

Morphohistological and molecular profiles during the developmental stages of somatic embryogenesis of *Musa acuminata* cv. 'Berangan' (AAA)

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Abstract The present study established a protocol for generating embryogenic cell suspension (ECS) cultures from an early stage embryogenic callus of *Musa acuminata* cv. 'Berangan'. The necessary baseline data for morphohistological recognition and the selected gene expression for differentiating embryogenic and non-embryogenic cells were defined to enable early selection, as well as for quality assessment, which could aid in scaling up the production of these cells. In this study, male bud-derived callus on Murashige and Skoog (MS) medium containing 23 μM 2,4-dichlorophenoxyacetic acid (2,4-D) was transferred to M1 medium containing different concentrations of 2,4-D and picloram for somatic embryo initiation. ECSs established in liquid MS medium (M2a) containing 4.1 μM biotin, 10 mg/l ascorbic acid, 100 mg/l glutamine, 100 mg/l malt extract, 4.5 μM 2,4-D, 1.0 μM zeatin, and 20 g/l sucrose were used to study embryogenic development. The frequency of embryo development and maturation was influenced by the culture media. The highest frequency of embryo development (96.4 %) and maturation (66.4 %) was achieved on solid MS hormone-free medium. Almost 69 % of embryos were induced to form shoots on the MS medium containing 34 μM 6-benzylaminopurine (BAP). Histological study

indicated that only embryos with distinct layer of protoderm having cells with prominent nucleus and dense cytoplasm were able to regenerate into plantlets. *Endochitinase* and *phenylalanine ammonia-lyase (PAL)* genes were expressed at all the developmental stages, and their expression level was tightly regulated during embryo development and reached its maximum in germinating embryos. Expression of these genes in embryogenic cells was higher than in non-embryogenic cells. The study findings suggested that these genes play an important role in the developmental stage and regeneration pathway of somatic embryos of banana.

Keywords Embryogenic cell suspension · *Endochitinase* · *Phenylalanine ammonia-lyase* · Somatic embryogenesis

Introduction

Banana is both a commercial and a staple food crop. Limitations in traditional breeding due to the parthenocarpic nature of the plant have necessitated strategies for improvement through alternative approaches. The ability to generate embryogenic cell suspensions (ECSs) for applications to these strategies is an advantage that would enable high-throughput methodologies. Somatic embryogenesis is also used as a model system for understanding the physiological, biochemical, and molecular biological events that occur during the development of plant embryos (Karami et al. 2009), yet much more remains to be elucidated on the mechanisms related to each plant type. The frequency of embryo production is not only dependent on the genomic group, but also differs among the varieties within the same genome (Strosse et al. 2006; Youssef et al. 2010).

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The ECS culture of banana was first reported by Novak et al. (1989) followed by numerous reports in both commercial and indigenous cultivars (Chong-Pérez et al. 2005; Ghosh et al. 2009; Meenakshi et al. 2011; Kulkarni and Bapat 2013; Remakanthan et al. 2014). Even though progress has been made in establishing ECS cultures of banana, the system is still hindered by a low frequency of induction of embryogenic callus and the subsequent induction of somatic embryos (Strosse et al. 2006; Remakanthan et al. 2014). Somatic embryogenesis in banana is still far from being considered a routine technology, has not been successfully applied to all cultivars, and warrants further improvement for many cultivars that are to be exploited as commercial crops. It is apparent that many morphological and physiological events go hand in hand with plant development during somatic embryogenesis, and the fate of cells in response to different conditions vary. Fundamental studies have not yet been fully pursued on the fate of meristematic cells during developmental stages affected by various physical and chemical factors in the somatic embryogenesis in banana.

Histological examination is an important tool to examine the effect of different conditions on cellular and embryonic development. Many investigators have studied the progression of specific stages in somatic embryogenesis histologically in comparison to non-embryogenic structures (Wong et al. 2006; Jalil et al. 2008). During the developmental stages in tissue culture systems, shoots can be regenerated through either direct or indirect pathways. In vitro regeneration, in particular somatic embryogenesis, can be considered as a model system to decipher events relating to the fate of cells during development. This system allows for studies of morphohistology and gene expression that could be used to study plant cell totipotency and subsequently to identify patterns and associations of gene expression. Changes in various biochemical and physiological events during the developmental pathway appear to be associated with the activation of a number of genes, some of which have been shown to be directly related to morphogenesis and development (Karami et al. 2009). Several genes, such as *SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK)*, *BABY BOOM (BBM)*, *LEAFY COTYLEDON (LEC)*, and *WUSCHEL (WUS)*, serve as molecular signatures of these changes during the different developmental processes (Yang and Zhang 2010; Neelakandan and Wang 2012). Given that somatic embryogenesis is highly genotypically dependent, elucidation of the regeneration pathway at the molecular level could lead to a better understanding of the processes that control the frequency of germination and embryogenesis. During the change from the somatic to the embryogenic state, plant cells have to dedifferentiate, activate their cell division

cycle, and reorganize their physiology, metabolism, and patterns of gene expression (Kurczynska et al. 2012; Neelakandan and Wang 2012). It has been observed that there are both genetic and physiological factors that trigger in vitro embryogenesis in different types of plant somatic cells (Karami et al. 2009).

Gene expression during the developmental process is controlled by the expression of a large number of genes that could be referred to as “developmental indicator genes”. The genes encoding endochitinase are such genes that have significant roles in plant developmental stages. Expression studies and mutational analyses of endochitinase in various plants, including model plants such as *Arabidopsis*, have underscored the importance of endochitinase in the control of development (Zhong et al. 2002; Grover 2012). It has been shown that various plant chitinases are involved in plant defensive mechanisms (Gerhardt et al. 2004). Although the main role of chitinases has been suggested to be in a defensive pathway primarily acting on fungal pathogens, chitinases have been reported to have a non-defensive role in plant development (Pirttila et al. 2002). Several reports have outlined their specific roles in different aspects of plant development, such as somatic embryogenesis and embryonic development (Wiweger et al. 2003; Gerhardt et al. 2004), and in distinct organs of the flower during the development of flowers (Takakura et al. 2000), seeds (Regalado et al. 2000), and fruit (Salzman et al. 1998). A second indicator gene family, the *PAL* genes, has been characterized in a number of species (Wang et al. 2007). These family members are differentially expressed in plant tissues in response to different stress conditions (Wang et al. 2007). *PAL* genes encode the phenylalanine ammonia-lyase enzyme, which is the first enzyme in phenylpropanoid synthesis (Kervinen et al. 1998). Previous study showed that disruption of *PAL* genes may cause a lethal phenotype in *Arabidopsis* (Huang et al. 2010) and male sterility in petunia (*Petunia hybrida*) anthers (van der Meer et al. 1992), suggesting the importance of phenylpropanoid pathway in plant growth and development. The regulation and expression of *PAL* genes have also been studied in many plant species to examine their function during plant development and defense-related activation (Ghanti et al. 2009).

In this study, morphohistological examinations during somatic embryogenesis and in response to culture factors were investigated in banana cv. ‘Berangan’ (AAA), a high-value commercial banana grown in Malaysia. The aim was to enable the selection and identification of suitable competent embryogenic cells leading to high rates of regeneration. In addition, the expressions of two indicator genes, *endochitinase* and *PAL*, in relation to these changes were also analyzed.

Materials and methods

Plant material and initiation of embryogenic callus

Male buds of *Musa acuminata* cv. 'Berangan' were trimmed to 6–8 cm in length, surface sterilized in 70 % (v/v) ethanol for about 15 min, and further trimmed to 1.5–2 cm in length. A total of 90 male inflorescences from positions 1–15 (1 being the immature flowers closest to the meristematic dome) were isolated and used individually as explants. The initiation of embryogenic callus was done initially either on solid or in liquid pre-treatment media (PM; Table 1). Solid PM media were solidified with 2 g/l gelrite and poured into 90 × 20 mm Petri dishes (25 ml per plate). Each plate contained 15 explants. The pH of the media was adjusted to 5.7 prior to sterilization. The liquid cultures were maintained in 50 ml shake flask containing 20 ml PM and were agitated on an orbital shaker at 60 rpm, and during this period, the medium was changed every 15 days by replacing half the volume with fresh medium. Each flask contained five explants. The cultures were maintained on PM for a period of 45 days. Subsequently, explants were transferred to Petri dishes containing M1 medium (25 ml per plate; Table 1) supplemented with different concentrations of 2,4-D and picloram for 3–4 months. The cultures were observed every 2 weeks. The frequency of somatic embryo formation (%) was calculated as the number of calluses with embryogenic response (whitish, translucent pre-embryos)/total number of calluses initially incubated × 100. The explants during incubation in the PM and M1 cultures were maintained in the dark at 25 ± 2 °C.

Initiation of suspension cultures

To establish cell suspensions of *M. acuminata* cv. 'Berangan', two culture media were tested: M2a (modified from Côte et al. 1996) and M2b (Dhed'a et al. 1991) (Table 1). Friable embryogenic calluses (0.2–0.5 g) with pro-embryos were selected and transferred to M2a or M2b liquid media. For the maintenance of the suspension culture, cell density was adjusted to 3 % settled cell volume (SCV) of the cell aggregates in 50 ml M2 medium. The suspension cultures were then sub-cultured every 15 days by adding 50 % fresh media to the total volume. The cultures were placed on an orbital shaker at 90 rpm at 25 ± 2 °C, with a 16-h photoperiod at a light intensity of 31.4 μmol m⁻² s⁻¹. To obtain homogeneous embryogenic cells during the maintenance period, the suspension cultures were filtered (450 μm pore size) at two-week intervals for 3 months. Meanwhile, immediately after removal of the flask, a small sample of the suspension was examined under a microscope to study embryonic development in different culture media: M3 (Strosse et al. 2003), modified M3 (MS3; Schenk and Hildebrandt 1972), solid MS without plant growth regulator (PGR; MS0), and liquid-based MS0 (MS0L) media (Table 1). For the development of the embryos, the suspension cells in the M2 medium were left to settle in a 50 ml graduated conical tube, and the cell density was adjusted to 20 % SCV. To ensure homogenous cell distribution, 200 μl of cells were pipetted and spread onto each solid medium. For the MS0 liquid medium, 300 μl of suspended cells were aliquoted into 30 ml of each liquid culture medium. These cultures were again maintained on an orbital shaker at 60 rpm. All cul-

Table 1 Media compositions for embryogenic callus and cell suspension cultures of *M. acuminata* cv. 'Berangan'

Medium	Composition
PM	Murashige and Skoog (1962) basal medium (MS) with 4.1 μM biotin, 100 mg/l glutamine, 10 mg/l ascorbic acid, 30 g/l sucrose, and 23 μM 2,4-dichlorophenoxyacetic acid (2,4-D)
M1	MS, 4.1 μM biotin, 10 mg/l ascorbic acid, 5.7 μM indole-3-acetic acid, 5.4 μM 1-naphthylacetic acid, 30 g/l sucrose, 2 g/l gelrite, pH 5.7
M2a	MS basal medium, 4.1 μM biotin, 10 mg/l ascorbic acid, 100 mg/l glutamine, 100 mg/l malt extract, 4.5 μM 2,4-D, 1 μM zeatin, 20 g/l sucrose, pH 5.7
M2b	MS basal medium, 10 mg/l ascorbic acid, 4.5 μM 2,4-D, 1 μM zeatin, 30 g/l sucrose, pH 5.7
M3	SH macro- and micro- nutrients, MS vitamins, 1 mg/l biotin, 100 mg/l L-glutamine, 100 mg/l malt extract, 230 mg/l proline, 0.2 mg/l NAA, 0.05 mg/l zeatin, 0.1 mg/l kinetin, 0.2 mg/l N6-2-isopentenyladenine (2ip), 45 g/l sucrose, 10 g/l lactose, 3 g/l gelrite, pH 5.7
MS3	MS basal medium, 1 mg/l biotin, 100 mg/l glutamine, 100 mg/l malt extract, 230 mg/l proline, 0.2 mg/l NAA, 0.05 mg/l zeatin, 0.1 mg/l kinetin, 0.2 mg/l 2ip, 45 g/l sucrose, 10 g/l lactose, 3 g/l gelrite, pH 5.7
MS0	MS basal medium, 30 g/l sucrose, 3 g/l gelrite, pH 5.7
MS0L	MS basal medium, 30 g/l sucrose, pH 5.7
M4	MS macro- and micro- nutrients, MS FeEDTA, 1 mg/l thiamine, 1 mg/l nicotinic acid, 1 mg/l pyridoxine, 2 mg/l glycine, 100 mg/l myo-inositol, 30 g/l sucrose, 2 g/l gelrite, pH 5.7

tures were placed at 25 ± 2 °C in the dark until the formation of somatic embryos was observed.

Regeneration of somatic embryos

Mature bipolar embryos were selected from embryonic development media (MS0) and regenerated on regeneration medium (M4; Table 1) consisting of various concentrations of 6-benzylaminopurine (BAP; 14, 24, and 34 μ M) and thidiazuron (TDZ; 3, 5, and 7 μ M). The MS0 was employed as a control. The cultures were incubated in the dark at 25 ± 2 °C until adventitious buds appeared and subsequently grown in 16 h daylight for shoot development. The regeneration frequency of embryos was calculated and recorded as a percentage of the number of plantlets over the number of mature embryos initially cultured after 8 weeks of culture.

In vitro rooting

Shoots of 2–3 cm in height were transferred to our previous established medium (MS0 supplemented with 0.1 % (w/v) activated charcoal, 30 g/l sucrose, and 2 g/l gelrite; Wong et al. 2006) for root induction.

Histological examinations

Histological examinations were performed on non-embryogenic callus, embryogenic callus, globular embryo, mature torpedo embryo, and germinated embryo. The samples were fixed for 24–48 h at room temperature in a glutaraldehyde–paraformaldehyde–caffeine (GPC) (Sigma Chemical Co., USA) fixative (50 ml 0.2 M phosphate buffer, pH 7.2; 20 ml 10 % (v/v) paraformaldehyde; 4 ml 25 % (v/v) glutaraldehyde; 1 g caffeine, and distilled water to a total volume of 100 ml). The samples were then dehydrated in a series of ethanol solutions (v/v): 30 %, 30 min; 50 %, 45 min; 70 %, 45 min; 80 %, 60 min; 90 %, 60 min; 95 %, 60 min; and twice in absolute ethanol for 60 min each. A vacuum was applied at a low pressure for approximately 10–20 min to ensure complete infiltration of the solution in the sample. The tissues were then ready for infiltration using Kit Resin LKB (Leica, Rueil-Malmaison, France) for 24 h at 4 °C for the small tissues. Larger tissues needed at least 1 week or more of the infiltration period. After infiltration, the specimens were embedded in basic resin and cut into 3.5 μ m sections using a microtome. Fine sections were stained for starch with 1 % (w/v) periodic acid for 5 min and then rinsed four times with distilled water at pH 4.5. Subsequently, they were submerged in Schiff's reagent (1 g basic fuchsin, 2 g disodium metabisulfite in 1 M HCl, and 0.5 g neutralized activated charcoal) for 20 min in the dark. The slides were

rinsed with distilled water (pH 4.5) four times and subsequently stained with Naphthol Blue Black (Sigma, USA) (1 g Naphthol Blue Black in 100 ml 7 % (v/v) acetic acid) for proteins at 60 °C for 5 min and rinsed again with distilled water. The slides were mounted with Cytoseal™ 60 mounting medium (Reichard-Allan Scientific, Kalamazoo, MI) and allowed to dry for 1 day. The slides were kept in the dark until microscopic observation. Photographs were taken using Leica DME microscope adapted with a Leica EC3 digital camera.

Analysis of gene expression

Genomic DNA was extracted from the young leaf tissues using a modified CTAB method (Doyle and Doyle 1990; 2 % (w/v) CTAB; 1.4 M NaCl; 20 mM EDTA; 100 mM Tris-HCl, pH 8.0; and 2 % PVP-40) and treated with 10 mg/ml RNase (Amresco, OH, USA). *Endochitinase* and *PAL* were selected as developmental indicators, and specific primers, forward and reverse, were designed to isolate the targeted gene sequences in the cv. 'Berangan' genome based on the gene sequences of other varieties of *M. acuminata* obtained from GenBank (www.ncbi.nlm.nih.gov). PCR amplification was performed to assess the presence of the *endochitinase* (Fwd ggcacgaggcgcacacgacgagcc; Rev gggccatgaactgttcagcttagctttta; AF416677) and *PAL* (Fwd agattccttaatgccggaatattcgctc; Rev gttgcaattatcatgcataagcacacaa; EU862233) genes. The expected sizes were 820 and 1930 bp, respectively. The 25 μ l PCR contained 1 μ g DNA, 5 pmol of each primer and master mix (Invitrogen, USA). The cycling conditions were: initial denaturation step of 95 °C for 5 min, followed by 35 cycles of 95 °C for 15 s, 68 °C (for *endochitinase*) or 57.8 °C (for *PAL*) for 30 s, and 68 °C for 90 s. Finally, the reaction was allowed to complete with an additional extension of 5 min at 68 °C before cooling to 25 °C. The PCR products were purified using a PCR purification kit (QIAquick; Qiagen) using the protocols provided by the manufacturer. DNA sequences were determined with a 3130xl genetic analyzer (Applied Biosystems, USA). The sequence identity was determined using the online BLAST algorithm on the GenBank database at NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Extraction of RNA and cDNA synthesis

A modification of the CTAB method by Zeng and Yang (2002) was used for RNA extraction. Tissues from different stages of somatic embryogenesis (0.3–0.5 g) were ground in liquid nitrogen with a pre-cooled mortar and pestle. The frozen tissue powder was quickly transferred to a 2 ml microcentrifuge tube containing a pre-warmed (65 °C) extraction buffer (2 % (w/v) CTAB; 2 % (w/v)

PVP-40; 100 mM Tris–HCl, pH 8.0; 25 mM EDTA; and 2 M NaCl; 20 μ l of 2-mercaptoethanol per ml of buffer were added shortly before use). The homogenate was mixed completely by vigorous vortexing and then incubated at 65 °C for 10 min with vigorous shaking. Washing steps were performed three times with chloroform-isoamyl alcohol (24:1). The supernatant was collected after centrifugation at 10,000 \times *g* for 10 min and carefully transferred to a fresh tube. The RNA was precipitated with 0.1 volume of 3 M sodium acetate and 3 volumes of absolute ethanol for overnight at –80 °C. The RNA was recovered by washing with 70 % ice cold ethanol and air dried for 10 min. The RNA was resuspended in 20 μ l DEPC-treated water. A DNase I kit (Invitrogen, USA) was used to eliminate the DNA from the purified total RNA to prevent amplification of genomic DNA targets during the qRT-PCR. Purified total RNA was obtained from each stage and subsequently used for the cDNA syntheses. The High Capacity cDNA Reverse Transcription with RNase Inhibitor Kit (Applied Biosystems, USA) was used to synthesize single-stranded cDNA from the total RNA.

Primer design for qRT-PCR

Primers were designed based on the gene sequences elucidated for the ‘Berangan’ variety. The banana gene encoding *Actin* was used as the candidate endogenous control. The gene-specific primer sets used for the qRT-PCR analysis were designed using Primer3: *Endochitinase* gene (Fwd TGGTTCTGGATGACTCCTCA; Rev CCATTGATGATGTTGGTGGT, 134 bp), *PAL* (Fwd GCTACACCCATCCAGGTTCT; Rev ACGAGCACCTGCCTTA ACTT, 129 bp), and *Actin* gene (Fwd ACGAGTAGCTAGGGCTCGCCG; Rev TCCAGCAAAGCCAGCCTTACC, 136 bp).

Relative quantification of gene expression

The qRT-PCR was performed using an Applied Biosystems 7500 real-time PCR system (Applied Biosystems, California, USA). The relative quantification of gene expression was performed using the Power SYBR[®] Green PCR Master Mix reagents kit (Applied Biosystems, California, USA). Meanwhile, the differential expression of selected genes was verified by relative quantification (RQ) using a comparative $\Delta\Delta C_T$ assay. The actin gene was used as the reference gene. The optimization of the qRT-PCR reaction was performed according to the manufacturer’s instructions (Applied Biosystems, Power SYBR[®] Green reagent protocol). The qRT-PCR reactions were performed using 100 ng of cDNA, 10 μ M of each primer, 12.5 μ l of the Power SYBR[®] Green master mix (Applied Biosystems, California, USA), and RNase-free water in a final volume of 25 μ l.

Forty cycles of amplifications consisting of 15 s at 95 °C, 1 min at 60 °C and 30 s at 72 °C, with an initial preheating at 95 °C for 10 min were performed for all primers tested. Three replicates of the qRT-PCR reactions were performed for each sample. Explants were examined in relation to morphohistological events that occur during developmental changes in the ECS regeneration pathway. The lowest level of expression of both genes was obtained in the regenerated plantlet, and this sample was used as the calibrator with an RQ value of 1. Samples were collected during the different developmental stages from the initiation of the culture of the cell suspensions to the regeneration of the plantlets (embryogenic cell aggregates; cell suspension containing non-embryogenic cells; globular, torpedo, and germinated embryos; and plantlets) and used for the gene-expression experiments. To differentiate embryogenic from non-embryogenic cells, fluorescein diacetate and double-staining tests were applied. In each experiment, the non-embryogenic cells that produced the lowest expression levels were used as the calibrator throughout the relative quantification analysis. All qRT-PCR data were analyzed using the RQ Study software (Applied Biosystems, California, USA). Each sample was replicated three times per experiment, and standard errors were calculated to define the region of expression within which the true expression level was likely to occur. Amplification and dissociation data were analyzed using SDS software (Applied Biosystems, California, USA) to determine the melting temperature (T_m) of a single-target nucleic acid sequence (specific product generated). Dissociation curve analysis was also performed to verify the specific or non-specific amplification, and in particular to check for the presence of primer dimers.

Statistical analysis

All experiments were repeated independently at least three times. The data were recorded and were analyzed statistically by one-way analyses of variation followed by Duncan’s multiple range tests at a significance level of $p < 0.05$ using SPSS version 16.0.

Results

Initiation of embryogenic callus

In this study, solid medium was more effective than liquid medium as a pre-treatment for the initiation of embryogenic callus (Table 2). The highest frequency of embryogenic callus (11.1 %) was observed from the explants pre-treated on solid PM medium containing 23 μ M 2,4-D for 45 days and subsequently transferred to M1 medium containing 10 μ M 2,4-D for 3–4 months. A lower percentage

Table 2 The effects of different concentrations of 2,4-D and picloram on callus initiation of *M. acuminata* cv. 'Berangan'

Media	Plant growth regulator (μM)		Percentage of callogenic clusters (%)	Percentage of embryogenic cluster (%)
	2,4-D	Picloram		
Solid PM	15	0	41.7 \pm 2.4	8.6 \pm 3.3
	10	0	53.3 \pm 3.1	11.1 \pm 2.6
	5	0	30.8 \pm 2.2	2.5 \pm 2.0
	0	15	32.5 \pm 2.9	3.9 \pm 2.5
	0	10	28.3 \pm 2.7	2.1 \pm 2.0
	0	5	21.7 \pm 3.0	0.0 \pm 0.0
Liquid PM	15	0	33.3 \pm 2.5	4.6 \pm 3.0
	10	0	39.8 \pm 2.7	6.7 \pm 3.2
	5	0	25.0 \pm 2.4	0.0 \pm 0.0
	0	15	24.4 \pm 2.6	2.5 \pm 2.0
	0	10	20.8 \pm 2.0	1.7 \pm 1.1
	0	5	16.0 \pm 2.4	0.0 \pm 0.0

Values represent the mean \pm SE

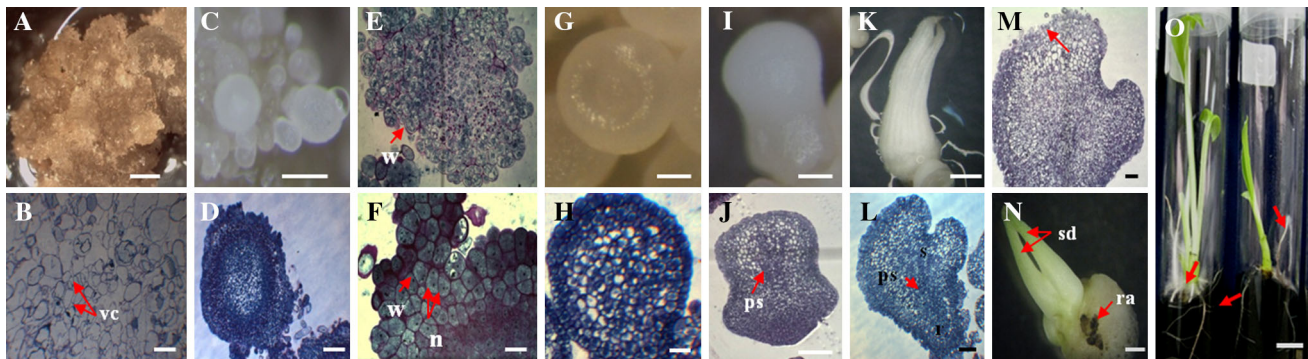


Fig. 1 Morphohistological changes in somatic embryogenesis. **a** Non-embryogenic callus (*bar* 1 mm). **b** Histology of non-embryogenic callus (*bar* 25 μm). **c** Embryogenic callus from male inflorescence (*bar* 200 μm). **d** Histology of embryogenic callus (*bar* 50 μm). **e** Cells with indistinct nuclei and poor protein storage when cultured in M2b medium (*bar* 50 μm). **f** Meristematic cells with distinct nuclei when cultured in M2a medium (*bar* 50 μm). **g** Globular embryo (*bar*

250 μm). **h** Histology of globular embryo (*bar* 100 μm). **i** Mature torpedo stage (*bar* 500 μm). **j** Histology of mature torpedo embryo (*bar* 200 μm). **k** Germinated embryo (*bar* 1 mm). **l** Histology of germinated embryo (*bar* 200 μm). **m** Irregular protodermal layer (*bar* 100 μm). **n** Mature germinated embryo (*bar* 1 mm). **o** Rooted plantlets derived from somatic embryos (*bar* 1 cm). *vc* vacuolated cells, *w* wall, *n* nucleus; *ps* procambial strand, *s* shoot pole, *r* root pole

of embryogenic callus formed (<4 %) when cultured on M1 medium supplemented with picloram after pre-treatment on PM solid medium.

The structures of the embryogenic and non-embryogenic calli were examined by histological examination. Friable embryogenic calli with translucent pro-embryos were observed after 5–6 months of culture. In comparison, non-embryogenic calli showed discrete tissue with unclear structure and vacuolated cells (Fig. 1a, b), whereas translucent globular structures were observed in embryogenic calli (Fig. 1c, d).

Establishment of ECSs

ECS was then established to obtain homogeneous populations that were initially composed of embryogenic-cell

aggregates and heterogeneous globules. However, a high frequency of browning was observed in ECSs, probably due to the accumulation of phenolic compounds. After about 3–4 months of culture, homogeneous, fine, and light-yellow ECSs containing uniformly shaped cells with dense cytoplasm and small vacuoles were observed, whereas non-embryogenic cells were observed as single or clumps of vacuolated cells.

A comparison of cell growth in M2a and M2b culture media was performed to determine the optimal medium for *M. acuminata* cv. 'Berangan'. M2b medium (Dhed'a et al. 1991) initially promoted cell division and growth, but the cells became sticky and caliginous with indistinct yellowish coloration after a few months of culture. Low sedimentation was observed from this medium, which might be due to fewer cytoplasmic cells. Histological

Table 3 Effect of different media and plant growth regulators on embryo development and maturation

Media	Percentage of embryo development (%)	Percentage of embryo maturation (%)
MS0	96.4 ± 1.7 ^c	66.4 ± 3.6 ^c
MSOL	80.2 ± 3.0 ^b	51.6 ± 3.9 ^b
M3	72.6 ± 4.9 ^b	44.2 ± 3.8 ^b
MS3	58.2 ± 3.9 ^a	33.4 ± 2.8 ^a

Values represent the mean ± SE. Values with different letters are significantly different at $p < 0.05$ level

analysis also indicated that the cells were poor in protein storage and contained indistinct nuclei (Fig. 1e). These characteristics might prevent subsequent cell development. In contrast, most of the cells grown in M2a medium were aggregated and had dense cytoplasm with small vacuoles, high protein storage, and distinct nuclei (Fig. 1f).

The process of differentiation and embryo development, from globular-shaped structures to regenerable mature embryos, were observed in both solid and liquid media (Fig. 1g–l). The highest frequency of embryo development (96.4 %) and maturation (66.4 %) was achieved by applying PGR-free MS solid medium, which subsequently produced the highest frequency of plant regeneration (69.3 %; Tables 3, 4). A lower frequency of embryo development and maturation was observed in the media containing PGR (M3 and MS3). Our studies also revealed that the liquid culture medium MSOL was not suitable for embryo maturation, because a lower frequency of embryos (51.6 %) was obtained compared to solid medium. Most of the embryos derived from liquid media did not regenerate into plants. Furthermore, abnormal structures were observed in some of the embryos.

Somatic embryos consisting of normal cells with nuclei, regular protoderm, and distinct procambium were able to regenerate into plants better than embryos with highly vacuolated cells and indistinct procambium. The latter form of embryo was mostly recorded in MSOL medium. Embryos with non-intact protoderm were unable to germinate in both solid and liquid media. Histological examination also revealed that undeveloped embryos consisted of vacuolated cells with disparate protoderm and no accumulation of either starch or protein (Fig. 1m).

Regeneration of somatic embryos

Embryos developed into shoots after 3–5 weeks on regeneration media (Fig. 1k). Morphological development of somatic embryos at the cotyledonary stage with shoots, root poles, and procambial strands were confirmed in the

Table 4 Effect of different media and plant growth regulators on shoot regeneration from embryo

Media	Plant growth regulator (μM)		Percentage of embryo forming shoots (%)	
	BAP	TDZ		
MS0	0.0	–	0.8 ± 0.5 ^a	
	14.0	–	25.0 ± 2.9 ^{g,h,i}	
	24.0	–	41.3 ± 4.8 ^j	
	34.0	–	69.3 ± 4.0 ^k	
	–	3.0	11.0 ± 1.6 ^{c,d,e}	
	–	5.0	23.8 ± 3.8 ^{f,g,h}	
	–	7.0	31.5 ± 5.2 ⁱ	
	MSOL	0.0	–	0.0 ± 0.0 ^a
		14.0	–	2.8 ± 0.9 ^{a,b}
		24.0	–	7.0 ± 0.9 ^{a,b,c,d}
34.0		–	12.5 ± 1.7 ^{d,e}	
–		3.0	0.8 ± 0.3 ^a	
–		5.0	2.8 ± 0.5 ^{a,b}	
–		7.0	4.5 ± 1.1 ^{a,b,c}	
M3	0.0	–	0.3 ± 0.2 ^a	
	14.0	–	10.5 ± 1.7 ^{c,d,e}	
	24.0	–	24.3 ± 2.70 ^{g,h,i}	
	34.0	–	38.8 ± 4.9 ^j	
	–	3.0	7.3 ± 1.3 ^{a,b,c,d}	
	–	5.0	16.8 ± 2.1 ^{ef}	
	–	7.0	26.0 ± 2.5 ^{g,h,i}	
MS3	0.0	–	0.3 ± 0.2 ^a	
	14.0	–	9.8 ± 1.5 ^{b,c,d,e}	
	24.0	–	22.5 ± 1.0 ^{f,g}	
	34.0	–	30.8 ± 4.6 ^{h,i}	
	–	3.0	7.8 ± 0.9 ^{a,b,c,d}	
	–	5.0	12.5 ± 1.0 ^{d,e}	
	–	7.0	23.5 ± 2.2 ^{f,g,h}	

Values represent the mean ± SE. Values with different letters are significantly different at $p < 0.05$ level

histological sections (Fig. 1l). Mature germinated embryos were obtained on regeneration media. Formation of chloroplasts and development of green shoots were observed after being transferred to light. The effect of different concentrations of BAP and TDZ on the regeneration of embryos was studied (Fig. 1n). The highest frequency of mature regenerated embryos (69.3 %) was observed on MS medium supplemented with 34 μM BAP (Table 4). In general, BAP was found to be more efficient (2.8–69.3 %) than TDZ (0.8–31.5 %) in enhancing embryo germination. The percentage of regeneration was proportional to the concentration of BAP used. All shoots from individual embryo and clumps of embryos started to root after 1 week of culture (Fig. 1o).

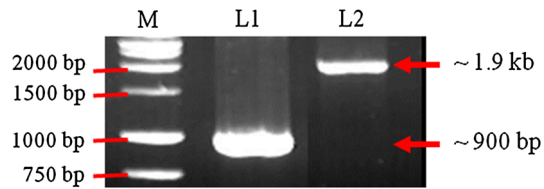


Fig. 2 PCR amplification of *endochitinase* and *PAL* gene sequences using specific primers. M: 1 kb DNA marker; lane 1 the *endochitinase* gene; lane 2 the *PAL* gene

Gene expression

The presence of endogenous *endochitinase* (~900 bp; accession AF416677) and *PAL* (~1,930 bp; accession EU862233) was confirmed using PCR analysis and DNA sequencing (Fig. 2). The amplified sequence of the ‘Berangan’ *endochitinase* gene showed high similarity (>80 %) to the *endochitinase* gene from other *M. acuminata* gene sequences in the database (accessions AF416677, AJ277279, FJ222750, FJ858155, and AJ277278) and more than 35 % similarity to those of the monocots *Oryza sativa* (accessions NM_001065161, AP004685, AP003685, and AK061280) and *Zea mays* (accessions EU724469 and EU724461). The partial sequence of *PAL* in the banana ‘Berangan’ genome also showed high similarity (>98 %) with other banana cultivars (accessions EU862232, EU862233, EU856393, EU856392, and EU856394) and 86 % similarity to *Oryza sativa* (accessions NM_001062143, AC135419, and AC135429).

The analysis of expression patterns of the indicator genes *endochitinase* and *PAL*

The expression patterns of *endochitinase* and *PAL* were evaluated in relation to morphohistological changes at the various developmental stages during somatic embryogenesis using qRT-PCR (Fig. 3). The results indicated that the expression of *endochitinase* was higher at the globular and torpedo stages of embryonic development and reached its maximum in germinating embryos. However, the expression level was significantly lower in the regenerated plantlets. *PAL* expression was detected at all stages of somatic embryogenesis, where higher expression was recorded at the torpedo stage but which gradually decreased during the germination and plantlet phases.

Discussion

Male inflorescences have been used previously in many banana cultivars for the establishment of ECS cultures (Wong et al. 2006; Bernardo et al. 2008; Ghosh et al.

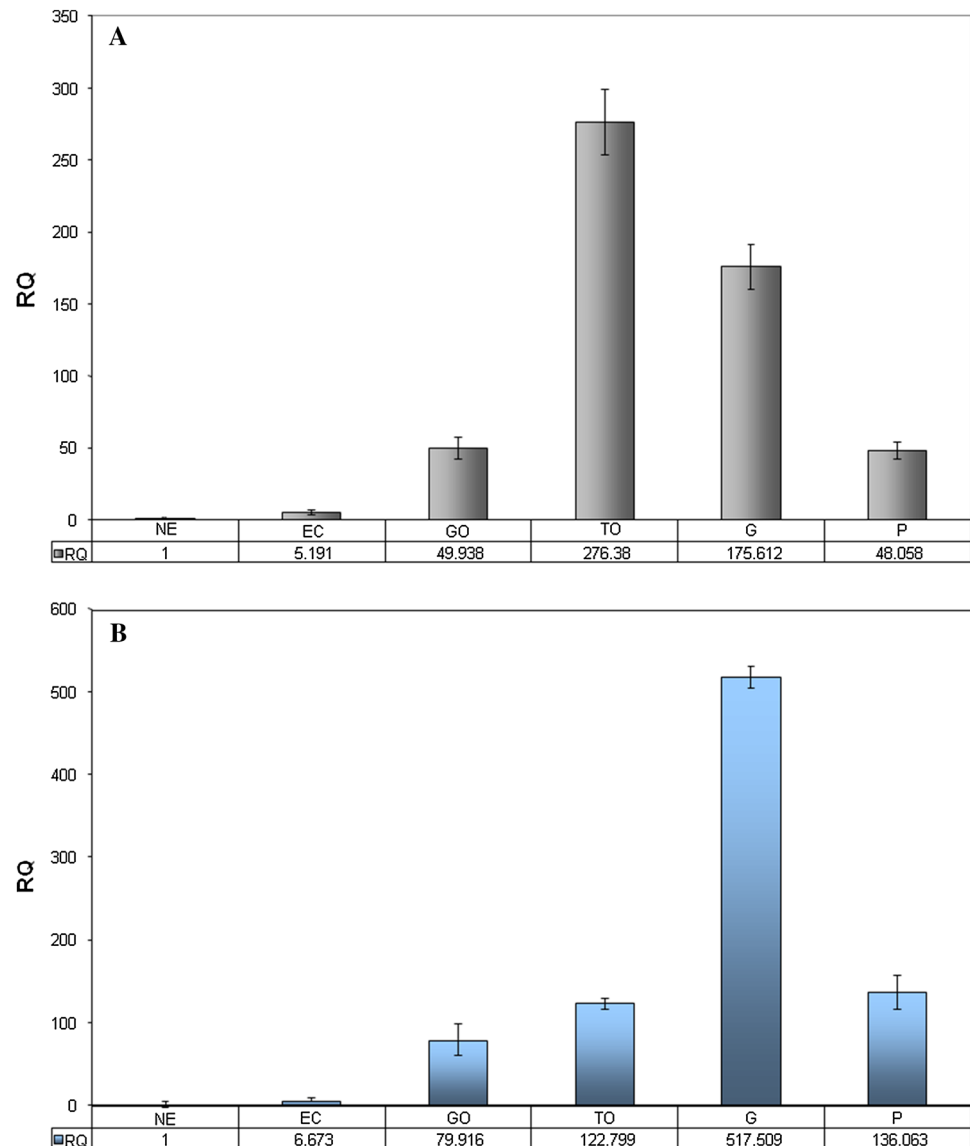
2009). Most male inflorescences were amenable to culture and formed callogenic clusters. However, production of calli with embryogenic structures was low. In this study, the influence of media on the different stages of somatic embryogenesis was investigated in tandem with histological analysis. Our results revealed that solid medium was more effective to initiate embryogenic callus compared to liquid medium, probably due to phenolic compounds which caused visible necrosis.

Appropriate use of PGR in media is critical for cell differentiation. Most techniques rely on the use of auxins to induce dedifferentiation and formation of embryogenic structures (Strosse et al. 2003; Karami et al. 2009). In this study, 2,4-D was more responsive compared to picloram in producing embryogenic callus, indicating the type of auxin used in culture media could affect embryogenesis. 2,4-D has been proven to be more effective in many plants for inducing somatic embryogenesis compared to other auxins (Venkatesh et al. 2009; Mousavizadeh et al. 2010). Previous studies reported that 2,4-D was effective to promote callus induction in the banana cultivar ‘Berangan’ (AAA), but prolonged culture in high concentrations of auxins prevented callus from dedifferentiating into embryogenic callus (Jalil et al. 2003; Kulkarni and Bapat 2013). Sharma and Millam (2004) reported that exposure of cells to an auxin shock for a limited period was sufficient to evoke somatic embryogenesis in potato. On the other hand, picloram induced the formation of callogenic clusters but did not favor the production of embryogenic callus. This was in agreement with the study by Ganapathi et al. (2001), where a high frequency of embryogenic callus was obtained on media supplemented with 2,4-D instead of picloram. In contrast, Smitha and Nair (2011) reported that MS containing picloram induced higher number of somatic embryos in banana cultivar ‘Njalipoovan’ that gave rise to plantlets rather than 2,4-D.

Homogeneous, fine, and light-yellow ECSs composed of actively dividing single cells, clusters, and small aggregates of cells have been established within 3–4 months in M2a medium using embryogenic callus with whitish, translucent proembryos as an inoculum. Dense cytoplasm containing small vacuoles, high protein storage, and distinct nuclei were observed in the aggregated cells under histological examination. Similar observations were reported in ECSs from the banana cultivars ‘Grand Naine’ (AAA) (Côte et al. 1996; Georget et al. 2000), ‘French Sombre’ (AAB) (Grapin et al. 1996), and ‘Mas’ (Jalil et al. 2003, 2008; Wong et al. 2006).

Cell differentiation and embryonic development were influenced by the type of media and the PGR treatments. PGR-free MS solid medium was able to produce maximal embryos in our study. This was in agreement with the study by Bozhkov et al. (2002), where the yield of mature

Fig. 3 Relative expression levels of the **a** *PAL* and **b** *endochitinase* genes during developmental stages of somatic embryogenesis, *NE* non-embryogenic callus, *EC* embryogenic callus, *GO* globular embryo, *TO* torpedo embryo, *G* germinated embryo, *P* plantlet. The expression levels were calculated using the comparative $\Delta\Delta C_T$ method and compared with non-embryogenic culture. Vertical bars indicate the standard error of the mean of three replicates



somatic embryos of Norway spruce was increased in the absence of growth regulators. This observation might be due to the inhibitory effect of PGRs during embryonic development. In contrast, Navarro et al. (1997) reported that zeatin and kinetin were necessary for embryonic maturation in *Musa* cv. 'Grand Naine' (AAA). However, the presence of cytokinins at the embryonic development stage suppressed the formation of pro-embryos and the subsequent somatic embryo development in our preliminary experiments (data not shown). Also, the accumulation of phenolic compounds in the culture media also inhibited the formation of somatic embryos.

Our studies also showed that the use of liquid culture media was not suitable for embryonic development compared to solid media. Most of these embryos did not regenerate when cultured on the regeneration media, and

some of the embryos became abnormal in structure. A similar finding was reported by Georget et al. (2000), where disorganized cell structures were observed in banana cell cultures, probably due to the shaking of the liquid culture. The prolonged shaking of suspension cultures could also lead to the breakdown of protoderm (Dhed'a et al. 1991). This is in contrast with the study by Wong et al. (2006), who reported that the liquid MS medium was suitable for embryonic development in banana cv. 'Mas' (AA). Our results for the embryo formation are comparable to previously reported studies for other cultivars (Côte et al. 1996; Strosse et al. 2006; Dai et al. 2010).

Green shoots developed on the regeneration media containing either BAP or TDZ. TDZ is a potent plant growth regulator and has been shown to promote cell differentiation and shoot regeneration (Blando et al. 2013).

From our preliminary results, we found that lower concentrations of TDZ induced multiple shoots, whereas higher concentrations of TDZ in medium reduced the frequency of shoot regeneration. Unlike BAP and Kn, TDZ is more effective at lower concentrations (Parveen et al. 2010; Devi et al. 2011). Therefore, direct comparisons between TDZ and the other cytokinins at equimolar concentrations are difficult to analyze statistically (Peddaboina et al. 2006). BAP has been considered to be one of the most effective cytokinins for the induction of shoot regeneration in plant tissue culture (Tan et al. 2011). In our study, the highest response of embryo forming shoots was observed on medium containing BAP. This was in conformity with the study carried out by Kulkarni and Bapat (2013), where a high frequency of banana mature somatic embryos was developed into plantlets on MS medium containing 0.5 mg/l BAP. Several morphologically developed embryos did not regenerate into plantlets, probably due to non-optimal physiological conditions or to donor plant genotype. A previous study demonstrated that the AAA genomic group of bananas was less responsive for somatic embryogenesis compared to AAB and ABB (Chung et al. 2006).

It is well accepted that plant embryogenesis is an organized process involving the development of somatic cells into embryos, embryonic maturation, and regeneration, which are developmentally regulated by a number of distinct groups of genes and involve both genomic and epigenetic changes (Karami and Sardi 2010; Neelakandan and Wang 2012). The identification of genes with altered expression patterns during somatic embryogenesis has allowed for a better understanding of the developmental process and can aid in formulating strategies to enhance regeneration and morphogenesis, particularly for recalcitrant species (Karami and Sardi 2010; Neelakandan and Wang 2012). In banana, little is known about the molecular events leading to somatic embryogenesis given that different species and cultivars have different efficiencies and levels of recalcitrance in achieving this state (Jalil et al. 2008). Studies in many different plants have shown that chitinase genes play specific roles in physiological events and in development in somatic embryogenesis. In this study, endochitinase genes were shown to be expressed at all stages of development during the somatic embryogenesis of banana cv. 'Berangan'. Low expression levels could be detected in non-embryogenic cells, potentially indicating the constitutive expression of this gene in the cells, albeit at low levels, prior to dedifferentiation into the somatic state. Consequently, a significantly higher level of expression was observed in embryogenic cells. The profile of endochitinase gene expression varied with gene expression in non-embryogenic culture in carrot, as reported by Van Hengel et al. (1998), but the endochitinase gene was not expressed in *Arabidopsis* (Passarinho et al. 2001).

We additionally showed that the endochitinase gene was most abundantly expressed during the globular and torpedo stages of embryonic development and appeared to reach its maximum in the regenerating embryo. The expression level significantly declined in plantlets, implying a process of developmental regulation.

In the banana 'Berangan' cultivar, *PAL* was also up-regulated in the embryogenic cells and during maturation of the somatic embryos. The expression patterns during banana somatic embryonic development related to *PAL* showed similarities with endochitinase in transcript accumulations during early stages of embryogenesis, although there were some differences during later developmental stages. In the present study, *PAL* expression sequentially increased in embryogenic cells and in the globular and torpedo stages but declined during the regenerating stage and was poorly expressed in plantlets. The most intense expression was seen at the torpedo stage. Similar observations have also been made in several other studies (Cviková et al. 1994; Ghanti et al. 2009). However, there are relatively few reports on the role of *PAL* during somatic embryogenesis. While it appears that *PAL* plays an important role in the process of embryonic development, its specific function in somatic embryogenesis is still unclear. Ghanti et al. (2009) hypothesized that the products of *PAL* may also be involved in the biosynthesis of lignin, which is necessary during embryogenesis. *PAL* and *endochitinase* might be involved in the control of histodifferentiation and morphogenesis. Given that the expression of these two genes declined during plantlet formation suggests that they may not be crucial for further morphological development. It is, however, expected that large numbers of genes must be expressed in a highly coordinated manner to complete development, resulting in regenerated embryos and eventually whole plantlets. These could be elucidated in future genome-based transcriptomic studies.

Conclusion

The biochemical changes and molecular mechanisms regulating somatic embryogenesis in banana are pivotal in crop improvement programs. In this study, we have successfully established an in vitro plant regeneration protocol and have identified the gene-expression patterns of *endochitinase* and *PAL* during somatic embryogenesis in banana cv. 'Berangan'. Our findings may offer an invaluable contribution on banana improvement programs since the established protocol enabled us to regenerate whole plants from cells after about 9–11 months.

Author contribution statement N. Jafari was responsible for the experimental design and conducted the

experiment. R.Y. Othman, B.C. Tan and N. Khalid helped in designing the study and drafted the manuscript. All authors read and approved the final manuscript.

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

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