC) which was similar to the qRT-PCR results, but unlike the qRT-PCR result, DC showed comparatively higher expression of *MET1* than WC and CS.

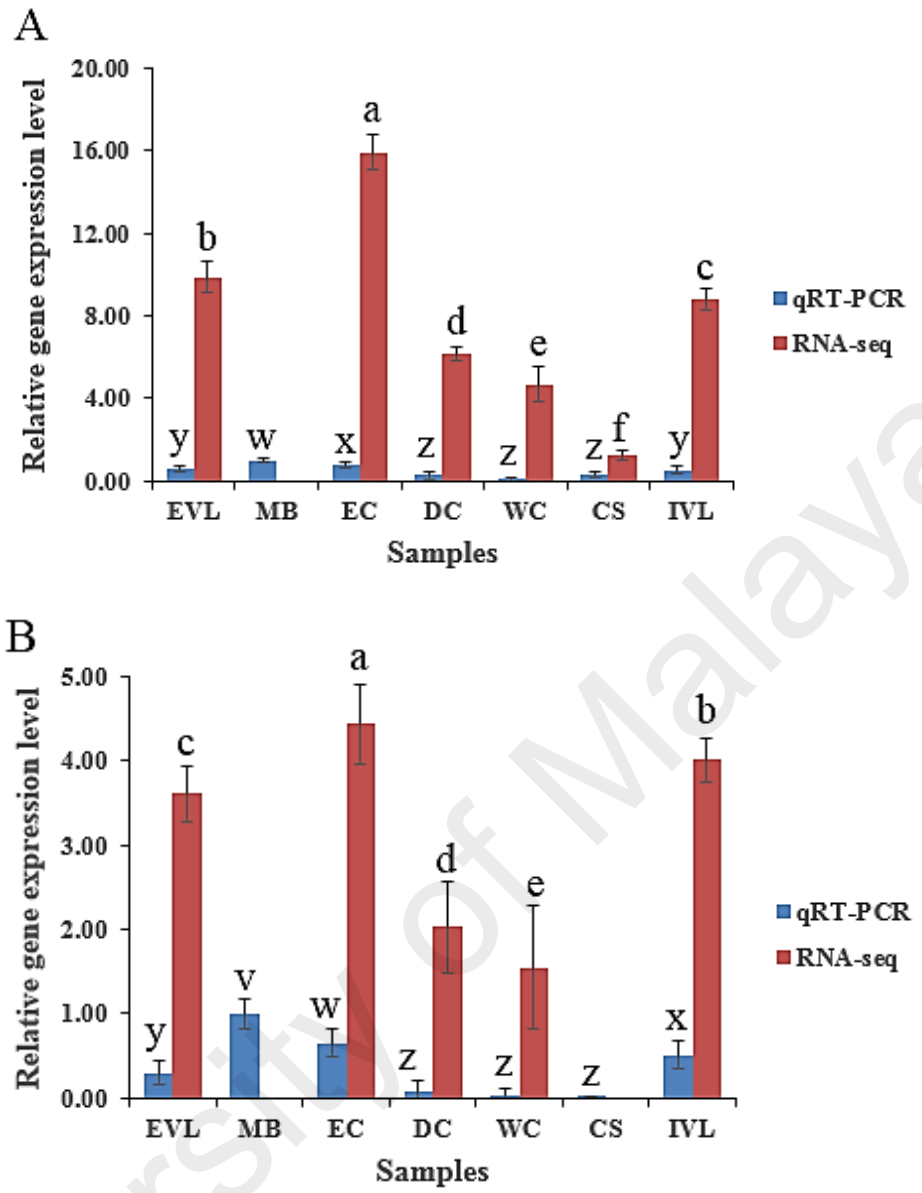

Figure 4.6: Relative gene expression of DNA methyltransferases in *ex vitro* and *in vitro* tissues and calli using qRT-PCR and RNA-seq. A: *MET1*; B: *CMT3* and C: *DRM2*. Blue bars represent relative expression levels determined by qRT-PCR using a $2^{-\Delta\Delta CT}$ method and red bars represent transcript abundance changes calculated by the TPM (transcript per million) method based on RNA-seq data sets. 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 qRT-PCR. Bars represent the standard error of three biological replicates. Letters indicate statistical significance, where the same letter indicates no significant difference between samples, according to Tukey's comparison test ($P > 0.05$), (v, w, x, y, z for qRT-PCR data; a, b, c, d, e, f for RNA-seq data). Note, the MB sample was not assayed by RNA-seq.

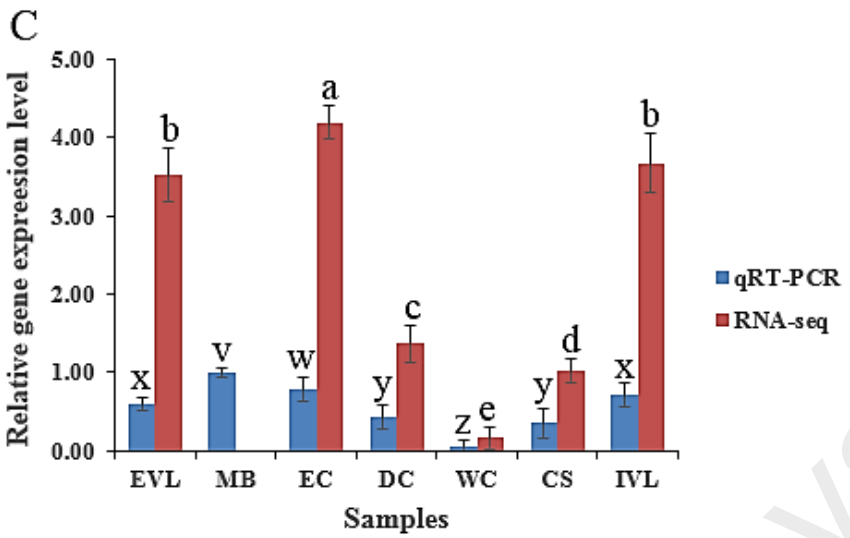

Figure 4.6, continued

The highest expression level of *CMT3* using qRT-PCR analysis was observed in MB, followed by EC and IVL (Figure 4.6B). The DC, WC and prolonged CS exhibited relatively low levels of transcripts; however, the expression in EC was higher than in DC, WC and CS. RNA-seq data exhibited a similar pattern of expression of *CMT3* to that determined by qRT-PCR, where the highest expression was observed in EC followed by IVL (Figure 4.6B). Among *in vitro* calli and cells, EC showed comparatively higher expression than DC and WC, and no expression was noticed in CS.

The pattern of expression of *DRM2* using qRT-PCR and RNA-seq among the samples was similar (Figure 4.6C). Based on qRT-PCR results, MB showed the highest expression level followed by EC, EVL and IVL. Unlike *MET1* and *CMT3*, EVL and IVL showed same level of expression of *DRM2* based on both methods. Among *in vitro* calli and cells (i.e. EC, DC, WC and CS), both qRT-PCR and RNA-seq results revealed that the highest expression of *DRM2* was in EC, like *MET1* and *CMT3* (Figure 4.6C). EC also showed markedly higher expression than CS, although CS was originated from EC.

4.5.2 Expression profile of somatic embryogenesis related genes, *SERK, BBM, LEC2* and *WUS*

To analyze the relationship between the expression of four somatic embryogenesis and plant regeneration related genes and embryogenic competence, the expression levels were compared in different types of calli or cells (embryogenic i.e. EC, and non-embryogenic i.e. DC, WC and CS); and the expression was also measured in *ex vitro* leaf (EVL), meristematic block (MB), and *in vitro* leaf of regenerated plants (IVL) using qRT-PCR and RNA-seq data. Meristematic block (MB) was used as the calibrator (value set as 1) for qRT-PCR, and housekeeping gene, *Actin* was used as for endogenous control.

4.5.2.1 *Somatic embryogenesis receptor-like kinase* (*SERK*)

qRT-PCR results revealed that among different types of calli and cells, the highest expression of *SERK* was in EC (Figure 4.7A). Although the CS was established from EC, the cells which showed loss of embryogenesis and regenerability after long-term maintenance, displayed a very low level of expression (Figure 4.7A). IVL, MB and EVL also showed comparatively higher expression than non-embryogenic calli and cells i.e. WC and CS, but lower than EC. After being normalized to transcript per million (TPM) of RNA-seq data, it was also observed that the expression of *SERK* was the highest in EC, and was not detectably expressed in CS, which agrees well with the qRT-PCR data. But, unlike the qRT-PCR data, DC showed comparatively higher expression than IVL and EVL in RNA-seq data.

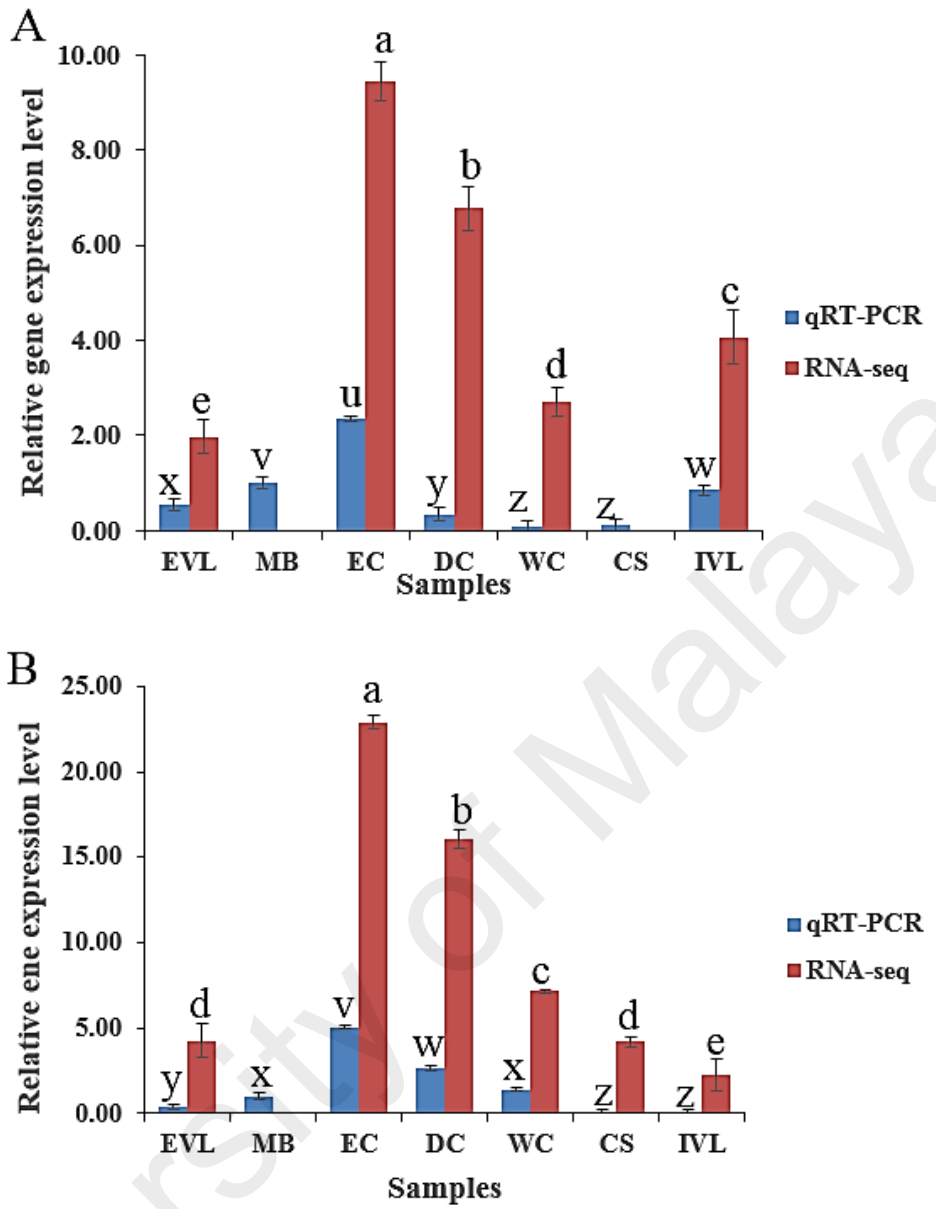

Figure 4.7: Relative gene expression of somatic embryogenesis related genes in *ex vitro* and *in vitro* tissues and calli using qRT-PCR and RNA-seq. A: *SERK*; B: *BBM*; C: *LEC2*; D: *WUS*. Blue bar indicates relative expression level determined by qRT-PCR using $2^{-\Delta\Delta CT}$ method and dark red bar indicates transcript abundance changes calculated by the TPM (transcript per million) 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) for qRT-PCR, and *Actin* was used as the internal control. Bars represent the standard error of three biological and three technical replicates for qRT-PCR, and three biological replicates for RNA-seq. Letters indicate statistical significance, where the same letter indicates no significant difference between samples, according to Tukey's comparison test ($P > 0.05$), (u, v, w, x, y, z for qRT-PCR data; a, b, c, d, e for RNA-seq data). Note, the MB sample was not assayed by RNA-seq.

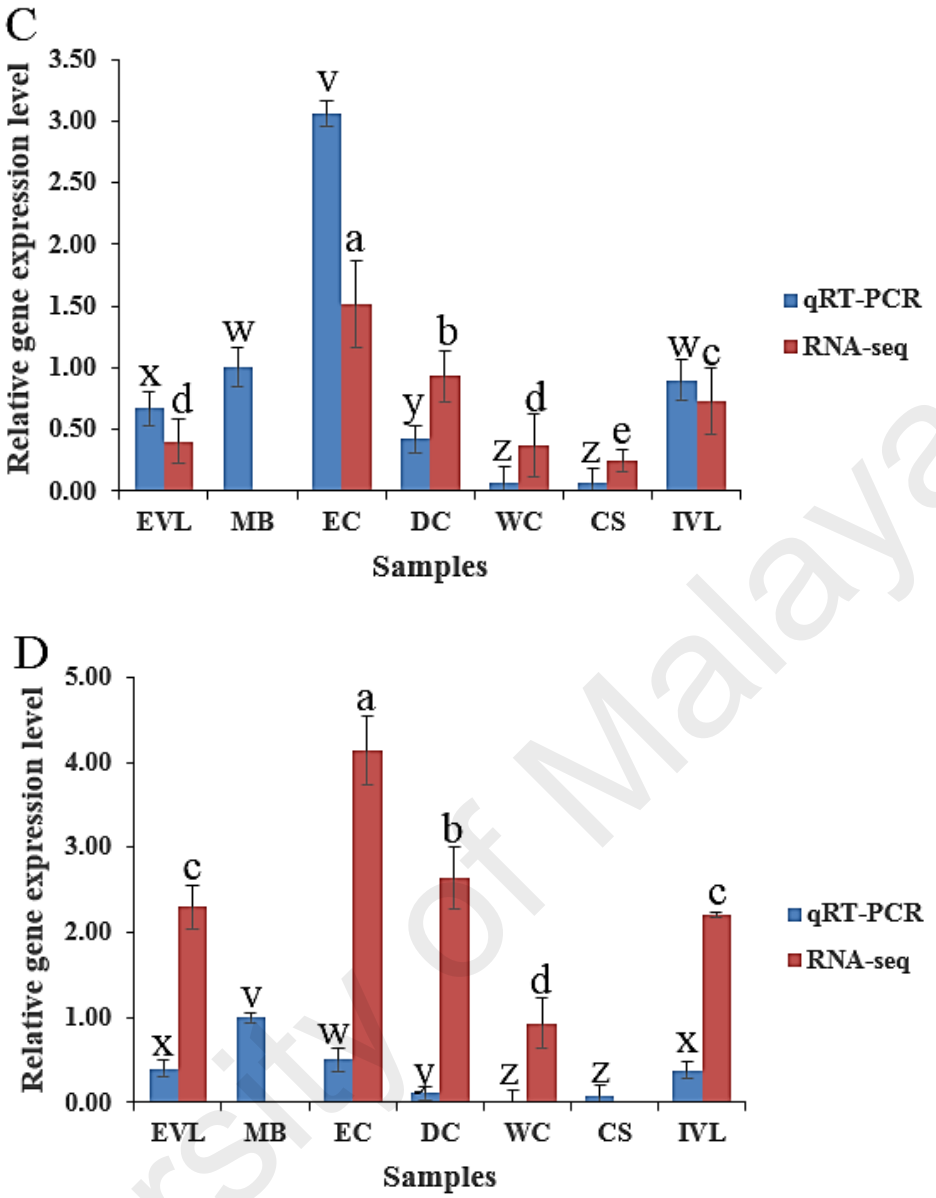

Figure 4.7, continued

4.5.2.2 *Baby boom* (*BBM*)

The expression of *BBM* determined by qRT-PCR and RNA-seq showed the highest expression in EC (Figure 4.7B). In contrast to *SERK*, non-embryogenic calli (i.e. DC, WC and CS) showed significantly higher expression of *BBM* than IVL, MB and EVL, but lower than EC (Figure 4.7B). Among all seven samples, IVL showed the lowest expression of *BBM* based on both methods.

4.5.2.3 *Leafy cotyledon 2* (*LEC2*)

Both qRT-PCR and RNA-seq results revealed that the expression pattern of *LEC2* was similar to that of *SERK*, where the highest expression was also observed in EC, the lowest expression was in CS. WC also showed comparatively lower expression (Figure 4.7C). In contrast to *BBM*, the qRT-PCR result revealed that the expression of *LEC2* was significantly higher in IVL, MB and EVL than in non-embryogenic calli (i.e. DC and WC). But, as observed for *SERK*, the RNA-seq results showed comparatively higher expression of *LEC2* in DC than in IVL and EVL (Figure 4.7C).

4.5.2.4 *Wuschel* (*WUS*)

qRT-PCR results exhibited the highest expression of *WUS* in MB followed by EC. RNA-seq results exhibited the highest expression in EC followed by DC. EVL and IVL showed same level expression of *WUS* based both methods. However, like *SERK* and *BBM*, among all the calli and cells, the highest and the lowest expression of *WUS* was noticed in EC and CS, respectively based on both qRT-PCR and RNA-seq (Figure 4.7D).

4.5.3 Dissociation curve analysis of DNA methyltransferase and somatic embryogenesis related genes

Since an important means of quality control in qRT-PCR is to check that all samples have a similar melting temperature (Tm), dissociation curve analysis step was done at the end of the amplification reactions to determine the melting point of the products, and to validate the specific amplification. All assays were performed using ExpressionSuite Software (version 1.0.4., Applied Biosystems, Life Technologies, Thermo Fisher Scientific, USA) with three biological replicates each with three technical replicates. Specific amplicons were identified for all *B. rotunda* genes tested in all seven samples (Appendix F). All peaks of biological and technical replicates in all seven samples for each gene were found at the same temperature, indicating single reaction products for all primers used in the analyses. Tm values of 79.8 °C, 79.0 °C and 82.0 °C were determined for *MET1*, *CMT3* and *DRM2*, and Tm values of 81.1 °C, 80.5 °C, 74.2 °C and 82.0 °C were determined for *SERK*, *BBM*, *LEC2* and *WUS* in all seven samples of *B. rotunda*. Dissociation curve analysis for *18S rRNA* and the *Actin* gene was also performed as the endogenous control with Tm values of 83 °C and 77.8 °C, respectively (Appendix F). These results confirmed that there was no unspecific amplification, primer dimer, mispairing or contamination in the reactions.

4.6 Exploring the changes in DNA methylation patterns in different types of calli / tissues of *B. rotunda*

4.6.1 DNA methylation analysis using Methylation Sensitive Amplified Polymorphism (MSAP)

MSAP markers were used to assess the changes in DNA methylation status and polymorphism in methylated DNA in different types of tissues and calli of *B. rotunda*. Nine combinations of selective MSAP primers (Table 3.2) with a 3-nucleotide specificity were used to detect cytosine methylation at the 5′-CCGG-3′ sequence context of genomic DNA extracted from EVL, MB, EC, DC, WC, CS and IVL of *B. rotunda*. Based on presence or absence of bands, four types of MSAP band patterns were observed (Figure 4.8A, B; Appendix G). These bands were classified according to the following scheme: bands were present in both *Eco*RI+*Hpa*II and *Eco*RI+*Msp*I lanes (class I); bands were present in *Eco*RI+*Msp*I lanes, but not in *Eco*RI+*Hpa*II lanes (class II); bands were present in *Eco*RI+*Hpa*II, but not in lanes *Eco*RI+*Msp*I (class III); and bands were absent from both *Eco*RI+*Msp*I and *Eco*RI+*Hpa*II lanes (Class IV) (Table 4.5 and Figure 4.8A, B). A DNA methylation event was detected when bands present in the electropherogram from the reaction *Eco*RI+*Msp*I (M) were absent from the reaction with *Eco*RI+*Hpa*II (H) or vice versa (Class II and III), and when bands were absent in one or more samples, but present in some of the samples (Class IV). The first case indicated that the internal cytosine was methylated (Hemi-methylation or fully methylation) (Class II) or the external cytosine of one DNA strand was methylated (Hemi-methylation) (Class III). In addition to class category, DNA methylation polymorphism were observed when at least one sample showed different banding pattern from the others, such as a band was present in both M and H in one sample, but was only present in M in other samples or absent in other samples (shown by box in Figure 4.8A, B). Methylation events where an obvious

difference in band intensity present were also shown DNA methylation polymorphisms (shown by arrow in Figure 4.8A, B).

In this study, the results revealed differential DNA methylation status and extensive changes in DNA methylation patterns, with a high frequency of polymorphism among all seven samples of *B. rotunda* tissues and *in vitro* calli. Nine primer combinations produced a total of 157 bands (Figure 4.8; Appendix G). Among these 157 bands, 135 (85.99%), 143 (91.08%), 117 (74.52%), 129 (82.16%), 128 (81.50%), 132 (84.08%), 138 (87.90%) bands provided evidence of DNA methylation events in MB, EVL, EC, DC, WC, CS and IVL, respectively (Table 4.6). The results indicated generally higher DNA methylation levels in MB, EVL and IVL than in the other samples. However, more replicates would be needed to confirm if there is a statistically significant difference. Among different types of calli or cells, it was observed that embryogenic callus (i.e. EC) showed a lower level of methylation than non-embryogenic calli (i.e. DC, WC and CS), and the highest and the least methylation was recorded in EVL (91.08%) and in EC (74.52%), respectively (Table 4.6). Considering all seven samples at a time, it was found that DNA methylation patterns were 100% polymorphic as no band was found at the same site in all seven samples (Figure 4.8; Appendix G). It was also noticed that class IV types of bands were the major source of DNA methylation polymorphism as the number of bands were much higher than class II and class III type of bands (Table 4.6 and Figure 4.8; Appendix G).

Table 4.5: DNA methylation sensitivity and restriction pattern of isochizomers

Class of Bands	Methylation status		Digestibility of isochizomers and banding patterns			
			***Hpa*II**	***Msp*I**	**H**	**M**
Class I	CCGG GGCC	Unmethylation	Active	Active	1	1
Class II	C**C**GG GGCC	Hemi-methylation	Inactive	Active	0	1
	CCGG GG**C**C	Hemi-methylation			0	1
	C**C**GG GG**C**C	Full methylation			0	1
Class III	**C**CGG GGCC	Hemi-methylation	Active	Inactive	1	0
	CCGG GGC**C**	Hemi-methylation			1	0
Class IV	**CC**GG GGCC	Hemi-methylation	Inactive	Inactive	0	0
	CCGG GG**CC**	Hemi-methylation			0	0
	CCGG GGC**C**	Full methylation			0	0
	CCGG GGC**C**	Full methylation			0	0
	CCGG GG**CC**	Full methylation			0	0
	CCGG GG**CC**	Full methylation			0	0
	Mutation	Unknown	Inactive	Inactive	0	0

H indicates the enzyme combination of *Eco*RI+*Hpa*II; M indicates the enzyme combination of *Eco*RI+*Msp*I; 1: band present, 0: band absent. Red-marked 'C' denotes methylated cytosine.

Table 4.6: Analysis of DNA methylation patterns detected by methylation-sensitive amplified polymorphism (MSAP) in calli or cells and tissues of *B. rotunda*

MSAP Band Types	MB	EVL	EC	DC	WC	CS	IVL
I (unmethylation)	22	14	40	28	29	25	19
II (methylation)	31	31	11	26	28	20	29
III (methylation)	14	20	7	15	16	15	24
IV (methylation)	90	92	99	88	84	97	85
Total bands	157	157	157	157	157	157	157
Total methylated bands[a]	135	143	117	129	128	132	138
Total methylation ratio (%)[b]	85.99	91.08	74.52	82.16	81.50	84.08	87.90

[a]Total methylated bands = II + III + IV

[b]Total methylation % = [(II + III + IV)/(I + II + III + IV)] × 100

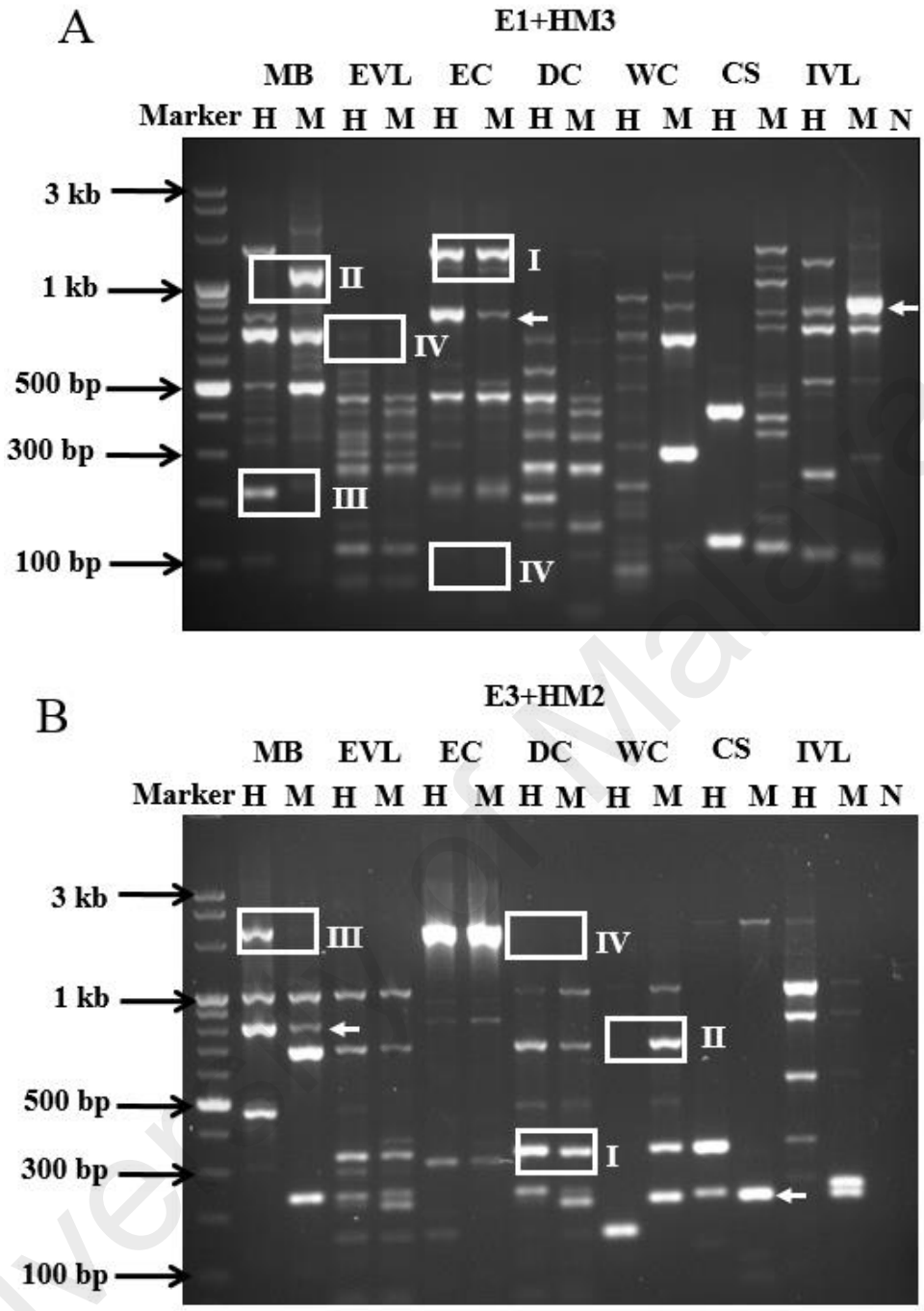

Figure 4.8: MSAP electrophoresis patterns in different types of calli and tissues of *B. rotunda* using the primer combination E1+HM3 (A) and E3+HM2 (B). EVL: *ex vitro* leaf; MB: meristematic block; EC: embryogenic callus; DC: dry callus; WC: watery callus; CS: cell suspension and IVL: *in vitro* leaf. H and M indicate digestion with *Eco*RI+*Hpa*II and *Eco*RI+*Msp*I, respectively. Rectangle indicates different types of methylation patterns and white arrow indicates methylation polymorphism due band intensity. Marker 100 bp ladder (Promega, USA).

4.6.2 DNA methylation analysis using Bisulfite Sequencing (BS-seq)

The differences of methylation patterns in different tissue and calli might be correlated with nature of *ex vitro* and *in vitro* cells and tissues. MSAP shows methylation level at 5′-CCGG-3′ sites, but is unable to provide complete cytosine methylation profiles. Therefore, bisulfite sequencing was performed to determine genome-wide methylation, and gene specific methylation at all three contexts (CG, CHG and CHH) based on mapping with *B. rotunda* transcriptome. The results are shown below.

4.6.2.1 Mapping of BS-seq data against *B. rotunda* transcriptome

After trimming low quality reads of BS-seq data, about 260.3, 225.6, 246.2, 249.0, 246.2 and 282.3 million reads were generated for EVL, EC, DC, WC, CS and IVL, respectively (BS-seq was not performed for MB) (Table 4.7). These reads were mapped to *B. rotunda* transcriptome sequence as a reference. The mapping output exhibited that about 5-10% reads were aligned to *B. rotunda* transcriptome (Table 4.7 and Figure 4.9A-F), where EVL and IVL showed the lowest alignment (5%) (Figure 4.9A, F) and EC showed the highest alignment (10%) (Figure 4.9 B).

Table 4.7: Mapping output of paired-end bisulfite sequencing (BS-seq) data with *B. rotunda* transcriptome

Searching Category	EVL	EC	DC	WC	CS	IVL
Total Reads	260341425.33	225630993.67	246221814.33	249030260.33	246216445.33	282391538.67
Aligned Reads	5813055.33	9039260.00	7541100.00	9244787.33	6042157.33	6645682.33
Unaligned Reads	247753649.67	203962014.67	228022000.67	226729626.00	231776333.67	267860714.00
Ambiguously Aligned Reads	6774720.33	12629719.00	10658713.67	13055847.00	8397954.33	7885142.33
No Genomic Sequence	54921.33	78896.00	68966.33	85547.33	55812.00	63434.33
Total Cytosines (C)	233024412.33	371635935.33	306655398.67	381388960.00	244966187.67	270898025.33
Methylated C at CG	15422950.67	7827398.00	11286485.33	11068246.67	10642939.67	14616830.67
Unmethylated C at CG	13416037.33	50185002.67	32742171.33	45330375.00	23577241.00	20770124.33
Methylated C at CHG	17696096.00	9161453.67	13715343.33	13306667.33	12796793.00	17150453.67
Unmethylated C at CHG	16877461.33	50410101.00	34319678.33	46736894.00	25057762.67	23661445.33
Methylated C at CHH	13686808.33	12911749.00	15037316.33	15956351.67	14543482.00	14517287.33
Unmethylated C at CHH	155925058.67	241140231.00	199554404.00	248990425.33	158347969.33	180181884.00

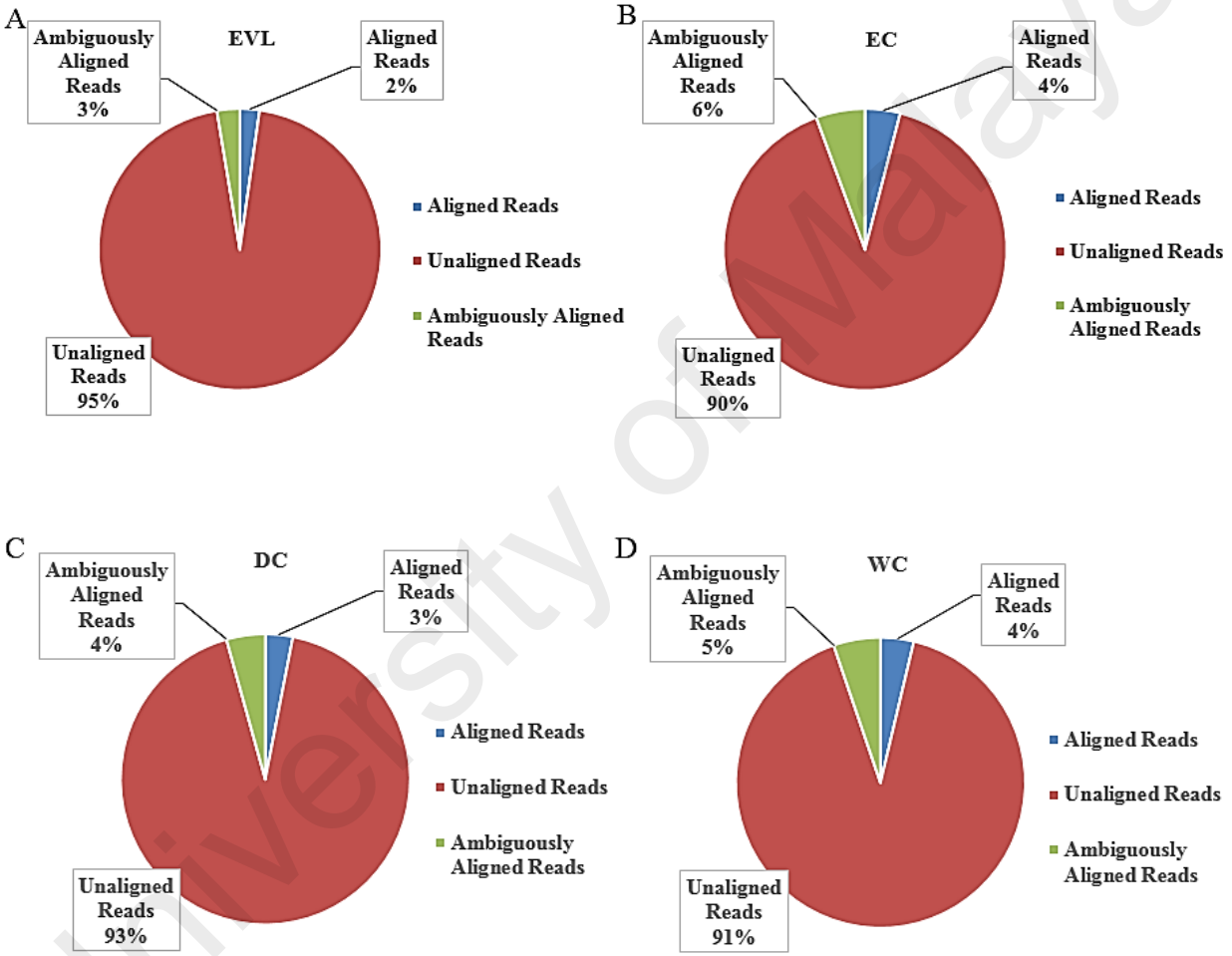

Figure 4.9: Alignment of BS-seq data with *B. rotunda* transcriptome data. A: EVL (*ex vitro* leaf); B: EC (embryogenic callus); C: DC (dry callus); D: WC (watery callus); E: CS (cell suspension) and F: IVL (*in vitro* leaf).

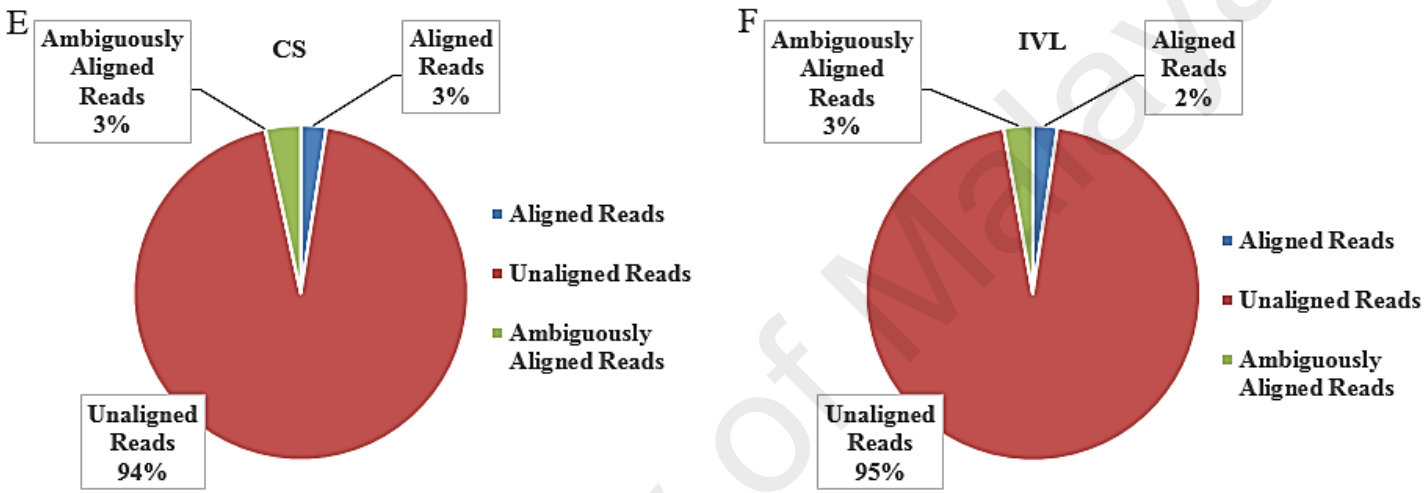

Figure 4.9, continued

4.6.2.2 Analysis of DNA methylation at CG, CHG and CHH contexts

BS-seq data revealed that the average values for 5-methylated cytosine (5mC) ranged from 7.8-15.4, 9.1-17.6 and 12.9-15.9 million at CG, CHG and CHH contexts, respectively (Table 4.7). EC showed the lowest number of 5mC among the samples at all three contexts (Table 4.7). EVL and IVL showed significantly higher numbers of 5mC than EC at both CG and CHG contexts. At CG and CHG contexts, non-embryogenic calli or cells (i.e. DC, WC and CS) showed higher numbers of 5mC than embryogenic callus (i.e. EC), but the differences were not statistically significant. At CHH context, there was no significant variation in number of 5mC among all six samples (Figure 4.10). The number of 5mC at CHG context in all six samples showed a similar pattern to the number of 5mC at CG context (Figure 4.10).

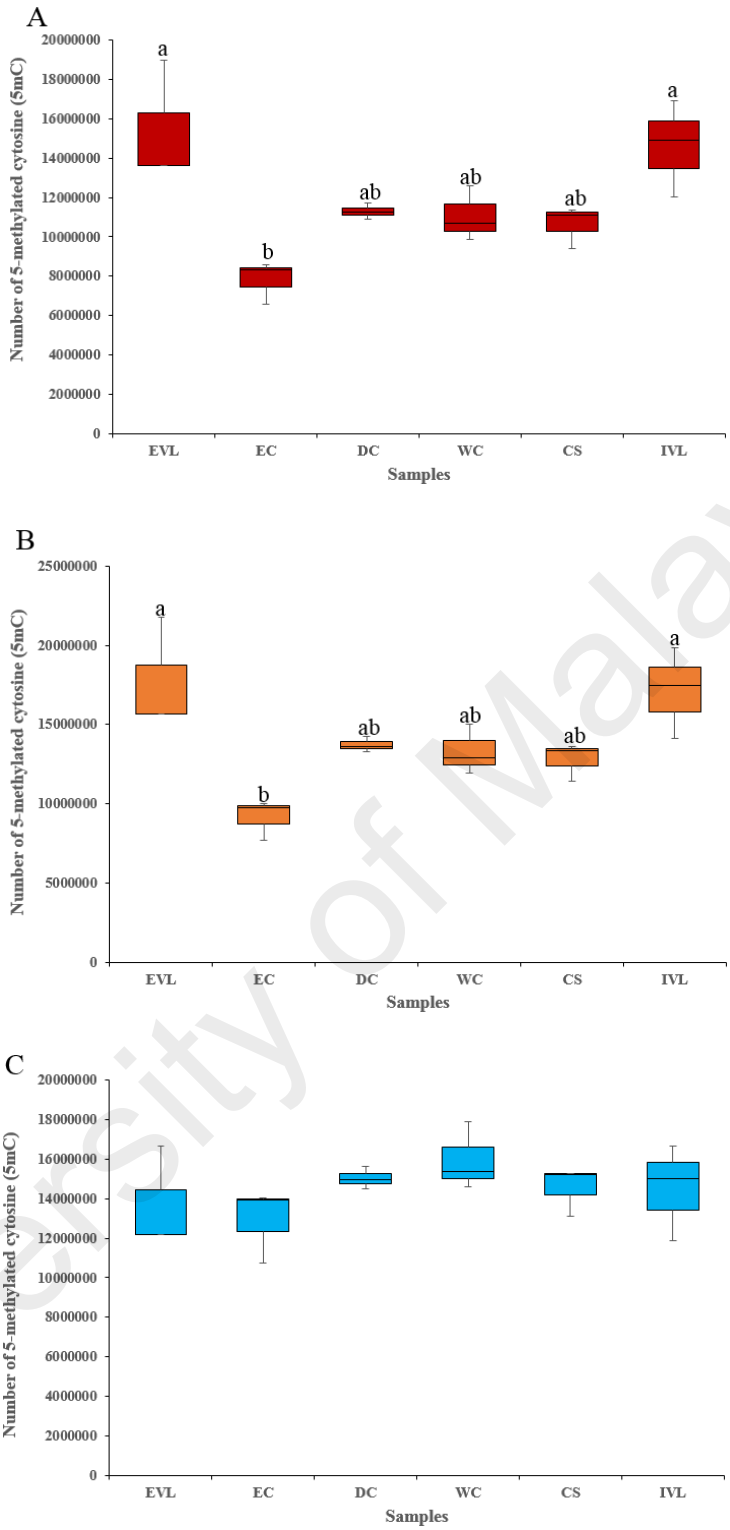

Figure 4.10: Number of methylated cytosines at CG, CHG and CHH contexts based on mapping of BS-seq data with *B. rotunda* transcriptome data. A: CG context; B: CHG context; C: CHH context. Boxes represent the interquartile range (IQR), the bar repspresents the median value (contains 50% of the data set) and lower and upper whiskers represent 1.5 × IQR values from the Q1 and Q3 value, respectively. Letters in A and B indicate statistical significance, where the same letter indicates no significant difference between samples, according to Tukey's comparison test ($P > 0.05$). There was no significant difference between any of the samples in the CHH context (C).

The average percentages of methylation for each sample ranged from 2.11-6.62, 2.47-7.59 and 3.47-5.94 in the CG, CHG and CHH contexts, respectively (Appendix H). All the samples were statistically significantly different from one another for CG and CHG methylation. For CHH methylation, EVL and CS showed similar level of methylation, whereas other samples showed statistically significant differences. The highest methylation levels were observed in EVL for CG (6.62%) and CHG methylation (7.59%), and in CS for CHH methylation (5.94%) (Figure 4.11A-C; Appendix H). The lowest methylation levels were observed in EC for all three types of methylation (2.11% for CG, 2.47% for CHG and 3.47% for CHH) (Figure 4.11A-C; Appendix H). CG and CHG contexts showed similar patterns of methylation for all samples (Figure 4.11A, B).

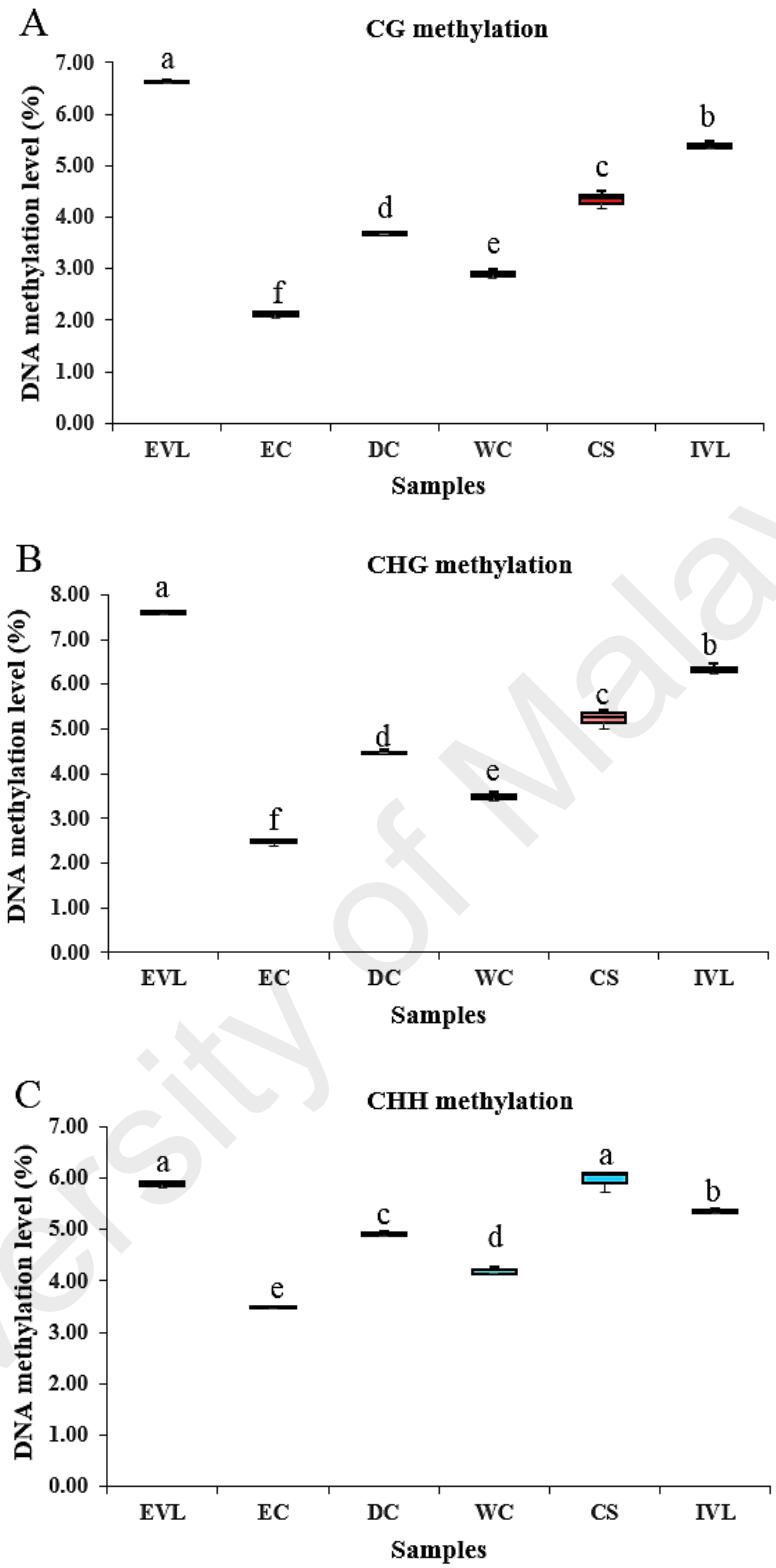

Figure 4.11: Percentage of global DNA methylation at CG, CHG and CHH contexts in different types of calli and tissues of *B. rotunda*. A: CG methylation; B: CHG methylation; C: CHH methylation. Boxes represent the interquartile range (IQR), bar meidan value (contains 50% of the data set) and lower and upper whiskers represent 1.5 × IQR values from the Q1 and Q3 value, respectively. Letters indicate statistical significance, where the same letter indicates no significant difference between samples, according to Tukey's comparison test ($P > 0.05$).

4.6.2.3 Gene specific DNA methylation

(a) DNA methyltransferase genes *MET1, CMT3* and *DRM2*

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 (Figure 4.12A-C). The frequency of CHH methylation was relatively lower than CG and CHG methylation (Figure 4.12A-C). 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 (Figure 4.12A, B), while WC and IVL showed the highest level of methylation in *DRM2* (Figure 4.12C).

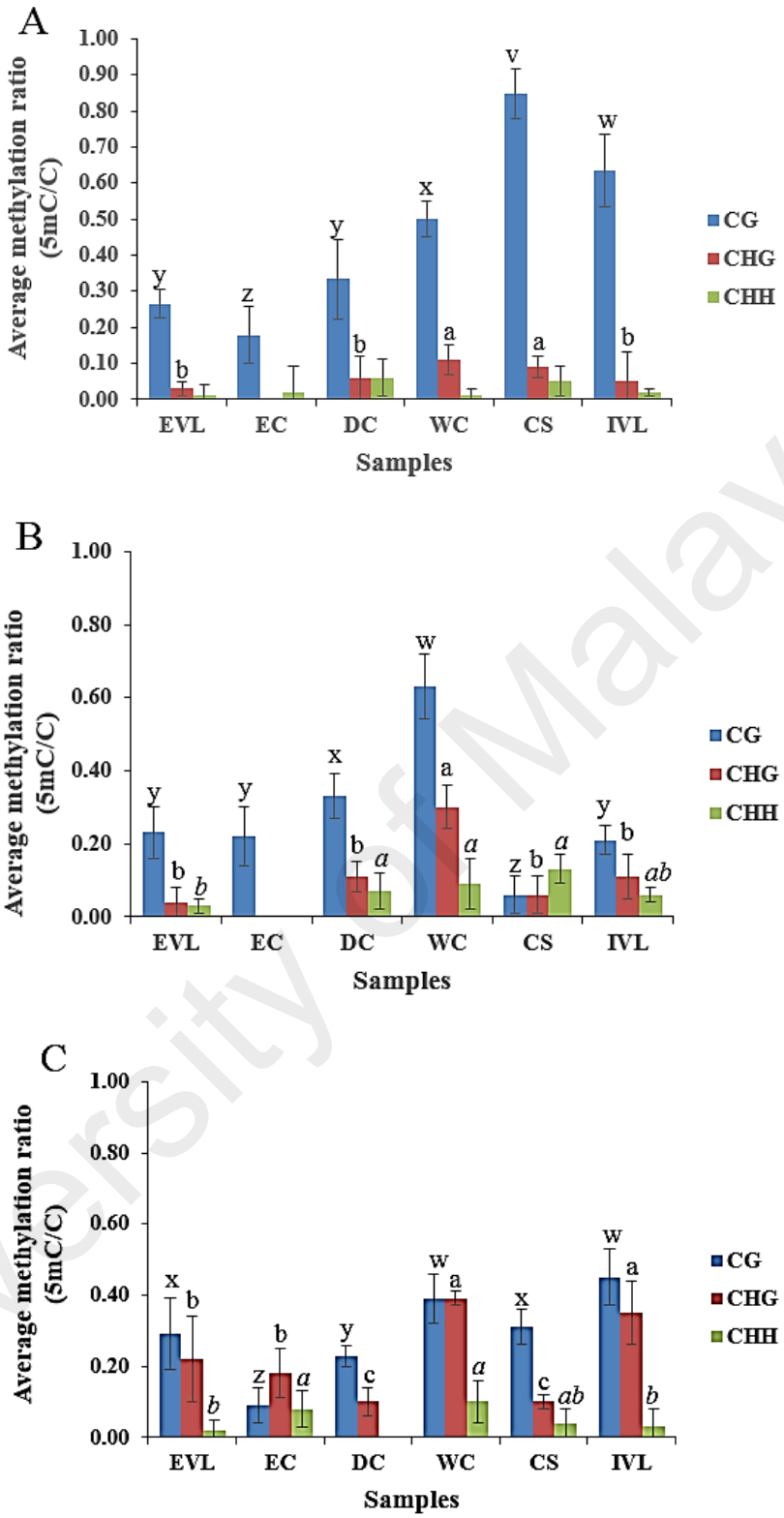

Figure 4.12: DNA methylation of *MET1*, *CMT3* and *DRM2* for *ex vitro* and *in vitro* calli and tissues of *B. rotunda*. A: *MET1*; B: *CMT3* and C: *DRM2*. EVL: *ex vitro* leaf; EC: embryogenic callus; DC: dry callus; WC: watery callus; CS: cell suspension and IVL: *in vitro* leaf. Bars represent the standard error 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).

(b) Somatic embryogenesis and plant regeneration related *genes SERK, BBM, LEC2* and *WUS*

Analysis of the level of methylation in each context (CG, CHG and CHH) of *SERK* gene revealed that CG methylation was generally higher than CHG and CHH methylation in all six samples. *SERK* showed a statistically significantly lower level of CG methylation in EC sample than EVL, DC, WC, CS and IVL (Figure 4.13A). EC lacked of CHG methylation (Figure 4.13A). Among the samples that showed CHG methylation, the highest level was found in EVL followed by DC. WC and CS showed relatively higher level of CHH methylation (Figure 4.13A).

DNA methylation analysis of *BBM* gene revealed relatively lower levels of methylation at all three sequence contexts (CG, CHG and CHH). Like *SERK*, CG methylation was predominant in all samples. CS showed the highest level of CG methylation, and EVL, EC and IVL showed similar level of CG methylation (Figure 4.13B). EC showed a lack of CHG methylation, whereas the rest of the samples showed low level of CHG and CHH methylation (Figure 4.13B).

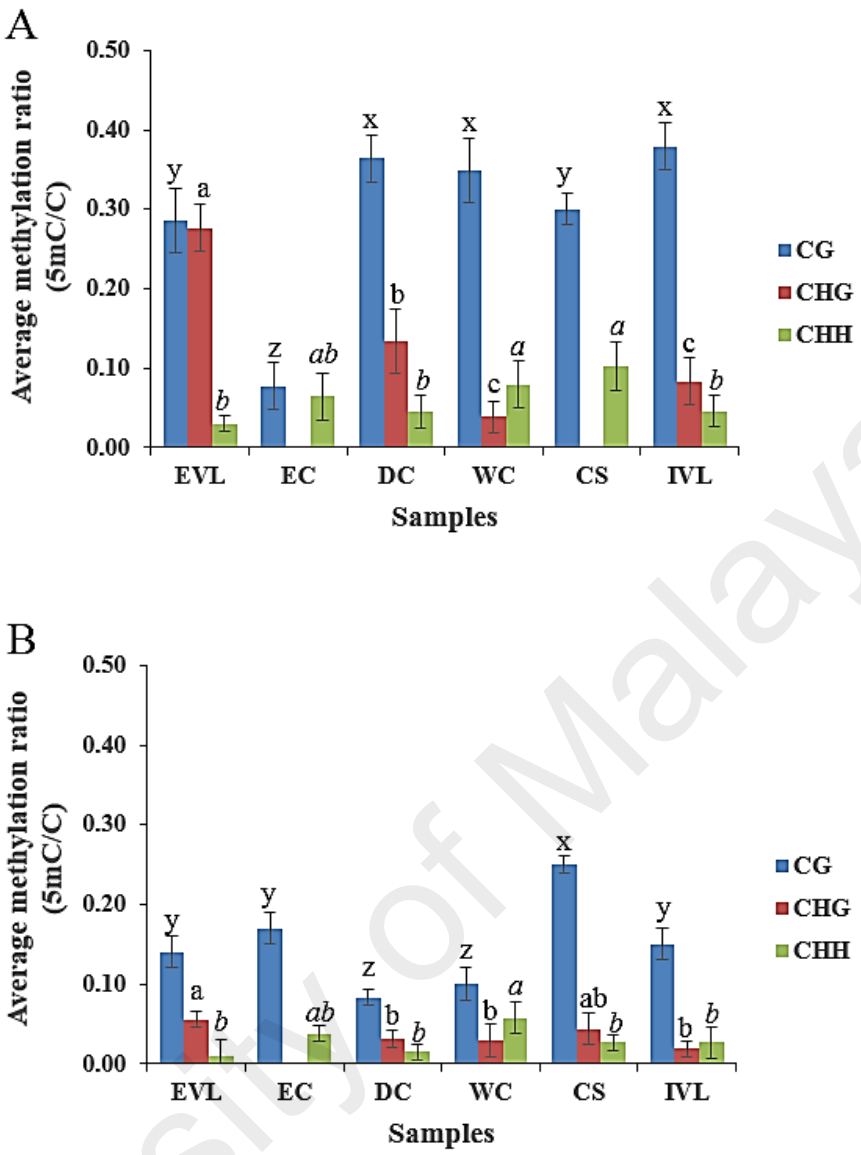

Figure 4.13: DNA methylation of *SERK*, *BBM*, *LEC2* and *WUS* for *ex vitro* and *in vitro* calli and tissues of *B. rotunda*. A: *SERK*; B: *BBM*; C: *LEC2*; D: *WUS*. EVL: *ex vitro* leaf; EC: embryogenic callus; DC: dry callus; WC: watery callus; CS: cell suspension and IVL: *in vitro* leaf. Bars represent the standard error 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 *a*, *b* for CHH methylation).

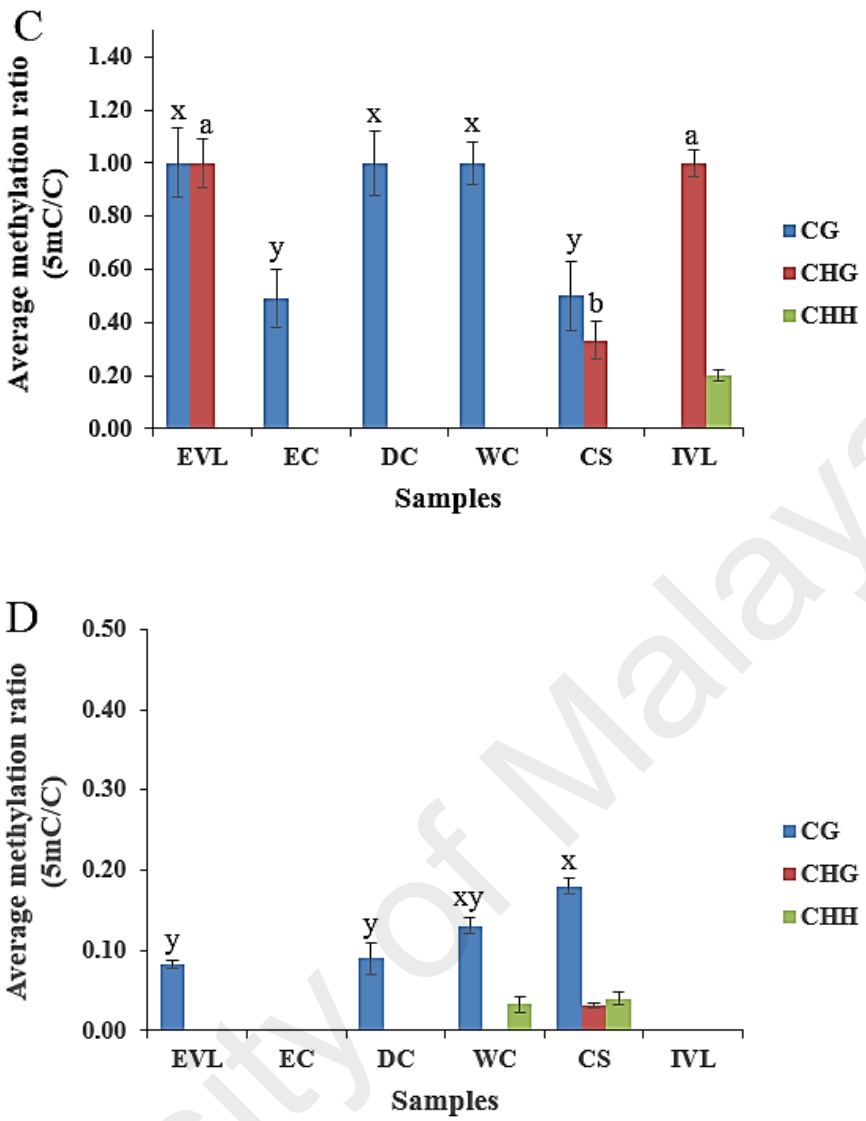

Figure 4.13, continued

Analysis of the DNA methylation of *LEC2* revealed the highest methylation ratio for CG in EVL, DC and WC. EC and CS showed a similar level of CG methylation, whereas IVL exhibited lack of CG methylation (Figure 4.13C). For CHG methylation, only EVL, IVL and CS showed CHG methylation, where the level of methylation was significantly higher in EVL and IVL than CS. In the case of CHH methylation of *LEC2*, it was observed that only IVL showed CHH methylation (Figure 4.13C). Although there were some variation of methylation patterns, overall EC showed a lower level of methylation than the other samples, and among the three types of methylation, CG methylation was higher than CHG and CHH methylation.

WUS showed very low frequency of methylation at all three contexts, where CG methylation was predominant (Figure 4.13D). WC and CS showed low level of CHH methylation, and only CS showed low level CHG methylation. EC and IVL showed lack of methylation at all three contexts (Figure 4.13D).

(c) Housekeeping genes *ACTIN, BETA-TUBULIN 1* and *ELONGATION FACTOR 1-ALPHA* (*EF1-α*)

To examine the potential role of DNA methylation in the gene expression responses in different types of calli and tissues of *B. rotunda*, the DNA methylation patterns of three housekeeping genes (*Actin, Beta-tubulin 1* and *EF1-α)* were also measured using bisulfite sequencing data in EVL, EC, DC, WC, CS and IVL. It was observed that all three housekeeping genes exhibited predominantly CG methylation (Figure 4.14A-C). Low levels of CHG and CHH methylation were also noticed in all three housekeeping genes, and *Beta-tubulin 1* and *EF1-α* showed similar patterns in all six samples. For *actin*, EC and IVL showed a significantly lower level of methylation than the other samples. DC and WC showed the highest level of CG methylation for all three genes (Figure 4.14A-C). *Actin* and *EF1-α* showed a similar pattern of methylation for all three types of methylation in all six samples (Figure 4.14A, C). Among all six samples *Beta-tubulin 1* showed more uniform methylation patterns in each context than *Actin* and *EF1-α*.

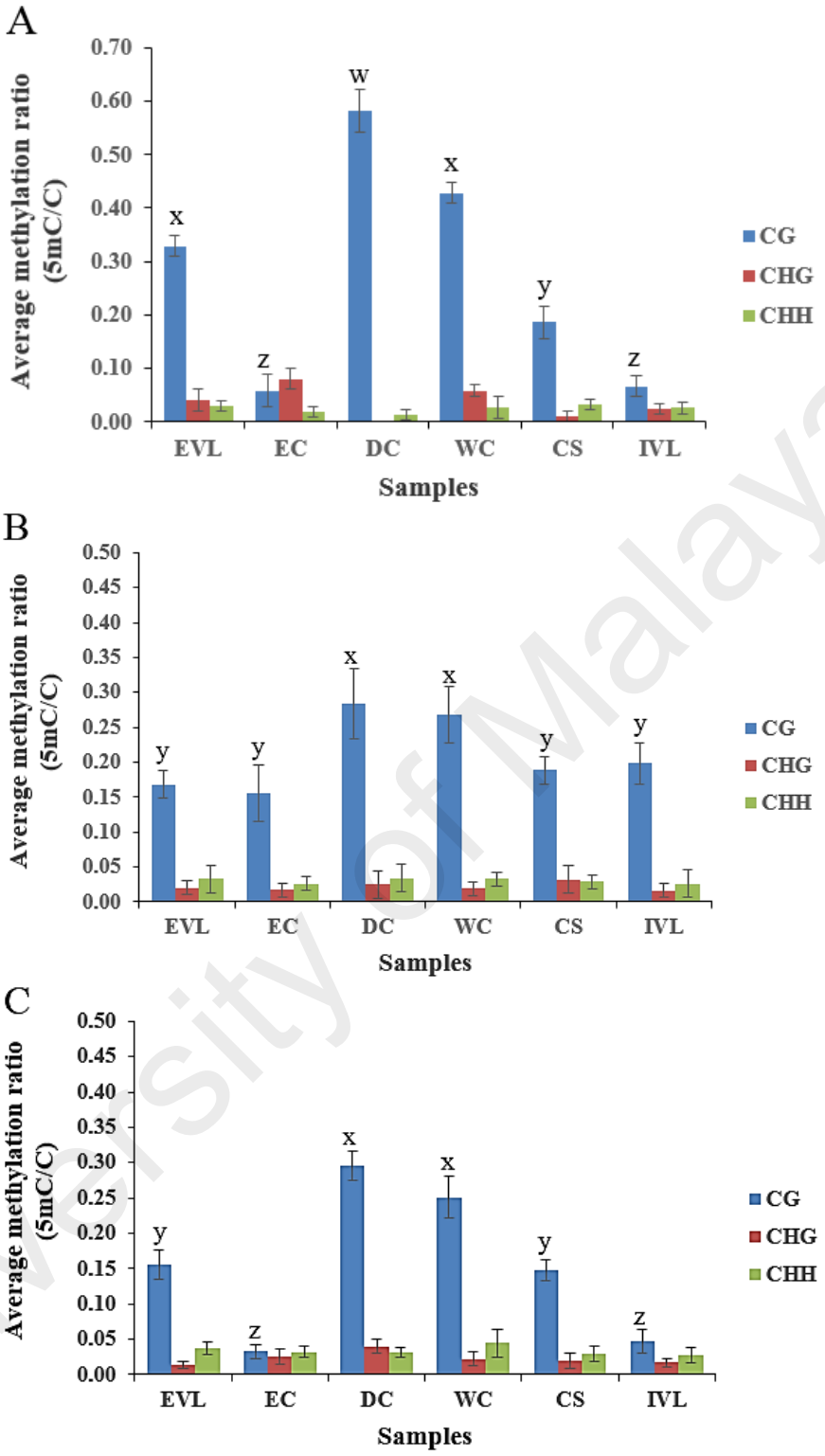

Figure 4.14: DNA methylation of housekeeping genes for *ex vitro* and *in vitro* tissues and calli of *B. rotunda*. A: *Actin*, B: *Beta-tubulin 1* and C: *EF1-alpha*. EVL: *ex vitro* leaf; MB: meristematic block; EC: embryogenic callus; DC: dry callus; WC: watery callus; CS: cell suspension and IVL: *in vitro* leaf. Bars represent the standard error 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 ($P > 0.05$).

4.7 Correlation between gene expression and DNA methylation

The association between the expression of genes (determined by qRT-PCR) and DNA methylation (determined by bisulfite sequencing (BS-seq)) data were compared for EVL, EC, DC, WC, CS and IVL samples using Pearson's correlation analysis. The results are described below.

4.7.1 Correlation of the expression of *MET1*, *CMT3* and *DRM2* with DNA methylation levels at CG, CHG and CHH contexts

While global DNA methylation levels at CG, CHG and CHH contexts determined by BS-seq data and the expression level of *MET1*, *CMT3* and *DRM2* determined by qRT-PCR data were not strongly correlated across all samples, the DNA methylation levels at all three sequence contexts showed a negative relationship with gene expression in callus and cell suspension samples (Figure 4.15A, B). The methylation level was significantly lower in all three contexts in EC samples than those of DC, WC and CS (Figure 4.15A) while expression of all three genes was significantly higher in EC compared to the other samples (Figure 4.15B).

For gene specific methylation, 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: Pearson's r = -0.47 (CG), -0.95 (CHG), -0.27 (CHH) for *MET1*; r = -0.29 (CG), -0.24 (CHG), -0.37 (CHH) for *CMT3* and r = -0.25 (CG), -0.18 (CHG), -0.45 (CHH) for *DRM2* (Figure 4.15C-E).

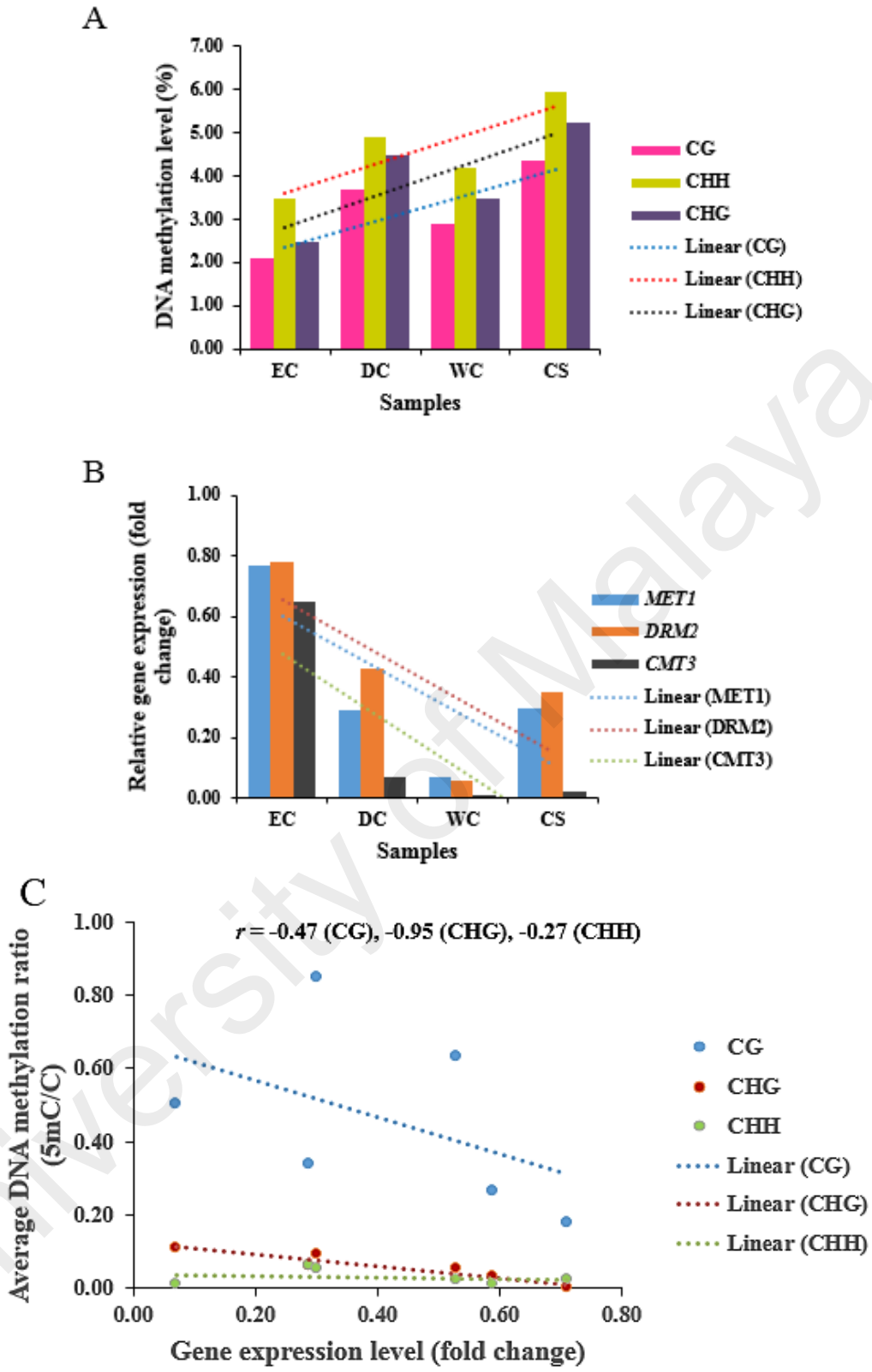

Figure 4.15: Correlation between gene expression and DNA methylation levels. Correlation of global DNA methylation level at CG, CHG and CHH contexts and the expression of *MET1*, *CMT3* and *DRM2* in *in vitro* calli and cell suspension culture (A, B). Correlation between gene specific methylation at CG, CHG and CHH contexts of *MET1* (C), *CMT3* (D) and *DRM2* (E). EVL: *ex vitro* leaf; EC: embryogenic callus; DC: dry callus; WC: watery callus; CS: cell suspension and IVL: *in vitro* leaf.

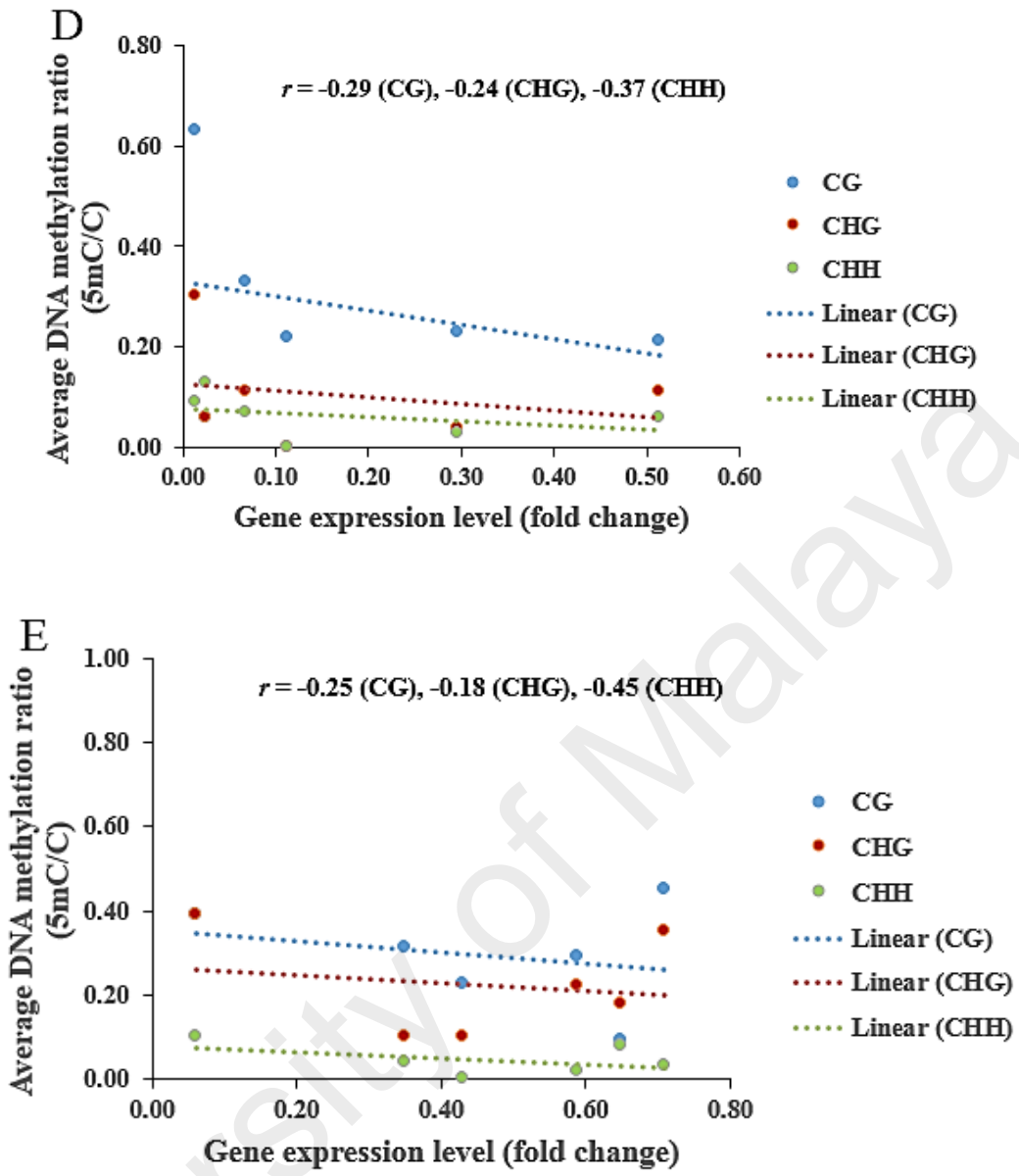

Figure 4.15, continued

4.7.2 Correlation of the expression of somatic embryogenesis related genes, *SERK, BBM, LEC2* and *WUS* with their DNA methylation status

Methylation levels at CG, CHG and CHH contexts of each gene related to somatic embryogenesis and plant regeneration (determined by BS-seq data) showed mostly negative correlation with their expression level (determined by qRT-PCR data): Pearson's *r* = -0.85 (CG), -0.24 (CHG), -0.20 (CHH) for *SERK*; *r* = -0.22 (CG), -0.72 (CHG), 0.24 (CHH) for *BBM*; *r* = -0.33 (CG), -0.15 (CHG), 0.02 (CHH) for *LEC2*; and *r* = -0.84 (CG), -0.43(CHG), -0.75 (CHH) for *WUS* (Figure 4.18A-D). Some weak positive correlation was also observed between gene expression and DNA methylation status at CHH contexts for *BBM* (*r* = 0.24) and *LEC2* (*r* = 0.02) (Figure 4.16B, C).

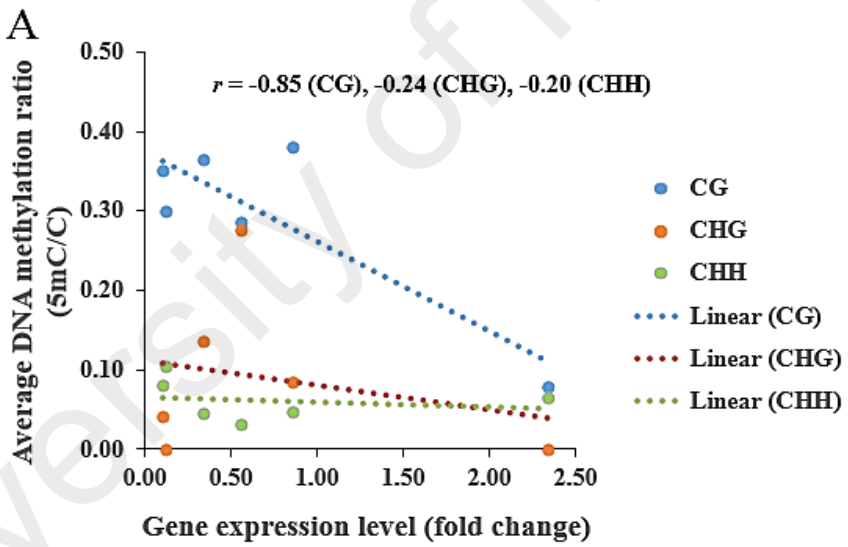

Figure 4.16: Correlation between gene expression and DNA methylation status. A: *SERK*; B: *BBM*; C: *LEC2* and D: *WUS*. Analysis was performed by Pearson's correlation coefficient.

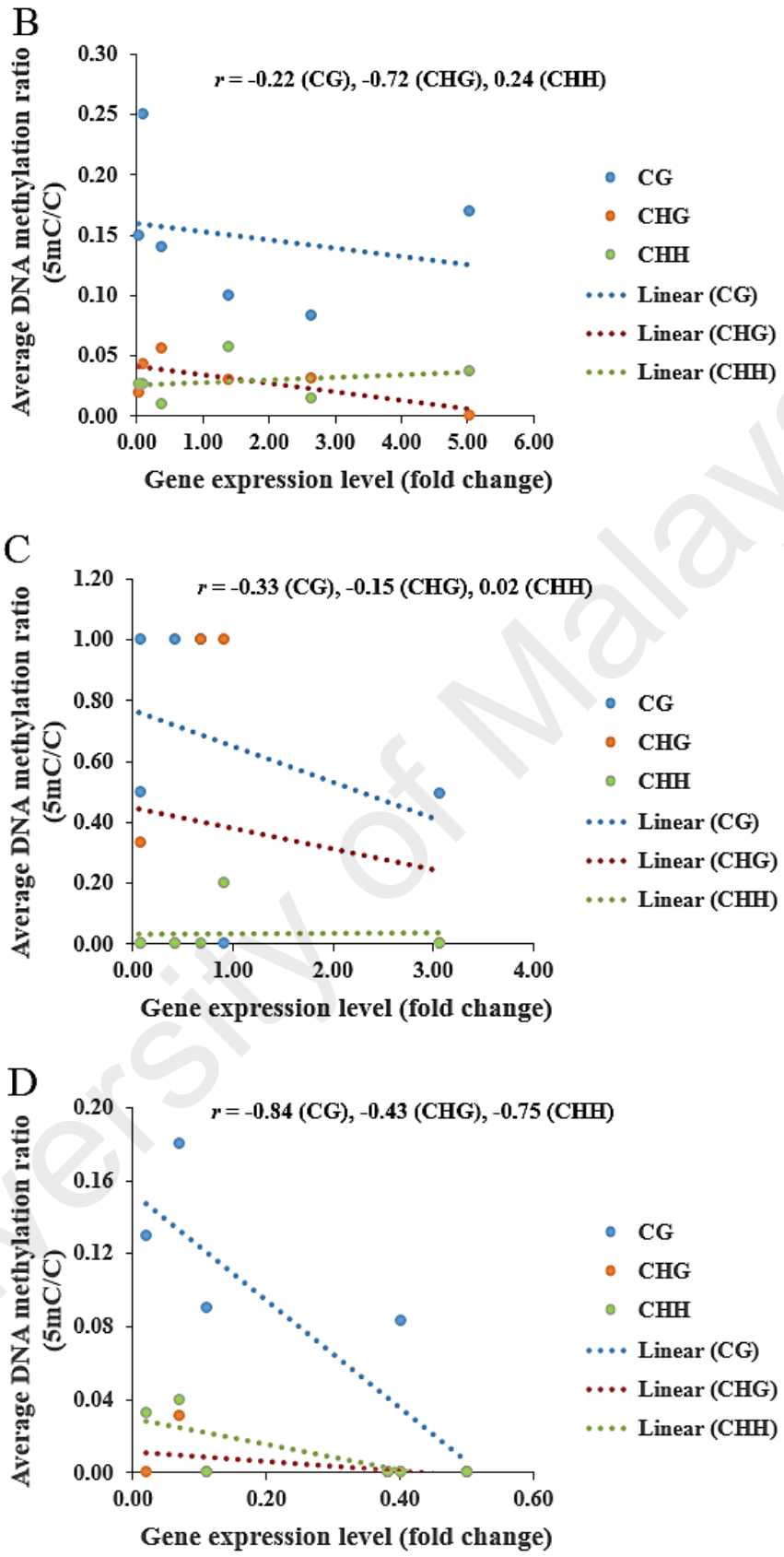

Figure 4.16, continued

CHAPTER 5: DISCUSSION

In vitro plant tissue and cell culture systems are important tools to be incorporated with other molecular techniques to overcome some of the challenges faced in the agricultural, industrial and medicinal sectors. *In vitro* multiplication of desired genotypes of plants with improved agronomic traits or with high industrial or medicinal values can be achieved due to the cellular totipotency of plant cells *via* organogenesis and somatic embryogenesis. Formation of somatic embryos is an important event, as during this unique developmental process, cells must undergo dedifferentiation, activation of cell division and reprograming of their metabolism, of their physiology and of their gene expression patterns (Yang & Zhang, 2010; Elhiti et al., 2013; Fehér, 2015). The processes of somatic embryogenesis and plant regeneration are also associated with changes in DNA methylation (Nic-Can & De la Peña, 2014). Some plant species or desired genotypes of plants are recalcitrant to the formation of somatic embryos and regeneration. Therefore, it is of interest to determine changes in gene expression and in DNA methylation during somatic embryogenesis and plant regeneration, and also to examine the relationship between the expression profile of specific genes associated with these processes and their DNA methylation status in different embryogenic and non-embryogenic cells and tissues.

Boesenbergia rotunda, an important medicinal plant in South-East Asia, India and Southern China, is used traditionally in folk medicine and as a spice. The ethnomedicinal usage of *B. rotunda* has drawn scientists' attention worldwide for further investigation to find out its medicinal properties. In recent years, *B. rotunda* has been shown to produce some important secondary metabolites with potential pharmaceutical value (Yusuf et al., 2013; Ng et al., 2016). *B. rotunda* contains active compounds that that have shown inhibition towards dengue 2 virus NS3 protease (Kiat et al., 2006), HIV-1 protease

(Tewtrakul et al., 2003), and also exhibited anti-mutagenic, anti-tumor and anti-inflammatory activities (Murakami et al., 1994; Trakoontivakorn et al., 2001; Tuchinda et al., 2002). Improvement in research on drug design has directed to develop lead compounds from *B. rotunda* metabolites using medicinal chemistry and bioinformatic studies (Eng-Chong et al., 2012). In addition, new insights on the biosynthetic pathways of *B. rotunda* metabolites can be elucidated because of the advancement of genomics, transcriptomics, proteomics, and metabolomics allowing researchers to foretell the potential bioactive compounds responsible for the medicinal properties of *B. rotunda* (Eng-Chong et al., 2012).

Based on these significances, in this study, *B. rotunda* was chosen as a model system in which the expression patterns and DNA methylation status of three DNA methylation pathway genes (*METHYLTRANSFERASE* (*MET1*), *CHROMOMETHYLASE 3* (*CMT3*) and *DOMAIN REARRANGED METHYLTRANSFERASE 2* (*DRM2*)), and four genes related to somatic embryogenesis and plant regeneration (*SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* (*SERK*), *BABY BOOM* (*BBM*), *LEAFY COTYLEDON 2* (*LEC2*) and *WUSCHEL* (*WUS*)) were examined in various cell samples representing different stages of the regeneration process, from explant through tissue culture and regeneration. The samples included two differentiated tissue samples i.e. *ex vitro* leaf (EVL) and *in vitro* leaf of regenerated plants (IVL) together with meristematic block of newly emerged shoots (MB) and four types of *in vitro* cultured cells, i.e. embryogenic callus (EC) and non-embryogenic calli, i.e. dry callus (DC), watery callus (WC) and prolonged cell suspension culture (CS) of *B. rotunda*.

5.1 Factors other than 2,4-D concentration show contribution to embryogenic competence of *B. rotunda in vitro* cultured cells

To produce embryogenic (EC) and non-embryogenic callus (DC, WC and CS), the auxin-supplementation method as used in reports by Yusuf et al. (2011) and Wong et al. (2013), was followed, and these samples were compared to *ex vitro* leaf (EVL), meristematic block of newly emerged shoots (MB) and *in vitro* leaf of regenerated plants (IVL) of *B. rotunda*. As previously reported (Wong et al., 2013), and seen in the current study, embryogenic and regenerable callus were produced in MS medium supplemented with only 3 mgL^{-1} 2,4-D, but when the callus was maintained in cell suspension culture supplemented with the same concentration of 2,4-D for 12 months, the cells lost their embryogenic ability and failed to regenerate (Table 4.1 and Figure 4.1). Watery callus (WC) and dry callus (DC) cultured on MS media supplemented with 1 mgL^{-1} 2,4-D and 4 mgL^{-1} 2,4-D, respectively, were also non-embryogenic and non-regenerable (Table 4.1 and Figure 4.1). In many plant species, exogenous 2,4-D (auxin) has been shown to be an effective inducer of somatic embryogenesis (Pasternak et al., 2002; Thomas & Jiménez, 2006; Kumar & Thomas, 2012; Kumar & Chandra, 2014). For instance, in *Clitoria ternatea,* optimum embryogenic callus formation (in 75 % of calli) was observed on MS medium supplemented with 2 mgL^{-1} 2,4-D (Kumar & Thomas, 2012). The authors also found a lower frequency of embryogenic callus on MS medium supplemented with relatively low (1 mgL^{-1}) or high (4 mgL^{-1}) concentrations of 2, 4-D, which was in similar pattern to the present study with *B. rotunda* meristematic block explants. Previous reports have suggested that 2,4-D affects somatic embryogenesis by altering DNA methylation. For example, in the presence of 2,4-D, the highest rate of DNA methylation was observed at the embryo stage in *Cucurbita pepo* (Leljak-Levanic et al., 2004), indicating that 2,4-D may enhance DNA methylation during embryogenesis. In a similar study on *Acca sellowianai,* Fraga et al. (2012) observed that the addition of 5-azacytidine (AzaC) to the

cultured cells caused decreased level of DNA methylation and embryogenesis, while a combination of 2,4-D and AzaC showed a contrary effect on methylation level, resulting the induction of somatic embryogenesis. Although plant growth regulators have been reported to induce somatic embryogenesis in most species (da Silva et al., 2009; Pavlovic et al., 2013; Rosa et al., 2015), the development of somatic embryos from *in vitro* cultured tissues has also been observed in the absence of plant growth regulators (Choi et al., 1998; Cosic et al., 2013). Thus, the previous reports indicated that the concentration of 2,4-D itself was not sufficient to maintain cells as embryogenically competent, and that duration of culture and / or other factors associated with this such as DNA methylation may also have an impact on embryogenesis. In this study, it was observed that although the EC and CS were supplemented with same concentration of 2,4-D (3 mgL^{-1}), after 12 months maintenance, the cells in CS lost their embryogenic ability which agrees well with the previous hypothesis that factors other than 2,4-D such as culture period or DNA methylation contribute to somatic embryogenesis process.

5.2 High quality and integrity of nucleic acids extracted from *B. rotunda*

In this study, a modified CTAB method described by Kiefer et al. (2000) was used to extract RNA from all samples. Since phenol is an effective reagent to remove protein contaminants from nucleic acids, phenolcholoroform-isoamylalcohol was used to enhance the purity of RNA. Purity of RNA is very important to ensure high quality of cDNA for library construction. In this work, RNA samples with A_{260nm}/A_{280nm} ratio about 2.0 indicated low protein contamination, and the A_{260nm}/A_{230nm} ratio greater than 1.0 indicated low contamination of polysaccharides and polyphenols (Table 4.2). Analysis using Agilent Bioanalyzer, also showed high RNA Integrity Number (RIN) for all samples as the ratio of 25S and18S rRNA of the isolated RNA was greater than 1.0 and RIN was higher than 7.0 (Table 4.3 and Appendix C). RNA integrity examined on

ethidium bromide-stained 1% (w/v) agarose gel showed sharp, intact 25S and 18S rRNA bands, with the earlier having more band intensity than the latter (Figure 4.2), which suggests little degradation of RNA (Salzman et al., 1999). High RNA integrity is an important indicator in the construction of cDNA library for next generation sequencing (RNA-seq), because degraded RNA sample may threaten the quality of the cDNA library as there may be higher proportions of undesirable RNA sequences, which compromises the quality of *de novo* transcriptome assembly: RNA with low integrity used for RNA-seq may prevent assembly of larger scaffolds (Johnson et al., 2012). Previous studies also reported that CTAB is a suitable method to extract RNA from calli and tissues of *B. rotunda* (Md-Mustafa et al., 2014; Ata et al., 2015) and from roots of *Musa acuminata* cv. Berangan (Lee, 2015).

Isolation of pure and high molecular weight genomic DNA is a prerequisite for genetic analysis of plants. However, the presence of polyphenols and polysaccharides in plants interferes the isolation of pure DNA and downstream analysis. A modified CTAB method described by Doyle (1990) was applied to isolate genomic DNA from calli and tissues used in this study. Neither protein contamination nor degradation of DNA was observed on the gel (Figure 4.3). The A_{260nm}/A_{280nm} ratios were between 1.7-1.9, and the A_{260nm}/A_{230nm} ratios were greater than 1.0 (Table 4.4), which indicate minimal contamination. Previously, Wong (2016) also isolated pure and high quality genomic DNA from *B. rotunda* calli and tissues using this CTAB method. Thus, taken the results obtained in this study and reports from previous studies together, it is suggested that CTAB-based methods are the appropriate and promising technique to isolate RNA and DNA from calli and tissue samples of *B. rotunda*.

5.3 *B. rotunda* gene sequences share high similarity and identity with reported sequences of the same functions in the database

As there is still lack of sequence information of *B. rotunda* genes, homology searching, conserved domains searching, sequence alignment and phylogenetic analysis of all seven genes used in this study, *MET1*, *CMT3*, *DRM2*, *SERK*, *BBM*, *LEC2* and *WUS* (with gene sequences obtained from our previous study on transcriptome analysis (Md-Mustafa et al., 2014) of *B. rotunda*) were performed before starting the gene expression and DNA methylation analyses in order to determine their similarity and identity with reported sequences of same functions. In view of this, it was observed that all seven genes showed high similarities with the available respective homologs with the target functions in the NCBI database (http://www.ncbi.nlm.nih.gov/) (Appendix D). All *B. rotunda* genes showed the highest similarity and identity scores with sequences from *Musa acuminata* subsp. malaccensis followed by *Phoenix dactylifera* and *Elaeis guineensis*. Conserved domains analysis of the predicted protein sequences for each gene, using NCBI Conserved Domains Search Tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) showed that BrMET1 (where Br indicates *B. rotunda*) contains domains from the BAH and DNMT1-RFD superfamily; BrCMT3 and BrDRM2 each contain domains from the Dcm superfamily; BrSERK contains Leucine-rich repeat (LRR) and Protein kinase (PKc) domains; BrBBM contains two Apetala2 (AP2) domains; BrLEC2 contains a B3 domain and BrWUS contains a homeobox domain (Figure 4.4). The conserved motifs identified for each sequence, match the general characteristics of their respective gene families as reported in previous studies such as for MET1, CMT3 and DRM2 (Ryazanova et al., 2012; Gu et al., 2016), for SERK (Baudino et al., 2001; Talapatra et al., 2014; Rupps et al., 2016), for BBM (Florez et al., 2015; Rupps et al., 2016), for LEC2 (Stone et al., 2001) and for WUS (Rupps et al., 2016), and thus, supporting the validity of the gene sequences those were

selected for this study from previous transcriptome data of *B. rotunda* reported in Md-Mustafa et al. (2014).

For further validation of sequence similarity and identity of *B. rotunda* genes with their homologs from other plants, in silico translated amino acid sequences of these transcripts were also aligned with 15 amino acid sequences reported to have the target functions (i.e. *MET1*, *CMT3*, *DRM2*, *SERK*, *BBM*, *LEC2* and *WUS* sequences were retrieved from the NCBI Non-redundant protein sequences (nr) database (https://www.ncbi.nlm.nih.gov/protein) (Appendix E). Phylogenetic analyses revealed that all seven genes of *B. rotunda* showed close relationships with their homologs from *Musa acuminata* subsp. malaccensis (Figure 4.5). As genes involved in the regulation of fundamental plant developmental processes, it was not unexpected to observe high similarity and conservation of functional domains between MET1, CMT3, DRM2, SERK, BBM, LEC2 and WUS from *B. rotunda* and other plant species. The validated *B. rotunda* gene sequences were next used for gene expression and DNA methylation analyses during *in vitro* culture and regeneration of *B. rotunda*.

5.4 *MET1, CMT3* and *DRM2* showed similar expression patterns across *in vitro* cell samples in *B. rotunda*

In the present work, the expression of three DNA methyltransferase genes, *MET1*, *CMT3*, and *DRM2* was performed to determine the pattern of expression during different stages of plant tissue culture using qRT-PCR and RNA-seq data. The enzymes encoded by *MET1*, *CMT3* and *DRM2* have been shown to 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 & Jacobsen, 2002; Cokus et al., 2008; Law & Jacobsen, 2010). Thus all three genes are expected to play a role in DNA methylation in actively dividing cells. Among the embryogenic-regenerable (EC) and non-embryogenic

and non-regenerable (DC, WC and CS) samples, it was observed that the expression levels of *MET1*, *CMT3* and *DRM2* were higher in EC than CS, WC and DC according to both qRT-PCR and RNA-seq data (Figure 4.6). The finding in this thesis agrees well with a report from *Arabidopsis thaliana* (Wickramasuriya & Dunwell, 2015), where higher levels of *MET1*, *CMT3* and *DRM2* transcripts were observed in somatic embryogenic cells compared to non-embryogenic cells. Thus based on the findings this study it is hypothesized that embryogenic-regenerable callus may have more actively dividing cells which requires higher activities of *MET1*, *CMT3* and *DRM2* to maintain and to mediate *de novo* methylation than non-embryogenic and non-regenerable calli.

Analysis of the expression of *MET1*, *CMT3* and *DRM2* revealed that their transcript levels were positively correlated with the embryogenic-regenerable (EC) and negatively correlated with non-embryogenic and non-regenerable (DC, WC and CS) nature of the calli (Figure 4.6). However, since different levels of 2,4-D were used to produce the different callus types, it has to be considered that 2,4-D level as well as cell type was varied between these samples. Although there are no previous reports about the effect of 2,4-D on the expression of DNA methyltransferases, since different concentrations of the synthetic auxin 2,4-D were required in order to generate the different types of callus and cells, the exposure to different concentrations of 2,4-D may be directly or indirectly responsible for the differences in gene expression observed in embryogenic and non-embryogenic samples, either alone or together with the different cell types.

5.5 Differentiated and dedifferentiated samples showed distinct profiles of gene expression and DNA methylation in *B. rotunda*

Gene expression and DNA methylation patterns may be varied due to the nature of tissues and cells because cellular activities are determined related to developmental stages. In view of this, the samples in this study were categorized into two types, i.e. one was differentiated tissues and another was dedifferentiated *in vitro* callus samples. The highest level of expression of all three DNA methyltransferases (*MET1*, *CMT3* and *DRM2*) was observed in MB (Figure 4.6) (according to qRT-PCR data only, RNA seq was not performed for MB as it was not feasible to obtain sufficient sample for the library construction), as would be expected for active cell division and growth in this sample. The leaf tissue samples with no exogenous growth factors before (*ex vitro* leaf: EVL) and following (*in vitro* leaf: IVL) *in vitro* culture, also showed relatively high expression of *MET1*, *CMT3* and *DRM2* genes, though this was lower that of MB tissue, which might be a consequence of relatively slower cell division and/or due to differentiation of the tissues. All three DNA methyltransferases showed relatively lower expression in the leaf tissues (EVL and IVL) than embryogenic callus (EC), whereas their expression levels were comparatively higher in EVL and IVL compared to non-embryogenic calli (DC, WC and CS). Previously, it has been reported that the expression of the *MET1*, and *CMT3* genes was significantly higher in embryo compared to endosperm in *Arabidopsis thaliana* (Ashapkin et al., 2016). Thus it can be suggested that the expression of *MET1*, *CMT3* and *DRM2* was higher in the cells which are actively dividing stage.

DNA methylation analysis based on MSAP and bisulfite sequencing data revealed that the leaf samples, EVL and IVL showed a higher level of DNA methylation than the other samples (i.e. EC, DC, WC and CS; BS-seq was not performed for MB as it was not feasible to obtain sufficient sample for the library construction) (Table 4.6 and Figure 4.11; Appendix H), which agreed well with the higher expression of DNA

methyltransferase genes in these samples. While being higher than those of the cell and callus samples, the levels of global DNA methylation at CG, CHG and CHH contexts were significantly lower in IVL than EVL (Figure 4.11), which may be due to *in vitro* conditions experienced by IVL. The results in this study agree well with reports on *Oryza sativa* (Stroud et al., 2013a), *Secale cereale* (González et al., 2013) and triticale (Machczyńska et al., 2014), where lower level of DNA methylation was noticed in regenerated compared to control plants.

The *in vitro* calli samples in this study showed different pattern in the expression of *MET1*, *CMT3* and *DRM2* and global methylation compared to EVL and IVL. This may happen due to two different types of cells or tissues, i.e. organized / differentiated leaf tissue (EVL and IVL) and unorganized / dedifferentiated callus and cells (EC, DC, WC and CS), and due to the presence or absence of growth regulators. In addition to organized and unorganized cell state, the association of methyltransferase genes expression (*MET1*, *CMT3* and *DRM2*) and global DNA methylation may vary depending on plant species or genotypes, or be influenced by additional factors like culture conditions, hormone types and concentrations.

5.6 Higher expression of DNA methyltransferase genes (*MET1*, *CMT3* and *DRM2*) and relatively lower global DNA methylation were associated with embryogenic-regenerable callus in *B. rotunda*

In the present study, it was observed that the expression level of *MET1*, *CMT3* and *DRM2* was significantly higher in embryogenic and regenerable callus (i.e. EC) than non-embryogenic and non-regenerable calli (i.e. DC, WC and CS) (Figure 4.6). On the other hand, the levels of global DNA methylation were significantly lower in EC than in CS, WC and DC (Table 4.6 and Figure 4.11; Appendix H) based on MSAP and BS-seq data, suggesting that the embryogenic nature and regeneration ability of calli were

associated not only with the higher expressions of DNA methyltransferase genes but also a relatively decreased level of global DNA methylation. This was also shown by the correlation data that the mRNA levels of all three DNA methyltransferases, *MET1*, *CMT3, DRM2* for EC, DC, WC and CS were negatively correlated with the global DNA methylation (Figure 4.15). The findings in this work agree well with a study on *Elaeis guineensis*, where it has been reported that the expression of *MET1*, *CMT3* and *DRM2* was higher in fast-growing calli than nodular calli, but the level of DNA methylation was lower in the earlier than the later (Rival et al., 2008). Thus, global DNA methylation can not be explained only by the expression of *MET1*, *CMT3* and *DRM2*, it may also be affected by the by plant growth regulators, calli or tissue types or demethylation events.

In this study, the higher level of methylation in DC and CS than EC may be the effect of a higher concentration of 2,4-D and long-term maintenance (12 months), respectively. Interestingly, although WC was grown on media containing a lower concentration of 2,4-D than that used for other types of calli and cells, it was observed that global DNA methylation were similar level to those for the DC and CS samples (Table 4.1 and Figure 4.11; Appendix H). Based on this, it is suggested that the global level of DNA methylation not only depends on levels of growth factors but also depends on the nature of calli, for instance, embryogenic and regenerable calli are associated with decreased methylation level, and non-embryogenic and non-regenerable calli are associated with increased DNA methylation level in *B. rotunda*. This hypothesis can be supported with some previous reports, for example, the global DNA methylation level was lower in embryogenic calli (11.20%) than non-embryogenic calli (16.99%) at the CCGG site in Siberian ginseng (*Eleutercoccus senticosus*) (Chakrabarty et al., 2003) similar to the data for global DNA methylation levels at CCGG sites determined by MSAP in this study, where the level of global DNA methylation was lower in EC compared to other samples. A similar pattern has also been reported for *Pinus nigra* Arn.

ssp Austriaca, in which embryogenic lines showed lower DNA methylation levels than non-embryogenic lines (Noceda et al., 2009). However, there were no significant differences in the amount of DNA methylation of embryogenic and non-embryogenic lines of *Pinus pinaster* (Klimaszewska et al., 2009), where the DNA methylation values were between 17.8 and 19.1%.

In contrast to the IVL, which was from a plant regenerated from EC, attempts to regenerate plants from WC, DC or CS samples were not successful, and it is suggested that these had a loss of regeneration ability (Table 4.1). In the case of the callus tissues, WC, EC and DC were exposed to increasing concentrations of 2,4-D. It is suggested that in addition to the well-established principle that the auxin concentration needs to be suitable to permit plant regeneration *via* somatic embryogenesis (Gaj, 2004; Carra et al., 2016), the auxin levels influence the activities of *MET1*, *CMT3* and *DRM2*, and thereby competence for regeneration. In the current study, it was observed that the intermediate concentration of 3.0 mgL^{-1} 2,4-D coincided with the highest levels of methyltransferases expression among the callus samples (EC>DC>WC, Figure 4.6) and also to competence for plant regeneration. While a few studies report an association of plant growth regulators and DNA methylation levels (Leljak-Levanic et al., 2004; Miguel & Marum 2011; Fraga et al., 2012, 2016), there are no previous reports for DNA methyltransferase expression in relation to differing concentrations of 2,4-D. DNA methylation levels were reported to be lower in the intermediate concentration of 2,4-D (and also for 6-BA) in callus of *Brassica napus* (Gao et al., 2014), however, a lower range of concentrations (0.05 to 0.20 mgL^{-1} 2,4-D) were tested. Most studies on DNA methylation levels report coincidence with just presence or absence of growth regulator, and as a result, the levels of methylation differ widely between reports due to differences in the experimental conditions. A key study in Arabidopsis from Berdasco et al. (2008) showed that the DNA methyltransferases *MET1* and *DRM2* play a role in the regulation of dedifferentiation

(establishment and maintenance of undifferentiated cells) which would correspond to the changes in expression between the MB and the calli samples (WC, EC and DC) in this current study, and the lower levels of *MET1* expression in the non-embryogenic and non-regenerable (WC and DC) calli. Taken altogether, it is suggested that relatively higher expression levels of *MET1*, *CMT3* and *DRM2* and lower levels of global methylation are associated with somatic embryogenesis than non-embryogenic and non-regenerability in *B. rotunda*. However, both higher expression of methyltransferases and relatively higher level of DNA methylation was noticed in EVL and IVL. Therefore, it is suggested that there is no general trend between the expression of *MET1*, *CMT3* and *DRM2* and the level of global DNA methylation at CG, CHG and CHH contexts.

5.7 *SERK, BBM, LEC2* and *WUS* are highly expressed during somatic embryogenesis and plant regeneration in *B. rotunda*

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); and by this process, certain somatic cells acquire the totipotency to produce embryogenic cells, which give rise to somatic embryos and regenerated plants (Feher et al., 2003; Rocha et al., 2016). 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 as these genes have been shown to be specific markers distinguishing embryo-forming cells during *in vitro* culture and plant regeneration in many plant species (Mahdavi-Darvari et al., 2015; Ikeuchi et al., 2016).

In the current study, the expression level of *SERK*, *BBM* and *LEC2* determined by both qRT-PCR and RNA-seq was the highest in EC, whereas the expression level of *WUS* was the highest in EVL among all seven samples (Figure 4.7). The lowest expression was

in CS and WC for *SERK*, *LEC2* and *WUS*, and was in EVL and IVL for *BBM* (Figure 4.7). In EVL, MB and IVL exhibited different patterns of expression which may be due to the different nature of tissues compared to *in vitro* calli and cells. Among *in vitro* calli, embryogenic-regenerable callus i.e. EC showed the highest expression, while non-embryogenic and non-regenerable calli i.e. DC, WC and CS (especially, WC and CS) showed significantly lower level of expression for all four genes (Figure 4.7), indicating that the expression levels of *SERK*, *BBM*, *LEC2* and *WUS* were determined by the nature of calli. This data agrees well with the previous studies, such as, for *SERK*, the expression was higher in embryogenic callus than non-embryogenic callus during *in vitro* culture of *Arabidopsis thaliana* (Singla et al., 2008), *Solanum tuberosum* (Sharma et al., 2008), *Zea mays* (Zhang et al., 2011), *Triticum aestivum* (Delporte et al., 2013), *Brassica napus* (Ahmadi et al., 2016), *Passiflora edulis* (Rocha et al., 2016) and *Trifolium nigrescens* (Pilarska et al., 2016). Similarly, for *BBM*, the high level of expression was noticed in embryogenic callus culture of *Brassica napus* and *Arabidopsis thaliana* (Boutilier et al., 2002), *Nicotiana tabacum* (Srinivasan et al., 2007), *Populus tomentosa* (Deng et al., 2009), *Capsicum annuum* (Heidmann et al., 2011; Irikova et al., 2012), *Zea mays* (Salvo et al., 2014), *Theobroma cacao* (Florez et al., 2015), *Coffea arabica* (Silva et al., 2015) and *Larix decidua* (Rupps et al., 2016). Like the findings in this work, a higher level expression of *LEC2* was observed in embryogenic callus than non-embryogeneic callus culture of *Brassica napus* (Malik et al., 2007), *Arabidopsis thaliana* (Stone et al., 2008; Ledwoń & Gaj, 2009; Wojcikowska & Gaj 2015), *Nicotiana tabacum* (Guo et al., 2013) and *Zea mays* (Salvo et al., 2014). The expression level of *WUS* was also found to be high in embryogenic callus of *Coffea canephora* (Arroyo-Herrera et al., 2008), *Panax ginseng* (Kiselev & Tchernoded, 2009), *Arabidopsis thaliana* (Bouchabke-Coussa et al., 2013), *Gossypium hirsutum* (Zheng et al., 2014) and *Larix decidua* (Rupps et al., 2016). The higher 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. As mentioned earlier, since different concentrations of 2,4-D were used to generate the different types of calli and cells in this thesis study (Table 4.1 and Figure 4.1), the expressions of *SERK*, *BBM*, *LEC2* and *WUS* might be affected by auxin supplementation (2,4-D) or other factors like DNA methylation which are discussed in the following sections.

In most previous studies, it has been reported that auxins and cytokinins were required for plant cell reprogramming, and were widely applied plant growth regulators to control callus induction, somatic embryogenesis induction and organ regeneration. Of these, auxin plays a central role in early and post-embryogenic development in plants (Cueva-Agila et al., 2016; Elhiti & Stasolla, 2016). However, an optimal amount of auxin is necessary for the induction of somatic embryogenesis. Among different auxins, exogenous 2,4-D was widely used in the culture medium during *in vitro* culture to trigger somatic embryogenesis (Halperin, 1964; Bai et al., 2013; Elhiti et al., 2013; Fujimura, 2014). Proper concentration is important for embryogenic competence of cells during *in vitro* culture. The level of concentrations of 2,4-D may affect the expression of high number of transcription factors including somatic embryogenesis and regeneration related genes directly or indirectly (Gliwicka et al., 2013). Gene expression data, from the current study support that embryogenic callus overall has relatively higher expression of *SERK*, *BBM*, *LEC*2 and *WUS* than non-embryogenic calli and cells which were generated by different concentrations of 2,4-D (Figure 4.7). Although the understanding of the action of auxins is complex, it can be assumed that the presence of an optimal concentration (3 mgL^{-1} in EC), of 2,4-D may promote the activation of *AUXIN RESPONSE FACTORS* (*ARF*), whereas in the presence of a relatively low (1 mgL^{-1}) or high concentration (4 mgL^{-1}) of 2,4-D, the functions of *ARF* may be blocked (Dinesh et al., 2016; Li et al., 2016). Although the MS medium used for CS contains the same

concentrations of 2,4-D as that used for EC ($3mgL^{-1}$), the expression for each of the four genes was very low. This may be because of a decrease in auxin responsive mechanisms, or may be affected by other genetic and epigenetic factors (DNA methylation is one of them considered in this study which is discussed in the following sections). Reduced embryogenic competence in old callus culture than new callus culture exposed to 2,4-D has also been reported in *Ipomoea batatas*, and it has been suggested that decrease in auxin responsiveness is correlated with ageing of the culture (Padmanabhan et al., 2001). However, a complex molecular network exists between auxin supplementation and somatic embryogenesis as well as plant regeneration. It will be helpful to determine the expression of genes associated with the auxins responsive pathway involving more concentration of 2,4-D and other auxins as well as with DNA methylation changes during somatic embryogenesis and plant regeneration in *B. rotunda* in future.

In order to establish efficient, stable, homogeneous embryogenic cultures and plant regeneration system with higher response during *in vitro* condition, it is of interest to gain better understanding of the epigenetic mechanisms involved in these processes. DNA methylation has been identified as a regulator of gene expression related to the induction of somatic embryogenesis and successful plant regeneration (Shibukawa et al., 2009; Vanyushin & Ashapkin, 2011; Nic-Can & De la Peña, 2014). Changes in cell differentiation during *in vitro* culture require cell reprogramming which involves changes in DNA methylation (Baroux et al., 2011). The current study investigated DNA methylation profiles in embryogenic and non-embryogenic calli and other tissues of *B. rotunda* using Methylation Sensitive Amplified Polymorphism (MSAP) and Bisulfite Sequencing (BS-seq) data.

5.8 *B. rotunda* calli and tissues showed high DNA methylation polymorphism and a relatively lower level of both global and gene specific DNA methylation was associated with somatic embryogenesis and plant regeneration in *B. rotunda*

DNA methylation changes and polymorphism in different types of calli and tissues of *B. rotunda* at CCGG site was assayed using MSAP as this technique has been applied in various studies and shown to be an efficient and robust technique to detect alterations of genome-wide DNA methylation patterns and levels at CCGG sites during tissue culture in many plants such as in *Cymbidium hybridium* (Chen et al., 2009), *Freesia hybrida* (Gao et al., 2010), *Secale cereale* (González et al., 2013), *Solanum tuberosum* (Tiwari et al., 2013) and *Coffea arabica* (Landey et al., 2015). In this study, differential DNA methylation patterns and levels were found using nine primer combination (Table 4.6 and Figure 4.8; Appendix G) indicating extensive changes in DNA methylation status in different types of calli and tissues of *B. rotunda*. EVL (91.08%) and IVL (87.90%) showed the highest level of methylation among all samples, but considering only *in vitro* calli and cell suspension, non-embryogenic callus, CS, showed the highest level of methylation (84.08%), and embryogenic callus, EC, showed the lowest level of methylation (74.52%) (Table 4.6 and Figure 4.8; Appendix G). Comparatively lower level of methylation in embryogenic callus than non-embryogenic callus and other tissues indicates that a decreased level of global methylation at CCGG sites is associated with somatic embryogenesis. The current study's results agree well with some previous reports where a lower level of methylation was noticed in embryogenic calli than non-embryogenic calli, such as in *Eleuterococcus senticosus* (Chakrabarty et al., 2003) and in *Pinus nigra* ssp Austriaca (Noceda et al., 2009). However, in several reports, it was noticed that an increased level of DNA methylation was associated with somatic embryogenesis such as in *Cucurbita pepo* (Leljak-Levanic

et al. 2004), in *Acca sellowianai* (Fraga et al., 2012), in *Coffea canephora* (Nic-Can et al., 2013) and in *Arabidopsis thaliana* (Kwiatkowska et al., 2014), and no significant difference was noticed in the amount of DNA methylation of embryogenic and non-embryogenic lines in *Pinus pinaster* (Klimaszewska et al., 2009), which suggest that this epigenetic modification varies depending on plant species and / or other factors involved during *in vitro* culture.

In this work, considering all seven samples in a data set, no band was found at the same loci, which suggests the high frequency of DNA methylation polymorphism in *B. rotunda* calli and tissues (Figure 4.8; Appendix G). Differences in band intensity (as suggested by Xiong et al., 1999) also suggest polymorphism. It was found that class IV types of bands were the major source of DNA methylation polymorphism in all seven samples as the number of bands were much higher than class II and class III type of bands (Table 4.6 and Figure 4.8; Appendix G). Many previous studies have been reported high and unexpected levels of DNA methylation changes during *in vitro* culture and plant regeneration (reviewed by Miguel & Marum, 2011). High DNA methylation polymorphism following meristem based *in vitro* culture was reported in *Gardenia jasminoides* (Wu et al., 2012) and in *Allium sativum* (Gimenez et al., 2016). In *Theobroma cacao*, MSAP analysis also have revealed high epigenetic variation (Rodríguez López et al., 2010). However, like genetic variation, these epigenetic polymorphisms could lead to induce somaclonal variation during *in vitro* cultures, as Ong-Abdullah et al. (2015) noticed abnormalities in the floral development of somatic embryo-derived plants in *Elaeis guineensis*, causing a mantled phenotype due to DNA methylation.

Since MSAP is not a straightforward technique and is unable to give complete methylation profile, overall global DNA methylation in terms of CG, CHG and CHH methylation, gene specific methylation status of three methyltransferase genes (*MET1*,

CMT3 and *DRM2*) and of four somatic embryogenesis and plant regeneration related genes (*SERK*, *BBM*, *LEC2* and *WUS*), and of three housekeeping genes (*Actin*, *Beta tubulin 1* and *EF1-α*) at all three cytosine contexts were determined using BS-seq data. As there is no completed genome sequence available for *B. rotunda*, the BS-seq data was mapped to a *B. rotunda* transcriptome assembly (described in another thesis paper headed by a PhD fellow colleague of our group (manuscript and thesis in preparation)), and the highest methylation levels were observed in EVL for CG methylation (6.62%) and CHG methylation (7.59%), and in CS for CHH methylation (5.94%). The lowest methylation levels were observed in EC for all three types of methylation (2.11% for CG, 2.47% for CHG and 3.47% for CHH) (Figure 4.11; Appendix H) which is in good agreement with the data from MSAP in this current study and supports a hypothesis of an association between decreased level of methylation (hypomethylation) and somatic embryogenesis during *in vitro* culture of *B. rotunda* (Table 4.6 and Figure 4.8, 4.11, Appendix G, H). In general, it has been reported that global hypomethylation is associated somatic embryogenesis (reviewed by De La Pena et al., 2015). The observations from this thesis study are also consistent with reports from *Eleuterococcus senticosus* (Chakrabarty et al., 2003) and *Pinus nigra* ssp Austriaca (Noceda et al., 2009), where global hypomethylation was associated with somatic embryogenesis. Although CS was established from EC, it showed higher levels of methylation than EC and also than other non-embryogenic calli samples i.e. DC and WC, which might be the effect of long-term maintenance in cell suspension culture (12 months) (Table 4.6 and Figure 4.8, 4.11). This result also coincides with reports from *Elaeis guineensis* (Rival et al., 2013) and *Arabidopsis thaliana* (Kwiatkowska et al., 2014) where higher level of methylation was associated with ageing of culture.

When considering the gene expression data together with the global DNA methylation levels in the same samples, there was a negative relationship between mRNA

levels of DNA methyltransferases (*MET1*, *CMT3* and *DRM2*) and the global methylation in the callus samples (as discussed in earlier in section 5.6). However, as there is not yet any genome sequence available for *B. rotunda*, it is difficult to map the location of the methylation and to determine if the differences in global genomic methylation in different samples affect gene space or are confined to repetitive parts of the genome. Due to this limitation, the current study focused on examining gene specific methylation using transcriptome data to identify the sequences of genes of interest that could be used together with BS-seq data to determine their DNA methylation status in the different samples used in this study. Among all samples, overall lower levels of methylation of *MET1*, *CMT3* and *DRM2* were seen in EC. CS and WC showed the highest level of methylation in *MET1* and *CMT3*, respectively, while WC and IVL showed the highest level of methylation in *DRM2* (Figure 4.12). Correlation analysis indicated that although there is no apparent simple correlation between the level of DNA methyltransferase genes *MET1*, *CMT3* and *DRM2* expression and global DNA methylation status at CG, CHG and CHH context in all samples used in this study, the gene specific DNA methylation levels of all three DNA methyltransferase genes at CG, CHG and CHH contexts showed overall negative correlation with their expression level in all samples.

According to global DNA methylation data and gene expression data, it can be suggested that for somatic embryogenesis and plant regeneration related genes (*SERK*, *BBM*, *LEC2* and *WUS*), a decreased level of global DNA methylation (as observed in EC) coincides with enhanced expression level which permits somatic embryogenesis and regeneration, whereas the relatively increased level of global methylation (in DC, WC and CS) reduced their expression level which represses the embryogenic competency and plant regeneration in *B. rotunda* (Figure 4.7, 4.11). This hypothesis can be supported with some previous observations on chromatin organization, as gene expression or repression depends on the degree of its compaction i.e. a decreased level of DNA methylation relaxes

the chromatin structure which allows gene expression, and the increased DNA methylation contracts the chromatin structure which represses the genes (Schones & Zhao, 2008; Tamaru, 2010; reviewed by De La Pena et al., 2015). When considering at the gene level of DNA methylation, embryogenic callus (EC) also showed a comparatively lower level of DNA methylation at CG, CHG and CHH contexts of *SERK*, *LEC2* and *WUS* than was seen for the other samples (Figure 4.13). In *BBM*, although EC exhibited comparatively higher level of CG methylation than DC and WC, it lacked CHG methylation (Figure 4.13). A lower level of methylation was also noticed in *BBM* and *WUS* than *SERK* and *LEC2* (Figure 4.13). It was observed that although decreased level of methylation within coding regions (transcript mapped) *SERK*, *LEC2* and *WUS* were associated with their higher expression, and showed negative correlation between methylation and their expression level, in the case of *BBM* and *LEC2*, some weak positive correlation was noticed for CHH methylation (Figure 4.16). Consistent with a decreased DNA methylation measured at the global level, it can be suggested that lower level of methylation at the coding regions of *SERK* and *LEC2* was associated with somatic embryogenesis and plant regeneration in *B. rotunda*. For *BBM* and *WUS*, as methylation level was very low and some samples lacked methylation, the expression of these two genes and their relevancy with somatic embryogenesis and regeneration may be affected by global DNA methylation level or methylation of upstream regions that could not be mapped without s reference genome sequence. The observation agrees with a report on *Arabidopsis thaliana*, where it was found that *met1* had the reduced DNA methylation levels and consequently with higher expression of *WUS* gene, resulting in an earlier shoot primordial initiation (Li et al., 2011). However, while a majority of studies of DNA methylation are based on estimated DNA methylation status using genome-wide methylation measurement, there are very few reports that have focused on the methylation of specific genes related with somatic embryogenesis and plant regeneration (reviewed

by Us-Camas et al., 2014; De-la-Peña et al., 2015). As in the current study, decreased levels of DNA methylation of *SERK* and *BBM* were found in embryogenic culture of *Coffea canephora* (Nic-Can et al., 2013). Although knowledge is limited on the methylation of genes related to somatic embryogenesis and regeneration during these processes, a decreased level of DNA methylation status of *SERK*, *BBM*, *LEC* and *WUS* has been reported to be associated with somatic embryogenesis and plant regeneration (reviewed by De-la-Peña et al., 2015; Mahdavi-Darvari et al., 2015). However, there have been no reports demonstrating gene specific methylation status of *SERK*, *BBM*, *LEC*2 and *WUS*, and also of DNA methyltransferase genes, *MET1*, *CMT3* and *DRM2* in different types of calli and tissues as studied in this thesis. The new data reported in this thesis indicated that although there was some positive correlation, gene specific DNA methylation showed mostly negative correlation between the expression of *SERK*, *BBM*, *LEC2* and *WUS* and their DNA methylation status during *in vitro* culture and plant regeneration.

For better understanding, the DNA methylation status of three housekeeping genes, *Actin*, *Beta-tubulin 1* and *EF1-α,* was determined as these genes are expressed at similar levels irrespective of tissue or culture conditions, and due to this are used for normalization of gene expression data. Predominantly CG methylation and low level of CHG and CHH methylation was found in *Actin*, *Beta-tubulin 1* and *EF1-α* (Figure 4.14). However, at the present time, it is not feasible to provide information on specific location of methylation such as in promoter or gene body or exons-introns since the complete genome sequence of *B. rotunda* has not yet been published. Nevertheless, it may be useful to determine full DNA methylation profiles of housekeeping and other specific genes associated with plant development, or of transposable elements, when *B. rotunda* genome sequence becomes available. As DNA demethylation cooperates side by side with the DNA methylation activities, it may be considered to determine the detail DNA

methylation profiles in the future studies. In addition, since other epigenetic mechanism such as histone methylation and acetylation are supposed to play important role to modify the chromatin organization, it will be better to consider in future design of epigenetic regulation of genes during *in vitro* culture and plant regeneration as well as different types of tissues during plant development.

In summary, the data shown in this thesis has addressed the study objectives as follows:

Objective 1: To determine the differences in expression of the DNA methylation pathway genes, *MET1*, *CMT3* and *DRM2* among *ex vitro* and *in vitro* tissues, embryogenic and non-embryogenic calli, and prolonged cell suspension culture of *B. rotunda*.

Findings of the study: A higher expression level of DNA methylation pathway genes *MET1*, *CMT3* and *DRM2* was associated with embryogenic callus, meristematic block tissue, *ex vitro* and *in vitro* leaf tissues, whereas a lower expression level was associated with non-embryogenic and non-regenerable calli. *MET1*, *CMT3* and *DRM2* maintain DNA methylation at CG, CHG and CHH contexts, respectively, while *DRM2* also mediates *de novo* methylation at all three contexts. Corresponding to the variation of DNA methylation among different tissues and plant development stages, the expression levels of DNA methyltransferases are also dynamic during plant development and it is hypothesized that expression of *MET1*, *CMT3* and *DRM2* was higher in the cells which are more dividing potential such as meristematic block tissue, embryogenic-regenrable calli.

Objective 2: To determine the differences in expression of the somatic embryogenesis and plant regeneration related genes, *SERK*, *BBM*, *LEC2* and *WUS* among

ex vitro and *in vitro* tissues, embryogenic and non-embryogenic calli, and prolonged cell suspension culture of *B. rotunda*.

Findings of the study: For *in vitro* calli or cells (i.e. EC, DC, WC and CS), the higher expression levels of *SERK*, *BBM*, *LEC2* and *WUS* were correlated with embryogenic callus, whereas lower expression levels were correlated with non-embryogenic and non-regenerable calli and cells. Meristematic block tissue, *ex vitro* and *vitro* leaf also showed relatively higher expression of than non-embryogenic and non-regenerable calli. The molecular mechanism for somatic embryogenesis and plant regeneration is still unclear, however, the findings of this thesis have added knowledge on somatic embryogenesis and plant regeneration and lead to a new hypothesis that the higher expression levels of *SERK*, *BBM*, *LEC2* and *WUS* are associated with these processes.

Objective 3: To determine the changes in DNA methylation patterns among *ex vitro* and *in vitro* tissues, embryogenic and non-embryogenic calli, and prolonged cell suspension culture of *B. rotunda*.

Findings of the study: A decreased level of both global and gene specific DNA methylation level (hypomethylation) at CG, CHG and CHH sequence contexts was associated with embryogenic callus, whereas the relatively increased methylation (hypermethylation) at those contexts was associated with non-embryogenic and non-regenerable calli. EVL and IVL showed relatively higher levels of global DNA methylation. This is a first report of gene specific methylation in *B. rotunda* and while there was a limitation for mapping methylation without a reference genome sequence, it can be hypothesized that a relatively lower level of both global and gene specific methylation are associated with somatic embryogenesis and plant regeneration in *B. rotunda*.

Objective 4: To determine the relationship between the gene expression and DNA methylation status.

Findings of the study: While *ex vitro* and *in vitro* leaf tissue showed positive correlation between the expression and DNA methylation level, for *in vitro* calli or cells (i.e. EC, DC, WC and CS), the expression of DNA methyltransferase genes (*MET1*, *CMT3* and *DRM2)* and somatic embryogenesis related genes (*SERK*, *BBM*, *LEC2* and *WUS)* showed mostly negative correlation with DNA methylation status, especially.

CHAPTER 6: CONCLUSIONS

During *in vitro* conditions, differentiated plant cells can be induced to generate organs, shoots, or somatic embryos, which can form new plants. Plant regeneration *via* somatic embryogenesis is used for clonal propagation and preferred over organogenesis for gene manipulation for a wide range of crops. In addition, somatic embryogenesis provides an interesting model to study gene expression and epigenetic changes during plant development. In this study, gene expression and DNA methylation during *in vitro* culture and plant regeneration of *Boesenbergia rotunda* were described to provide an insight of these processes. DNA sequence alignment and phylogeny characterization showed that the DNA methylation pathway genes (*METHYLTRANSFERASE 1* (*MET1*), *CHROMOMETHYLASE 3* (*CMT3*) and *DOMAIN REARRANGED METHYLTRANSFERASE 2* (*DRM2*)), and four somatic embryogenesis and plant regeneration related genes (*SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* (*SERK*), *BABY BOOM* (*BBM*), *LEAFY COTYLEDON 2* (*LEC2*) and *WUSCHEL* (*WUS*)) from *B. rotunda* share high sequence similarity and identity with their respective homologs from *Musa acuminata* subsp. Malaccensis.

Based on the expression and methylation analyses of methyltransferase genes, it was observed that the higher expression level of *MET1*, *CMT3* and *DRM2*, and the decreased global and gene specific methylation level (hypomethylation) at CG, CHG and CHH sequence contexts were associated with embryogenic callus, whereas the lower expression level and relatively increased methylation (hypermethylation) at those contexts were associated with non-embryogenic and non-regenerable calli. On the other hand, the samples those were an organized tissue, EVL, IVL showed both higher level of gene expression and relatively higher level of global DNA methylation. Based on these findings, it can be concluded that although there was positive correlation between

expression levels of DNA methyltransferase genes and global DNA methylation level in EVL and IVL, for *in vitro* calli or cells (i.e. EC, DC, WC and CS), gene expression and both global and gene specific DNA methylation at CG, CHG and CHH contexts were mostly negatively correlated. Different to *in vitro* calli and cells, as EVL and IVL are organized and differentiated tissue, it can behave differently, however, and it can be further validated by gene expression and DNA methylation analysis considering other differentiated tissues compared to *in vitro* calli samples.

For *SERK*, *BBM*, *LEC2* and *WUS*, specific genes related to somatic embryogenesis and regeneration, it was observed that the higher expression levels of *SERK*, *BBM*, *LEC2* and *WUS* were correlated with embryogenic callus, whereas lower expression levels were correlated with non-embryogenic and non-regenerable calli and cells. Meristematic block tissue, *ex vitro* and *vitro* leaf also showed relatively higher expression of than non-embryogenic and non-regenerable calli. It was also observed that decreased levels of both global and gene specific DNA methylation of *SERK, BBM, LEC2* and *WUS* at CG, CHG and CHH sequence contexts were associated with their higher expression level and showed negative correlation between DNA methylation and their expression level. Although there was no apparent correlation between gene expression and DNA methylation among all samples, for *in vitro* calli or cells (i.e. EC, DC, WC and CS), it can be concluded that the higher expression level and overall lower methylation level at CG, CHG and CHH sequence contexts were associated with embryogenic callus, whereas lower expression level and overall higher methylation level at those contexts were associated with non-embryogenic and non-regenerable calli and cells.

Somatic embryogenesis mediated plant regeneration is a complex process involving hormone actions, transcription factors and epigenetic regulations (Yang & Zhang, 2010). The controlling mechanisms for somatic embryogenesis and plant

regeneration are still unclear. However, since 2000, more than 100 plant genomes have been sequenced, 63% of which are crop species (Michael & VanBuren, 2015). This new availability of genome sequences of crop species provides more scope for the retention of key agronomic traits by using gene expression and DNA methylation profile. With the advent of information relating to regulation of gene expression, epigenetics and somatic embryogenesis, there is a tangible possibility to employ DNA methylation profiles related to embryogenesis and plant regeneration for selection and modification of genotypes from recalcitrant to somatic embryogenesis and regeneration of high value crops. The data in this work may be useful to the study considering more concentration of 2,4-D as well as other plant growth regulators, different time points, and detailed DNA methylation profile of promoter region or exon-intron of genes related to DNA methylation pathway or of genes associated with somatic embryogenesis and plant regeneration when *B. rotunda* genome sequence will be available in future. Other epigenetic mechanisms such as histone methylation and acetylation may also be considered in future studies. The findings of this thesis could form the foundation for future research and could provide more research avenues on genetic and epigenetic control of plant somatic embryogenesis and regeneration during *in vitro* culture.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Journal papers

Published / accepted

Rezaul Karim, Mohammed Nuruzzaman, Norzulaani Khalid, Jennifer Ann Harikrishna (2016). Importance of DNA and histone methylation in *in vitro* plant propagation for crop improvement: A review". *Annals of Applied Biology*, *169*(1), 1-16. (*ISI-indexed journal*).

Conference presentations

Rezaul Karim, Norzulaani Khalid and Jennifer Ann Harikrishna. Genes associated with DNA methylation, histone modification and plant regeneration during *in vitro* culture. Oral presentation at the 18th Biological Science Graduate Congress (18th BSGC), 6-8 January 2014, University of Malaya, Kuala Lumpur, Malaysia.

Rezaul Karim, Norzulaani Khalid and Harikrishna. An overview of alterations in DNA methylation and histone modifications pattern during *in vitro* culture and plant regeneration. Poster presentation at the 19th Biological Science Graduate Congress (19th BSGC), 12-14 December 2014, National University of Singapore (NUS), Singapore.

Rezaul Karim, Yew Seong Tan, Pooja Singh, Mohammed Nuruzzaman, Norzulaani Khalid and Jennifer Ann Harikrishna (2016). Expression of DNA methyltransferase genes *MET1*, *CMT3* and *DRM2* during *in vitro* culture, somatic embryogenesis and plant regeneration of *Boesenbergia rotunda*. Poster presentation at the 23rd Malaysian Society for Molecular Biology and Biotechnology (MSMBB) Scientific Meeting, 9-11 March 2016, Connexion@Nexus, Bangsar South, Kuala Lumpur, Malaysia.