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# Characterization of *Aquilaria malaccensis* Callus Cells using SEM and Somatic Embryogenesis Associated Genes Identification

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**Abstract.** *Aquilaria malaccensis* or gaharu is one of Malaysia's top tropical resources that is being protected and conserved. Somatic embryogenesis is a plant tissue culture method used most extensively in commercial micropropagation systems and conservation purposes. This study aims to optimize callus induction techniques for somatic embryogenesis study using Scanning Electron Microscope (SEM) and Polymerase Chain Reaction (PCR) analysis. In this study, callus was induced on Murashige and Skoog's (MS) medium supplemented with a combination of plant growth regulators and optimized parameters (explant used, sterilization techniques, media content, pH media, plant growth regulators, incubation condition, and incubation temperature). The cells were subcultured for long-term callus maintenance and subjected to SEM analysis for somatic embryogenesis confirmation. Four set of genes associated to somatic embryogenesis (SERK, BBM, LEC1, and WOX) were studied based on National Centre for Biotechnology Information database and literatures. The data obtained were used for primer design and gene amplification using 3 different tissues (leaf, stem, callus). The highest frequencies of callus induction were observed on Murashikage and Skoog medium supplemented with 6-Benzylaminopurine and 1-Naphthaleneacetic acid together with optimized growth parameters. SEM analysis showed embryogenic characteristics in cells of the yellow compact calli evidenced by the presence of small and isodiametric cells. Only SERK gene was successfully amplified and enable to proceed with *in silico* analysis. This study provides fundamental results for genetic conservation effort of *A. malaccensis* related to somatic embryogenesis study.

## 1. Introduction

*Aquilaria malaccensis* is one of Malaysia's agarwood or gaharu species producing valuable resin-impregnated heartwood and essential oil that are being used as incense, perfumery and traditional medicine [1,2]. *A. malaccensis* is also known as karas or depu. It is highly exploited as it is claimed to be source of high quality agarwood [1,3]. *A. malaccensis* is the largest tree in size among the Malaysia's *Aquilaria* species and produces highest amount of agarwood per tree that resulted to overexploitation. Thus, this species was included in Appendix II of Convention on International Trade



in Endangered Species (CITES) and continue to decrease in nature [4]. The Forest Department of Peninsular Malaysia (FDPM) reported the number of wild *Aquilaria* trees in natural forest of Peninsular Malaysia stood at more than 3 million based on nationwide inventory conducted between 2002 to 2004 and about 766 ha are cultivated with *Aquilaria* trees [5]. The Conservation Action Plan for *Aquilaria malaccensis* was established for *in situ* and *ex situ* conservation, management of the resource, artificial propagation, enforcement, research and development and strengthening cross-sectoral enabling factors [6]. Therefore, conservation efforts are vital to ensure the continued survival of this species as Malaysia is being one of the countries with naturally occurring *Aquilaria* trees.

There are several *in vitro* technologies that had been applied for *Aquilaria sp.* research for different purposes. Successful *in vitro* plant regeneration system had been reported by [7,8,9] for *Aquilaria agallocha*, *Aquilaria malaccensis* and *Aquilaria hirta* respectively for rapid planting materials propagation as well as for conservation purposes. There were also several studies conducted to establish *Aquilaria calli* and cell suspension cultures to study the functional genes for agarwood fragrant constituents and sesquiterpene biosynthesis [2,10,11,12,13,14]. In 2008, [15] reported the development of *in vitro* interspecific micrografting of *A. malaccensis* for white rot free disease seedlings while new breeds of enhanced phytochemical production *A. malaccensis* for commercial plantation had been developed by [16], through *in vitro* polyploidization in 2014. Plant calluses derived from many different cell types can differentiate into a whole plant, a process called regeneration, through addition of plant hormones to the culture medium. By manipulation of hormonal constituents in the medium, the cells of calli can be transformed into embryonal mass and the whole plant can be regenerated.

Somatic embryogenesis is an *in vitro* technology to generate embryogenic cells by somatic cells that are being induced and undergone morphological and biochemical changes [17]. These somatic embryos can then be germinated into new plants with identical genotypes to the original somatic cells. It was first observed in *Daucus carota* suspension cultures by [18,19] and later, many researchers studied embryo development and the morphological characteristics in various species, such as conifers and other pines [20,21]. Generally, there are three stages of development from the explant to somatic embryos: initiation, proliferation, and maturation [22]. Somatic cells of explants can be induced to proliferate and produce somatic embryos in suitable media supplemented with different plant growth regulators and some hormones have a primary role in somatic embryogenesis but may cross-talk with others [23]. The culture conditions and juvenility of explant are major embryogenesis induction determinants as shown in several species and genotypes [24]. This technology able to produce large number of plantlets in a short time, and makes possible for the large-scale production by using bio reactor [25]. Nowadays, it has been applied as a tool for understanding the mechanisms of embryo development and a method for developing crops with ideal characteristics especially for the forestry industry. [26] succeed to obtain somatic embryos presenting a high plant regeneration frequency in *Eucalyptus dunnii* and having been subject of property rights under the Patent No. (PI 9801485-4 INPI).

Understanding somatic embryogenesis would not only solve the problem of micropropagation but also will assist in plant improvement. Moreover, recent advances in technologies such as advanced microscopic imaging, omics and computational biology have opened more windows to elucidate the mechanism. Scanning electron microscope (SEM) analysis revealed that induction of morphogenesis is linked to the formation of a fibrillar network referred to as the extracellular matrix (ECM) or extracellular matrix surface network (ECMSN). The structure was proposed as subcellular marker for embryogenic tissues [27]. The ability to produce somatic embryos would allow genetic manipulation which could lead to improvement of the crop importance of investigating embryonic characteristics at the molecular level to ensure that the obtained structures are somatic embryos [28]. These include

combination of plant growth regulators, media, pretreatments and culture environments, which relate to various molecular events encompassing gene expression and signal transduction pathways. A number of genes that expressed differentially during somatic embryogenesis induction have been isolated which were *LEAFY COTYLEDON* genes, *LEC1* [29] and *LEC2* [30], *WUSCHEL (WUS)* [31] *BABY BOOM (BBM)* [32] and *SOMATIC EMBRYOGENESIS RECEPTOR-like KINASE (SERK)* [33,34] These genes encode transcription factors and when overexpressed, somatic embryogenesis is promoted.

This study was aimed to optimize callus induction techniques for somatic embryogenesis study using Scanning Electron Microscope (SEM) and Polymerase Chain Reaction (PCR) analysis. This study provides fundamental results for genetic conservation effort of *A. malaccensis* related to somatic embryogenesis study.

## 2. Materials and method

### 2.1 Explant Collection and sterilization

Mature and young leaf were taken from new sprouting branches of mature trees of *Aquilaria malaccensis* in Endau, Rompin forest. The sample were kept in liquid nitrogen during sample collection and kept in -20 freezer for long term storage in the laboratory. Method for sterilization was optimised based on [13]. The explants were rinsed under slow running tap water for 15 minutes and rinsed in Tween-20 for 1 minute inside the laminar air flow cabinet. Later, the explants were rinsed three times with sterilized distilled water and dipped in the 70% alcohol for 30 seconds. They were then rinsed twice with sterilized distilled water and dipped in 5% sodium hypochlorite (Clorox) for 2 minutes. The explants again were rinsed 3-4 times using sterilized distilled water and later were dipped in 0.2% BENEX Imaspro Benomyl fungicide for 2 minutes, and finally were rinsed 3-4 times with sterilized distilled water.

### 2.2 Somatic embryo induction

All basal media contain 4.4 g/L Murashikage and Skoog (MS) salts and vitamins (Duchefa, Netherlands), 15 g/l sucrose and 2.75 g/l gelrite (Sigma Aldrich, USA). The pH was adjusted to  $5.7 \pm 0.1$  before autoclaving at 121°C, 15 psi for 15 min. After autoclaving, a total of 25 mL of sterile medium was poured into 90 mm 15 mm Petri dish in the laminar air flow and allowed to solidify. Plant hormones used were indole-3-butyric acid (IBA), 1-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP) (Duchefa, Netherlands). Five different hormone concentration and combinations were used in this experiment; BAP 1.5  $\mu\text{mol/L}$ , IBA 0.5  $\mu\text{mol/L}$ , NAA 0.5  $\mu\text{mol/L}$ , BAP 2.2  $\mu\text{mol/L}$ , and NAA 1.1  $\mu\text{mol/L}$  according to protocol developed by [9,13,14,35]. Cultures were maintained at  $25 \pm 2^\circ\text{C}$  under two different conditions which were in dark and 16h photoperiod of 50  $\mu\text{Mol m}^{-2}\text{s}^{-1}$  in the growth chamber. Subcultures were routinely performed at 3 weeks intervals during somatic embryo induction and 3 weeks intervals during maintenance of embryogenic tissue. Morphological development of somatic embryo induction was observed each week.

### 2.3 Electron microscopy

Native samples (free of chemical fixation and drying) of embryogenic tissue were placed on a cooled specimen holder (Peltier stage) and their temperature was gradually decreased and then maintained between -18 and -22°C. Due to the relatively low heat conductivity of the samples (dimensions of 2–3 mm<sup>2</sup> and thickness of 2 mm), the real temperature of the sample surface can be higher. At the beginning of the pumping the pressure was equal to the atmospheric pressure in all parts of the microscope. The pumping process started approximately one minute after the decrease of the sample holder temperature. Imaging was started after ten minutes when the sample was inserted. Every one of

selected parts were observed approximately 8-10 minutes. The microstructures for fresh calli were observed using FEI QUANTA 450 Scanning Electron Microscope equipped with Everhart Thornley Detector (ETD). High resolution images were obtained with a magnification range from 10-500x.

#### 2.4 DNA extraction and PCR analysis

100 mg of *A. malaccensis* leaves, stem, and calli were pulverized in liquid nitrogen. The plant genomic DNA was extracted using the FavorPrep™ Plant Genomic DNA Extraction Mini Kit (Favorgen, Taiwan), according to the manufacturer's protocol. The concentration and quality of the DNA samples were determined with the OPTIZEN NanoQ spectrophotometer. Absorbance of A260nm/A280nm was taken to determine the yield and purity ratio of the DNA samples. The concentration of the DNA sample was obtained directly from the reading of the spectrophotometer in the unit of µg/mL. Homologous regions among SERK, BBM, LEC1, and WOX gene sequences from NCBI database were identified using the ClustalW multiple alignment programme and used for constructing primers. Each of the forward primers were combined with each of the reverse primers in an attempt to amplify putative gene fragments (Table 1). PCR was conducted in a final reaction volume of 25 µL, containing 12.5 µL of 2x PCRBIO Taq Mix Red (PCRBiosystems, UK), 1.0 µM of each primer, and 25 ng of genomic DNA as template. For negative control, distilled water was added instead of DNA to verify absence of contamination. PCR amplification was conducted on a BIORAD T100 Thermal Cycler, programmed for 1 min at 95°C; 35 cycles for 15 s at 95°C, 15 s at Ta and 1 min at 72°C; and a final 3 min extension at 72°C (Table 3).

**Table 1.** Primer used for Somatic Embryogenesis associated genes identification

Genes	Sequence Source Accession No.	Forward Primer	Reverse Primer	Amplicon Size (bp)
SERK	XM_002325978.3	CGTGGTACAATAGGGCACATAG	AGCAGTGCAACCTGGATTAG	275
BBM	XM_018121249.1	CTCTGGCTCTAACTCTGTCATC	GACCCATCTCTCTCCAATCTC	385
LEC1	AM494833.1	GTCCGAGTACATCAGCTTCATC	GCAACACTCAAAGCTCCATAATC	237
WOX2	AM286747.1	GGCAGAAGGTCAATCCACTATG	TGTGAATGCTGGAAGGTGTAAG	387

#### 2.5 Isolation and sequencing of gene fragments

PCR products were separated on a 1.5% agarose gel, and DNA fragments with the expected sizes were sent to Apical Scientific Sdn Bhd Laboratory for sequencing. The forward and reverse sequences retrieved after sequencing were analysed by using chromatograms for each sequence and manually edited in order to reduce the noise. Nucleotide sequences were compared to available sequences in Gen-Bank at the National Centre for Biotechnology Information (NCBI). Deduced protein sequences were compared with desired proteins from other species using Constraint-based Multiple Alignment Tool (COBALT) available on the NCBI home page.

### 3. Results and discussion

#### 3.1 Optimization of somatic embryo induction

Parameters that had been optimised in this experiment were type of explants, sterilization method, media components, pH media, combination of plant growth regulators, incubation conditions, and incubation temperature.

**Table 2.** Optimized parameters for somatic embryo induction

	Parameters had been tested	Best parameters obtained
Explant		

	Parameters had been tested	Best parameters obtained
Young leaf/shoot	/	/
Mature leaf	/	
Mature Seed	/	
Node	/	
<b>Sterilization method</b>		
Running tap water for 15 minutes	/	/
Tween-20 for 1 minute	/	/
70% EtOH for 30 sec	/	/
5% Clorox for 2 minutes	/	/
0.2% Benomyl for 2 minutes	/	/
<b>Media</b>		
MS media + gelrite	/	
MS media + gelrite + sucrose	/	/
<b>pH Media</b>		
5.7-5.8	/	/
<b>Plant Growth Regulators</b>		
BAP 1.5 $\mu\text{mol/L}$	/	
IBA 0.5 $\mu\text{mol/L}$	/	
NAA 0.5 $\mu\text{mol/L}$	/	
BAP 2.2 $\mu\text{mol/L}$	/	/
NAA 1.1 $\mu\text{mol/L}$	/	/
<b>Incubation condition</b>		
Light	/	
Dark	/	/
<b>Incubation Temperature</b>		
25-26°C	/	/

The best parameters obtained were using young leaf as the explant, sterilisation using running tap water for 15 minutes, Tween-20 for 1 minute, 70% EtOH for 30 sec, 5% Clorox for 2 minutes and 0.2% Benomyl for 2 minutes while media contained MS media, gelrite, sucrose, BAP 2.2  $\mu\text{mol/L}$ , NAA 1.1  $\mu\text{mol/L}$  at 5.7-5.8 pH and incubated in dark at 25-26°C were successfully induce the callus and somatic embryo formation. There are thousands of journal articles that describe research using varied explants, culture media, plant growth regulator levels and combinations, as well as other addendum to the culture medium, and varied culture conditions to induce callus and regenerable callus from specific plant species. Two major problems in developing in vitro technology for woody plants are difficulty in obtaining suitable plant material for isolation and the tissues may be slow to respond in culture. Optimally, the material should be in active growth and obtained from seeds, seedlings, or newly propagated plants. Once established, callus cultures may be used for a variety of experiments. The callus culture can be used to study protoplast isolation, cell type, cellular selection, somatic embryogenesis, organogenesis, and secondary metabolite production, as well as for genetic transformation [36].

Five different hormone concentration and combinations were used in this experiment; BAP 1.5  $\mu\text{mol/L}$ , IBA 0.5  $\mu\text{mol/L}$ , NAA 0.5  $\mu\text{mol/L}$ , BAP 2.2  $\mu\text{mol/L}$ , and NAA 1.1  $\mu\text{mol/L}$  according to protocol developed by [9,13,14,35]. [13] found that compact and friable types of calli could be induced from leaf explants of *A. malaccensis* when using NAA singly or in combination with BAP

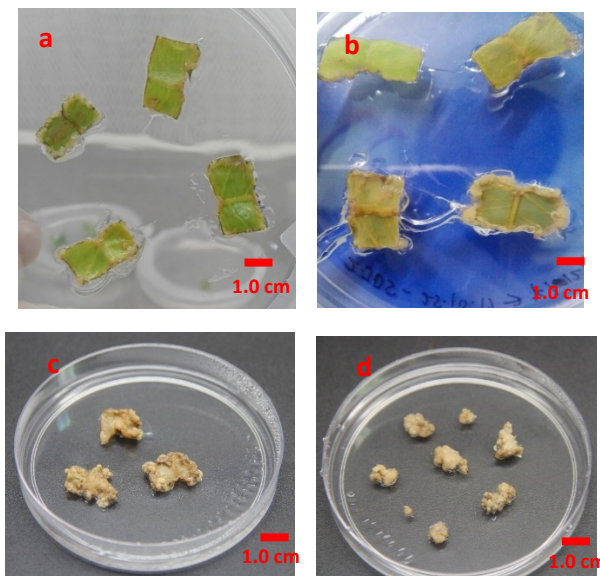
hormone respectively. Specific auxin to cytokinin ratios in plant tissue culture medium give rise to an unorganized growing and dividing mass of callus cells. Callus cultures are often broadly classified as being either compact or friable. Friable calluses fall apart easily, and can be used to generate cell suspension cultures. For compact callus, the cells are densely packed and do not favor rapid cell division.

Besides plant growth regulators, sucrose plays an important role by supplying the energy for *in vitro* plant tissue cultures as these have insufficient autotrophic ability. Sucrose not only acts as an external energy source but also contributes to the osmotic potential of the medium [37]. These would permit the absorption of mineral nutrients present in the medium, essential to the cells' growth. The significant effect of carbon source concentration in culture media on the frequency of callus formation has been well studied in many plants like rice [38] and olives [39].

### 3.2 Somatic Embryo induction

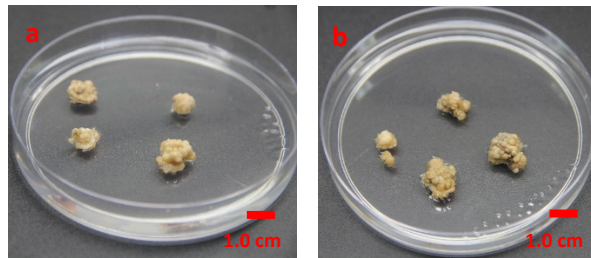
Figure 1 shows images of callus formation after 30 days, 50 days, 3 and 4 months incubation in dark while Figure 2 shows images of embryogenic callus formation after 5 and 6 months incubation in dark. 6 months was a long period for the induction of embryogenic callus since only 8 weeks time taken for sandalwood and eucalypts [14,40].

The use of plant growth regulators is a fundamental importance in directing the organogenesis response of any plant tissue or organ under *in vitro* condition. Further optimisation need to be conducted for reliable and reproducible somatic embryogenesis system in *A. malaccensis*. It took a year to induce somatic embryo in sandalwood and low frequency proliferation obtained during their preliminary study using different steps and hormonal combinations [14]. The treatment initiated compact, cream coloured calli but after close observation under the electron microscope, they were observed to actually occur in isodiometric.



**Figure 1.** Images of Callus formation a) after 30 days incubation in dark b) 50 days incubation in dark c) 3 months incubation in dark d) 4 months incubation in dark



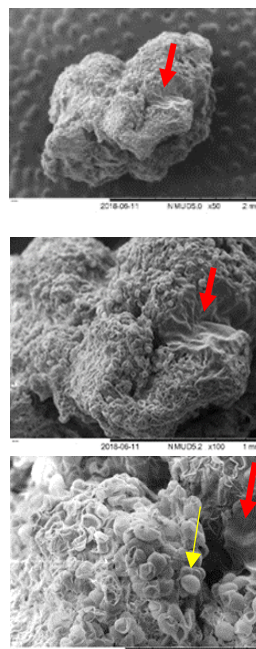


**Figure 2.** Images of Embryogenic Callus formation a) after 5 months incubation in dark b) 6 months incubation in dark

### 3.3 Scanning Electron Microscopic (SEM) Analysis

Figure 3a-c show images of extracellular matrix surface network (ECMSN) on embryogenic callus formation (red arrow) after 6 months incubation in dark viewed under SEM at 50 x, 100 x and 300 x magnification. Embryogenic characteristic in the calli also detected by the presence of small and isodiametric cells (Figure 3c- yellow arrow).

The ECMSN plays an important morphoregulatory role during somatic embryogenesis and organogenesis through cell adhesion [27]. The adhesion of plant cells to one another is fundamental to the formation and maintenance of plant structure. ECMSN was described as net-like structure covering the embryogenic cell complex as a response to stress and protection against external factors that were specific to the culture conditions [41]. This paper introduces the native structure of *A. malaccensis* embryogenic tissues investigated using electron microscope. To our knowledge, this is the first report on ECM layer in the embryogenic cells of the *A. malaccensis*. It can be used as an early structural marker for screening embryogenic competent cells and improved current tissue culture techniques.



**Figure 3a-c.** Images of embryogenic callus formation after 6 months incubation in dark viewed under SEM at 50 x, 100 x and 300 x magnification.



### 3.4 PCR Optimization and Sequencing Analysis

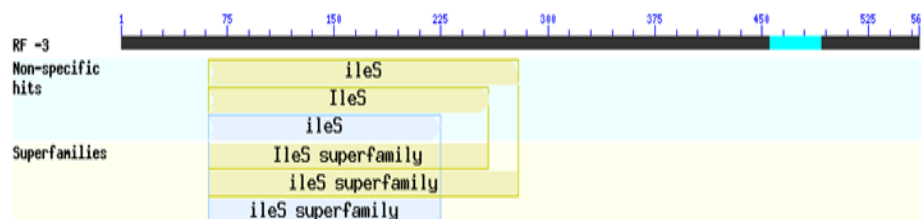
4 set of genes associated to somatic embryogenesis (SERK, BBM, LEC1, and WOX) were studied based on NCBI database and literatures. The data obtained were used for primer design and gene amplification using 3 different tissues; leaf, stem, callus (Table 3). However, only SERK gene was successfully amplified and enable to proceed with *in silico* analysis.

Thus, further optimization need to be conducted to characterize these genes particularly the PCR cycles to increase the successful amplification rate. PCR cycle is important to increase the potential of primer binding toward the DNA template and also to amplify more targeted DNA.

The partial fragment of sequenced PCR product hit on Isoleucine transferase (IleS) conserved domain using Basic Local Alignment Search Tool (BLAST) from NCBI database (Figure 4). Sequence comparisons revealed 30-40 % identical with sequences from other species (Figure 5). The isoleucyl tRNA synthetase (IleS) is a class I amino acyl-tRNA ligase and is particularly closely related to the valyl tRNA synthetase.

**Table 3.** PCR Optimization for Somatic Embryogenesis associated genes identification

Annealing temperature (T <sub>a</sub> ) (°C)	PCR cycles	DNA samples	Targeted Genes
45	35	Callus	BBM LEC1
50			SERK WOX2
55		Leaf	BBM LEC1
60			SERK WOX2
65		Stem	BBM LEC1 SERK WOX2



**Figure 4.** Graphical summary conserved domain of IleS superfamily retrieved from NCBI Conserve Domains database

XP_020589307.1	1	MVVAVPPFFSS[5]RPSA-----LSRRN-----QLSFNKTSS	LS---LFLPFLGYHTEFRE	50
XP_017229288.1	1	MASQM-----SSYKVMMSRS-F--SSFRNETSVFIINSRRSS	SanfIAFLNMTYYSTNSGN	53
XP_010024695.1	1	-----MVVIHASPYKVLTRQRTyScLG--KTASVNNLFYLRASS[4]FS---	LLGRLRPFCSFSSAD	56
XP_024467114.1	1	MSLLRKS LAAN[5]PSEaatVAMIHSASHRVLLRRS-S-SSSLRTATSVDLFYRRGSA	LS---LFNTVTHYSTSSSD	74
XP_011005092.1	1	MSLLRKS LAAN[5]PSEaatVAMIHSASHRVLLRRS-ScSSSLRTATSVDLFYRRGSV	LS---LFSTVTNYSTSSSD	75
BAF00309.1	1	MSSFFKSAFEN PREAaaMAMVQSSSYRVLSGKS---CSNLRRTPLDSFLAKGRS[4]FS---	FLYVSRFSTEPNN	71
Query_10001		-----	-----	
XP_020589307.1	51	-FFPFGIKQRAHGFPVISAKKSSKSGKQDSEMYKHTIDLPKTTFGMRANSI	VREPEIQKFWEDNDVFKRV-AEKNIGETFI	128
XP_017229288.1	54	DYHFSS-KRRSRGPFVMAAKKASKGEKLEDGKYKHTVDLPKTKFGMRANST	TREPEIQKLWEENQVFKRL-SDRNNGGSFV	131
XP_010024695.1	57	--VPSS-KRRSRGPFVMAAKAAEGGKQDEGKYKHTVALPRTSFGMRANAS	VREPEIQKSWDENQVFERI-VSRNNGESFV	132
XP_024467114.1	75	DFPSS-KQRSRGPFVMAAKKASGGKQEEGRYKHTVDLPKTTFGMRANAL	VREPEIQKLWEDNQVFKRV-VDKNIGEHFV	152
XP_011005092.1	76	DFPSS-KQRSRGPFVMAAKKASGGENQEEGRYKHTVDLPKTTFGMRANAL	VREPEIQKLWEDNQVFKRV-VDKNIGEHFV	153
BAF00309.1	72	EPGHSS-KRRSRGPFVMAAKKASEGKQEDGKYKQTVDLPKTKFGMRANSL	TREPEIQKLWEENQVFKRV-SDNNNGGSFI	149
Query_10001	1	-----YRQTVFLPQTEFPMKAGLAKEPALPDRWARIDLYGKLRADRAKAERFL		49
XP_020589307.1	129	LHDGPPYANGKLHMGHALNKILKDIINRYKLLQNYKVQFIPGWDCHGLPIELKVLQ	SMDQAAEKDLTPLTLRELAAKFAK	208
XP_017229288.1	132	LHDGPPYANGDLHMGHALNKILKDMINRYKLLQNFVKVHYVPGWDCCHGLPIELKVLQ	SLDQEAARKALTPKLRAKAFAK	211
XP_010024695.1	133	LHDGPPYANGDLHMGHALNKILKDIINRYKLLQNYKVHYVPGWDCCHGLPIELKVLQ	SLDKDARKDLTPIKLREKAFAK	212
XP_024467114.1	153	LHDGPPYANGELHMGHALNKILKDVINRYKILQNYKVHYVPGWDCCHGLPIELKVLQ	SMDQEAARKDLTQSKLRAKAFAK	232
XP_011005092.1	154	LHDGPPYANGELHMGHALNKILKDVINRYKILQNYKVHYVPGWDCCHGLPIELKVLQ	SMDQEAARKDLTQSKLRAKAFAK	233
BAF00309.1	150	LHDGPPYANGDLHMGHALNKILKDIINRYKLLQNYKVQYVPGWDCCHGLPIELKVLQ	SMDQEVKELTPLKLRAKAFAK	229
Query_10001	50	VHDGPPYANGX-----		60
XP_020589307.1	209	ETVDVQMKSFKRYGVWADWSDPYLTLFPEYEAQIEVFGQMATKGYIYRGRKPVHWS	SSHTALAEAELEYPDGHVSKSI	288
XP_017229288.1	212	TTVKAQMSFVKRYGVWADWHPYLTLDPEYEAQIEVFGEMVFGYIFRGRKPVHWS	SSRTALAEAELEYPEGHISKSM	291
XP_010024695.1	213	STVKTQMASFKRYGVWAWNPNYLTLDPEYEAQIEVFGQMALRGYIYRGRKPVHWS	SSRTALAEAELEYPEGHVSRSI	292
XP_024467114.1	233	ATVKTQMASFKRFGVGDWERPYLTLDPEYEAQIEVFGQMAFQGYIYRGRKPVHWS	SSRTALAEAELEYPEGHVSRSI	312

**Figure 5.** Alignment of partial fragment of SERK gene hit on Isoleucine transferase (IleS) conserved domain using Constraint-based Multiple Alignment Tool (COBALT) from NCBI database. Amino acids in red colour indicate conserved regions

#### 4. Conclusion

The highest frequencies of callus induction were observed on MS medium supplemented with BAP and NAA together with optimized growth parameters. SEM analysis showed embryogenic characteristics in cells of the yellow friable calli evidenced by the presence of small and isodiametric cells. Only targeted SERK gene was successfully amplified and enable to proceed to *in silico* analysis. However, the partial fragment of sequenced PCR product hit on Isoleucine transferase (IleS) conserved domain. This study provides fundamental results for genetic conservation effort of *A. malaccensis* related to somatic embryogenesis study. In future, further optimization need to be conducted to characterize these genes particularly the PCR cycles and study on full length gene isolation will be carried out for gene expression analysis towards biomarker and functional genomic study.

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