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Cloning, Characterization, and Expression Pattern Analysis of the *BBM* Gene in Tree Peony (*Paeonia ostii*)

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Abstract: BABY BOOM (BBM) is one of the members of the plant-specific APETALA2/ethylene-responsive factor (AP2/ERF) transcription factor superfamily. It acts as a key regulator of plant cell pluripotency, playing a significant role in promoting somatic embryogenesis. In this study, a *BBM* gene named *PoBBM* was screened, cloned, and identified from the third-generation full-length transcriptome data of *Paeonia ostii*. Its open reading frame was 2136 bp, encoding 711 amino acids. Sequence feature analysis revealed that it possessed two AP2 conserved domains and eight motifs, including bbm-1. The phylogenetic tree indicated that PoBBM clusters with AtBBM in the euANT group of the Arabidopsis AP2 family, which is most closely related to grape VvBBM and may have the same ancestry as grape. Subcellular localization demonstrated that the PoBBM protein was localized in the nucleus. Semi-quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to assess the PoBBM transcript levels during ten developmental stages of somatic embryos and in five tissue types of peonies. The results indicate that *PoBBM* was highly expressed in the early stages of peony somatic embryo development. The expression on 0–15 d was the highest and decreased gradually with somatic embryogenesis. The gene is almost not expressed after 40 d since somatic embryo formation. PoBBM was expressed in roots, stems, leaves, seeds, and calli, with the highest levels in seeds, followed by leaves and calli. The PoBBM protein displayed transcriptional self-activation activity, which may facilitate further research on its relationships with other proteins. The above results provide a key gene *PoBBM* for somatic embryogenesis in peonies, which is significant for advancing the establishment of a stable and efficient regeneration and genetic transformation system for peonies.

Keywords: Peony (*Paeonia ostii*); *PoBBM*; subcellular localization; expression pattern

Citation: Zhang, X.; Zhang, W.; Chang, Y.; Ma, Y.; Deng, Y.; Zhang, N.; Bai, Y.; Jiang, Z.; Hu, T. Cloning, Characterization, and Expression Pattern Analysis of the *BBM* Gene in Tree Peony (*Paeonia ostii*). *Forests* **2024**, *15*, 36. https://doi.org/10.3390/f15010036

Academic Editor: Ivar Wendling

Received: 30 November 2023
Revised: 15 December 2023
Accepted: 21 December 2023
Published: 23 December 2023

1. Introduction

Peony (*Paeonia suffruticosa*) of the Paeoniaceae family is an esteemed ornamental and medicinal woody plant native to China. It is well known for its large, vibrant flowers with an enticing fragrance, meriting its epithets "king of flowers" and "national beauty with a heavenly scent" [1–3]. This species represents a significant cultural and botanical asset, embodying the confluence of esthetic appreciation and pharmacological utility in the Chinese floricultural tradition. However, its lengthy growth cycle and low regeneration efficiency have constrained the development of new peony cultivars by traditional breeding methods, tissue culture techniques, and genetic breeding, failing to meet the demands of the peony flower market [4–6]. Molecular breeding offers an effective strategy to overcome the limitations of conventional breeding and reduce the breeding timeline [7,8]. Plant

growth and development are genetically determined, and the advent of high-throughput sequencing technologies has laid the groundwork for the discovery of previously unknown functional genes [9]. Current molecular studies of peonies primarily focus on traits such as flower and leaf color, flower morphology, flowering period, abiotic stress resistance, and dormancy, with limited foundational research on the molecular mechanisms of somatic embryogenesis [9,10]. The identification and functional verification of key genes during somatic embryogenesis can elucidate the regulatory patterns of peony somatic embryogenesis and establish a stable and efficient regeneration and genetic transformation system for peonies.

Plant cells exhibit a high degree of developmental plasticity, readily regenerating new tissues or organs (pluripotency) and even embryos (totipotency) from ex vitro cultured explants [11,12]. Somatic embryogenesis represents a form of plant cell totipotency and can be induced in vitro by the ectopic expression of embryonic transcription factors (TFs) or the application of plant hormones [12–15]. The complex yet orderly process is influenced and regulated by various internal and external environmental factors. In recent years, researchers have investigated the molecular mechanisms of somatic embryogenesis, identifying several key genes involved in the process [16–18]. Genes such as *LEAFY COTYLEDON 1/2 (LEC1/2)*, *FUSCA3 (FUS3)*, *WUSCHEL (WUS)*, *AGAMOUS-LIKE15 (AGL15)*, *BABY BOOM (BBM)*, and *SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK)*, along with auxin- and cytokinin-related genes, play a crucial role in somatic embryo development [12,16,19–22]. Notably, BBM is at the top of the gene regulatory network associated with somatic embryo regeneration, encoding an AP2/ERF TF that has multifaceted functions in regulating cell proliferation and plant growth and development [23–25].

The AP2/ERF family is a major family of TFs exclusive to plants, characterized by at least one AP2 domain comprising a highly conserved sequence of approximately 60 amino acids. These regulatory proteins are involved in controlling primary and secondary metabolism, growth and developmental programs, and responses to environmental stimuli [26,27]. The two classification systems based on the number of AP2 domains categorize families in the AP2/ERF family into five (Sakuma) and three (Nakano) subfamilies. Both classification systems assign genes encoding two AP2 DNA-binding domains in the AP2 family [27–30]. Within the AP2 family, genes are divided into the ANT and euAP2 groups, with proteins encoded by these genes featuring a 10-amino acid insertion (euANT1 motif) within the AP2-R1 domain and a single histidine (H) insertion within the AP2-R2 domain. The ANT group comprises the basal ANT and euANT clusters [17,26,31]. The euANT cluster includes eight members: BBM, AIL1, AIL2, AIL3, AIL4, AIL5, AIL6, and AIL7. Among these, BBM is distinguished by an additional, highly conserved bbm-1 motif, which is unique to it and plays an important role in the transcriptional regulation of plant growth and development [32,33]. Genes within the AP2 family are principally involved in regulating plant growth and development, such as floral organ formation, embryogenesis, and root development [34]. Notably, BBM is a key regulator of plant cell totipotency, primarily functioning in somatic embryogenesis and genetic transformation in plants [19,25,34].

BBM was initially isolated from embryogenic cells in microspore cultures of *Brassica napus*, and its ectopic expression in *Arabidopsis thaliana* showed characteristics such as the formation of calli and adventitious buds, changes in leaf morphology, and hormone-independent regeneration of explants [20,35]. Subsequently, the *BBM* gene has been cloned or its function confirmed in many plant species, including *Arabidopsis thaliana* and *Nicotiana tabacum*, among others [20,36]. In the latest report on BBM, MdBBM1 overexpression enhanced bud regeneration and transformation, suggesting BBM efficiency even in recalcitrant woody species in the 'Royal Gala' apple [37]. However, to date, there has been no report on the peony *BBM* gene, which is significant for research on this species, known for its transformation challenges and regeneration difficulties.

The objectives of this study were to clone the *PoBBM* gene and analyze its sequence characteristics, investigate its expression patterns across different developmental stages

of somatic embryos and tissue types, and determine its subcellular localization. Understanding the role of the *PoBBM* gene in somatic embryogenesis and genetic transformation of peonies will contribute to identifying a key gene for peony ex vitro regeneration and genetic transformation, providing guidance for subsequent research on the regeneration and genetic transformation system in peonies.

2. Materials and Methods

2.1. Plant Materials

The experimental material selected for this study is 'Fengdan' (*P. ostii*), which is a cultivated variety of Yangshan peony (*P. ostii*), a wild species of peony formed after a long period of cultivation and evolution with stable genetic traits, and known for its ornamental, medicinal and edible properties. Mature 'Fengdan' seeds of the current year were sourced from Heze, Shandong. The peony tissue culture seedlings were developed from zygotic embryos as explants, which, after 7 days of dark culture at 24 ± 1 °C, were transferred to an alternating light cycle of 16 h light and 8 h dark conditions, with a light intensity of 40 μmol·m^{-2}·s^{-1} at 24 ± 1 °C. Subculturing was performed every 20 days. Seeds and tissue samples from roots, stems, leaves, and calli of the peony tissue culture seedlings were flash-frozen in liquid nitrogen for use in qRT-PCR experiments. *Nicotiana benthamiana* was grown in a controlled environment chamber at 24 ± 1 °C and 75% humidity, with a light intensity of 40 μmol·m^{-2}·s^{-1} and alternating light cycle of 16 h light followed by 8 h darkness.

2.2. Screening and Cloning of the PoBBM Gene

Preliminary full-length transcriptome data were obtained from the mixed sample sequencing of somatic embryos of 'Fengdan' at ten developmental stages. We conducted a local BLAST search against the NCBI database using the AtBBM (AT5G17430.1) gene sequence as the query and setting the E-value threshold to 1×10^{-5}. The sequences retrieved were initially filtered by removing duplicates and applying identity ≥ 50% and AlignmentLen ≥ 100 criteria. Sequences containing two AP2 conserved domains were identified using the conserved domain database Pfam (http://pfam-legacy.xfam.org/, accessed on 28 August 2023) with the HMMER (https://www.ebi.ac.uk/Tools/hmmer/search/hmmscan, accessed on 28 August 2023) tool. Subsequent removal of redundant sequences with overlapping regions was performed using ClustalW multiple sequence alignment, combined with expression level filtering from the third-generation transcriptome data to select sequences for further gene amplification studies. RNA was extracted from somatic embryos of peony seedlings at different developmental stages using the Quick RNA Isolation Kit (Hua Yueyang, Beijing, China). RNA concentration was measured with a microvolume spectrophotometer (Thermo Scientific, Waltham, MA, USA) and RNA integrity and quality were assessed by 1% agarose gel electrophoresis (Bio-Rad, Hercules, CA, USA). The extracted RNA was then used as a template for first-strand cDNA synthesis using a reverse transcription kit (TaKaRa, Kusatsu, Japan). Suitable primers were designed according to primer design principles using SnapGene software (v.2.3.2) (San Diego, CA, USA) (Table 1), with the designed cDNA serving as a template. Initial amplification of the target sequence was performed in a 10-μL reaction system using the 2× Phanta® Max Master Mix kit (Vazyme, Nanjing, China), followed by a 50-μL large volume PCR amplification using the amplification product as the template. The PCR products were then resolved on a 1% agarose gel electrophoresis, and the bands corresponding to the target sequence were excised and purified using a DNA purification and recovery kit (Tiangen, Beijing, China).

The target sequence was inserted into a pCE2 TA/Blunt-Zero vector (Vazyme, Nanjing, China) using a cloning kit at 25 °C in a metal bath. The ligation product was then transformed into *Escherichia coli* DH5α competent cells (Coolaber, Beijing, China) and cultured overnight at 37 °C. The following day, ten single clone colonies were selected, numbered, streaked, and subjected to colony PCR. Positive colonies from the colony PCR were sent

for Sanger DNA sequencing to Suzhou Azenta Company. Sequence results were aligned and analyzed using SnapGene software. Finally, the colonies with correct sequence alignment were cultured in a shaking incubator, and plasmids were extracted using a plasmid extraction kit (Tiangen, Beijing, China) and stored at −20 °C for future use.

Table 1. Primer sequences of PCR amplification.

Primers Name	Primers Sequence (5′–3′)
PoBBM-F	ATGGGTTCTATGAACAACTGGT
PoBBM-R	TTAAGTATCATTCCACACTGTGAAAGT
Q-PoBBM-F	GGAAGCAGCAGAAGCATA
Q-PoBBM-R	TGGTGTTGTCGTCATCAG
Q-Poubiquitin-F	TCCTCCACCTCCTACCTTCCGACTC
Q-Poubiquitin-R	CGATCCTCCTGAGCCAAGCGTCAT
PHG-PoBBM-F	TCTCTCTCTCAAGCTTATGGGTTCTATGAACAACTGGT
PHG-PoBBM-R	CGGGTCATGAGCTCCTGCAGAGTATCATTCCACACTGTGAAAGT

2.3. Analysis of the Physicochemical Properties and Phylogenetic Tree of PoBBM

The fundamental physicochemical properties of the PoBBM protein sequence were analyzed using ProtParam (https://web.expasy.org/protparam/, accessed on 24 October 2023) [38]. The secondary structure of the PoBBM protein was assessed using SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html, accessed on 24 October 2023) [39]. The presence of transmembrane domains in PoBBM was predicted online using TMHMM v.2.0 (https://services.healthtech.dtu.dk/services/TMHMM-2.0/, accessed on 24 October 2023) [40]. SignalP v.6.0 was used to predict the presence of signal peptides in PoBBM (https://services.healthtech.dtu.dk/services/SignalP-6.0/, accessed on 24 October 2023) [40]. Subcellular localization of the PoBBM protein was predicted using Plant mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/, accessed on 24 October 2023) [41]. Homologous BBM protein sequences from other species were searched using the Plant Transcription Factor Database and the NCBI public database. Multiple sequence alignment of peony BBM with BBM protein sequences from *A. thaliana*, *B. napus*, *Glycine max*, *Medicago truncatula*, *Rosa canina*, *Vitis vinifera*, *Zea mays*, and others was performed using ClustalW software v.2.0.11 [42]. A phylogenetic tree was constructed with the BBM protein sequences of the other species and PoBBM using the neighbor-joining method in MEGA 11 software, with 1000 bootstrap replicates and the other parameters set to default [43]. The phylogenetic tree was visualized and embellished using the Interactive Tree of Life (iTOL, https://itol.embl.de/upload.cgi, accessed on 24 October 2023) online platform.

2.4. Multiple Sequence Alignment and Analysis of Conserved Domains of PoBBM

DNAMAN 9.0 software (LynnonBiosoft, USA) was used to perform multiple sequence alignment of BBM protein sequences from peonies, *A. thaliana*, *B. napus*, *G. max*, *M. truncatula*, *V. vinifera*, *Theobroma cacao*, *Gossypium hirsutum*, *Z. mays*, and *R. canina*. The MEME online tool was used to predict motifs in the PoBBM and BBM proteins of 15 other species and to analyze the motifs within the PoBBM and homologous BBM protein sequences, setting the number of motifs to 10 (https://meme-suite.org/meme/, accessed on 31 October 2023) [44]. The saved XML files and the Newick files obtained from phylogenetic tree construction were input into Gene Structure View (Advanced) of TBtools v.2.4.0.119028 software (South China Agricultural University, China) for visualization and analysis [45].

2.5. Real-time Fluorescent Quantitative Analysis

Tissue samples from ten developmental stages of somatic embryos (0 d; 5 d; 10 d; 15 d; 20 d; 30 d cotyledon growth stage, 40 d globular embryo formation stage, 60 d secondary embryo initiation stage, 70 d secondary embryo growth stage, and 80 d globular embryo mature stage) and five tissues of peony tissue (seeds, roots, stems, leaves, and calli) of culture seedlings were flash-frozen in liquid nitrogen. Total RNA was extracted for

the reverse transcription of cDNA, which was then diluted tenfold with deionized water. Specific primers for the quantitative PCR of the *PoBBM* gene were designed using Primer Premier v.6.0 (Table 1).

Quantitative real-time PCR (qRT-PCR) was performed using TB Green® Premix Ex Taq™ II (TaKaRa, Kusatsu, Japan) on a qTOWER RT-PCR system (Analytik, Jena, Germany). The PCR mixture consisted of 5 µL TB Green Premix Ex-Taq, 1 µL of 10x diluted cDNA template, and 0.4 µL primers (F + R) (Table 1), with double-distilled water (ddH_2O) added to bring the total volume to 10 µL. Four technical replicates were established with the following cycling conditions: an initial denaturation at 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 15 s. The melting curve analysis ranged from 60 °C to 95 °C, with a temperature increment of 1 °C every 15 s. The peony ubiquitin ligase gene was used as an internal reference gene, and the relative gene expression levels of PoBBM in five peony tissues were analyzed using the $2^{-\Delta\Delta CT}$ method [46], with the expression in 0-day peony somatic embryos and peony root tissue serving as control groups.

2.6. Subcellular Localization of PoBBM Protein

The coding sequence (CDS) of PoBBM was inserted into a pHG vector containing an enhanced green fluorescent protein (EGFP) tag to construct a plant expression vector using the seamless cloning method. Seamless cloning primers (Table 1) were designed using SnapGene, with homology arms between 15 and 20 bp. The pCE2 TA/Blunt-Zero vector plasmid containing the PoBBM fragment was used as the template. The cloning of the PoBBM insert with homology arms was performed using the 2× Phanta Max Master Mix high-fidelity enzyme kit (Vazyme, Nanjing, China). The cloning reaction system was as follows: 2 µL plasmid template, 2 µL seamless cloning primers (F + R), 25 µL 2× Phanta Max Master Mix, and ddH_2O up to 50 µL. The PCR product was run on a 1% agarose gel, and the insert was purified from the gel using a universal purification kit (Tiangen, Beijing, China). The pHG plasmid was digested with *Hind*III and *Pst*I restriction enzymes (New England BioLabs, UK) to obtain linear vector fragments. The double-digestion reaction system was as follows: 1 µg pHG vector, 5 µL CutSmart Buffer, 1 µL *Hind*III, 1 µL *Pst*I, and ddH_2O up to 50 µL, followed by incubation at 37 °C for 1 h and heat inactivation at 65 °C for 20 min in a PCR system.

The plant expression vector 35S::PoBBM-EGFP was constructed by homologous recombination of the insert fragment with the pHG linear vector using a one-step cloning kit (Vazyme, Nanjing, China). The 5 µL ligation system consisted of 1 µL gel-purified insert fragment and 4 µL mixture of digested linear vector, which was incubated at 37 °C for 30 min and placed on ice or kept at 4 °C. The ligation product was transformed into *E. coli* DH5α competent cells and cultured inverted on Luria–Bertani (LB) solid medium containing kanamycin (50 µg/mL) at 37 °C for 12–16 h. Positive clones were identified by colony PCR and sent for sequencing to Suzhou Azenta Company. Plasmid DNA from the sequence-verified colonies was extracted using a plasmid mini-preparation kit and stored at −20 °C.

The constructed plant expression vector plasmid 35S::PoBBM-EGFP was transformed into *Agrobacterium tumefaciens* competent cell strain GV3101 (Weidibio, Shanghai, China) by heat shock, and then incubated inverted on LB solid medium containing kanamycin (Kan, 50 µg/mL) and rifampicin (Rif, 50 µg/mL) at 29 °C for 2–3 days. Single colonies identified as positive by PCR were inoculated into 5 mL of LB liquid medium and incubated with shaking at 200 rpm/min at 29 °C for 16 h. When the optical density (OD_{600}) of the culture reached approximately 0.8, the sample was centrifuged at 4000 rpm for 15 min at room temperature to collect the bacterial cells. The supernatant was discarded, and cells were resuspended in suspension solution (10 mM MES-KOH, 10 mM $MgCl_2$, 200 µM acetosyringone, pH 5.7) to an $OD_{600} \approx 0.8$. The solution was allowed to stand at room temperature in the dark for 2–3 h.

On the day before infection, we refrained from watering *Nicotiana benthamiana* plants. Using a 1 mL syringe (needle removed), the infection solution was injected into the abaxial

surface of *N. benthamiana* leaves and labeled accordingly. After infection, the plant was watered thoroughly and placed in a 25 °C controlled environment chamber for 1 day of dark culture, followed by 1–2 days of light culture. The infected *N. benthamiana* leaves were cut and stained with 4′,6-diamidino-2-phenylindole (DAPI) solution at room temperature (10 mg/mL) for 10–15 min, followed by rinsing 3–5 times with sterile water. The fluorescence signals of DAPI and EGFP were observed and photographed under light excitation at 465 and 509 nm, respectively, using an Axio Imager M2 microscope (Zeiss, Oberkochen, Germany).

3. Results

3.1. Basic Physicochemical Properties and Phylogenetic Analysis of PoBBM

Using the AtBBM sequence as a query, 78 gene sequences were obtained by homologous BLAST searches using a previously sequenced peony full-length transcriptome database. Domain identification revealed that 34 of these sequences contained two conserved AP2 domains. After multiple sequence alignment, motif analysis, and expression level screening, one candidate PoBBM sequence was selected for amplification. Using cDNA from different developmental stages of peony somatic embryos, the CDS of this *PoBBM* gene was amplified, and the protein was obtained.

The basic physicochemical properties of the obtained PoBBM protein were analyzed, including protein length, molecular weight, theoretical isoelectric point, instability index, aliphatic index, and overall average hydrophilicity (Table 2). The PoBBM protein comprised 711 amino acids, had a molecular weight of approximately 77.9 kDa, and was classified as an unstable hydrophilic protein. Subcellular localization prediction revealed that it was located in the cell nucleus.

Table 2. Basic physicochemical properties of *PoBBM* genes.

Basic Physicochemical Properties	PoBBM
GenBank Accession	OR711905
Open Reading Frame (bp)	2136
Protein Length (aa)	711
Molecular Weight (kDa)	77.92892
Isoelectric Point (PI)	5.90
Instability Coefficient (II)	47.81
Aliphatic Index	50.14
GRAVY	−0.794
Subcellular Localization	Nucleus

The secondary structure of the PoBBM protein consisted of alpha-helices, extended sheets (beta-strands), beta-turns, and random coils. Specifically, alpha-helices constituted 19.83% of the structure, extended sheets made up 16.03%, beta-turns accounted for 3.23%, and random coils represented 60.90%. The conserved amino acid structure was characterized by a "helix-turn-sheet-turn-helix" configuration (Figure 1a). The PoBBM protein lacked transmembrane domains (Figure 1b) and a signal peptide (Figure 1c).

To further understand the evolution and phylogenetic relationships of the peony *BBM* gene with BBM members from other species, a phylogenetic tree was constructed using the obtained peony *BBM* gene and BBM sequences from eight members of the euANT group in *Arabidopsis*, as well as BBM sequences from 15 other species (Figure 2). The phylogenetic tree was largely consistent with biological evolution, with peony BBM clustering with AtBBM from the eight members of the euANT group in *Arabidopsis* on the same branch, indicating a close phylogenetic relationship with grapes.

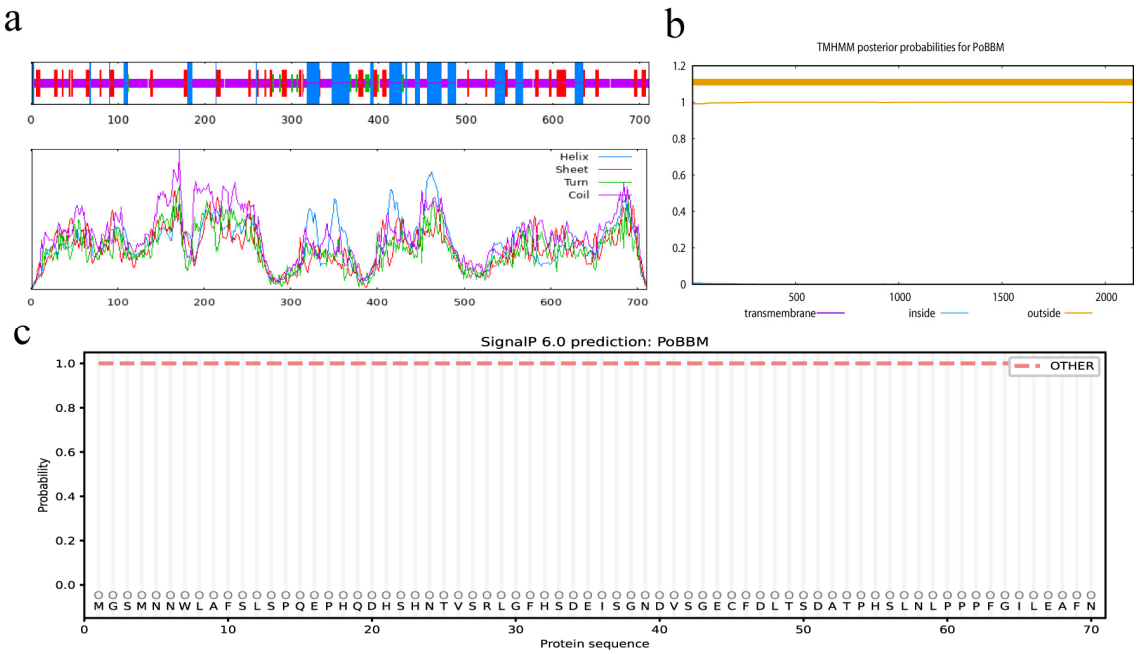

Figure 1. Secondary structure, transmembrane domain prediction, and signal peptide prediction of the PoBBM protein. (**a**) For secondary structure prediction of PoBBM proteins, blue represents alpha-helices, red represents beta-strands, green represents beta-turns, and purple indicates random coils. (**b**) In the transmembrane domain prediction of PoBBM protein, orange indicates intracellular regions, blue indicates extracellular regions, and purple represents transmembrane structures. (**c**) The signal peptide prediction, where the pink line represents the likelihood of the absence of a signal peptide.

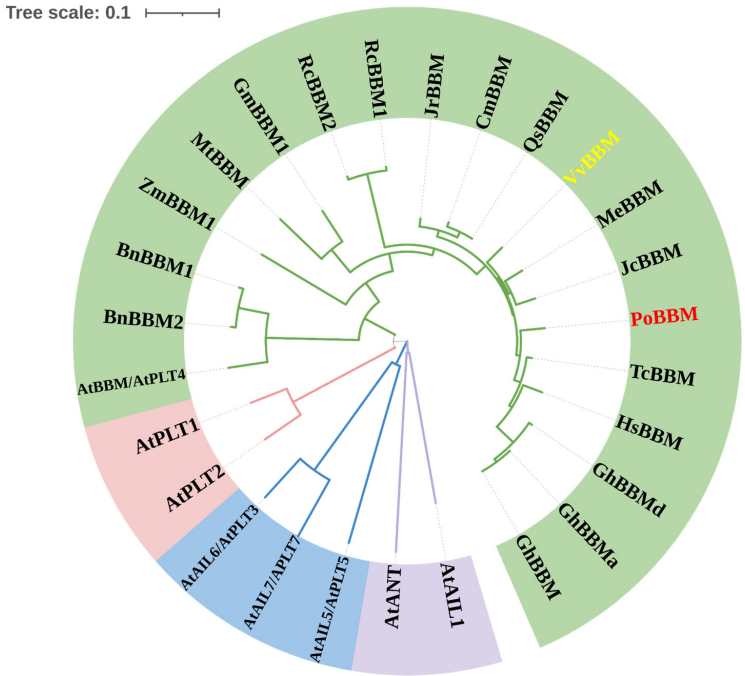

Figure 2. Phylogenetic tree of peony *BBM* gene in comparison with BBM from other species. The phylogenetic tree analysis of the full-length sequences of BBM proteins was constructed using the Neighbor-Joining (NJ) method in MEGA 11. The peony BBM is marked in red, and the grape BBM, which is most closely related to peony BBM, is highlighted in yellow.

3.2. Multiple Sequence Alignment and Sequence Feature Analysis of the PoBBM Protein

To analyze the similarity of PoBBM with other BBM proteins, we performed a multiple sequence alignment of the amino acid sequences of PoBBM with BBM members from seven other species. PoBBM contained two AP2 domains (AP2-R1 and AP2-R2) with a linker region between them, indicating that it belonged to the AP2 family. It also contained the bbm-1 motif and all typical conserved motifs in the euANT group: euANT1, euANT2, euANT3, euANT4, euANT5, euANT6, and an amino acid insertion (H) in the AP2-R2 domain (Figure 3). NCBI BLAST analysis revealed that PoBBM had sequence similarities of 43%, 56%, 44%, 60%, 57%, 49%, 49%, 71%, and 50% with AtBBM, BnBBM1, BnBBM2, GmBBM1, MtBBM, RcBBM1, RcBBM2, VvBBM, and ZmBBM, respectively.

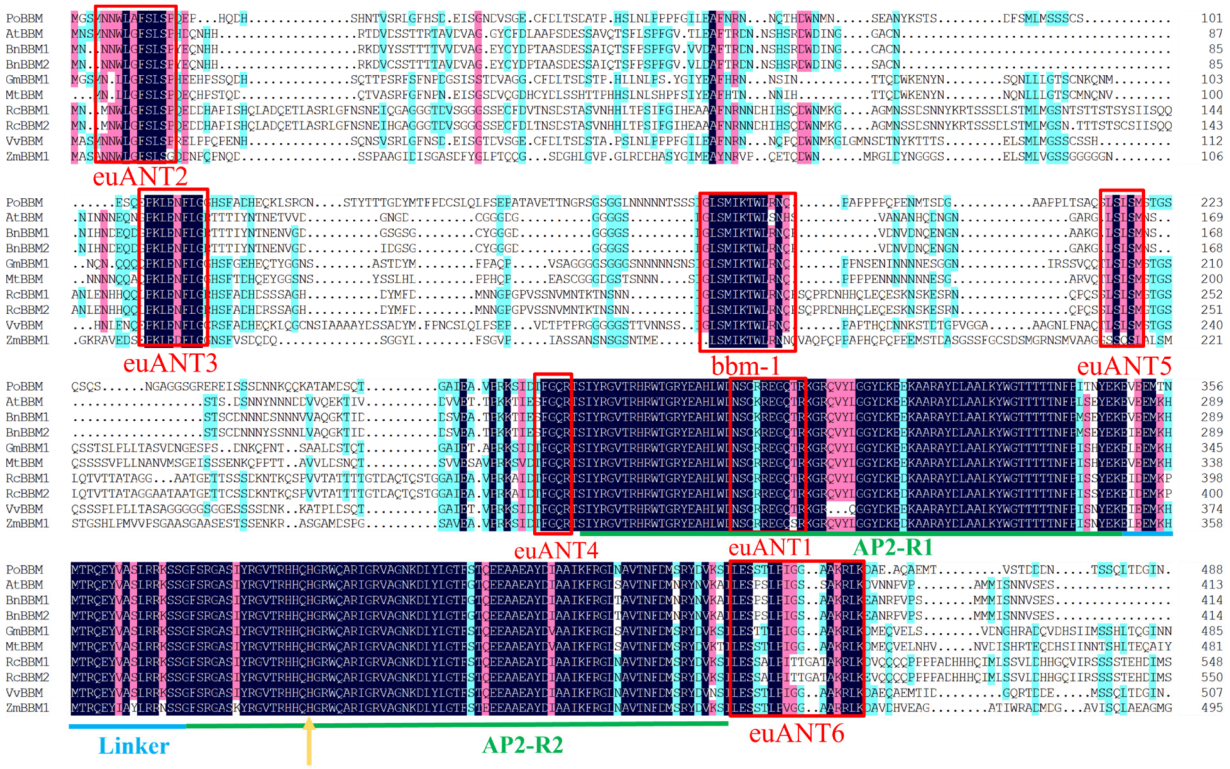

Figure 3. Sequence alignment of amino acid sequences of PoBBM and other BBM proteins. The numbers on the right side of the image indicate the amino acid positions in the entire protein. Classic motifs of *BBM* genes, including euANT1, euANT2, euANT3, euANT4, euANT5, euANT6, and bbm-1, are marked with red rectangles. AP2-R1 and AP2-R2 are indicated by green underlines, and the linker between the two AP2 domains is marked with a blue underline. An insertion of an H amino acid in the AP2-R2 domain is indicated by a yellow arrow. Accession numbers and original species are as follows: *Arabidopsis thaliana* AtBBM (NM_121749.2), *Brassica napus* BnBBM1 (AF317904), BnBBM2 (AF317905), *Glycine max* GmBBM1 (HM775856), *Medicago truncatula* MtBBM (AY89909), *Rosa chinensis* RcBBM1 (KC429673), RcBBM2 (KC429674), *Vitis vinifera* (XP_019074893.1), *Zea mays* (NM_001154063.1).

Based on amino acid sequences, MEME was used to predict and analyze the conserved motifs of BBM proteins in 16 species, with a search for 10 motifs (Figure 4). PoBBM contained all of the motifs of BBM proteins, with motif 7 corresponding to euANT2, motif 6 to euANT3, motif 5 to bbm-1, and motifs 3, 2, 10, 1, and 4 collectively representing euANT5, euANT4, AP2-R1, euANT1, Linker, AP2-R2, and euANT6. Previous motif analyses have revealed that AtBBM, BnBBM1, BnBBM2, and ZmBBM1 do not contain motifs 8 and 9.

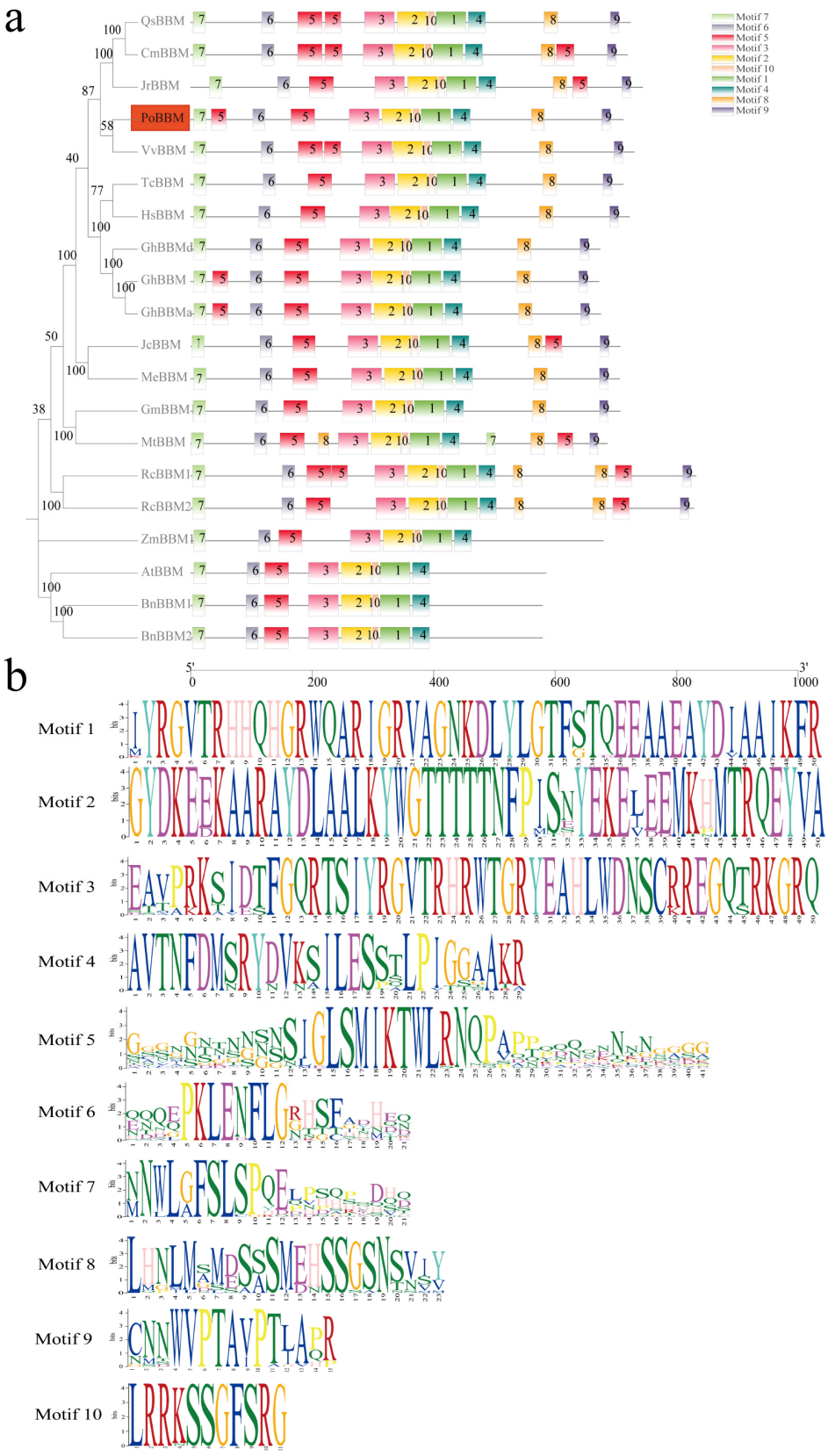

Figure 4. Motif analysis of BBM proteins across 16 species and identification of 10 conserved motifs. (**a**) The phylogenetic tree of 16 species combined with conserved motif analysis is depicted, with the PoBBM protein from peony highlighted with a red rectangular background, and each of the 10 conserved motifs represented by a unique number and color. (**b**) The 10 conserved motifs. The other 15 species

and their accession numbers are as follows: *Arabidopsis thaliana* AtBBM (NM_121749.2), *Brassica napus* BnBBM1 (AF317904.1) and BnBBM2 (AF317905), *Glycine max* GmBBM1 (HM775856), *Medicago truncatula* MtBBM (AY89909), *Rosa canina* RcBBM1 (KC429673) and RcBBM2 (KC429674), *Vitis vinifera* (XP_019074893.1), *Zea mays* ZmBBM1 (NM_001154063.1), *Hibiscus syriacus* HsBBM2 (KAE8678638.1), *Juglans regia* JrBBM (XP_018814163.1), *Manihot esculenta* MeBBM2 (XP_021631367.1), *Jatropha curcas* JcBBM (XP_012085806.1), *Gossypium hirsutum* GhBBM (OP918666.1), GhBBMa (MW836956.1), GhBBMd (MW836957.1), *Castanea mollissima* CmBBM (KAF3971867.1), *Quercus suber* QsBBM1 (XP_023872279.1), and *Theobroma cacao* TcBBM (XP_017976738.1).

3.3. Analysis of the Expression Pattern of the PoBBM Gene

Expression pattern analysis is beneficial for elucidating potential biological functions of target genes. To explore the role of the peony *BBM* gene in embryogenesis and tissue development, we performed an expression pattern analysis of the *PoBBM* gene by qRT-PCR. Based on the expression pattern of PoBBM in different developmental stages of peony somatic embryos in vitro (Figure 5a), the expression level of PoBBM was higher in the 1st to 3rd periods of early somatic embryo development, with the highest expression in the 1st period. It gradually decreases with somatic embryogenesis. In particular, it is hardly expressed after the 7th somatic embryo formation stage. According to the expression pattern of PoBBM in different tissue types (Figure 5b), PoBBM was expressed in roots, stems, leaves, seeds, and calli, with the highest expression level in seeds, followed by leaves and calli.

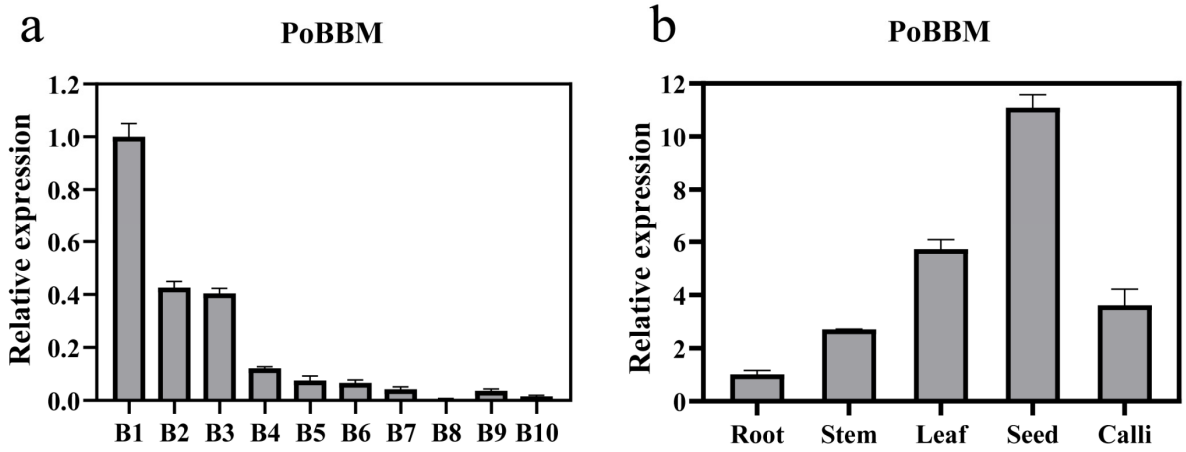

Figure 5. Expression levels of the *PoBBM* gene at various developmental stages of peony somatic embryogenesis and in different tissues. (**a**) The expression levels of the *PoBBM* gene at 10 developmental stages of peony somatic embryogenesis (indicated as B1–B10 representing 0 d, 5 d, 10 d, 15 d, 20 d, 30 d cotyledon growth stage, 40 d globular embryo formation stage, 60 d secondary embryo initiation stage, 70 d secondary embryo growth stage, and 80 d globular embryo mature stage [47], with B1 as the control group. (**b**) The expression levels of the *PoBBM* gene in peony roots, stems, leaves, seeds, and callus tissues, with expression in peony roots as the control group.

3.4. Localization Results of PoBBM Protein in N. benthamiana

The prediction results of the Plant-mPLoc server showed that the PoBBM protein was localized in the cell nucleus. Thus, a 35S::PoBBM-EGFP fusion expression vector was constructed using the pHG plant expression vector to detect the subcellular localization of the PoBBM protein. The green fluorescence signal of the PoBBM protein was only observed in the cell nucleus of *N. benthamiana* epidermal cells (Figure 6), further confirming the nuclear localization of the PoBBM protein and indicating that the PoBBM protein might function as a TF.

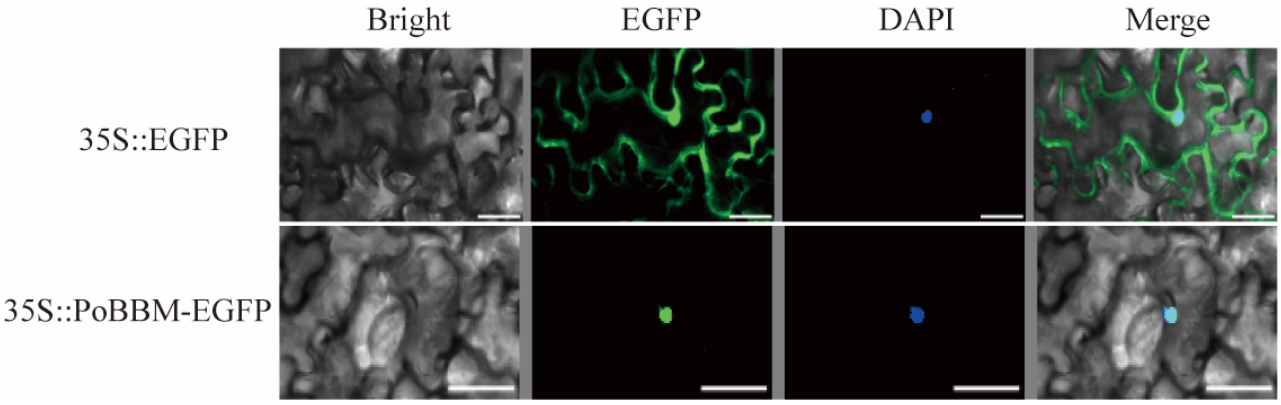

Figure 6. Subcellular localization analysis of the 35S::PoBBM-EGFP fusion protein. The 35S::PoBBM-EGFP vector was introduced into *Nicotiana benthamiana* leaves via Agrobacterium GV3101-mediated infiltration, and the expression of the fusion protein was observed under a Zeiss Axio Imager M2 microscope. The positive control was 35S::EGFP empty vector. The positive control 35S::EGFP fusion protein was expressed in both the cell nucleus and cytoplasm, while the PoBBM protein was expressed in the cell nucleus. Cell staining of tobacco leaf cells showed the location of the fusion protein, with green fluorescence indicating the fusion protein's position and blue fluorescence representing the nuclear signal labeled with DAPI. Brightfield, EGFP, DAPI, and Merge channels are shown. The scale bar is 50 μm.

4. Discussion

The traditional breeding of peonies by cutting and grafting is inefficient, while tissue culture techniques face challenges such as explant browning, rooting difficulties, bottlenecks in inducing shoots from calli, and a lack of a somatic embryogenesis system. With the advancement of modern molecular biotechnology, many functional genes and TFs have been detected and applied in the research on the recent molecular breeding of peonies. To introduce target trait-related functional genes into homologous plants and carry out directional breeding, it is imperative to establish a stable genetic transformation system for peonies as soon as possible [10]. Genes associated with somatic embryogenesis are key to accelerating the establishment of a peony genetic transformation system [25]. In particular, *BBM* is at the top of the genetic network promoting somatic embryogenesis and has been studied in many ornamental plants, achieving good results in somatic embryogenesis and genetic transformation [23]. However, the function of the peony *BBM* gene remains unclear.

In this study, the *PoBBM* gene was cloned, and its full-length CDS was 2136 bp, encoding a protein with 711 amino acids and a relative molecular mass of 77,928.92 Da. It was classified as an unstable hydrophilic protein, lacking transmembrane structures and signal peptides, with subcellular localization within the nucleus. This is consistent with the role of BBM as a TF, regulating the expression of genes involved in embryonic development and root formation, thereby modulating embryogenesis. PoBBM contained two AP2 conserved domains, six typical motifs of the euANT group of the AP2 family, and the classical bbm-1 motif. Compared with seven other members of the euANT group, BBM functions to promote embryonic development. However, because of the indispensable role of the bbm-1 motif during embryonic development, BBM is the most central member. Experimental results from the deletion of the bbm-1 motif and domain swapping have demonstrated that the bbm-1 motif is a highly conserved sequence specific to the BBM-like genes across different species, is crucial for somatic embryogenesis, and acts synergistically with at least one other motif [20,32]. This sequence in the *PoBBM* gene may play a significant role in embryonic development. In addition, PLETHORA1 (PLT1) and PLT2 in *Arabidopsis* are essential for the specification and maintenance of stem cells within the root meristem [48,49]. *BBM* and *PLT2* genes exhibit functional redundancy in the proliferation and cellularization of the endosperm, as well as in zygotic embryo development [23]. This is consistent with evolutionary analysis results that place PoBBM in the same group as

PLT1 and PLT2 of the euANT group of the *Arabidopsis* AP2 family. In addition, peony PoBBM is most closely related to grape VvBBM, which is consistent with the findings that Paeoniaceae, represented by 'Fengdan' peony, and core true dicotyledons, such as grapes, may have shared their ancestral chromosome doubling events [50]. BBM may play different roles at various developmental stages and induce distinct developmental pathways depending on the genetic and cellular context [32]. BBM regulates early embryo and endosperm development as well as being used to engineer asexual embryo development in dicotyledonous plant species [23]. In cocoa, the overexpression of the *TcBBM* gene was observed throughout the process of embryonic development, from the globular, heart-shaped, early torpedo, or late torpedo embryo stage to the mature somatic embryo stage [14]. In an indirect embryogenesis system (i.e., one that passes through a callus stage), BBM induces localized and ectopic *YUC* gene expression in seedlings, thereby promoting growth hormone biosynthesis. BBM activates LEC1 expression, which may be required for the activation of early embryonic developmental genes [12].

To investigate the spatiotemporal specificity of the *PoBBM* gene, quantitative analyses were performed across ten stages of peony somatic embryo development in seedlings and five different tissue types. The *PoBBM* gene exhibited higher expression levels in the early stages of peony somatic embryo development (Figure 5a). The expression of PoBBM was higher at 0–10 d and gradually decreased with embryonic development, suggesting that PoBBM may play a promotional role in early somatic embryo development. The *PoBBM* gene was expressed in roots, stems, leaves, seeds, and calli, with the highest expression level in seeds (Figure 5b). This result is in line with earlier research findings that BBM, an AP2/ERF family TF, is expressed in seeds and root meristematic tissues [51]. *BBM* gene was expressed during syncytial embryogenesis from the syncytial period and is also expressed in bead heart and endosperm seed tissues [23].

To further investigate the function of the *PoBBM* gene, it is necessary to overexpress the 35S::PoBBM-EGFP plant expression vector in *Arabidopsis* and to perform complementation tests in *Arabidopsis BBM* knockout mutants. Observations of transgenic *Arabidopsis* relative to the wild type for any new phenotypes should be made, including root callus induction and histological sectioning. In addition, the 35S::PoBBM-EGFP plant expression vector could be introduced into the peony callus by *Agrobacterium*-mediated transformation to explore its potential promotive effects on callus-induced differentiation and its function in embryo and root development. Currently, the systems for peony in vitro regeneration and genetic transformation are not perfect, which hinders the progress of genetic engineering or breeding efforts in peony. By cloning and functionally validating key genes during peony somatic embryogenesis and utilizing molecular biology techniques to improve the efficiency of peony in vitro regeneration, it is possible to overcome the bottlenecks in peony genetic transformation, achieve directed breeding or improve efficiency, and accelerate the acquisition of a foundation of knowledge for peony molecular breeding.

5. Conclusions

The *BBM* gene, termed *PoBBM*, was cloned from peony somatic embryos using PCR and identified by bioinformatics methods. PoBBM was found to have an important function in peony somatic embryonic development and showed tissue-specific expression in different parts of the plant. Subcellular localization analysis revealed that PoBBM is localized in the nucleus. The PoBBM protein showed transcriptional self-activation activity; therefore, reasonable hypotheses about the regions causing self-activation were proposed, providing a basis for the experimental design for further studies on PoBBM.

Author Contributions: Conceptualization, X.Z., W.Z., Z.J. and T.H.; methodology, X.Z, W.Z., Y.C. and Y.M.; resources, X.Z., W.Z. and Y.D.; software, X.Z., N.Z., Y.B. and Y.C.; validation, X.Z., W.Z., Y.C. and Y.D.; data curation, X.Z., N.Z. and Y.B.; writing—original draft preparation, X.Z. and W.Z.; writing—review and editing, X.Z., W.Z. and T.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by ICBR Fundamental Research Funds Grant (No. 1632023009 and No. 1632020001).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data in this study can be found in the manuscript.

Acknowledgments: We thank all the colleagues who helped with the development of different parts of this manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Gao, J.; Xue, J.; Xue, Y.; Liu, R.; Ren, X.; Wang, S.; Zhang, X. Transcriptome sequencing and identification of key callus browning-related genes from petiole callus of tree peony (*Paeonia suffruticosa* cv. Kao) cultured on media with three browning inhibitors. *Plant Physiol. Biochem.* **2020**, *149*, 36–49. [CrossRef] [PubMed]
2. Xie, L.; Niu, L.; Zhang, Y.; Jin, M.; Ji, D.; Zhang, X. Pollen Sources Influence the Traits of Seed and Seed Oil in *Paeonia ostii* 'Feng Dan'. *HortScience* **2017**, *52*, 700–705. [CrossRef]
3. Zhao, D.; Zhang, X.; Fang, Z.; Wu, Y.; Tao, J. Physiological and Transcriptomic Analysis of Tree Peony (*Paeonia* section *Moutan* DC.) in Response to Drought Stress. *Forests* **2019**, *10*, 135. [CrossRef]
4. Xia, M.; Zhang, W.; Chang, Y.; Ma, Y.; Deng, Y.; Fan, K.; Zhang, X.; Jiang, Z.; Hu, T. A Preliminary Investigation on the Functional Validation and Interactions of *PoWOX* Genes in Peony (*Paeonia ostii*). *Horticulturae* **2022**, *8*, 266. [CrossRef]
5. Zhang, K.; Yao, L.; Zhang, Y.; Baskin, J.M.; Baskin, C.C.; Xiong, Z.; Tao, J. A review of the seed biology of *Paeonia* species (Paeoniaceae), with particular reference to dormancy and germination. *Planta* **2018**, *249*, 291–303. [CrossRef]
6. Guo, Q.; Xue, X.; Hu, X.; He, Y.; Wei, S.; Liu, S.; Zhao, G.; Wang, Z.; Hou, X. Genetic Diversity and Population Structure Analysis of Tree Peony (*Paeonia* Section *Moutan* DC.) Germplasm Using Sixteen Functional SSR Markers. *Forests* **2023**, *14*, 1945. [CrossRef]
7. Daler, S.; Cangi, R. Characterization of grapevine (*V. vinifera* L.) varieties grown in Yozgat province (Turkey) by simple sequence repeat (SSR) markers. *Turk. J. Agric. For.* **2022**, *46*, 38–48.
8. Yazici, K.; Sahin Cevik, M. Development of DNA markers associated with sunburn resistance in pomegranate (*Punica granatum* L.) using bulk segregant analysis. *Turk. J. Agric. For.* **2022**, *46*, 955–965. [CrossRef]
9. Fan, Y.; Wang, Q.; Dong, Z.; Yin, Y.; Teixeira da Silva, J.A.; Yu, X. Advances in molecular biology of *Paeonia* L. *Planta* **2019**, *251*, 23. [CrossRef]
10. Yang, Y.; Sun, M.; Li, S.; Chen, Q.; Teixeira da Silva, J.A.; Wang, A.; Yu, X.; Wang, L. Germplasm resources and genetic breeding of *Paeonia*: A systematic review. *Hort. Res.* **2020**, *7*, 107. [CrossRef]
11. Horstman, A.; Bemer, M.; Boutilier, K. A transcriptional view on somatic embryogenesis. *Regeneration* **2017**, *4*, 201–216. [CrossRef] [PubMed]
12. Li, M.; Wrobel-Marek, J.; Heidmann, I.; Horstman, A.; Chen, B.; Reis, R.; Angenent, G.C.; Boutilier, K. Auxin biosynthesis maintains embryo identity and growth during BABY BOOM-induced somatic embryogenesis. *Plant Physiol.* **2021**, *188*, 1095–1110. [CrossRef] [PubMed]
13. Fehér, A. Callus, Dedifferentiation, Totipotency, Somatic Embryogenesis: What These Terms Mean in the Era of Molecular Plant Biology? *Front. Plant Sci.* **2019**, *10*, 536. [CrossRef] [PubMed]
14. Florez, S.L.; Erwin, R.L.; Maximova, S.N.; Guiltinan, M.J.; Curtis, W.R. Enhanced somatic embryogenesis in Theobroma cacao using the homologous BABY BOOM transcription factor. *BMC Plant Biol.* **2015**, *15*, 121. [CrossRef] [PubMed]
15. Karami, O.; Rahimi, A.; Mak, P.; Horstman, A.; Boutilier, K.; Compier, M.; van der Zaal, B.; Offringa, R. An Arabidopsis AT-hook motif nuclear protein mediates somatic embryogenesis and coinciding genome duplication. *Nat. Commun.* **2021**, *12*, 2508. [CrossRef] [PubMed]
16. Horstman, A.; Li, M.; Heidmann, I.; Weemen, M.; Chen, B.; Muino, J.M.; Angenent, G.C.; Boutilier, K. The BABY BOOM Transcription Factor Activates the LEC1-ABI3-FUS3-LEC2 Network to Induce Somatic Embryogenesis. *Plant Physiol.* **2017**, *175*, 848–857. [CrossRef]
17. Riechmann, J.; Meyerowitz, E. The AP2/EREBP family of plant transcription factors. *Biol. Chem.* **1998**, *379*, 633–654.
18. Stone, S.L.; Kwong, L.W.; Yee, K.M.; Pelletier, J.; Lepiniec, L.; Fischer, R.L.; Goldberg, R.B.; Harada, J.J. LEAFY COTYLEDON2 encodes a B3 domain transcription factor that induces embryo developmen. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 11806–11811. [CrossRef]
19. Karim, R.; Tan, Y.S.; Singh, P.; Khalid, N.; Harikrishna, J.A. Expression and DNA methylation of *SERK*, *BBM*, *LEC2* and *WUS* genes in in vitro cultures of *Boesenbergia rotunda* (L.) Mansf. *Physiol. Mol. Biol. Plants* **2018**, *24*, 741–751. [CrossRef]
20. Kulinska-Lukaszek, K.; Tobojka, M.; Adamiok, A.; Kurczynska, E.U. Expression of the *BBM* gene during somatic embryogenesis of Arabidopsis thaliana. *Biol. Plant* **2012**, *56*, 389–394. [CrossRef]
21. Li, K.; Wang, J.; Liu, C.; Li, C.; Qiu, J.; Zhao, C.; Xia, H.; Ma, C.; Wang, X.; Li, P. Expression of AtLEC2 and AtIPTs promotes embryogenic callus formation and shoot regeneration in tobacco. *BMC Plant Biol.* **2019**, *19*, 314. [CrossRef] [PubMed]

22. Wójcik, A.M.; Wójcikowska, B.; Gaj, M.D. Current Perspectives on the Auxin-Mediated Genetic Network that Controls the Induction of Somatic Embryogenesis in Plants. *Int. J. Mol. Sci.* **2020**, *21*, 1333. [CrossRef] [PubMed]
23. Chen, B.; Maas, L.; Figueiredo, D.; Zhong, Y.; Reis, R.; Li, M.; Horstman, A.; Riksen, T.; Weemen, M.; Liu, H.; et al. BABY BOOM regulates early embryo and endosperm development. *Proc. Natl. Acad. Sci. USA* **2022**, *119*, e2201761119. [CrossRef] [PubMed]
24. Passarinho, P.; Ketelaar, T.; Xing, M.; van Arkel, J.; Maliepaard, C.; Hendriks, M.W.; Joosen, R.; Lammers, M.; Herdies, L.; den Boer, B. BABY BOOM target genes provide diverse entry points into cell proliferation and cell growth pathways. *Plant Mol. Biol.* **2008**, *68*, 225–237. [CrossRef] [PubMed]
25. Yavuz, C.; Tillaboeva, S.; Bakhsh, A. Apprehending the potential of BABY BOOM transcription factors to mitigate cotton regeneration and transformation. *J. Cotton Res.* **2020**, *3*, 29. [CrossRef]
26. Ghorbani, R.; Zakipour, Z.; Alemzadeh, A.; Razi, H. Genome-wide analysis of AP2/ERF transcription factors family in Brassica napus. *Physiol. Mol. Biol. Plants* **2020**, *26*, 1463–1476. [CrossRef]
27. Licausi, F.; Ohme-Takagi, M.; Perata, P. APETALA2/Ethylene Responsive Factor (AP2/ERF) transcription factors: Mediators of stress responses and developmental programs. *New Phytol.* **2013**, *199*, 639–649. [CrossRef]
28. Li, K.; Sun, X.; Han, H.; Zhang, S. Isolation, characterization and expression analysis of the *BABY BOOM* (*BBM*) gene from Larix kaempferi×L. olgensis during adventitious rooting. *Gene* **2014**, *551*, 111–118. [CrossRef]
29. Nakano, T.; Suzuki, K.; Fujimura, T.; Shinshi, H. Genome-Wide Analysis of the ERF Gene Family in Arabidopsis and Rice. *Plant Physiol.* **2006**, *140*, 411–432. [CrossRef]
30. Sakuma, Y.; Liu, Q.; Dubouzet, J.G.; Abe, H.; Shinozaki, K.; Yamaguchi-Shinozaki, K. DNA-Binding Specificity of the ERF/AP2 Domain of Arabidopsis DREBs, Transcription Factors Involved in Dehydration- and Cold-Inducible Gene Expression. *Biochem. Biophys. Res. Commun.* **2002**, *290*, 998–1009. [CrossRef]
31. Shigyo, M.; Ito, M. Analysis of gymnosperm two-AP2-domain-containing genes. *Dev. Genes Evol.* **2004**, *214*, 105–114. [CrossRef] [PubMed]
32. El Ouakfaoui, S.; Schnell, J.; Abdeen, A.; Colville, A.; Labbé, H.; Han, S.; Baum, B.; Laberge, S.; Miki, B. Control of somatic embryogenesis and embryo development by AP2 transcription factors. *Plant Mol. Biol.* **2010**, *74*, 313–326. [CrossRef] [PubMed]
33. Yang, H.F.; Kou, Y.P.; Gao, B.; Soliman, T.M.A.; Xu, K.D.; Ma, N.; Cao, X.; Zhao, L.J. Identification and functional analysis of *BABY BOOM* genes from *Rosa canina*. *Biol. Plant* **2014**, *58*, 427–435. [CrossRef]
34. Xie, Z.; Nolan, T.M.; Jiang, H.; Yin, Y. AP2/ERF Transcription Factor Regulatory Networks in Hormone and Abiotic Stress Responses in Arabidopsis. *Front. Plant Sci.* **2019**, *10*, 228. [CrossRef] [PubMed]
35. Boutilier, K.; Offringa, R.; Sharma, V.K.; Kieft, H.; Ouellet, T.; Zhang, L.; Hattori, J.; Liu, C.M.; van Lammeren, A.A.; Miki, B.L.; et al. Ectopic Expression of BABY BOOM Triggers a Conversion from Vegetative to Embryonic Growth. *Plant Cell* **2002**, *14*, 1737–1749. [CrossRef] [PubMed]
36. Srinivasan, C.; Liu, Z.; Heidmann, I.; Supena, E.D.J.; Fukuoka, H.; Joosen, R.; Lambalk, J.; Angenent, G.; Scorza, R.; Custers, J.B.M.; et al. Heterologous expression of the BABY BOOM AP2/ERF transcription factor enhances the regeneration capacity of tobacco (*Nicotiana tabacum* L.). *Planta* **2006**, *225*, 341–351. [CrossRef] [PubMed]
37. Chen, J.; Tomes, S.; Gleave, A.P.; Hall, W.; Luo, Z.; Xu, J.; Yao, J.-L. Significant improvement of apple (Malus domestica Borkh.) transgenic plant production by pre-transformation with a Baby boom transcription factor. *Hort. Res.* **2022**, *9*, uhab014. [CrossRef]
38. Wilkins, M.R.; Gasteiger, E.; Bairoch, A.; Sanchez, J.C.; Williams, K.L.; Appel, R.D.; Hochstrasser, D.F. Protein Identification and Analysis Tools in the ExPASy Server. *Methods Mol. Biol.* **1999**, *112*, 531–552.
39. Geourjon, C.; Deléage, G. SOPMA: Significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments. *Bioinformatics* **1995**, *11*, 681–684. [CrossRef]
40. Krogh, A.; Larsson, B.; Von Heijne, G.; Sonnhammer, E.L. Predicting transmembrane protein topology with a hidden markov model: Application to complete genomes. *J. Mol. Biol.* **2001**, *305*, 567–580. [CrossRef]
41. Chou, K.-C.; Shen, H.-B. Plant-mPLoc: A Top-Down Strategy to Augment the Power for Predicting Plant Protein Subcellular Localization. *PLoS ONE* **2010**, *5*, e11335. [CrossRef] [PubMed]
42. Thompson, J.D.; Higgins, D.G.; Gibson, T.J. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **1994**, *22*, 4673–4680. [CrossRef] [PubMed]
43. Tamura, K.; Stecher, G.; Kumar, S. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Mol. Biol. Evol.* **2021**, *38*, 3022–3027. [CrossRef] [PubMed]
44. Bailey, T.L.; Johnson, J.; Grant, C.E.; Noble, W.S. The MEME Suite. *Nucleic Acids Res.* **2015**, *43*, W39–W49. [CrossRef] [PubMed]
45. Chen, C.; Chen, H.; Zhang, Y.; Thomas, H.R.; Frank, M.H.; He, Y.; Xia, R. TBtools: An Integrative Toolkit Developed for Interactive Analyses of Big Biological Data. *Mol. Plant* **2020**, *13*, 1194–1202. [CrossRef]
46. Livak, K.J.; Schmittgen, T.D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods* **2001**, *25*, 402–408. [CrossRef]
47. Wei, X.R. *Cloning and Analysis of PoWUS and PoAGL15, Somatic Embryogenesis and Related Genes in Peony*; Chinese Academy of Forestry Sciences: Beijing, China, 2019. (In Chinese)
48. Aida, M.; Beis, D.; Heidstra, R.; Willemsen, V.; Blilou, I.; Galinha, C.; Nussaume, L.; Noh, Y.S.; Amasino, R.; Scheres, B. The *PLETHORA* Genes Mediate Patterning of the Arabidopsis Root Stem Cell Niche. *Cell* **2004**, *119*, 109–120. [CrossRef]

49. Santuari, L.; Sanchez-Perez, G.F.; Luijten, M.; Rutjens, B.; Terpstra, I.; Berke, L.; Gorte, M.; Prasad, K.; Bao, D.; Timmermans-Hereijgers, J.L.; et al. The *PLETHORA* Gene Regulatory Network Guides Growth and Cell Differentiation in Arabidopsis Roots. *Plant Cell* **2016**, *28*, 2937–2951. [CrossRef]
50. Yuan, J.; Jiang, S.; Jian, J.; Liu, M.; Yue, Z.; Xu, J.; Li, J.; Xu, C.; Lin, L.; Jing, Y.; et al. Genomic basis of the giga-chromosomes and giga-genome of tree peony *Paeonia ostii*. *Nat. Commun.* **2022**, *13*, 7328. [CrossRef]
51. Nole-Wilson, S.; Tranby, T.L.; Krizek, B.A. *AINTEGUMENTA-like (AIL)* genes are expressed in young tissues and may specify meristematic or division-competent states. *Plant Mol. Biol.* **2005**, *57*, 613–628. [CrossRef]