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ANALYSIS OF BBM, LEC, AND SERK EXPRESSIONS IN CALLUS OF SUGARCANE (*Saccharum officinarum* L.) AT SOMATIC EMBRYOGENESIS DEVELOPMENT STAGES

Analisis Gen BBM, LEC, dan SERK terhadap Kalus Tebu (*Saccharum officinarum* L.) pada Tahap Perkembangan Somatik Embriogenesis

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ABSTRACT
Callus formed in somatic embryogenesis is divided into two types, embryogenic and non-embryogenic callus. Embryogenic callus can be distinguished by the expression of genes as markers related to somatic embryogenesis which are expected to be used as markers to detect callus that has embryogenic capabilities in sugarcane. The aim of this study was to determine the expressions of somatic embryogenesis-related genes in sugarcane. The genes analysis was carried out using somatic embryogenesis callus and using a kit for RNA analysis. Results showed that there were expressions of Baby Boom (BBM), Leafy Cotyledon (LEC), dan Somatic Embryogenesis Receptor Like-Kinase (SERK) gene with specific primer of those three genes, which were collected from embryogenic callus in mass, globular, scutelar, and coleoptilar pre-embryo stages, meanwhile there were no expressions of those genes collected from non-embryogenic callus. Amplification result from PCR product of cDNA using three gene primers detected were in 500 bp for BBM, 400 bp for LEC, and 700 bp for SERK gene.

Keywords: *auxin, coleoptilar, embryogenic, globular, scutelar*

ABSTRAK
Kalus yang terbentuk pada embriogenesis somatik terbagi menjadi 2 jenis, yatu kalus embriogenik dan non embriogenik. Kalus embriogenik dapat dibedakan dengan adanya ekspresi gen sebagai penanda karena adanya proses somatik embriogenesis yang nantinya diharapkan dapat digunakan sebagai marka untuk mendeteksi kalus yang memiliki kemampuan embriogenik pada tanaman tebu. Tujuan dari penelitian ini yaitu untuk mengetahui ekspresi gen yang diduga terkait proses embriogenesis somatik pada tanaman tebu. Analisis gen terkait proses embriogenesis tersebut dilakukan dengan menggunakan kalus hasil embriogenesis somatik, dan penggunaan kit untuk analisis RNA. Hasil penelitian menujukkan bahwa terdapat ekspresi gen *Baby Boom* (BBM), *Leafy Cotyledon* (LEC), dan *Somatic Embryogenesis Receptor Like-Kinase* (SERK) dengan primer spesifik ketiga gen tersebut, yang diambil dari sampel kalus embriogenik fase pre-embryo mass, globular, skutelar, dan koleoptilar, namun tidak adanya ekspresi ketiga gen tersebut pada kalus non embriogenik. Hasil amplifikasi sampel produk PCR dari cDNA menggunakan ketiga primer gen yang terdeteksi memiliki ukuran sebesar 500 bp untuk gen BBM, 400 bp untuk gen LEC, dan 700 bp untuk gen SERK.

Kata Kunci: auksin, embriogenik, globular, koleoptilar, skutelar

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INTRODUCTION

Somatic embryogenesis is one of the tissue culture techniques for plant propagation. It refers to a process of embryo formation from a somatic cell that developed into a whole new plant without gamete fusion (Herawan et al. 2014). A somatic embryogenesis technique has a high multiplication rate and become the potential to use as a plant propagation method. Factors affecting this method are the source of explant, media composition, plant growth regulator, and culture condition (Anggraeni et al. 2012, Damayanti et al. 2018, Yelnititis 2018). It may have resulted in different responses for different tissue, genotype, or species of explant to the same plant growth regulator that was treated.

Auxin and cytokinin are the most studied plant growth regulator in the somatic embryogenesis method (Hendaryono et al. 2012). Auxin has an important role during the process of callus induction and embryogenic ability development. The callus formation is dependent on a series of complex processes from transcription factors that have been induced by auxin which leads to cell regulation. In somatic embryos development from embryogenic callus, a decreased level or even removal of auxin in the culture media is needed for the next culture development (Reis et al. 2021). Indah and Ermavitalini (2013) stated that the addition of appropriate auxin to the culture media could trigger a cell differentiation process and callus induction. The appropriate auxin for different genotypes of plant might be different in concentration that should be given. Several genotypes of plant with different variety might require a diiferent concentration of auxin consisted in the media. Auxin from 2,4-Dichlorophenoxyacetic acid (2,4-D) is the most used plant growth regulator for somatic embryogenesis in the sugarcane plant. Embryogenic callus was highest formed in the callus induction stage with culture media containing 4 mg L^{-1} 2,4-D (Sholeha et al. 2015, Ningtiyas et al. 2016, Dewanti et al. 2021). In addition to that, a study from Dewanti et al. (2020) used 1 mg L^{-1} 2,4-D in the proliferation stage to trigger somatic embryos development in sugarcane.

Somatic embryo development in the sugarcane plant is divided into 4 stages which are pre-embryogenic mass (PEM), globular, scutellar, and coleoptilar (Alfian et al. 2019). PEM stage is occured in an early stage of embryo development which has a dense and shiny structure. It was formed in the 3 weeks old callus. A PEM structure develops into a globular stage that is formed after cell division. The most visible change is there are nodules formed on the surface of the callus (Widuri et al. 2016). Scutelar is the next stage formed, marked by dense cytoplasm cells and at this stage, a leaf primordial started to form. The scutelar was developed to coleoptilar structure marked by a clear visible leaf primordial (De Alcantara et al. 2014). Coleoptilar structure hinted at the end of the somatic embryo stage before growing as a plantlet (Damayanti et al. 2018).

Table 1. Primer sequence for gene transcriptor analysis

Gen	Primer
BBM	Forward: CGATTTACCGTGGCGTGACA
	Reverse: CGTGAAGAGCATCCTGGACA
LEC	Forward: CGATCCAGGAGTGCGTGTCG
	Reverse: AGCCACTACCTGCCTTACGC
SERK	Forward: GCTGCAGAGTTGGGATCCAA
	Reverse: ACTTCAGGATCCTCCTCAGC

Callus induced through somatic embryogenesis methods is separated into two types, which are embryogenic and non-embrogenic callus. Morphological analysis under the microscope is one of the basic analyses to define the type of callus formed based on characterization that has been published. A molecular analysis could be a piece of very useful information which more effective and precise to support the information from the result of morphological analysis to differentiate embryogenic and non-embryogenic callus. The callus could be differentiated with gene expression as a gene marker for somatic embryogenesis. Several genes related to somatic embryogenesis in the plant that has been found with molecular analysis are *Baby Boom* (*BBM*), *Leafy Cotyledon* (*LEC*), and *Somatic Embryogenesis Receptor-Like Kinase* (*SERK*) among other genes which has been analyzed. *BBM* plays a role in signaling in the cell differentiation process and somatic embryo formation alongside growth and development in the proliferation stage (Yavus et al. 2020). *LEC* has the role for manage

embryo development and activating embryo maturity (Song et al. 2021). *SERK* get in all the mechanical aspect of somatic embryogenesis which showed by its high concentration in all stages of summer tulip embryogenic callus (Sucharitakul et al. 2014).

A study for gene expression related somatic embryogenesis in sugarcane variety Bululawang has been previously studied by Maulidiya et al. (2020), where the *BBM* and *LEC* expressed highly in the embryogenic callus at the callus proliferation stage. However, the previous study was only conducted at the callus proliferation stage, without *SERK* gene expression result. This research was conducted to complete the study early which analyzed the gene expression of *BBM*, *LEC*, and more *SERK* in some of the different stages of the somatic embryo which are PEM, globular, scutellar, and coleoptilar structure which was include at callus induction and callus proliferation stage.

MATERIALS AND METHODS

Location and time

This research was conducted in Laboratorium of Molecular Biology and Biotechnology Division of Center for Development of Advanced Science and Technology (CDAST) of Jember University from July 2020 to July 2021.

Plant materials

Explant material used for this research was the spindle leaf of 4 to 6 months old grown sugarcane var. Bululawang collected from research field of Agrotechnopark Jubung, Jember University.

Callus induction stage

Culture media used for callus induction stage was MS media containing 4 mgL^{-1} 2,4-D + 300 mgL^{-1} casein hydrolisate + 30 g L^{-1} sucrose + 5 gL^{-1} agar. The pH was adjusted at 6,2 and sterilization process was set at 121ºC and 17,5 psi for 15 minutes using autoclave. The 0,2 cm height and 0,5 cm diameter of spindle leaf explant transverse section was planted in a callus induction media at laminar air flow cabinet from Biobase aseptically. The culture was placed in a dark condition with a room temperature of 22-24ºC for 6 weeks which was subcultured ever 3 weeks on the same media composition. The final result of callus induction stage was an embryogenic callus in pre-embryo mass (PEM) structure and a few of globular structure.

Callus proliferation stage

Culture media used for callus proliferation stage was MS media containing 1 mgL^{-1} 2,4-D + 300 mgL^{-1} casein hydrolisate + 560 mgL^{-1} proline + 30 g L^{-1} sucrose + 5 gL^{-1} agar. The pH was adjusted at 6,2 and sterilization process was set at 121ºC and 17,5 psi for 15 minutes using autoclave. The callus produced from induction stage was then transferred to the callus proliferation media. Callus proliferation is aimed to push the somatic embryogenesis stage development. Culture was placed in a dark condition for a week then moved in a light condition for 6 weeks with room temperature of 22-24ºC.

Callus harvest for genes analysis

Embryogenic and non-embryogenic callus was used for the *BBM*, *LEC*, and *SERK* genes expressions analysis. The calluses used were harvested microscopically. The embryogenic callus was taken at PEM, globular, scutelar, and coleoptilar structure, with the PEM structure was collected from the callus induction stage, whereas the globular, scutelar, and coleoptilar structure was collected from callus proliferation stage. Meanwhile, the non-embryogenic callus was picked up from callus proliferation stage. All the harvested callus was storaged in a –80ºC cold room.

RNA isolation and cDNA synthesis

RNA was isolated from embryogenic and non-embryogenic calluses sample material according to the protocol contained in the instructions from rneasy plant mini kit of Tiangen that used in this study. Primer used for sequencing of *BBM*, *LEC*, and *SERK* genes were shown in Table 1, designed by Maulidiya et al. (2020) from the previous study. The resulting RNA concentration was measured with a spectrophotometer and synthesized to cDNA with the iScript cDNA synthesize kit. The cDNA synthesized was applicated according to the protocol contained in the instructions from iScript cDNA that used in this study. The cDNA was amplified with 30 cycles of

PCR reaction, include the initial denaturation process at 95ºC for 3 minutes, the denaturation process at 94ºC for 10 seconds, the annealing process with *BBM* primer at 53ºC, *LEC* primer at 55ºC, and *SERK* primer at 52ºC, each for 30 seconds, the extention process at 72ºC for 1 minute, and the elongation process at 72ºC for 5 minutes. The PCR product was then visualized with electroprhoresis in agarose gel 1% and visualized by UV transilluminator.

RESULTS AND DISCUSSION

Induction and callus proliferation stage

Callus embryogenic initiation under 4 mg L^{-1} 2,4-D was evaluated after 6 weeks of incubation at 22-24ºC. The addition of 2,4-D had the main role in initiating the early stage of somatic embryogenesis stage (Mendez-Hernandez et al. 2019). Stimulation of cell division, plant growth, and morphogenesis response is significantly affected by 2,4-D application (Wojcik et al. 2020).

A lower auxin concentration was applied to initiate somatic embryos. Somatic embryos initiation was marked by the emergence of globular, scutelar, and coleoptilar structures. In this study, the callus induction stage was performed with 4 mgL^{-1} 2,4-D and the callus proliferation stage was performed with 1 mgL^{-1} 2,4-D. The use of higher 2,4-D concentration than 1 mg L-1 resulted in lower of scutellar and coleoptilar stage development caused by the failure of callus cell differentiation according to Dewanti et al. (2020) study. Plant growth regulator composition in the media is highly significant in inducing somatic embryo development (Inderiati et al.2021). According to Mendez-Hernandez et al. (2019), stress factor also influenced the success of somatic embryo development during the proliferation stage. Additionally, L-proline application as a desiccation substance was also required to trigger somatic embryo development (Alfian 2015).

Two types of callus structure resulted in the present study, namely embryogenic and non-embryogenic. Observation of callus morphology was carried out to describe the structure and callus colour. Non-embryogenic callus (NE) had a brown color and a watery structure indicating necrotic tissue (Figure 1). This structure was classified as non-regenerative tissue with a low ability to grow into a whole new plant (Alfian et al. 2019). In the other hand, the embryogenic callus exhibited a nodular shape similar to the globular stage with a crumb structure. The color of this embryogenic structure was yellowish, greenish-yellow, and green similar to the result from Sari et al. (2018) study. In this

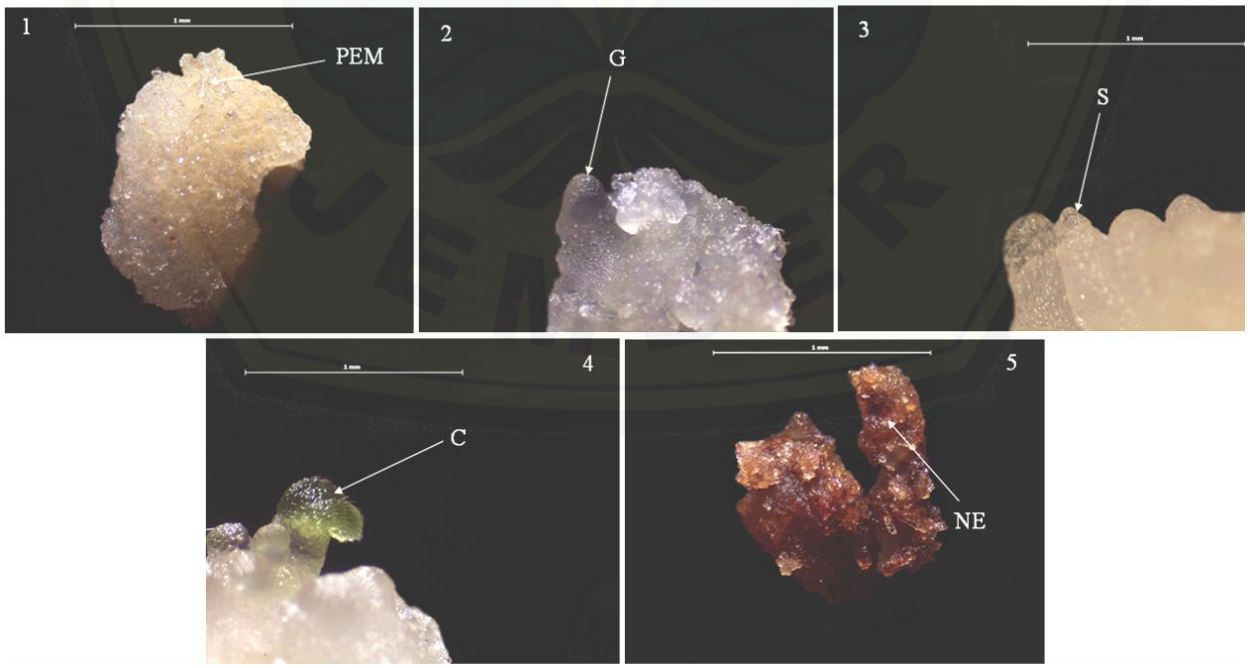

Figure 1. Callus at induction and proliferation stage: 1). Pre-embryonic mass (PEM) (P) 6 weeks old; 2). Globular (G) 8 weeks old; 3). Scutelar (S) 10 weeks old; 4). Coleoptilar (C) 12 weeks old; 5). Non-embryogenic (NE) 8 weeks old (a white horizontal line = 1 mm)

Table 2. Callus morphology at sugarcane somatic embryogenesis development stage

Indication	PEM	Globular	Scutelar	Coleoptilar	Non-Embryogenic
Stage	induction	proliferation	proliferation	proliferation	proliferation
Color	5Y 8/2	5Y 8/2	5Y 8/2	7,5 GY 6/8	7,5 YR 4/4
Structure	solid	crumb	crumb	crumb	solid
Age of callus	6 weeks old	8 weeks old	10 weeks old	12 weeks old	8 weeks old

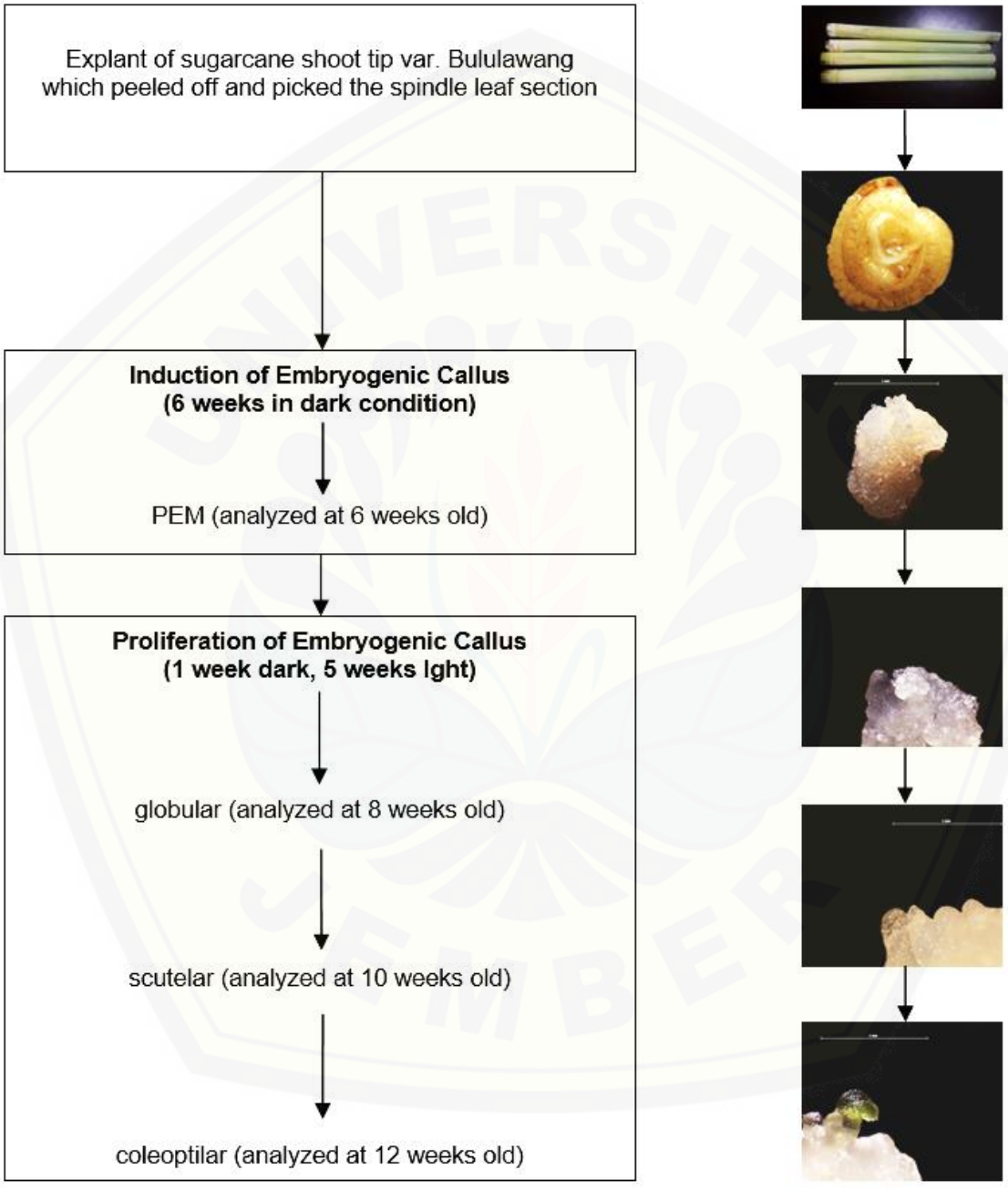

Figure 2. Flowchart for somatic embryogenesis system in sugarcane plant (*Saccharum officinarum* L.)

present study, there were several stages during somatic embryo transformation into a new plant: pro embryo mass (PEM), globular (G), scutellar (S), and coleoptilar (K). Initially, the PEM structure formed after 3 weeks of callus induction.

The PEM structure was characterized by a compact and soft structure. The globular

stage developed from PEM structure into nodular shape. The globular embryo was proliferated under the light conditions until 8 weeks for RNA analysis sample. Nodular shape indicated a multicellular cell that demonstrated a dense cytoplasm structure. Globular structure containing meristematic cells that further develop into the scutellar node. Scutellar (S) had a pointed surface with whitish color and a crumb structure. An RNA analysis sample for scutellar stages was conducted after 10 weeks of incubation. Based on Munsell Colour Chart Plant Tissue Culture we observed that PEM had a similar colour with globular and scutellar stages, 5Y 8/2. 5Y 8/2 indicated the colour was yellow with bright saturation (Table 2).

The coleoptilar structure was observed after 12 weeks of incubation, characterized by a crumb structure with obvious leaf primordia like-structure similar to the study performed by Dewanti et al. (2020) . The color code observed was 7.5 GY 6/8, which indicated the coleoptilar had green yellowish with bright saturation. As comparison, non-embryogenic colour had 7.5 YR 4/4, indicated yellow reddish colour with darker saturation. As explained by Nofita et al. (2020), code and notation in Munsell Colour Chart Plant Tissue Culture showed variable spectrum from hue, value, and chroma. Hue explained red, green, or yellow spectrum and their wavelength. Value demonstrated darkness or brightness of the colour according to the amount of reflected light. The closer value to 0/ indicated the darker saturation. Otherwise, the closer value to 10/ indicated the brighter saturation. Chroma explained the purity and the power of intensity of colour transformation. The process of somatic embryogenesis system in sugarcane was shown in Figure 2.

BBM, LEC, and SERK gene expression analysis

The RNA was isolated from non-embryogenic callus at proliferation stage and embryogenic callus in a different stage, which were PEM structure at 6 weeks old in callus induction stage, globular structure at 8 weeks old in callus proliferation stage, scutelar structure at 10 weeks old in callus proliferation stage, and coleoptilar structure at 12 weeks old in callus proliferation stage. The isolated RNA was synthetized to cDNA using a specific primer designed from the previous study by Maulidiya et al. (2020). Gene primer of *BBM*, *LEC*, and *SERK* genes were used for cDNA synthesis. The primer tested in this study had different size of cDNA band. *BBM*, *LEC*, and *SERK* gene were amplified at length 500 bp, 400 bp, and 700 bp respectively. Based on the result, embryogenic callus expressed *BBM*, *LEC*, and *SERK* in all embryogenesis stage including PEM, globular, scutellar, and coleoptilar. On the contrary, there was no targeted gene expressed in non-embryogenic callus (Figure 2). This result informed that morphology structure of non-embryogenic in this study was admittedly verified. The morphology characterization of non-embryogenic callus of sugarcane could be used for determined the embryogenic property of the callus, since it has been proved in the gene level that the non-embryogenic callus did not express the genes related embryogenic, in this study were *BBM*, *LEC*, and *SERK*. This non-embryogenic structure had low to none for the ability to

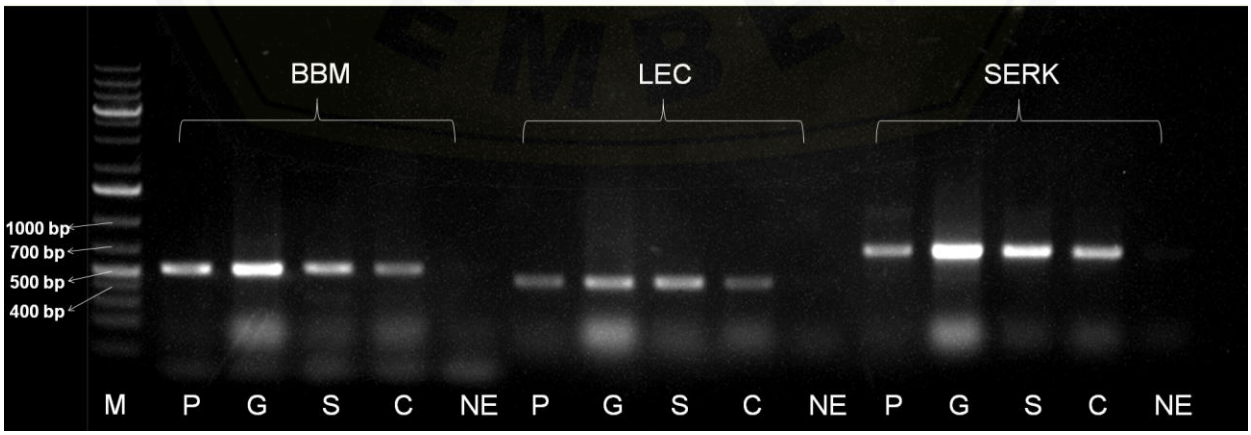

Figure 3. Expression of *BBM*, *LEC*, and *SERK* gene in embryoganic callus: Marker (M); (P = pre-embryonic mass (PEM); G = globular; S = scutelar; C = coleoptilar), and NE = non-embryogenic

regenerate into new plant. It was caused by the structure of non-embryogenic callus with watery and brown color indicating a high phenolic compound that inhibit the cell growth (Ningtiyas et al. 2016).

Results revealed that the highest expression of the *Baby boom* (*BBM*) gene was in globular (G) stage (Figure 3) seen from the thickness of cDNA band visualized. Interestingly, *BBM* was still expressed but showed lower tendency until late somatic embryogenesis stage especially in developing green spot callus structure that indicated leaf primordia development (coleoptilar stage). Embryogenic callus initiation was triggered by *BBM* gene expression (Lowe et al. 2016). A higher *BBM* expression induced callus formation and somatic embryo development during induction and proliferation stage in corn (Du et al. 2019). Previous study also mentioned that *BBM* gene expression increased significantly during callus induction in barley (Suo et al. 2021). Maximum gene expression played important role for somatic embryogenesis development. The *BBM* gene was only binded and activated several genes involved in somatic embryogenesis process (Horstman et al. 2017). *BBM* gene expressed in embryo and root meristem to regulate cell identity and totipotency capacity.

Figure 3 showed *Leafy cotyledon* (*LEC*) gene also expressed from PEM to coleoptilar stage. *LEC* was highly expressed at globular and scutellar stage, although the level of expression was not as high as *BBM*. Embryogenic callus development and shoot regeneration also promoted by *LEC* gene expression, for example in tobacco plant (Li et al. 2019). Initiation of somatic embryo development and biological process at embryo maturation stage was determined by the presence of *LEC* gene (Kumar et al. 2020). During callus initiation and maturation of somatic embryogenesis in cassava, the level of *LEC* expression change simultaneously. *LEC* actively involved in early to late embryo morphogenesis (Brand et al.2019). *LEC* gene was detected at early PEM stage and callus proliferation as reported by Orlowska et al. (2017) in Medicago truncatula Gaertn plant. According to Horstman et al. (2017), *LEC1* and *LEC2* directly regulated gene expression from seed maturation, biosynthesis, and plant response to auxin. *LEC1* was higher expressed in shoot tip and the edge of cotyledon structure, particularly during shoot swelling and callus development. *LEC1* was also found at somatic embryo attached in tip and the edge of cotyledon.

High expression of *Somatic Embryogenesis Receptor Kinase* (*SERK*) gene expression was also identified in this study (Figure 3). The *SERK* gene had high expression level in globular (G) stage (Figure 3). Another study also reported that *SERK* detectedat early stage of somatic embryogenesis in Spanish cedar plant (*Cedrela odorata* L.) (Porras-Murillo et al.2018). This report is in line with Lu et al. (2017) that showed high expression of *SERK* in Castanea mollissima. The *SERK* gene detected during proliferation stage in *Ananas comosus*. In concurrence with earlier reports, *SERK* gene also expressed at globular, scutellar, and coleotilar stage. Embryogenic callus at 8 weeks of globular stage had high level of three gene primers (*BBM*, *LEC*, and *SERK*) expression as explained by Karim et al. (2018) in *Boesenbergia rotunda* L. Embryogenic callus transition during globular to suctellar stage often occur in short time. It proved by the expression of *BBM* from cell under scutellar epithelium that indicated the existence of mitosis cell activity (Lowe et al. 2018). *SERK* and *LEC* also have role to induce somatic embryogenesis during callus initiation and transition from cell to embryogenic tissue (Lu et al. 2017).

Gene expression during somatic embryogenesis divided into several category including stress gene, plant growth regulator gene, and transcription factor (Mendez-Hernandez et al. (2019). The addition of plant growth regulator especially auxin has been widely used to induce somatic embryogenesis. 2,4-D has high effectivity to induce somatic embryogenesis (about 78% of existing protocols). This synthetic PGR has long lasting effect due to its stability in plant cell. In the other hand, 2,4-D also influence plant development process in response to stress condition (Wojcik et al. 2020).Somatic embryogenesis process integrates endogen signal and gene activity to initiate embryogenic process. Application of single auxin, combination with other PGR, or stress condition are capable of inducing different gene expression by modifying genetic

program from somatic cell and regulating the transition of each stage during somatic embryogenesis development (Mendez-Hernandez et al. 2019).

CONCLUSION

Based on the results, there was expression of genes with primer of *BBM*, *LEC*, and *SERK* genes in sugarcane embryogenic callus from PEM, globular, scutelar, and coleoptilar structures. In contrast, it was not expressed in non-embryogenic callus of sugarcane. These three genes have found in different sizes, which are *BBM* in 500 bp, *LEC* in 400 bp, and *SERK* in 700 bp.

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