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ORIGINAL RESEARCH

Expression of somatic embryogenesis-related genes in sugarcane (*Saccharum officinarum* L.)

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Abstract

The low frequency of plantlet regenerates from the somatic embryogenesis (SE) callus in sugarcane becomes a problem to produce its seed. Plant growth regulators were able to increase the regeneration frequency of SE to normal plantlets, such as 2,4-dichlorophenoxyacetic acid (2,4-D) as promoting callus induction. Since molecular mechanisms involved SE in sugarcane have not been reported, expression of *Baby Boom* (*BBM*) and *Leafy Cotyledon* (*LEC*) genes related to SE had investigated. The effect of difference concentration of 2,4-D on callus induction and expression of somatic embryogenesis-related genes (*BBM* and *LEC*) in sugarcane were important information for the increasing quantity and quality of seed production. The percentage of callus formation and embryogenic callus in the Murashige and Skoog's (MS) medium contained 4 mg L^{-1} 2,4-D after 6 week cultivation were 76% and 86%, respectively. The MS + 4 mg L^{-1} 2,4-D medium was recommended for the large-scale embryogenic callus production from sugarcane explant. The high-level expression of *BBM* and *LEC* was shown in the embryogenic callus, which suggested that the expressions of both genes were believed to play on the somatic embryogenesis regulation in sugarcane.

Keywords Somatic embryogenesis · *BBM* · *LEC* · Sugarcane

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BBM	*Baby boom*
CH	Casein hydrolysate
DC	Dry callus
EC	Embryogenic callus
LEC	*Leafy cotyledon*
MS	Murashige and Skoog's
NEC	Non-embryogenic callus
RT-PCR	Reverse-transcriptase PCR
SE	Somatic embryogenesis
WC	Watery callus

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Introduction

The success of plant embryogenesis required an appropriate media that contained the sufficient nutrition such as plant growth regulators (PGRs) (Iqbal et al. 2016), carbon sources (Kaur and Kapoor 2016), culture conditions (Aslam et al. 2008), vitamins (Reyes-Diaz et al. 2017), and amino acids (Gerdakaneh et al. 2011). Furthermore, the increasing of plant embryogenesis frequency caused by the genotype factor of explant (Narvaez et al. 2019). The 2,4-dichlorophenoxyacetic acid (2,4-D) was able to increase the induction of callus (Mostafiz and Wagiran 2018; Naz et al. 2017; Zamir et al. 2012; Zang et al. 2016).

Embryogenic capacity is modulated by changes in gene expression that affect the somatic embryogenesis (SE) response (Yang and Zhang 2010). Some genes associated with embryogenesis have been identified and believed play on the regulation of SE formation are *Wuschel* (*WUS*), *Baby Boom* (*BBM*), *Agamous-Like15* (*AGL15*), *Auxin Response*

Factor (*ARF*), *Somatic Embryogenesis Receptor Kinase* (*SERK*), *Aintegumenta-Like5* (*AIL5*), and *Leafy Cotyledon* (*LEC*) (Ahmadi et al. 2015; Bouchabké-Coussa et al. 2013; Junker et al. 2012; Silva et al. 2014; Wojcikowska and Gaj 2017; Zhai et al. 2016; Zheng et al. 2016). Some studies suggested that *BBM* and *LEC* genes have a role for embryogenesis induction in soybean (Ouakfaoui et al. 2010) and European larch (Rupps et al. 2016) required at the initially of SE.

The *BBM* gene has an important function for induced SE (Jha and Kumar 2018), stimulated the cell proliferation and development (arabidopsis and coconut) (Bandupriya and Dunwell 2012; Passarinho et al. 2008), and improved the potential regeneration (tobacco, rose, and Arabidopsis) (Lutz et al. 2015; Srinivasan et al. 2007; Yang et al. 2014). For induced SE, *BBM* activates *LEC1*, *ABI3*, *FUS3*, and also encoded AP2/ERF that promoted proliferation of cell and morphogenesis (Horstman et al. 2017).

The *LEC* gene has an important role for SE induction (cotton and fern) (Li et al. 2017; Min et al. 2015), initiation of the vegetative phase to embryonic modulation in tobacco (Guo et al. 2013), regulation of embryo formation and regeneration in rapeseed (Elahi et al. 2016). *LEC* is expressed in SE from early through late stages (Brand et al. 2019).

The information about molecular detection of SE in sugarcane is limited, therefore, the present study was conducted to investigate the influence of 2,4-D concentration in medium for the callus induction and identify of *BBM* and *LEC* genes in sugarcane embryogenesis.

Materials and methods

Preparation of somatic embryogenesis callus

Spindle leaf of sugarcane var. Bululawang obtained from the sugarcane plantation on 4–6 months old. It was sterilized by spraying ethanol (70%) and burned on the Bunsen flames until the fire spread on the surface for 3 min, and then removed the 4–5 layers of green leaves. The inner part of spindle leaf was sliced into ± 3 mm.

The explants were inoculated on MS medium supplemented with 2.4 D (2, 4, 5 mg L^{-1}), 30 g L^{-1} sucrose, 300 mg L^{-1} CH, and 5 g L^{-1} agar, and incubated in the darkroom at 22–24 °C for 6 weeks. The percentage of callus formation and embryogenic callus were recorded. For the callus proliferation, the callus was transferred to MS medium-containing 560 mg L^{-1} proline, 1 mg L^{-1} 2,4-D, 300 mg L^{-1} CH, and 5 g L^{-1} agar. All of the data were analyzed by the analysis of variance (ANOVA) followed by Fisher's Least Significant Difference (LSD) test at $P \leq 0.05$ using SPSS software 16.0.

After successful establishment, callus and embryo structures were observed under a stereomicroscope from Leica EZ4HD (Prescotts Inc. U.S). Samples consisted of embryogenic callus at different stages including globular (G), scutellar (S), coleoptilar (C), and non-embryogenic callus (dry and watery calluses) were collected for histological observation and RNA extraction to identify gene expression.

Histology observation

The samples were fixed in FAA for 24 h, and then transferred into ethanol (70%) and dehydrated in an increasing ethanol series. The samples were infiltrated and embedded in paraffin wax. Transverse sections (10 µm) were cut using a rotary microtome and stained with safranin and fast green 1%. The object was observed under a stereomicroscope from Leica DM 2500 (Prescotts Inc. U.S).

Primer design

As the *BBM* and *LEC* gene sequence for sugarcane was unavailable in the database, thus primers were designed based on conservative regions from other plant sequences that are in the same family (*Zea mays*, *Dactylis glomerata*) in the NCBI database. Primer pairs were designed for BBM (F: CGATTTACCGTGGCGTGACA, R: CGTGAAGAG CATCCTGGACA) and for LEC (F: CGATCCAGGAGT GCGTGTCG, R: AGCCACTACCTGCCTTACGC).

RNA isolation and cDNA synthesis

Total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen, USA) following the manufacturer's instructions. To avoid DNA contamination, DNAse treatment was used for all samples. The RNA was stored at − 80 °C. The RNA quantity was observed using Nanovue plus spectrophotometer (GE Healthcare, USA), while the quality with agarose gel (1%) electrophoresis in gel doc system (Major Science, USA).

cDNA was converted from total RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, CA), from 1 µg total RNA in a 20 µL final volume. The program of RT-PCR reactions was 25 °C of priming reactions for 5 min, 46 °C of reverse transcription for 20 min, 95 °C of RT inactivation for 1 min, and an optional step with 4 °C for 5 min.

Gel purification and sequence analysis

The primer was validated by cutting and purifying band of cDNA embryogenic callus amplified using specific primer, with Zymoclean Gel DNA Recovery Kit (Zymo Research, CA, USA), and submitted for sequencing. Sequences of *BBM* and *LEC* were submitted for similarity search in the

NCBI database using the BLAST program. ORF Finder was used to predicting amino acids. Multiple sequence alignment (MSA) was analyzed using CLC Sequencer. MEGA 5 was used to construct a phylogenetic tree following the UPGMA method.

RT-PCR analysis

GoTaq Green Master Mix (Promega, USA) was used for PCR reaction, with a 95 °C of initial denaturation phase for 3 min, followed by 35 cycles of denaturation (95 °C, 30 s), annealing (53 °C for *BBM* and 55 °C for *LEC*, 30 s), and extension (72 °C, 1 min) with a elongation step of 72 °C for 5 min. Products of PCR were run in agarose gel (1%) electrophoresis, and then visualized by UV light using Gel Documentation System (Major Science Co. Ltd., USA).

Results

Effect of 2,4-D on somatic embryogenesis induction

The induction callus in MS medium-containing different 2,4-D concentrations resulted in a significant difference on the percentage of callus formation (Fig. 1). The MS medium-containing 2, 4, and 5 mg L^{-1} of 2,4-D induced callus of 29%, 76%, and 57%, respectively, at 6 weeks after cultivation showed the addition 4 mg L^{-1} of 2,4-D produced higher callus at 2–6 weeks after cultivation. Generally, the increase of the percentage of callus formation in the difference medium has the same tendency during it developed at 2–6 weeks after cultivation.

The different concentration of 2,4-D (2, 4, and 5 mg L^{-1}) into MS medium effected to the percentage of EC about 34%, 86%, and 43%, respectively. Especially for EC, it showed that the MS medium-containing 4 mg L^{-1} of 2,4-D produced the normal EC was higher than other concentration at 5 and 6 weeks after cultivation (Fig. 2).

Morphological and histological observation of somatic embryogenesis calli

The embryogenic callus (EC) characteristics of the spindle leaf explant was appeared a yellowish, transparent, and friable callus when it was cultured in MS medium-containing 4 mg L^{-1} of 2,4-D (Fig. 3a). On the other case, the explant was induced became non-embryogenic callus (NEC) which consisted of watery callus (WC) and dry callus (DC). MS medium-containing 2 mg L^{-1} of 2,4-D produced more WC with a spongy, compact, and slightly browned characteristics (Fig. 3b). The DC formed in MS medium-containing 5 mg L-1 of 2,4-D with appearances of milky white, nodular, and dry. It was very slowly regenerated into planet and had the possibility of being a plantlet greater than WC (Fig. 3c). EC developed into embryogenesis stages consists of globular, scutellar, and coleoptilar in proliferation medium (Fig. 3d–f).

The arrangement of cell density showed that EC had the high-density cells with a large and clear nucleus (Fig. 3g). WC had a large vacuole and intercellular space, with no or only small nuclei (Fig. 3h). Dry callus appeared nodular structure with obscured nuclei (Fig. 3i). However, the

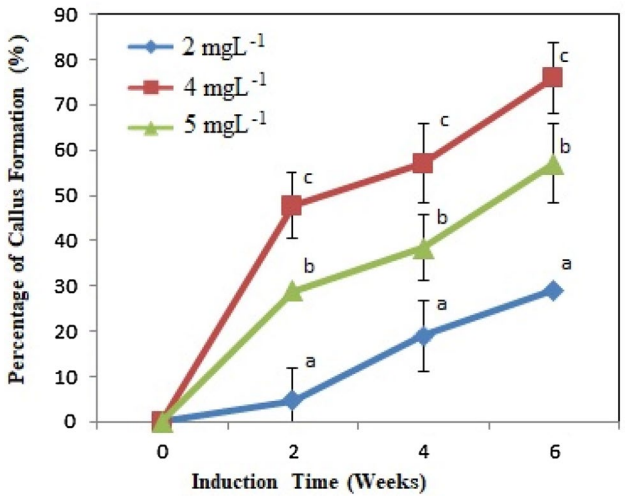

Fig. 1 Effect of different 2,4-D concentration on the callus formation percentage until 6 weeks. Different letters in vertically represent the statistical significance of mean differences between the percentage of callus formation at a given time by LSD test ($P \leq 0.05$)

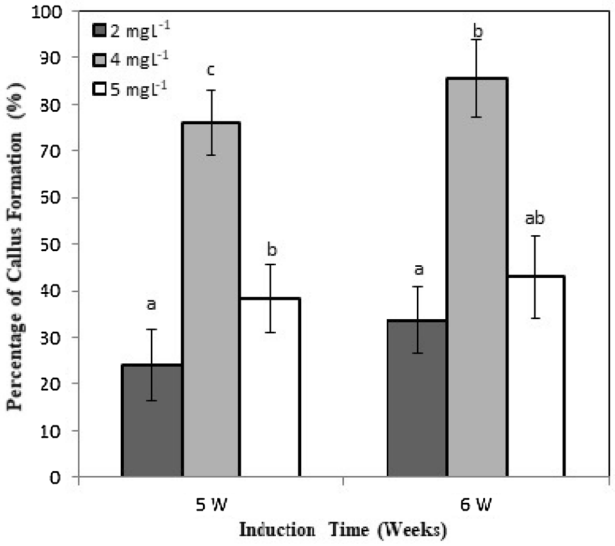

Fig. 2 Effect of different 2,4-D concentration on the embryogenic callus percentage on 5 and 6 weeks. Different letters in columns indicate a significant difference according to LSD test at $P \leq 0.05$, and values represent mean ± SD

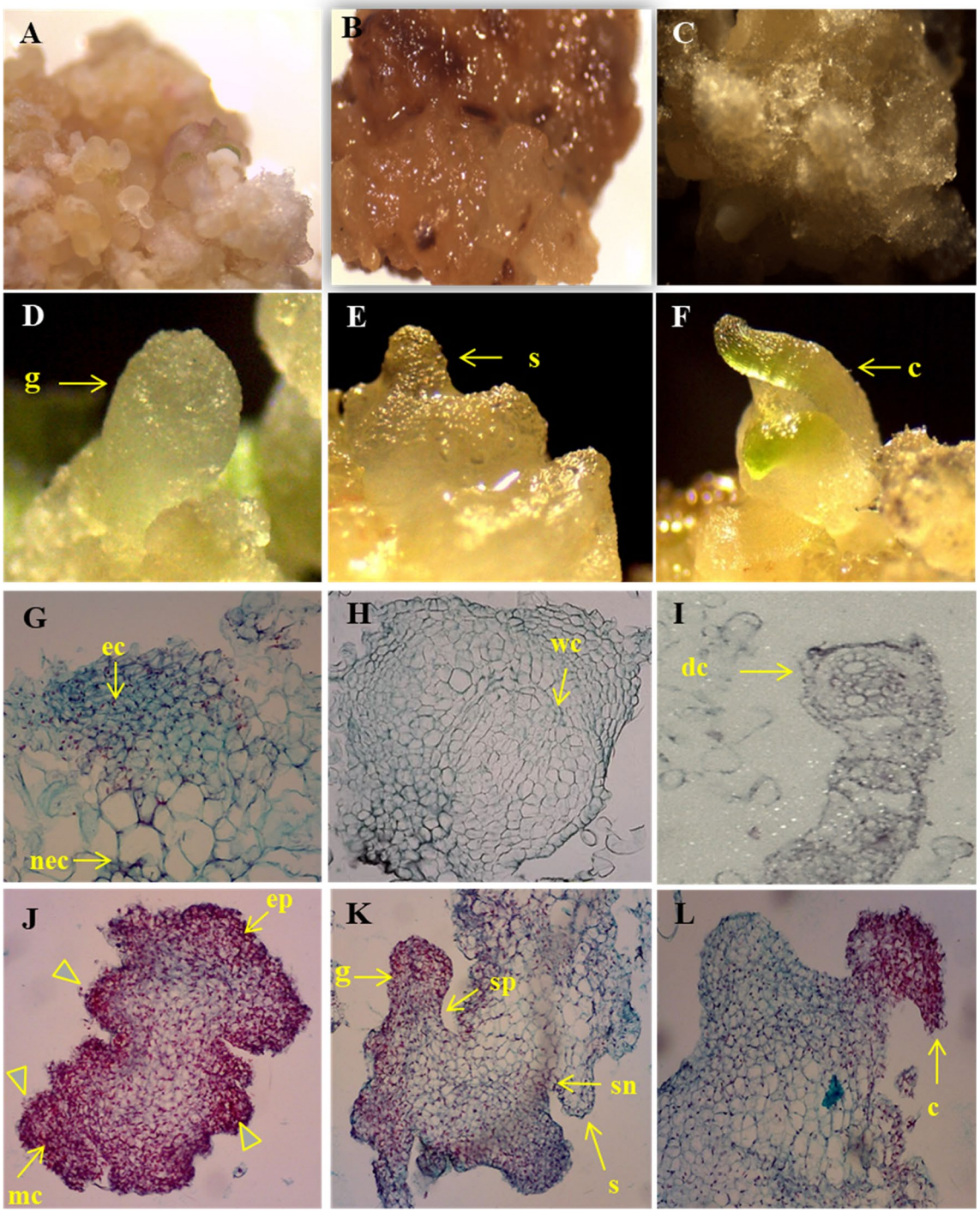

Fig. 3 Morphological and histological observation of SE in sugarcane. **a** Embryogenic callus (EC). **b** Watery callus (WC). **c** Dry callus (DC). **d** Globular structure (g). **e** Scutellar structure (s). **f** Coleoptilar structure. **g** The callus showing the group of embryogenic (EC) and non-embryogenic callus (NEC). **h** Watery callus with large vacuole and intercellular space. **i** Dry callus with nodular and obscured nuclei. **j** Pro-embryogenic mass of nodular embryogenic callus (arrowed) with meristematic cells (mc) and epidermal cells (ep). **k** A globular embryo (g) with a suspensor (sp) and scutellar embryo (s) with scutellar notch (sn). **l** A coleoptilar embryo (c)

embryogenic callus develops into various stages of SE. Meristematic cells in the periphery of the callus form pre-embryo mass (PEM) (Fig. 3j). Globular embryos are the development of PEM and had a suspensor, but in this case, not clearly distinguishable and then the globular develop to form of scutellar notch indicated the initially of scutellar

formation (Fig. 3k). The scutellar has an increased number of cells and elongates to form coleoptilar embryo (Fig. 3l).

Sequence analysis of *BBM* and *LEC* genes in sugarcane

The result of cloning and sequencing showed that the *BBM* gene was amplified at length 494 bp and *LEC* gene at 390 bp. BLAST NCBI analysis showed that *BBM* in sugarcane had 98% similarity with BBM at *Sorghum bicolor* (XM_021457893.1), 96% with *Zea mays* (XM_008676474.3), 95% with *Setaria italica,* and 94% with *Panicum hallii* (XM_025962827.1). While, the *LEC* in sugarcane showed 98% similarity with *LEC* at *Dactylis glomerata* (JN191353.2), 90% with *Oryza sativa* (AY264284.1), 88% with *Zea mays* (AF410176.1), and 85% with *Bixa orellana* (AY264284.1).

The amino acid sequence of BBM in sugarcane had high homology with *S. bicolor*, *Z. mays, S. italica*, *P. hallii*, *Brassica napus, Raphanus sativus*, and *Aegilops tauschii* sequences (Fig. 4a). While LEC was highly homology with *Z. mays* and lowly homology with *D. glomerata* (Fig. 4b). The BBM sequence starts in the consensus amino acid sequence 417–496, whereas the LEC starts at 106 until 205. Therefore, our findings indicated that the *BBM* and *LEC* genes in sugarcane and partial sequences of these genes were successfully identified. According to a phylogenetic tree construction, the BBM of sugarcane had similarity with *Sb*BBM and *Zm*BBM (Fig. 4c) and LEC was closely similar with *Dg*LEC (Fig. 4d).

Expression analysis of EC and NEC by RT-PCR

The expression of *BBM* and *LEC* genes found in the all developing stages of embryogenic callus (globular, scutellar, and coleoptilar). The highest *BBM* expression was found in the globular then showed tendencies decrease in scutellar and coleoptilar stages. *LEC* gene showed a high-level expression in EC. *BBM* gene appeared a low-level expression in DC and did not appear in WC. *LEC* gene showed a low-level expression in NEC (Fig. 5). *LEC* gene expressed obviously in DC compared to WC, which showed that gene expression-related SE was also detected in NEC, but the presence of both genes was unable to regenerate callus for embryogenesis development. In further developments, watery callus showed stagnantly and failed to regenerate. Dry callus exhibited a slower growth rate, and only a few calli developed to the complete plantlet.

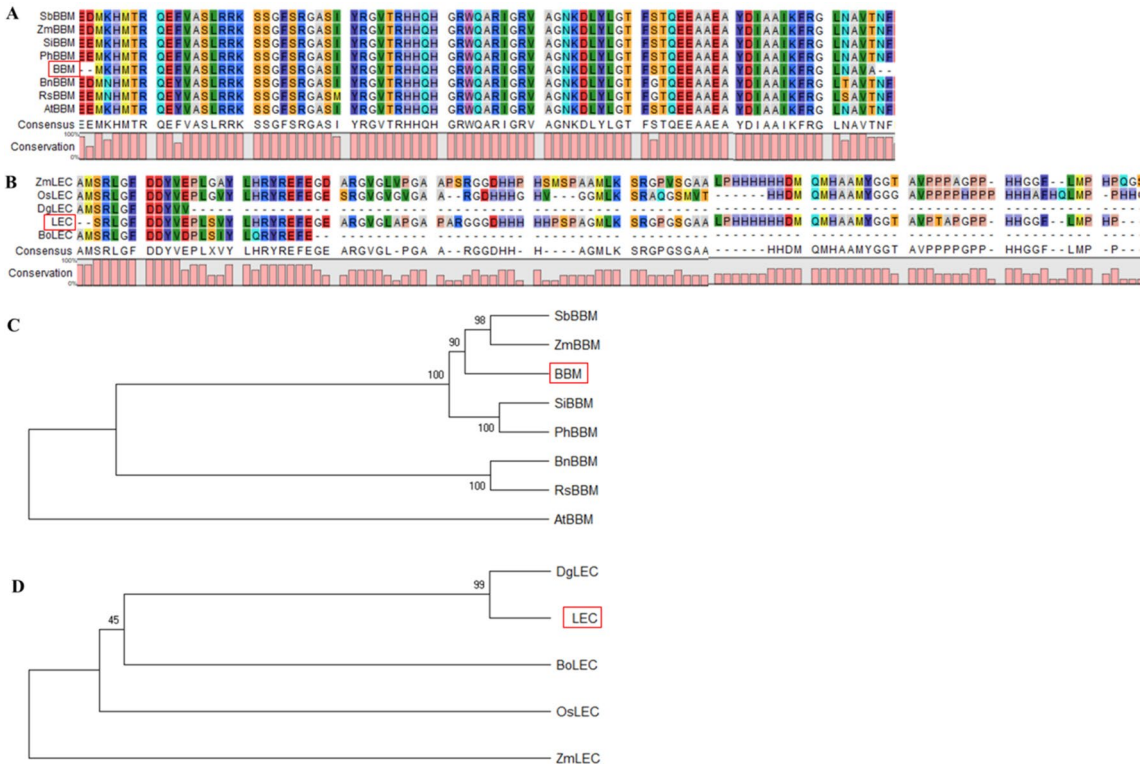

Fig. 4 Alignment amino acid sequences: **a** BBM and **b** LEC, and phylogenetic tree construction for partial sequences: **c** BBM and **d** LEC

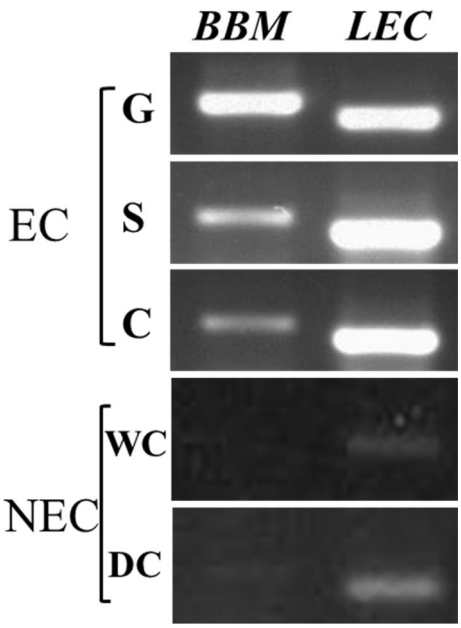

Fig. 5 Expression of *BBM* and *LEC* genes in embryogenic callus (G = globular; S = scutellar; C = coleoptilar) and non-embryogenic callus (WC = watery callus; DC = dry callus)

Discussion

The 2,4-D is one of the plant growth regulators for cell elongation and determines the success of culture induction process. Our findings showed that the 4 mg L^{-1} of 2,4-D provides the best response for callus induction, with a high percentage of embryogenic callus (EC). EC can be proliferated for many generations and also has the potential to develop into somatic embryos (Hu et al. 2017; Thorat et al. 2017). The friable callus is one of the callus cells that easily divided into many new cells, continue to grow, and differentiate through several stages of SE (Sari et al. 2018), including globular, scutellar, and coleoptilar (Burrieza et al. 2012).

Morphologically, the globular embryo structures are a variable number of cells, including nodular callus, and had a small suspensor (Smertenko and Bozhkov 2014). In the next development, the globular callus will form a lateral notch (in the terminal leaf node) which indicated the scutellar stage (de Alcantara et al. 2014). The coleoptilar stage was characterized by the differentiationof scutellum, and early development of shoot and root meristems (Borji et al. 2018).

In another side, non-embryogenic callus cannot develop into embryos (Dewanti et al. 2016). Watery callus had the dark brown-colored callus, wet, and compact structure; this characteristic was referred to NEC (Jamil et al. 2017). The brown color in callus cultures caused by the accumulation of phenolic compound was unable to regeneration (Jones and Saxena 2013).

At the high concentration of 2,4-D more than 4 mg L^{-1} cause a low percentage of EC and reduce the callus induction ability in the process of sugarcane embryogenesis. The high 2,4-D concentration caused a high accumulation of O_2 and antioxidant enzyme activity which affects the inhibition of callus formation, somatic embryo, and normal embryo development (Fraga et al. 2012; Orlowska and Kepcyzynka 2020).

The SE formation depends on the role of genes in its process. In our study, the BBM gene appeared at a high level of expression for the globular stage and become lower on the next stages, which showed that the expression of *BBM* occurs the high-level expression during early stages of SE (Salvo et al. 2014). *BBM* plays a role in cell division, developing SE, shoot-like structures, and callus (Kulinska-Lukaszek et al. 2012). AP2 transcription factor is encoded by *BBM* which functions for cell proliferation in soybean (Ouakfaoui et al. 2010).

Inhibition of cell differentiation can occur, because the lowest of *BBM* dose, the lower BBM dose stimulates root, and shoot organogenesis and the high BBM dose induce embryogenesis (Horstman et al. 2017). In poplar and chillies, the efficiency of the induction increased when the *BBM* gene was over-expressed (Deng et al. 2009), *BBM* gene stimulates transformation in sorghum, sugarcane, and rice (Lowe et al. 2016).

In sugarcane embryogenesis, the *LEC* expression gene was detected in the embryogenic stage including G, S, and C stages, as found in Chinese chestnut (Lu et al. 2017) and mangosteen (Fadryn et al. 2018). This expression profile concurs with the role of LEC induced in hypocotyl elongation by mediating auxin accumulation (Junker et al. 2012). *LEC* gene encodes an HAP3 subunit, known as NF-Y (NUCLEAR FACTOR-Y) (Uddenberg et al. 2011). In Arabidopsis, this gene was important for the differentiation and development process of the embryo somatic (Ledwon and Gaj 2011). Because the *LEC* gene was essential for SE induction, this gene presumed as a molecular marker of SE for embryogenic development regulation (Rupps et al. 2016).

Our finding also revealed that EC has high-level expression of *BBM* and *LEC* compared to NEC (WC and DC), and it was found in pistachio (Ghadirzadeh-Khorzoghi et al. 2019). The high-level expression of both claimed to be associated with low DNA methylation (Karim et al. 2018). Decreased of DNA methylation triggers cellular dedifferentiation to obtain cell totipotence (Nic-Can et al. 2013). *BBM* and *LEC* are the main regulators of plant embryo formation and totipotency (Irikova et al. 2012). Although the mechanism of these two genes is unclear, we suggested that *BBM* and *LEC* involved in embryogenesis process.

Generally, MS medium-containing 4 mg L^{-1} 2,4-D provide the best response for callus induction. *BBM* and *LEC* expressions were detected in all SE stage. In

the early stages of SE, *BBM* expression was high. Maximum expression of *LEC* observed in the scutellar stage of SE. *BBM* and *LEC* had high-level expression in EC compared to NEC. This result indicated that the *BBM* and *LEC* genes involved in the initiation of embryogenesis process in sugarcane.

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Author contributions AUKM performed cultivation, analysis gen expression, data analysis, and preparing a manuscript. BS supported for the chemicals and facilities during research. TH contributed for the discussion to improving the final manuscript. PD, supervising professor of AUKM, contributed for the discussion about somatic embryogenesis and corresponding author.

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Availability of data and materials All data sets and software used to support the conclusions of this article are available and can be accessed through correspondents.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interest in writing this paper.

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