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Full Length Article

Histological Examination and Gene Expression Profiles of *CpLAX2*, *CpLAX3* and *CpPIN4* during Somatic Embryogenesis in *Carica papaya*

Baker Al-Shara[1,2*], Jamaludin Mohamad[2], Rosna Mat Taha[2], Wesam Al Khateeb[3], Hashimah Elias[4], Asif Khan[2] and Mohammad Jawarneh[3]

[1]*Department of Medical Laboratory Sciences, Faculty of Allied Medical Sciences, Jadara University, Irbid, Jordan*
[2]*Institute of Biological Sciences, Faculty of Science, University Malaya, 50603 Kuala Lumpur, Malaysia*
[3]*Department of Biological Sciences, Faculty of Science, Yarmouk University, Irbid 21163, Jordan*
[4]*Faculty of Resource Science and Technology, University Malaysia Sarawak (UNIMAS), 94300 Kota Samarahan, Sarawak, Malaysia*
[*]For correspondence: alshara.baker@yahoo.com; b.alshara@jadara.edu.jo
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Abstract

Histological and molecular examinations were performed on somatic embryos derived from 'Eksotika' papaya; including immature zygotic embryos, throughout somatic embryogenesis process. To overcome the challenges in regenerating somatic embryos from immature zygotic embryos of "Eksotika", this study specifically focused on understanding callus development at the bases of somatic embryos. Somatic embryogenesis systems serve as valuable models for studying the molecular, regulatory, and morphogenetic processes involved in plant embryogenesis. Light microscopy and quantitative real-time PCR were employed to examine the histological and molecular changes during the developmental stages of somatic embryogenesis and callus formation at the bases of somatic embryos. The accumulation of secondary somatic embryos on the root surface, particularly in the root cap zone, represents the most significant obstacle to successful somatic embryo formation. Different developmental stages, starting from pro-embryos to cotyledonary phases, were found to be quite similar to the ontogeny of zygotic embryos. All selected genes: (*Carica papaya* PIN-FORMED (auxin efflux carriers) *CpPIN4*, *Carica papaya AUXIN RESISTENT 1/LIKE AUX1 (AUX/LAX)* (auxin influx carriers) (*CpLAX3* and *CpLAX2*) were clearly expressed in somatic embryos formed during the induction phases of somatic embryogenesis. *CpLAX3* exhibited the highest expression levels among the selected tested genes, while *CpPIN4* showed the lowest. The formation and development of somatic embryos involved the expression of all selected genes.

Keywords: Somatic embryogenesis; *Carica papaya;* Gene expression*;* Auxin efflux transporters; Auxin influx transporters

Introduction

Carica papaya (*C. papaya*) 'Eksotika' cultivar was developed through the hybridization of two distinct cultivars: Hawaiian Sunrise Solo (an American cultivar) and Subang 6 (an old Malaysian cultivar) (Chan 2009). Most *C. papaya* crops are propagated using seeds collected from residential farms due to their simplicity and low cost. However, farmers often produce poor-quality seeds from farm-grown crops. This traditional practice has contributed to the broadening of harmful infections in *C. papaya* plantations (Wu *et al.* 2012). The inability to develop healthy roots is one obstacle preventing the successful propagation of *C. papaya*. Callus formation at the root tips during *in vitro* propagation prevents propagation *C. papaya* commercially (Al-Shara *et al*. 2018; Posada-Perez *et al.* 2022).

Somatic embryogenesis (SE) provides exceptional model for revealing and understanding the molecular and cellular changes that occur during plant development (Feher *et al.* 2003; Rajan *et al.* 2025). It serves as an appropriate model for studying plants ontogeny (Pescador *et al.* 2008). Additionally, SE is an efficient system for large-scale commercial plant propagation and development of artificial seeds (Jain and Gupta 2018; Botini *et al* 2021). SE encompasses the generation of complete plants, with root and shoot axes, from somatic cells, and it can be separated into four stages. The first stage is the initiation or induction of somatic embryos (SEs), where the proembryogenic cells are initiated. The second stage is the maturation of SEs,

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during which the embryogenic callus proliferates and develops. The third stage is germination, and finally, the fourth stage encompasses plant regeneration, where SEs develop into complete plants (Girón-Ramírez *et al.* 2024).

The induction stage is critical phase for achieving ideal SEs and improving the following SE stages (Bevitori *et al.* 2014). During this phase, the somatic cells undergo reprogramming and differentiate into new cell types. This reprogramming is restricted to somatic cells that are competent to respond appropriate inductive stimuli (Su *et al*. 2021).

The main concern with SE is the high prevalence of aberrant embryos that cannot sprout or grow into healthy plants. These anomalies may result from genetic or epigenetic modifications to DNA, triggered by external factors and environmental stresses such as high temperatures, drought, salinity, heavy metals, mutagenic chemicals, or plant growth regulators. In most cases, aberrant embryos may develop into normal plants because epigenetic modifications are reversible. On the other hand, genetic changes that cause abnormalities are normally irreversible (Garcia *et al*. 2019).

Histological examinations provide strong evidence for tracking the structural transformations during SE, that assist in understanding plant ontogeny, morphology, and development (Saeed and Shahzad 2015; Jain and Gupta 2018). In most varieties of *C. papaya*, immature zygotic embryos (IZE) are considered the most suitable explant source for proembryogenic cells for SE (Fitch and Manshardt 1990). Additionally, the auxin hormone, 2, 4-Dichlorophonexyacetic acid (2, 4-D), in particular, is regarded as a superlative growth regulator for inducting the growth of proembryogenic cells (Mandal and Dutta 2003).

Generally, not all plant cells are capable of producing auxin. The intrinsic auxin transfer among cells relies on two processes: auxin influx (AIT) and auxin efflux (AET), which are mediated by specific transporters (Konstantinova *et al.* 2021). The first type of AET transmembrane proteins, the PIN protein, facilitates auxin transfer between cells (Křeček *et al.* 2009). Another type of AIT transmembrane protein is AUX1/LAX, which facilitates auxin transport across cell membranes. Intercellular auxin flow is regulated by the AUX1/LAX and PIN proteins, which is essential for SE and plant growth (Estrella-Maldonado *et al.* 2016). These proteins are essential in SEs development, regulate auxin movement, and contribute in root growth and development (Bainbridge *et al.* 2008). Examining the expression of these genes can provide insights into the challenges of SE in *C. papaya*, particularly the barriers to normal root development, such as the callus formation observed at the base end of SEs.

This study aimed to address the challenges of regenerating SEs from the IZE of "Eksotika," particularly focusing on callus formation at the base of the SEs. This work is designed to monitor histological and gene expression changes during SE in *C. papaya* L. 'Eksotika', elucidate root development and clarify the growth of callus at the base of SEs during induction stages.

Materials and Methods

Explant source

Papaya fruits of the 'Eksotika' cultivar were obtained from organic farms (Latitude 1.684107, Longitude 104.217907) in Johor Bahru, Malaysia, 391 kilometers from Kuala Lumpur. The climate of Johor is a tropical rainforest with high annual temperatures, little temperature change, and steady annual precipitation with an average of 2600 mm of rainfall early (Tan *et al*. 2021; Talib *et al*. 2024). The soils of Johor Bahru, according to their parent material, have been classified into three broad groups: sedentary, alluvial, and miscellaneous. The type of soil in the *Carica papaya* collection site was alluvial, consisting of organic soil with Gley soils (Department of Agriculture Peninsular Malaysia 1973; Saleh *et al*. 2015). The immature fruits were harvested 95 to 100 days after pollination. They were immediately spurted with ethanol (70% v/v) and stored in a polystyrene icebox (4-6°C) for 8 h before transportation to the laboratory. The immature papaya fruits showered under tap water with detergent for 15-20 minutes. Finally, fruits were spurted for a second time with ethanol (70% v/v) under a horizontal laminar flow hood, sliced release with a sterile blade, and the immature seeds (white seeds) were gently detached applying a sterile spoon before being incubated in the induction.

Media constituents for induction and maturation

The induction medium is half-strength Murashige and Skoog medium (½ MS) (Duchefa Biochemie) (Murashige and Skoog 1962) supplemented with vitamins 10 mg/L 2, 4-D growth regulator (Sigma-Aldrich), 100 mg/L L-glutamine (Sigma-Aldrich), 45 mg/L adenine hemisulfate (Sigma-Aldrich), 50 mg/L myo-inositol (Sigma-Aldrich), 6% sucrose (Duchefa, Biochemie) and 3.3 mg/L Gelrite. After eight weeks, the induced SEs were transported to the maturation medium, (comprised of ½ MS medium with, 68 mg/L adenine hemisulfate (Sigma-Aldrich), 100 mg/L L-glutamine (Sigma-Aldrich), 100 mg/L myo-inositol (Sigma-Aldrich), 3% sucrose and 3.3 g/L Gelrite (Duchefa, Biochemie). The initial 2, 4-D concentration in the maturation medium was reduced to half that of comparable induction medium (5 mg/L 2, 4-D for the first subculture). For second subculture, the 2, 4-D strength was further reduced by half (2.5 mg/L) and third subculture was prepared without growth regulator (2, 4-D).

Histological examination

For histological examination, somatic callus samples were fixed at different time points, starting from day 25 until day 80 of culturing. The combined stains (Acetocarmine and

Evan blue) showed that SE induction began after 21 days of culture. At each time point, at least three explants (calli) were randomly selected and processed for histological analysis. Callus samples (0.5 cm) were fixed in cold FAA (5: 5: 90 v/v/v) solution at 4°C for 48 h (Huang and Yeung 2015). The samples were then dehydrated with a gradual concentration of tert-butyl alcohol for 2 h per stage. Then, paraffin wax (melting point: 57°C) was used to embed the samples. A rotary microtome (Leica RM2125, Wetzlar, Germany) was used to cut serial ten μm sections. The sample slides were stained with 0.05% (w/v) Toluidine Blue O (TBO) (Sigma-Aldrich) using Johansen (1940) method and Safranin-O and Fast Green (Sigma-Aldrich). The stained slides were examined using a Zeiss Axioscope microscope and OLYMPUS CX22 microscope (Tokyo, Japan). The images were cropped, combined, and optimized using Adobe Photoshop CS6 Extended 13.0.1.

RNA extraction and cDNA synthesis

SE calli were collected after 59, 61, 63, 70, 90, 100, 105, 115, 130, 135, 143 and 158 days of culture, following the transfer of SE calli from induction to maturation media. Total RNA was extracted from three separate replications. Calli were crushed in liquid nitrogen before RNA extraction with the RNeasy Plant Mini Kit (Qiagen, Germany) (Urasaki *et al.* 2012). RNA concentrations and purity were determined by using the Nanodrop 2000 (Eppendorf, Germany). A gel electrophoresis apparatus operating at 100 volts and 200 amps for 30 min was used to assess the integrity of the extracted RNA samples.

Genomic DNA was eliminated prior to cDNA synthesis using a genomic DNA removal kit (Qiagen, Germany). The extracted RNA was then used as a template for cDNA synthesis and the QuantiNova Rev kit (Qiagen, Germany) was used for reverse transcription of 0.2 μg of total RNA.

Primers design and PCR reaction system

Arabidopsis thaliana genes (AtLAX2, AtLAX3 and AtPIN4) were first searched on the "bioinformatics" website (https://bioinformatics.psb.ugent.be/plaza), the orthologs were identified using the integrated orthology viewer. Cpa.g.sc115.56, Cpa.g.sc36.16, and Cpa.g.sc37.169 were the most suitable orthologs for the Arabidopsis genes (*AtPIN4*, *AtLAX3* and *AtLAX2*), respectively. After that, the sequence of Arabidopsis genes (without intron) was then blasted utilizing the website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with genomic data from *C. papaya*. Afterward, the ortholog sequences were used to navigate to GenPept and locate RefSeq to select primers. Integrated DNA Technologies (Malaysia) generated these primers (Table 1). The primers' gene specificity was evaluated using cDNA clones with primers for the *EIF*, *PIN4*, *LAX3* and *LAX2* genes.

Quantitative real-time PCR (qRT-PCR) conditions

Gene expression analysis was performed using a SYBR Green PCR Kit (QuantiNova, Qiagen, Germany) on a qRT-PCR machine (Stratagene Mx 3005P, USA). A final volume of 10 μL was used for each sample. The components included 0.7 μM of each primer, 2.6 μL cDNA (26 ng), 5 uL of 2X SYBR Green PCR Master Mix, and 1 μL of RNase-free water. All samples were run in triplicate. The real-time PCR conditions were as follows: initial heat of polymerase activation was 5 min at 95°C, followed by 35 cycles of 45 sec at 95°C, 45 sec at 62°C and 30 sec at 72°C. A crucial first step in every RT-qPCR experiment is selecting appropriate reference genes to normalize target gene expression. EIF (Eukaryotic Initiation Factor 4A) (FJ644949.1) was one of the internal reference genes that performed well in most experiments (Zhu *et al*. 2012). Therefore, the EIF gene was selected as a reference gene in this study. Each experiment was performed in triplicate with a no-template control. The threshold cycle of the principal amplification curve was used for computations. Gene expression levels were determined using ΔΔCt and relative quantification techniques as described by Livak and Schmittgen (2001) ($2^{-\Delta\Delta Ct}$) were used to analyze the data.

Results

Histological examination

The histological changes of SEs were examined at various times through induction stage. At day 25, histological examination with Acetocarmine and Evan blue showed that embryogenic cells had differentiated. However, histological examination with Safranin and Fast Green staining showed the pro-embryonal complex, confirming the origin of SEs (Fig. 1A). As shown in Fig. 1B, the embryogenic calli have contained tiny compressed cells with compacted nuclei and tiny vacuoles. Larger, more vacuolated, diversely shaped parenchymal cells made up the inner explant cells, while the marginal cells displayed elongated structures and wider intercellular gaps (Fig. 1C-D).

The cells that formed the meristematic core were uniformly shaped, tiny, and lacked intracellular spaces. They also featured a central nucleus, thick cell walls, and dense cytoplasm (Fig. 1E-F). Somatic embryo initiation occurred in the explant's epidermal cells through periclinal division, resulting in two cells: an exterior apical cell and an internal basal cell. Subsequently, the internal basal cell divided anticlinally and later periclinally, while the apical cell retained its primary state (Fig. 1F-G). The apical cell then split, producing a globular shape (Fig. 1H-I). In the later stages of development, the protoderm developed into a globe-like structure, with no differentiation observed in the inner cells (Fig. 2, Fig. 3A).

Table 1: Primers used for RT-PCR analysis, primer name, sequence, melting temperatures (Tm) and GC content

Primer name	(5' to 3')	Sequence	Tm	GC%
LAX2	Forward	TGTGCTCACACTGACACTCC	59.90	55.0
	Reverse	CAGCCATGTCTCGGAAAGGT	60.04	55.0
LAX3	Forward	GGCCAGCCAAAATGGTTCTG	60.04	55.0
	Reverse	TCCACTGTCACAGCATGTCC	59.96	55.0
PIN4	Forward	CCAACTCCGGCCTTTCTCTT	59.96	55.0
	Reverse	TAGAGCATCTTGGCGTTGGG	60.11	55.0
EIF	Forward	CGCGGAAGACAAACCCAATC	59.83	55.0
	Reverse	CTGGAGCAAAACGGCTGATG	59.83	55.0

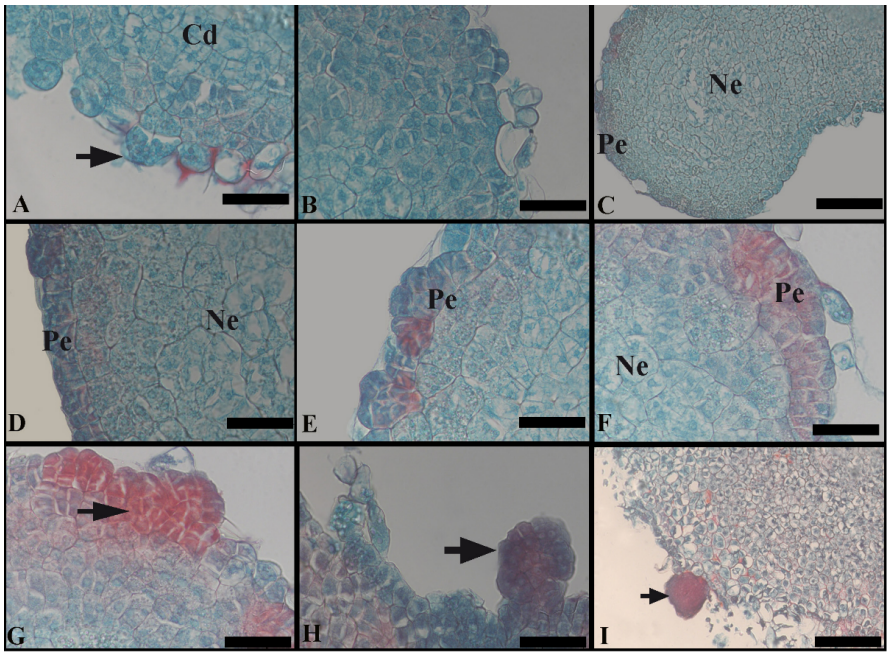

Fig. 1: Histological slices after 25 days of culture, stained with double safranin O and quick green. (**A** and **B**) Pro-embryonic (pe) cells (arrow) began to organize and develop. (**C**) The marginal region of the callus reveals pro-embryonic (pe) cells in their early stages, distinguishable from other non-embryonic (ne) cells. (**D**) The magnified epidermis (pro-embryonic (pe)) cells of the callus were uneven in shape, with interior cells proliferating and developing. (**E** and **F**) Cells continued to divide, forming meristematic zones. (**G**) A cluster of epidermal and subepidermal cells (arrow). Illustration of the globular embryo in its early stage at 28 days of culture. (**H** and **I**) histological longitudinal sections after 32 days of culture. Illustration of the globular embryo (arrow). Scale bar A-B, D-H = 50 μm, C, I = 200 μm

Evagination developed in the globular shape, indicating the onset of heart-shaped stage (Fig. 3B). At this stage, the embryo's root pole could be identified as a clump of dense cytoplasmic cells, with no vascular connection linking the mother explant to the SEs (Fig. 3C-D). In the later stage of heart-shaped embryos development, the procambia were evolved. The evagination then developed continually in heart stage progressing to the torpedo shape and cotyledonary stage (Fig. 3E). Most mature SEs exhibited bilateral symmetry, with visible and stained apical shoot and root meristems (Fig. 3F). In the longitudinal segment, the developed SEs displayed a concave shoot meristem with multilayers (Fig. 3F).

Somatic callus development, particularly in the root pole, was observed through the cotyledonary stage of SEs (Fig. 4A). Histological study showed the growth of a somatic callus at root base of SEs (Fig. 4A). Most somatic calli were observed at the root cap of embryos at cotyledonary stage (Fig. 4A-D). Numerous new embryos at various developmental stages were discovered. These newborn embryos developed from the peripheral zones of primary SEs, particularly during the cotyledonary stage. Some anomalies in the developing SEs were identified, including variations in cotyledon numbers (Fig. 4E-I) and fused SEs (Fig. 4H).

Gene expression of *CpPIN4, CpLAX3* and *CpLAX2* genes

The housekeeping gene, *CpEIF*, was used to compare the relative expression levels of the *CpLAX2*, *CpLAX3* and *CpPIN4* genes in SE produced by IZE at different times using quantitative RT-PCR.

Table 1 lists the gene names, primer sequences, accession numbers, and amplicon properties such as Tm, length, and amplification efficiency. Fig. 5 shows that the maximum expression level of *CpLAX2* gene was observed

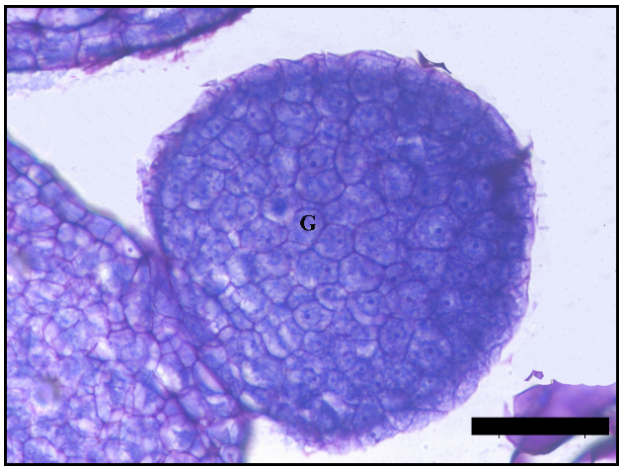

Fig. 2: Late stages of development after 30 days of culture, the globular embryo is composed of tiny, densely cytoplasmic cells. The protoderm was identified without observing any organization in the inner cell during the globular stage. Scale bar = 50 μm

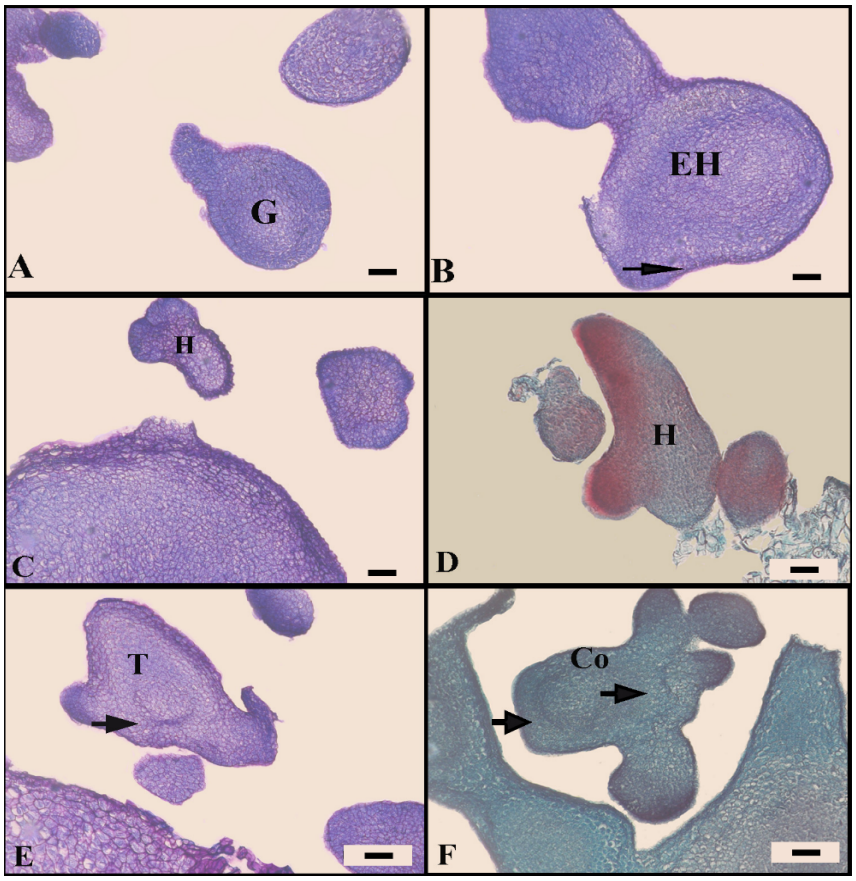

Fig. 3: A histological section after 52 or 56 days of culture (**A**) A globular embryo (g). (**B**) A globular embryo begins evagination, forming an early heart shape (eh). (**C** and **D**) Heart-shaped (h) embryos with no visible vascular tissue in the shoot or root. (**E**) The late heart shape or early torpedo (t) form, in which evagination is greater and procambial tissue (arrow) first appears. (**F**) The mature cotyledon (co) embryo has the root and shoot poles and a distinct procambial in the shoot and root poles (arrow). Scale bar = 100 μm

in samples examined after 158 days of culturing. In contrast, the lowest gene expression level was observed in samples examined after 59 and 135 days of culturing. The expression pattern of *CpLAX2* gene can be divided into three stages: The first stage begins on day 59, increases until day 63 and then declines until day 70. In the next stage, the expression level of *CpLAX2* gene increases after day 70 peaks around day 115 and then begins to decline until day 135.

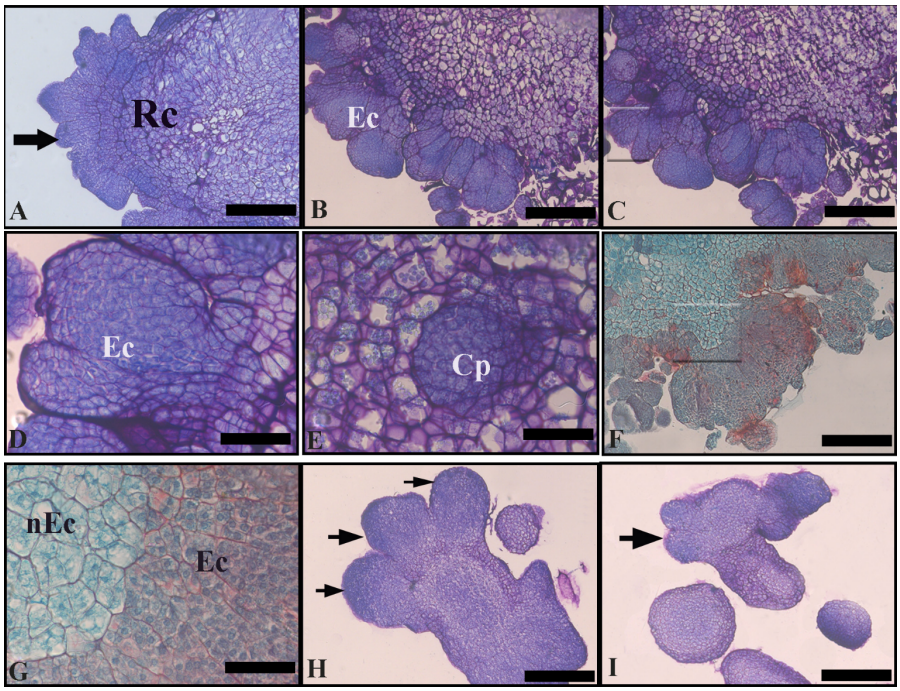

Fig. 4: (**A**) histological section after 56 days of culturing, exhibiting callus development entrenched in the root cap (rc). (**B** and **C**) Embryonic callus (ec) on the margin of the root zone. (**D**) Zoom in on c. (**E**) Cellular protrusions (cp) are hindered in the root zone. (**F**) Some embryonic calli (ec) are inhibited in the root zone where non-embryonic callus (nec). (**G**) Zoom in on f. (h and i) A histological section of a somatic embryo after 52 days of culturing shows various anomalies discovered during somatic embryogenesis. (**H**) Changed the number of cotyledons (arrow). (**I**) Fused somatic embryos (arrow). Scale bar = A-C, F, H-I = 200 μm, D, E, G = 50 μm

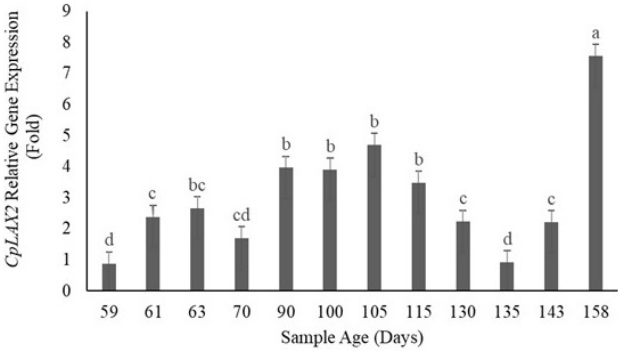

Fig. 5: Relative expression level of *CpLAX2* (*Carica papaya Like-AUX2)* at various developmental stages of SE. Error bars represent standard deviation (n = 5). Different letters indicate significant differences ($P < 0.05$, ANOVA, Duncan's test)

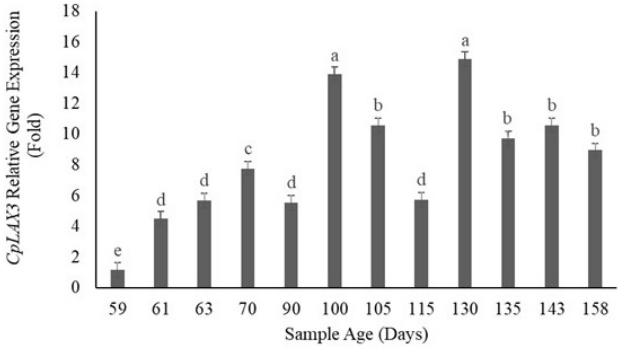

Fig. 6: Relative expression of *CpLAX3* (*Carica papaya Like-AUX3)* at various developmental stages of SE. Error bars represent standard deviation (n = 5). Different letters indicate significant differences ($P < 0.05$, ANOVA, Duncan's test)

The third stage begins on day 135 and ends on day 158 when the expression level reaches its highest peak.

In contrast, as shown in Fig. 6, the maximum gene expression level of *CpLAX3* was observed in samples examined after 100 and 130 days of culturing. The lowest expression level was noted in samples examined after 59 days. The expression pattern of *CpLAX3* gene can also be divided into three stages: the first stage begins on day 59, with expression increasing until day 70, after which it diminishes until day 90. The second stage occurs when *CpLAX3* expression level begins to rise after 90 days and peaks around day 100. The final stage is marked by increased expression starting on day 115 and peaking after 130 days, then decreasing until day 158.

For the *CpPIN4* gene, the maximum gene expression level was observed in samples examined after 70 days of culturing, while the lowest expression level was observed after 130, 135 and 143 days of culturing IZE (Fig. 7).

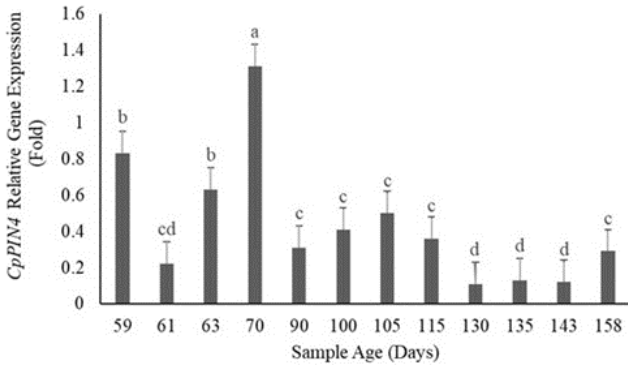

Fig. 7: Relative expression level of *CpPIN4* (*Carica papaya Like-AUX4)* at various developmental stages of SE. Error bars represent standard deviation (n = 5). Different letters indicate significant differences ($P < 0.05$, ANOVA, Duncan's test)

The expression levels decreased from day 59 to day 61, then increase until day 70. Afterward, they begin to drop again by day 143. By day 143, the expression level increases until day 158 (Fig. 7).

Discussion

The primary challenges in cultivating transgenic 'Eksotika' plants include poor root production from shoots (Sekeli *et al.* 2013). Histological and molecular investigations were performed to monitor SE development. These analyses facilitate the early detection of SEs and provide a deeper understanding of their developmental stages and the root formation process, especially the growth of callus at the base of SEs during the induction stages.

After three weeks of culture, SE from the IZE of 'Eksotika' was induced on an auxin-enriched medium. Highly influential somatic cells derived directly from SEs (competent cells) were observed in the protoderms. The findings of the current study align with those of Kurczyńska *et al.* (2007), who reported that Arabidopsis IZE led to direct SE when cultivated on an auxin-enriched medium.

Spherical cells formed the embryogenic callus, characterized by a prominent nucleus and thick cytoplasm. On the other hand, non-embryogenic calli were produced by irregular or elongated cells with a tiny nucleus and light cytoplasm. These findings are consistent with Silveira *et al.* (2013), whose histological investigation on continuous embryogenic cultures revealed that specific cells on the surface of SEs had a prominent nucleus and thick cytoplasm, which were believed to be the origin of daughter embryos. Similarly, Pinto *et al.* (2011) examined SEs from *Passiflora edulis* Sims and observed specific epidermal cells with rich cytoplasm and large nuclei, which distinguished embryogenic cells from other cell types.

Our findings revealed that the majority of embryonic cells, shared meristematic characteristics such as dense cytoplasm and a conspicuous nucleus through the globular embryo stage. However, these properties began to disappear during the early heart stage. Ma *et al.* (2012) observed similar histological characteristics in embryogenic cells. According to Feher *et al.* (2003), cells' capability to respond to particular hormonal or environmental signals is correlated to formation of meristematic centers and organs.

Many researchers have reported contradictory findings regarding the formation of SEs in the early stages of SE. Mikuła *et al*. (2021) and Ferreira *et al.* (2022) state that SEs can arise from unicellular or multicellular cells. Typically, SEs with a multicellular origin were commonly directly attached to parental tissue, whereas those with a unicellular origin were linked to the parental *via* a suspensor (Ferreira *et al.* 2022). According to Jayasankar *et al.* (2003), the suspensor regulates and promotes germination, development, and regeneration rates. Santos *et al.* (2006) found that the key distinction between SEs from IZE and zygotic embryos was the lack of suspensors and delays in the development of internal arrangements.

In our investigation, daughter embryos at varied times and sizes were observed on the external surface of mother SEs. These daughter SEs arose from cells characterized by large nuclei and thick cytoplasm and were attached to the mother tissue without suspensors. This finding aligns with Bharathy and Agrawal (2008), who reported that new (daughter) embryos formed on the root-shoot zone, hypocotyls in cotyledon, and occasionally developed into complete embryos in grapevine SEs. Fehér (2005) demonstrated that explants maintained in auxin-enriched conditions, such 2, 4-D, could retain the embryogenic potential of meristematic cells. When the embryogenic callus was transferred to conditions without growth regulators after exposure to auxin, it produced SEs. The 2, 4-D is an artificial auxin that functions similarly to natural auxins but exerts a broader range of effects. It influences various genes involved in cellular transport, protein mobility, signal transduction, and subcellular localization (Fehér 2005). Kurczyńska *et al.* (2007) and coworkers investigated the dispersion of auxin in SEs during different stages of embryogenesis to illustrate the embryogenic characteristics of protodermal and subprotodermal tissues. Auxin was found to accumulate in the protodermis of globular SEs and was subsequently transported to cotyledon primordia and the root pole through heart and cotyledonary phases (Wu *et al.* 2020).

The histological investigation reveals that root was developed at an early heart-shaped stage. These conclusions agree with Sun *et al*. (2024) discovery that root development is activated in late globular stage, when the top suspensor cell undergoes asymmetric division, creating small cells that form the core of embryonic root.

The present research uncovered anomalies during SEs development, including existence of fused SEs and variations in the number of cotyledons. During cotyledonary stage of SEs, somatic callus growth was observed, particularly at the root pole. Sekeli *et al.* (2013) explained that the development of callus at this position inhibits normal root formation, resulting in incomplete plants.

According to Kong *et al.* (2012), these defects can be recognized by abnormal root morphology and the inability to develop into mature plants.

The histological analysis of *Acca sellowiana* (pineapple guava) revealed that abnormal SEs might be created either directly or indirectly from calli generated on the explant. This result implies that the growth of anomalous SEs reflects changes in genetics, physiology, or both, caused by the existence of 2, 4-D in media (Pescador *et al.* 2008). Our finding agrees with previous research, which reported that SEs of most species exhibited various morphological anomalies, including variations in size, cotyledon number, and shape. Oliveira *et al*. (2022) uncovered those abnormal SEs regeneration increased with time and 2, 4-D concentration. A study by Araujo *et al.* (2005) demonstrated that explants treated with synthetic auxin significantly influenced the expression of diverse transposable elements.

Based on the preceding findings, the production of anomalous SEs, which leads to poor conversion rates in *C. papaya* may result from the presence of auxin (2, 4-D) in the medium. The auxin may alter normal physiological and genetic activities. Hence, adequate levels of exogenous auxin in explants play an important role in the growth of normal SEs (Al-Shara *et al*. 2020). The climate of Johor is a tropical rainforest with little temperature change and steady annual precipitation with an average of 2600 mm of rainfall early (Tan *et al*. 2021; Talib *et al*. 2024). Also, the type of soil in the *C. papaya* collection site was alluvial, consisting of organic soil with Gley soils (Saleh *et al*. 2015; Agriculture Peninsular Malaysia 1973). These anomalies may result from genetic or epigenetic modifications to DNA, triggered by external factors and environmental stresses such as salinity, heavy metals, mutagenic chemicals in soil. In most cases, aberrant embryos may develop into normal plants because epigenetic modifications are reversible. On the other hand, genetic changes that cause abnormalities are normally irreversible (Garcia *et al*. 2019).

Auxin is a hormone that regulates plant growth as well as its response to abiotic stressors. Yu *et al.* (2017) identified three protein families responsible for auxin transport in plants: auxin-resistant 1/LIKE AUX1 (AUX/LAX) influx carriers, ABCB/MDR/PGP efflux/condition carriers, and PIN-FORMED efflux carriers. Auxin content in root tissues is regulated by AET (PIN) and AIT (AUX1/LAX) genes, which control development and the mechanisms of lateral root formation. Therefore, this investigation was designed to analyze the expression pattern of key genes involved in root development (*CpLAX2*, *LAX3* and *CpPIN4*) at various times of SEs development.

During the early stage of SE (induction stage), *LAX3* plays a crucial role in auxin uptake, as suggested by Wójcik *et al.* (2020) and Karami *et al.* (2021, 2022). The gene expression of *CpLAX3* continues to play a role in auxin transport, which is essential for the maturation of SEs. *CpLAX2* is involved in auxin influx, similar to *CpLAX3* during the initiation of SEs, but it has a slightly lower impact than *CpLAX3*.

The *CpLAX2* also contributes to auxin transport during maturation but to a lesser degree than *CpLAX3*. The *CpLAX3* and *CpLAX2* are both involved in auxin influx, with *CpLAX3* showing a stronger correlation with SEs than *CpLAX2*. *CpPIN4* is critical for establishing auxin gradients necessary for embryo initiation, as highlighted by Loyola-Vargas (2022) and Monroy-González *et al.* (2023) De-la-Peña and which essential for the early maturation of SEs.

In Arabidopsis, the AUX-LAX gene family plays a vital role in embryonic root and plant development (Bainbridge *et al.* 2008; Ugartechea-Chirino *et al.* 2009). Yang *et al.* (2006) discovered that both *AtLAX3* and AtAUX1 genes have a role in regulating lateral root and apical hook development. Péret *et al.* (2012) found that when explant was grown in a medium enriched with 2, 4-D, both *AtLAX1* and *AtLAX3* genes were significantly expressed in the roots. The *AtLAX2* gene regulates cell division and vascular development in the quiescent center (QC). PIN family genes have vital functions in different plant developmental processes (*e.g*., embryo development, vascular bundle differentiation, root meristem modeling, lateral root development, root hair growth, and phototropism) (Ganguly *et al.* 2010; Robert *et al.* 2013). The *ClLAX*, *ClPIN* and *ClABCB* genes are unevenly expressed in the cotyledons, roots, flowers, leaves, and shoots of *Citrullus lanatus*. The uneven expression of these genes in diverse tissues is thought to be a robust indicator that they participate in plant development and maturation (Kaneda *et al.* 2011; Péret *et al.* 2012). Our research found that the AIT (*CpLAX 2, 3*) and AET (*CpPIN 4*) genes were expressed across all stages of SE (from globular to cotyledonary), suggesting that these genes are involved in plant growth and development, as supported by the above-mentioned studies. *CpLAX3* is essential for auxin absorption and transport during the induction and maturation phases of SE, and its influence is greater than that of *CpLAX2*, which also plays a part, but to a lesser extent. According to current research, *CpPIN4* is also necessary for the establishment of auxin gradients during embryo initiation and early maturation. A deeper study is needed to explain the mechanism of action of *LAX3* gene in SEs development in *C. papaya*.

Conclusion

The ontogeny and structural features of SEs in *C. papaya* were investigated in this work at the induction stage. Somatic callus induction was successfully improved by adding 10 mg/L 2, 4-D to MS media; however, this also promoted callus formation at the base of SEs, which impeded appropriate SE development and maturation. All SEs showed expression of the genes *CpLAX2*, *CpLAX3*, and *CpPIN4* at various times of development. *CpLAX3* was the most highly expressed, while *CpPIN4* was the least

expressed. These findings suggest that all three genes play crucial roles in the formation and proliferation of SEs in papaya, highlighting their potential involvement in regulating SE in this species. *CpLAX3* is essential for auxin absorption and transport during the induction phases of SE, and its influence is greater than other genes studied.

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Author Contributions

RMT, JM and BA designed and supervised the research. BA conducted experiments and data collection. BA, WA, and HE conducted the statistical analysis. BA wrote the original manuscript. AK, WA and MJ reviewed and edited. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

Authors declare no conflict of interest.

Data Availability

Data will be available upon request.

Ethics Approval

Not applicable to this paper.

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