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ANALYSIS OF PUTATIVE *EgBBM* PROMOTER BINDING PROTEINS DURING OIL PALM SOMATIC EMBRYOGENESIS

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ABSTRACT

BABY BOOM (BBM) gene has been identified as a crucial factor in regulating somatic embryogenesis in plants. During this process, high levels of BBM expression are observed. Nonetheless, the mechanisms regulating the expression of BBM during somatic embryogenesis in oil palm remain unclear. In this study, we cloned and examined the cis-regulatory elements of the EgBBM promoter. Our analysis revealed the presence of cis-regulatory elements, such as ABRE, as-1, G-box, Sp1, STRE, ARE, CAT-Box and MYB, which are similar to those found in the promoters of AtBBM and OsBBM1. In addition, we conducted a DNA-protein pull-down assay followed by mass spectrometry to identify the potential binding proteins of the EgBBM promoter. Several proteins were related to histone modification through direct and indirect interaction for controlling the EgBBM expression. Therefore, this study highlighted a complex regulatory network formation of histone modification and putative EgBBM binding proteins through both DNA-protein and protein-protein interactions. Our findings provide valuable insights into the factors that influence the role of EgBBM in oil palm tissue culture during somatic embryogenesis.

Keywords: *BABY BOOM (BBM)*, DNA-protein pull-down assay, somatic embryogenesis.

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INTRODUCTION

Elaeis guineensis Jacq., commonly known as the African oil palm, is a widely cultivated plant species used for oil production. The oil palm has three types of fruit: *Dura* (thick-shell), *Pisifera* (shell-less), and *Tenera* (thin-shell), which is a hybrid between *Dura* and *Pisifera* and is preferred for commercial palm oil production. Because *Tenera* oil palm produces segregated yields through seed production, it is more effective to grow elite oil palm plants true to their type through tissue culture, which also increases oil yield compared to commercial *Dura* x *Pisifera* seedlings (Wahid *et al*., 2015). However, the indirect somatic embryogenesis process used in oil palm tissue culture takes a long time (Konan *et al*., 2010), and regulating gene expression associated with this process can improve its efficiency.

Previous reports have established that *BABY BOOM (BBM)*, a member of the AP2/ERF family of transcription factors, is a crucial regulator of plant embryogenesis (Boutilier *et al*., 2002). The *BBM* gene, which encodes two AP2 DNA-binding domains, is expressed at high levels during somatic embryogenesis in certain plant species (Irikova *et al*., 2012; Kulinska-Lukaszek *et al*., 2012; Nic-Can *et al*., 2013). Moreover, *BBM* ectopic expression in specific plant species can induce somatic embryo

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formation (Deng *et al*., 2009; El Ouakfaoui *et al*., 2010; Passarinho *et al*., 2008). In oil palm, *EgBBM* gene was investigated in the expression through RT-PCR and in-situ hybridisation. *EgBBM* gene was highly expressed in somatic embryos and proliferating tissues such as the early development of leaf primordia, root initials and provascular tissues (Morcillo *et al*., 2007; Wachananawat *et al*., 2017). The expression of the *BBM* gene can be regulated by various factors, including epigenetic mechanisms such as DNA methylation and histone modifications, which can influence chromatin accessibility and gene expression and are commonly observed during somatic embryo development (Duarte-Aké and De-la-Peña, 2016; Kumar and Van Staden 2017; Osorio-Montalvo *et al*., 2018). Previous studies have reported that the expression of *BBM* gene can be regulated by epigenetic modifications, such as DNA methylation and histone modification. For instance, H3K27me3, a mark associated with transcriptional repression, is detected at different levels on the *BBM* gene during somatic embryogenesis in certain plant species like coffee and *Arabidopsis* (Borg *et al*., 2020; Nic-Can *et al*., 2013). Furthermore, the binding of various proteins to the cis-regulatory elements in the *BBM* promoter is crucial for regulating its expression. However, the proteins that control the expression of *BBM* during oil palm somatic embryogenesis remain unknown. Therefore, this study focused on the analysis of cis-regulatory elements in *EgBBM* promoter and the identification of the putative *EgBBM* binding protein with DNA-protein pull-down assay followed by mass spectrometry.

The DNA-protein pull-down assay is a technique that can be utilised to identify DNA-binding proteins (Chaparian and van Kessel, 2020). This method is effective in identifying promoter-binding proteins for gene regulation (Schlag *et al*., 2020). In this study, we analysed the promoter sequence of the oil palm *BBM* (*EgBBM*) gene and utilised the *EgBBM* promoter pull-down assay to identify potential proteins that may regulate *EgBBM* expression. Ultimately, understanding the regulation of *EgBBM* expression may help to shorten the somatic embryogenesis process in oil palm tissue culture.

MATERIALS AND METHODS

DNA Extraction and *EgBBM* Promoter Cloning

To obtain the *EgBBM* promoter sequence, DNA was extracted from oil palm leaf using the C-TAB method (Semagn, 2014), and a specific region 2458 base pairs upstream of the transcriptional start site was amplified using the pEgBBM_F (5′-ACCCTCCGTACTTAAATCAGTG-3′) and pEgBBM_R (5′-TTTGAGGAGAAGAGAAGG GAAG-3′) primers (NCBI accession number NM_001303564.1). The PCR reaction consisted of 35 cycles of 98°C for 10 s, 56°C for 45 s, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. The resulting *EgBBM* promoter sequence was cloned into the pGEM®-T Easy vector (Promega, Wisconsin, USA), and the positive clones were selected for DNA sequencing using a barcode-tagged sequencing service provided by U2Bio Thailand.

Analysis of *EgBBM* Promoter Sequence

The PlantCARE database (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to search for cis-regulatory elements in the *EgBBM* promoter sequence (*EgBBM* promoter sequence was submitted in an appendix). Tbtools software (https://github.com/CJ-Chen/TBtools) was then used to visualise these cis-regulatory elements.

Preparation of Biotinylated dsDNA Fragment of *EgBBM* Promoter

A specific region 2458 base pairs of the *EgBBM* promoter fragments were amplified from the pGEM®-T Easy vector containing *EgBBM* promoter using biotinylated primers; pEgBBM_biotin_F (5′-biotin-ACCCTCCGTACTTAAATCAGTG-3′) and pEgBBM_biotin_R (5′-biotin-TTTGAGGAGAA GAGAAGGGAAG-3′). The PCR reaction conditions were as follows: 35 cycles of 98°C for 10 s, 56°C for 45 s, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. The resulting biotinylated dsDNA fragment of the *EgBBM* promoter was purified using the MEGAquick-spin™ Plus Total Fragment DNA Purification Kit (iNtRON Biotechnology, Seongnam, Republic of Korea), and its quantity was determined using Nanodrop 2000 (Thermo Fisher, Massachusetts, USA).

Protein Extraction

Fifteen-week-old *Tenera* zygotic embryo explants from the Golden *Tenera* (KB) variety were cultured on callus induction medium. After three months, embryogenic callus was transferred to somatic embryo maturation medium until somatic embryo appeared (Thuzar *et al*., 2011). Total proteins were extracted from oil palm somatic embryo tissues at three to five months, including globular, torpedo, and cotyledon stages, which is the *EgBBM*-expressed stage (*Figure 1*). The tissue sample was ground in liquid nitrogen and dissolved in an ice-cold extraction buffer containing 10% glycerol, 25 mM Tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, 2% w/v PVPP, 10 mM dithiothreitol, 1X protease inhibitor cocktail (Sigma), and 0.1% Tween 20 (Sigma). The mixture was then centrifuged at

4200 x g for 30 min at 4°C, and the resulting supernatant was collected and filtered through a 0.45 µm syringe filter (De la Concepcion *et al*., 2018). The concentration of soluble protein was determined using the Bradford's method (Bio-Rad protein assay, Bio-Rad Laboratory, CA) and stored at -80°C. To perform SDS-PAGE, 10 µg of protein sample was separated on a 15% denaturing discontinuous polyacrylamide gel at 150V for 50 min and then stained with Bio-Safe Coomassie G-250 stain (Bio-Rad Laboratories, Inc., USA).

Promoter Pull-down Assay

A DNA-protein binding pulldown assay was conducted to identify proteins that bind to the *EgBBM* promoter according to Wu (2006). The assay involved mixing 4 µg of biotinylated dsDNA fragment of *EgBBM* promoter, 400 µg of total extracted proteins, and 40 uL of streptavidin agarose beads with 70% slurry in 500 µL of PBSI buffer. The mixture was incubated on a rocking platform at gentle speed for 3 hr at 4ºC and then centrifuged at 550 x g for 1 min at 4ºC. The pellet was collected and washed with 500 µL of PBSI buffer for three times, resuspended in 50 µL of 2X Laemmli sample buffer, and incubated at 95ºC for 5 min. The supernatant was collected and separated using 15% SDS-PAGE gel at 150V for 50 min and stained with Bio-Safe Coomassie G-250 stain. The concentration of promoter-binding proteins was measured by the Bradford's method (Bio-Rad protein assay, Bio-Rad Laboratory, CA), and 300 µg of these proteins were identified by Mass spectrometry (MS).

In-solution Digestion, Nano-LC-MS/MS Analysis, and Data Analysis

The proteomic analysis was carried out at the Proteomics Services Center, Center for Research and Innovation, Faculty of Medical Technology, Mahidol University, Nakhon Pathom, Thailand. The promoter-binding proteins were cleaned up using Clean-up kit (GE Healthcare, USA) before dissolving the protein pellets in 8 M urea. The reduced proteins were then alkylated and digested with Trypsin, Gold (Promega, USA) for 16 hr at 37°C. The samples were dried in a CentriVap DNA Concentrator (Labconco Co., Kansas City, Missouri, USA), resuspended in 0.1% formic acid (FA), and cleaned up by C18 Zip tip. The cleaned peptide was then dried in the CentriVap and stored at -80°C until further processing. Finally, the sample was resuspended in 0.1% formic acid and the peptide concentration measured by Nano drop 1000 (Thermo Fisher Scientific, Bremen, Germany).

Peptides were analysed on an LC-MS/MS system including a Nano-liquid chromatograph (Dionex Ultimate 3000, RSLCnano System, Thermo Scientific) in combination with a CaptiveSpray source/Quadrupole ion trap mass spectrometer (Model Q-ToF Compact, Bruker, Germany). A linear gradient elution of Solvent B was used over 90 min at a flow rate of 300 nL/min and a column temperature of 60°C. The MS acquisition rate was set at 6 Hz, and AutoMSn CID fragmentation experiments were performed.

The peptide sequence was searched on the uniprot-*Elaeis guineensis* database using the MASCOT (Version 2.3) searching engine. Search parameters in MASCOT MS/MS Ions search were followed by carbamidomethyl at cysteine residues as fixed modification, oxidation on methionine were set as variable modifications, peptide tolerance ±1.6 Da, MS/MS fragment tolerance ±0.8 Da.

RESULTS AND DISCUSSION

***EgBBM* Promoter Sequence Analysis**

The promoter region is responsible for regulating the expression of a gene by integrating signals from various transcription factors (TFs)

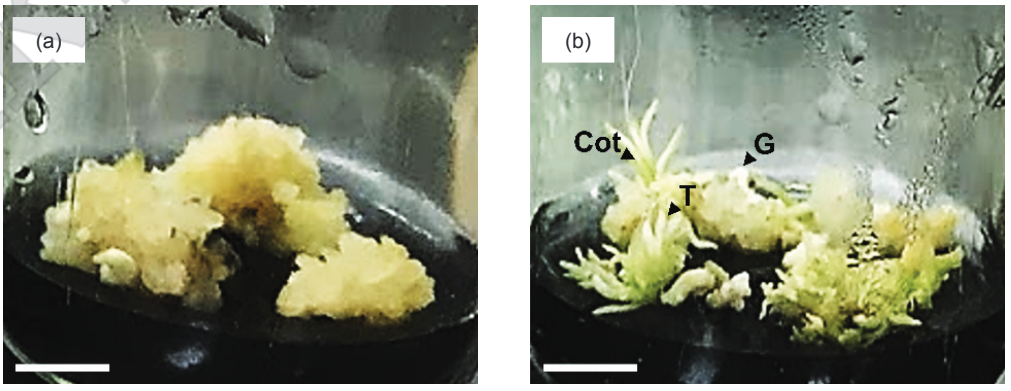

Figure 1. The oil palm tissues; (a) embryogenic callus at 3 months on callus induction medium and (b) somatic embryo tissues at 3-5 months including globular (G), torpedo (T), and cotyledon (Cot) stages on somatic embryo maturation medium used in this study, scale bar = 10 mm.

to establish a specific pattern of gene expression. In this study, the *EgBBM* promoter was analysed for cis-regulatory elements using the PlantCARE database. The promoter region of *EgBBM* gene contains several cis-regulatory elements involved in hormone response, light response, condition specificity, plant tissue specificity, transcription factor binding, unnamed factors, and common elements. The presence of these cis-regulatory elements suggests that they may be involved in the regulation of *EgBBM* expression by recruiting factor binding in the promoter region. It is noteworthy that some cis-regulatory elements are shared with the promoters of *AtBBM* and *OsBBM1*, which have been shown to be active in promoting gene expression.

The promoter region of *EgBBM*, which comprises cis-regulatory elements involved in hormone response, was analysed in this study. ABRE elements, known to play a role in ABA responsiveness, were found predominantly in the promoter regions of *EgBBM*, *AtBBM*, and *OsBBM1* (*Figure 2a*). These elements are recognised and bound to by the ABRE-BINDING PROTEIN/FACTOR (AREB/ABF) bZIP transcription factors, which help regulate ABA-responsive gene expression. In *Arabidopsis*, ABI5, a member of the AREB/ABF transcription factor family, has been found to be involved in seed maturation and germination by regulating the expression of *Late embryogenesis abundant* (*LEA*) genes. ABI5 directly binds to the ABRE element in the *AtEM6* promoter region in response to ABA, thereby activating late embryogenesis pathways following seed germination. ABI5 has also been shown to interact with the embryogenesis-related genes *LEC1* and *FUS3*, which regulate germination sensitivity to ABA and suppress vivipary. The TGA protein belongs to the basic region leucine zipper (bZIP) family and contains a bZIP DNA binding domain that interacts with activation sequence-1 (as-1) element for MeJA and SA responsiveness in regulating defense responses. TGA1 and TGA4 are required for shoot apical meristem (SAM) maintenance, flowering, and inflorescence architecture through their interaction with BLADE-ON-PETIOLE1 (BOP1) and BOP2.

The promoter regions of *EgBBM*, *AtBBM*, and *OsBBM1* contain shared cis-regulatory elements involved in light response, such as the G-box element and Sp1 element (*Figure 2b*). The G-box element is bound by bHLH and bZIP families, which regulate cell elongation, seed germination, and gene expression, while the Sp1-binding element is recognised by zinc finger proteins Sp1, although it has not been extensively studied in plants. These promoter regions also contain condition specific elements such as STRE and ARE elements (*Figure 2c*), which induce various stresses through zinc finger transcription factors and activate hypoxia-responsive genes, respectively (Gasch *et al*., 2016; Kuang *et al*., 2017; Walley *et al*., 2007). The CAT-box element, essential for meristem expression, is also present (*Figure 2d*). Moreover, MYB transcription factor binding element is common in the promoter regions of all three plant species (*Figure 2e*). MYB transcription factors have a wide range of biological functions, including cell division and differentiation, hormone responses, environmental factor responses, plant development, and metabolism (Li *et al*., 2019). For example, MYB118 promotes the formation of somatic embryos and upregulates the expression of *LEC1* gene, which is associated with embryonic development (Wang *et al*., 2009).

Identification of Putative Proteins Regulating *EgBBM* Expression

The study aimed to identify proteins that interact with the *EgBBM* promoter during the oil palm somatic embryo stages. DNA-protein pull-down assay was conducted, and the result showed that proteins with molecular weights of approximately 25 kDa were detected in both treatment and negative control, while a protein with a molecular weight of 15 kDa was detected only in the treatment group. This protein was considered a potential candidate for binding to the *pEgBBM* fragment. To further investigate the differences in protein profiles between the two groups, total eluted-DNA binding proteins in the treatment and negative control were identified using mass spectrometry (MS) (*Figure 3*).

The DNA-protein pull-down assay identified 495 proteins specifically observed in the pEgBBM-binding protein treatment, and several putative proteins were reported to be involved in embryogenesis as shown in *Table 2*. These candidate proteins were presented as putative binding partners to the *EgBBM* promoter or as proteins that indirectly regulate *EgBBM* gene expression through protein interactions.

Histone deacetylases and histone acetyltransferases are involved in the regulation of gene expression through multi-subunit protein complexes, which achieve specific cellular functions (Kurdistani and Grunstein, 2003). Histone deacetylation is commonly associated with the suppression of gene expression. In grapevine, the effect of NaB, an inhibitor of histone deacetylases, on *BBM* gene expression during somatic embryogenesis was investigated, and the result showed a significant upregulation of *BBM* gene expression in the presence of 0.5 mM NaB (Martínez *et al*., 2021).

TABLE 1. FUNCTIONAL CATEGORY OF CIS-REGULATORY ELEMENTS OF *EGBBM* PROMOTER USING PLANTCARE

Name	Conserved sequence	Number	Function	*AtBBM*	*OsBBM1*
Hormone response element					
ABRE	ACGTG	5	ABA responsiveness	✓	✓
ERE	ATTTTAAA	3	Ethylene responsiveness		✓
as-1	TGACG	2	MeJA and SA responsiveness	✓	✓
JERE	AGACCGCC	1	MeJA responsiveness		
TCA	TCATCTTCAT	1	SA responsiveness	✓	
AuxRR-core	GGTCCAT	1	Auxin responsiveness		
Light response element					
G-Box	CACGTT	5	Light responsiveness	✓	✓
GA-motif	ATAGATAA	1	Light responsiveness		✓
GATA-motif	AAGGATAAGG	1	Light responsiveness	✓	
ATCT-motif	AATCTAATCC	1	Light responsiveness		
TCT-motif	TCTTAC	1	Light responsiveness	✓	
TCCC-motif	TCTCCCT	1	Light responsiveness		✓
Sp1	GGGCGG	1	Light responsiveness	✓	✓
Condition specific element					
STRE	AGGGG	6	Stress responsiveness	✓	✓
ARE	AAACCA	2	Anaerobic induction	✓	✓
DRE	TACCGACAT	1	Dehydration responsiveness		
DRE core	GCCGAC	1	Dehydration responsiveness		✓
WRE3	CCACCT	1	Wounding responsiveness		✓
GC-motif	CCCCCG	1	Anoxic specific inducibility	✓	
O2-site	GATGACATGG	1	Zein metabolism regulation	✓	
Plant tissue specific element					
A-Box	CCGTCC	4	Meristem specific activation		✓
CAT-Box	GCCACT	1	Meristem expression	✓	✓
Transcription factor binding element					
MYB	TAACTG/CAACCA	5	MYB binding	✓	✓
MYC	CAT(T/G)TG	4	MYC binding	✓	✓
W-Box	TTGACC	4	WRKY binding	✓	✓
HD-ZIP III	GTAAT(G/C)ATTAC	2	HD-ZIP III binding		
OCT	CGCGGATC	2	Octamer binding		

Note: Number indicated the number of cis regulatory element contained in 2458 bp of *EgBBM* promoter. *AtBBM* indicated the cis-regulatory element in the promoter region of *BBM* gene in *Arabidopsis*. *OsBBM1* indicated the cis-regulatory element in the promoter region of *BBM1* gene in rice. ✓ indicated sharing of the cis-regulatory element in the promoter region of *AtBBM1* gene and/or *OsBBM1*.

Furthermore, SCL15, a transcription factor in the GRAS family, has been reported to interact with histone deacetylases as the protein complex to repress the expression of embryonic genes during the embryo-to-seedling transition (Guo *et al*., 2015; Jaiswal *et al*., 2022). SCL14, on the other hand, acts as a transcriptional coactivator with TGA transcription factors to promote the SCL14 target gene expression (Fode *et al*., 2008). TGA2.2 has been reported to promote the defense-responsive genes, while TGA2.1 has a crucial role in plant development (Thurow *et al*., 2005). Both SCL and TGA proteins are considered to play an important role in regulating *EgBBM* expression during somatic embryogenesis.

In addition, previous studies have shown that TGA2.2 and TGA2.1 can interact with Nonexpressor of Pathogenesis-Related 1 (NPR1) to activate the expression of *PR* genes (Niggeweg *et al*., 2000; Thurow *et al*., 2005). NPR1 has been proposed to deactivate Suppressor of NPR1-1, Inducible 1

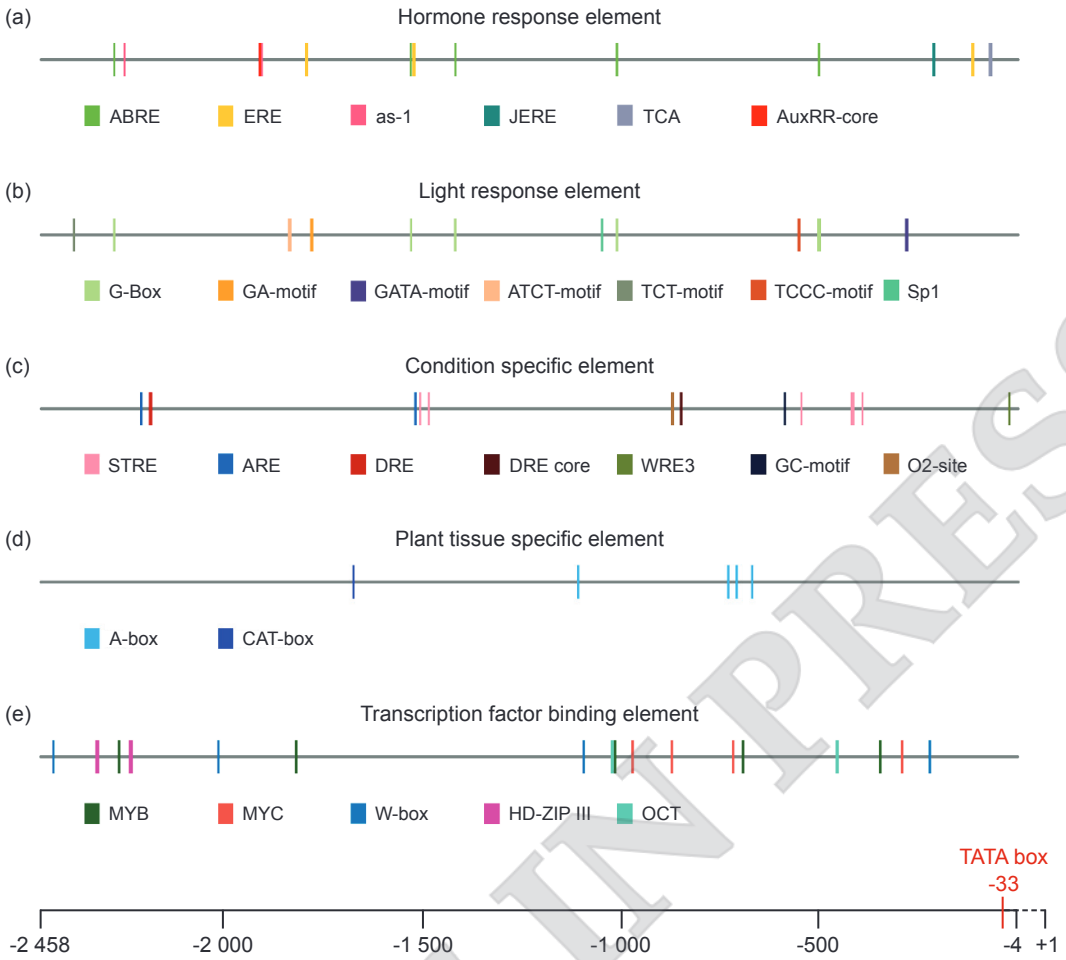

Figure 2. Distribution of cis-regulatory elements within 2458 base pairs upstream of the transcriptional start site of the EgBBM promoter sequence. Different elements indicated by different box colors. (a) Hormone response elements included ABRE element (ACGTG), ERE element (ATTTTAAA), as-1 element (TGACG), JERE element (AGACCGCC), TCA element (TCATCTTCAT), and AuxRR-core element (GGTCCAT). (b) Light response elements included G-Box element (CACGTT), GA-motif element (ATAGATAA), GATA-motif element (AAGGATAAGG), ATCT-motif element (AATCTAATCC), TCT-motif element (TCTTAC), TCCC-motif element (TCTCCCT), and Sp1 elements (GGGCGG). (c) Condition specific element included STRE element (AGGGG), ARE element (AAACCA), DRE element (TACCGACAT), DRE core element (GCCGAC), WRE3 element (CCACCT), GC-motif element (CCCCCG), and O2-site element (GATGACATGG). (d) Plant tissue specific element included A-Box element (CCGTCC), and CAT-Box element (GCCACT). (e) Transcription factor binding element included MYB (TAACTG/CAACCA), MYC (CAT(T/G)TG), HD-ZIP III (GTAAT(G/C)ATTAC), WRKY (W-Box; TTGACC), and Octamer (OCT; CGCGGATC) binding elements.

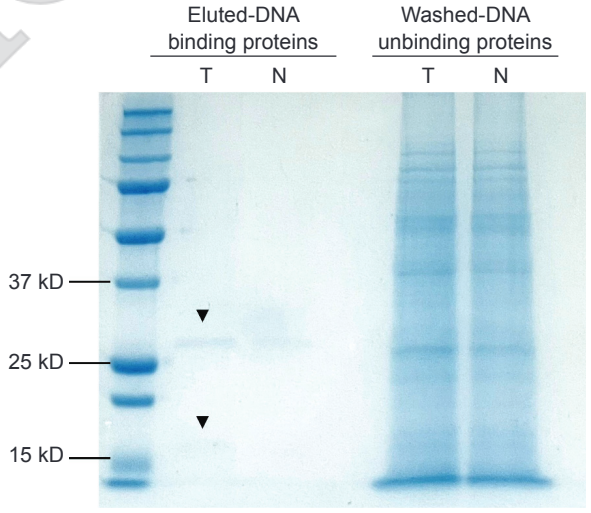

Figure 3. Separation of pEgBBM*-binding proteins and* pEgBBM*-unbinding protein by DNA-protein binding pull-down assay using 15% SDS-PAGE at 150V for 50 min. T (as treatment) indicated the mixture of extracted-somatic embryo protein,* pEgBBM *fragment, and streptavidin agarose bead. N (as negative control) indicated the mixture of extracted-somatic embryo protein and streptavidin agarose bead without* pEgBBM *fragment. Arrow indicated protein detection.*

(SNI1), as the phenotype of the *npr1* mutant can be restored by the *sni1* mutation (Durrant *et al*., 2007; Li *et al*., 1999). SNI1-mediated transcriptional repression involves a highly conserved mechanism that affects chromatin modification (Mosher *et al*., 2006). Interestingly, SNI1 shares structural similarity with Armadillo repeat proteins, homologous to ARABIDILLO (Coates *et al*., 2006; Mosher *et al*., 2006; Moody *et al*., 2016), which were identified as putative *EgBBM* promoter-binding proteins in this study. ARABIDILLO 1 and 2 have been reported to promote lateral root development and seed germination in *Arabidopsis*, similar to the role of the *BBM* gene (Coates *et al*., 2006; El Ouakfaoui *et al*., 2010; Galinha *et al*., 2007; Moody *et al*., 2016). Furthermore, CRF5, another putative *EgBBM* promoter-binding protein identified in this study, has been shown to play an important role in growth and development in *Arabidopsis* by upregulating the expression of Suppressor of NPR1-1, Constitutive1 (SNC1) (Zou *et al*., 2017).

RCF3 is a small subunit of the RFC complex that has been shown to regulate cell proliferation and pathogen resistance in *Arabidopsis* (Xia *et al*., 2009). The *rcf3-1* mutation can enhance the expression of *pathogenesis-related* (*PR*) genes, and the phenotype of *rcf3-1* is similar to that of *sni1*. Although the interaction between RFC3 and SNI1 could not be detected through the yeast-two hybrid assay, RFC3 may negatively regulate *PR* genes by indirectly interacting with SNI1 and affecting chromatin modification (Mosher *et al*., 2006; Xia *et al*., 2009).

In this study, WRKY71, a putative *EgBBM* promoter-binding protein, is functionally related to leaf senescence and overlapped with WRKY53 in *Chimonanthus praecox* (Huang *et al*., 2019). CpWRKY71 is highly expressed in flowers and senescing leaves, and its overexpression in *Arabidopsis* promotes precocious leaf senescence (Huang *et al*., 2019). Furthermore, WRKY71/EXB1 plays an important role in axillary meristem (AM) initiation in the control of shoot branching by regulating the auxin pathway in *Arabidopsis* (Guo *et al*., 2015).

RBR is a vital protein for cell division, differentiation, and stem cell maintenance in *Arabidopsis* root, and it is regulated by the GRAS family member SCR (Borghi *et al*., 2010; Wildwater *et al*., 2005). RBR also cooperates with PRC2 during cell differentiation and development, and PRC1 acts downstream of PRC2 to maintain gene repression (Kuwabara and Gruissem, 2014; Margueron and Reinberg, 2011). PRC1 represses the embryonic trait by suppressing the *BBM* gene expression during vegetative growth in *Arabidopsis*, but PRC2 subunit mutation does not affect *BBM* expression (Chen *et al*., 2010; Ikeuchi *et al*., 2015; Mozgová *et al*., 2017).

AHL10, a nuclear-localised protein with an AT-hook motif, binds to AT-rich DNA and recruits other transcription factors to regulate transcriptional regulation. It interacts with ADM to reestablish histone modifications after fertilisation in the endosperm (Jiang *et al*., 2017). Additionally, it is directly dephosphorylated by HAI1 to control plant growth under drought stress and regulate gene expression (Wong *et al*., 2019). RAV1 and RAV2-like proteins contain both AP2 and B3 DNA binding domains and are putative *EgBBM* promoter-binding proteins. RAV1 is a negative regulator in ABA signaling and interacts with SnRK2 to modulate gene expression (Feng *et al*., 2014). The ABRE cis-regulatory element, bound by ABI5 protein, is present in the *EgBBM* promoter, suggesting that the RAV1-ABI5 regulatory network may play a crucial role in *EgBBM* expression during somatic embryogenesis.

PIF1 is a bHLH transcription factor that binds to the G-box cis-regulatory element during light-dependent seed germination inhibition in *Arabidopsis* (Oh *et al*., 2009). PIF1 activates ABA and jasmonic signaling, which inhibit seed germination, while it represses GA and brassinosteroid signaling, which promote seed germination (Oh *et al*., 2009).

TRN1 plays a crucial role in cell-specific differentiation of radial dimension during early leaf and root development in *Arabidopsis* (Cnops *et al*., 2006). The establishment of radial symmetry in roots occurs during embryogenesis and is maintained throughout postembryonic growth. This process requires the participation of the SCR protein, which is essential for embryonic stem cell niche specification (Salvi *et al*., 2018).

Histone modifications play a critical role in regulating *EgBBM* gene expression during somatic embryogenesis. FRI, a scaffold protein, recruits various chromatin modifications to modify chromatin structure, including H3K4 methyltransferase complex COMPASS-like, Histone acetyltransferase of the MYST family1 (HAM1), SWR1 chromatin remodeling complex (SWR1-C), and ubiquitin-conjugating enzyme 1 (UBC1), forming a FRI-containing super complex that promotes the transcriptional activation of the flowering gene, *FLOWERING LOCUS C* (*FLC*) gene during embryogenesis in *Arabidopsis* (Choi *et al*., 2009; 2011; Li *et al*., 2018). Furthermore, MRG1 is a protein localised to euchromatin that interacts with H3K36me3 and histone H4-specific acetyltransferases (HAM1 and HAM2) to recruit histone acetylation at the promoter region of the *FLC* gene, leading to the activation of *FLC* gene expression (An *et al*., 2020; Xu *et al*., 2014).

Several bZIP transcription factors have been identified as potential *EgBBM* promoter-binding proteins involved in seed and embryo development. bZIP53 is a transcriptional regulator of the seed maturation (*MAT*) gene in *Arabidopsis* and is expressed in embryos and endosperm of maize

TABLE 2. PUTATIVE *EgBBM* PROMOTER-BINDING PROTEINS

Accession No.	Protein name	Protein mass (kDa)	Protein sequence coverage	MOWSE score	Peptide sequence	Putative binding cis-regulatory element
LOC105032345	Scarecrow-like protein 15 (SCL15)	59	9.8	69	APPPGLR	-
LOC105032336	Histone deacetylase 15	70	22.3	30	GDPLGCCDVTPAGYAQMTHMLTGLSQGK	-
LOC105053122	Replication factor C subunit 3 (RCF3)	40	7.9	29	LSNGDMR	-
LOC105053961	ARABIDILLO 1 protein	100	4.0	28	IVLRNLEHHQLEASI	-
LOC105053872	AT-hook motif nuclear-localised protein 10 (AHL10)	37	44.0	27	DDRDSPR	AT-rich element binding
LOC105047044	AP2/ERF and B3 domain-containing transcription repressor RAV2-like	41	17.0	25	RNMPAAAAAGGLGLSQALGGAQR	ABRE cis-regulatory element (Indirect binding)
LOC105033317	TORNADO 1 protein (TRN1)	16	13.1	24	DGVVAILR	-
LOC105035615	Transcription factor TGA2.2	40	8.9	22	MASLDMAASAVGAELDMGKLAFPR	-
LOC105039935	bZIP transcription factor 53	17	15.2	22	MSSVPAPQTSGSEEDAQLIVDER	G-box cis-regulatory element
LOC105053422	Histone acetyltransferase	63	18.6	22	GATGPSGQQNAAAAASLMPGIVVK	-
LOC105057643	Chromatin remodeling 5 protein (CRF5)	198	6.1	22	IVQQHEVSYKQTR	-
LOC105038489	Transcription factor MYB54-like	37	13.4	21	SHFHDLSLRSSYHPYR	MYB cis-regulatory element
LOC105051995	bZIP transcription factor 12-like	32	39.7	21	NIYGDGTAAAASPATPFGGDAGEKAAPPPLAR	G-box cis-regulatory element
LOC105047023	bZIP transcription factor 11	17	17.0	20	ASSNGTSSGSSALFPGSGEAVMDRK	G-box cis-regulatory element
LOC105051512	FRIGIDA-like protein (FRI)	63	10.3	18	VSQIKAGNMSPGGQNETNER	-
LOC105051764	Histone deacetylase 14	40	28.7	18	GSGEGTTLNLPLPGGSVMMLMR	-
LOC105042616	Transcription factor bHLH93-like	38	31.8	17	QEMGMDEQSLLQELFAPWR	G-box cis-regulatory element
LOC105046537	AP2/ERF and B3 domain-containing transcription factor RAV1	40	11.2	17	RSMPAGGYGGGLGLR	ABRE cis-regulatory element (Indirect binding)
LOC105054681	Homeobox-leucine zipper protein ROC5-like	86	34.5	16	GQDEMSQLGVIGGVGDGSGDK	-
LOC105055793	Phytochrome interacting factor1 (PIF1)	40	27.0	16	MGSSAVAEAVRQLFMEEDDIASWIHYQIGEDGGGDMYR	G-box cis-regulatory element
LOC105035176	Retinoblastoma-related protein (RBR)	115	12.7	15	VLETMCR	-
LOC105047232	WRKY transcription factor 71	38	4.5	14	GFNTSCSSPEMFGPR	W-box cis-regulatory element
LOC105059243	Phytochrome interacting factor-like 15 (PIL15)	53	42.3	14	PMVPSFRSPNMGMAMADQTR	G-box cis-regulatory element
LOC105061003	MRG1 protein	37	11.7	14	DAKSAEK	-

grains (Alonso *et al*., 2009; Lv *et al*., 2021). bZIP11 is co-expressed with bZIP53 and highly expressed in the chalazal pole and globular seed in *Brassica napus*, where it binds to G-box cis-regulatory elements to modulate auxin-induced transcription by interacting with histone acetylation machinery (Ehlert *et al*., 2006; Khan *et al*., 2022; Weiste and Dröge-Laser, 2014; Ziegler *et al*., 2019). Additionally, bZIP12, which is related to ABA signaling, was specifically and highly expressed in the early embryo stage during zygotic embryo development in oil palm, and was found to be co-expressed with the *BBM* gene (Zhang *et al*., 2022).

CONCLUSION

In this study, we cloned the *EgBBM* promoter to investigate the cis-regulatory elements in this region and identify putative *EgBBM* promoter-binding proteins using a DNA-protein pull-down assay. Our findings revealed that developmental processes, hormone response, and transcription factor binding were represented in *EgBBM* promoter. Moreover, several proteins were involved in histone modification, leading to the establishment of a complex regulatory network involving interactions between DNA-proteins and protein-proteins. Through our research, we have gained valuable insights into the factors that impact the function of *EgBBM* in oil palm tissue culture, specifically during the process of somatic embryogenesis.

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