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Identification of the Embryogenesis Gene *BBM* in Alfalfa (*Medicago sativa*) and Analysis of Its Expression Pattern

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Abstract

Apomixis-mediated fixation of heterosis could transform hybrid breeding in alfalfa (*Medicago sativa*), a globally important forage crop. The parthenogenesis-inducing morphogenetic regulator BABY BOOM (BBM) represents a promising candidate for enabling this advancement. Here, we identified *BBM* homologs from three alfalfa genomes, characterized their promoter regions, and cloned a 2082 bp *MsBBM* gene encoding a 694-amino acid nuclear-localized protein. Three alfalfa *BBM* gene promoters primarily contained light- and hormone-responsive elements. Phylogenetic and conserved domain analyses of the MsBBM protein revealed a high sequence similarity with *M. truncatula* BBM. Expression profiling demonstrated tissue-specific accumulation of *MsBBM* transcripts, with the highest expression in the roots and developing pods. Hormonal treatments differentially regulated *MsBBM*. Expression was upregulated by GA_3 (except at 4 h) and SA, downregulated by NAA, MeJA (both except at 8 h), and ABA (except at 4 h), while ETH treatment induced a transient expression peak at 2 h. As an AP2/ERF family transcription factor showing preferential expression in young embryos, *MsBBM* likely participates in reproductive development and may facilitate apomixis. These findings establish a molecular framework for exploiting *MsBBM* to enhance alfalfa breeding efficiency through heterosis fixation.

Keywords: *Medicago sativa*; *BBM* gene; gene cloning; conserved motif; expression pattern

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1. Introduction

Alfalfa (*Medicago sativa*) is a perennial herbaceous plant of the Fabaceae family. It possesses high forage value due to its high yield, rich nutritional content, and strong regenerative capacity. It also plays a significant ecological role in soil and water conservation, soil improvement, and vegetation restoration. It is one of the preferred forage crops in China for implementing agricultural restructuring, protecting cultivated land ecology, and achieving sustainable agricultural development [1]. However, over 80% of China's alfalfa seeds rely on imports, creating an urgent need to develop new high-quality forage varieties with independent intellectual property rights suitable for local growth conditions in China [2]. Hybrid breeding serves as a cornerstone strategy in the development of high-yielding alfalfa cultivars. Although utilizing heterosis can significantly enhance alfalfa yield and adaptability, the impossibility of fixing parental genotypes of a hybrid variety by inbreeding (due to strong inbreeding depression) or cloning (due to excessive cost) has prevented the use of hybrids.

Apomixis in plants produces seeds without the fusion of male and female gametes. This asexual seed formation allows for the spontaneous fixation of any desired genotype and maintains the stable transmission of the heterozygous F_1 genotype, enabling the long-term fixation of heterosis [3]. Although the mechanism of apomixis in angiosperms is not yet fully understood, breakthroughs have been made in artificially creating the apomixis process. The *BBM* gene belongs to the AP2 transcription factor family [4] and was initially isolated from immature pollen grains of rapeseed (*Brassica napus*) [5]. This gene not only induces plant somatic embryogenesis [5–7], promotes cell proliferation and regeneration [8,9], and enhances genetic transformation efficiency [10–12], but has also been successively demonstrated to induce parthenogenesis in both monocot and dicot plants [13,14]. *AtBBM* is crucial for regulating early embryo development and promoting cell proliferation and morphogenesis in *Arabidopsis* [5]. Ectopic expression of *AtBBM* in *Arabidopsis* egg cells initially failed to induce parthenogenesis [15]. Afterward, researchers replaced *AtBBM* with complementary DNA (cDNA) of *Brassica napus BBM* (*BnBBM1*) and introduced a translational enhancer to promote ectopic expression of *AtBBM* in *Arabidopsis* egg cells and induce parthenogenesis, albeit with low efficiency in obtaining haploid offspring [14]. Liu et al. demonstrated that AtRKD5 inhibits *AtBBM* expression in egg cells and that the ectopic expression of *AtBBM* in *atrkd5* mutant egg cells achieved 0.28% parthenogenesis with haploid offspring production [16].

Compared to dicot plants, research on *BBM* gene-induced parthenogenesis is more common in monocot plants. Through genetic mapping of hybrid progeny derived from crosses between sexual and apomictic materials in the genus *Pennisetum*, researchers progressively identified the apomixis-specific genomic region ASGR (apospory-specific genomic region) [17–19]. The ASGR harbors members of the *BBM* clade within the *AINTEGUMENTA-LIKE* (*AIL*) gene family [20]. The *PsASGR-BBML* (*BABYBOOM-LIKE*) gene from apomictic *P. squamulatum*, when expressed under the control of the *Arabidopsis* egg cell-specific promoter AtDD45 [21], induces parthenogenesis in sexual pearl millet (*P. glaucum*) [22], maize (*Zea mays*) [15], rice (*Oryza sativa*) [15], sorghum (*Sorghum bicolor*) [11], and tobacco (*Nicotiana tabacum*) [13]. The rice gene *OsBBM1* is an ortholog of *PsASGR-BBML* [23]. Researchers first achieved the replacement of meiosis by mitosis (MiMe) in rice using gene editing, generating unreduced female gametes [24]. Subsequent ectopic expression of *OsBBM1* in egg cells induced parthenogenesis in diploid egg cells, successfully yielding doubled haploid seeds containing solely maternal genetic information [25]. Furthermore, three phylogenetically distinct *SiBBM* genes from *Setaria italica*, when specifically expressed in egg cells, all induced parthenogenesis in rice [26]. Wang's team demonstrated that specific expression of the rice *OsBBM4* gene in egg cells similarly induced parthenogenesis, with a haploid induction rate of 3.2% [27].

The *BBM* gene in alfalfa has not yet been identified. To explore its fundamental characteristics and verify whether alfalfa possesses a functional *BBM* ortholog with conserved motifs, this study retrieved candidate genes from three published alfalfa genome databases: 'XinJiangDaYe' (*M. sativa* L. 'XinJiangDaYe'), 'Zhongmu No.1' (*M. sativa* L. 'Zhongmu No.1'), and 'Zhongmu No.4' (*M. sativa* L. 'Zhongmu No.4'). Bioinformatic screening aimed to identify genes containing both two AP2 domains and the bbm-1 motif, features indicative of functional conservation with known embryogenesis *BBM* genes. Cis-acting elements in the promoters of the obtained target genes were analyzed to detect embryogenesis-associated regulatory motifs, providing insight into potential spatiotemporal expression regulation. The *MsBBM* gene was cloned from alfalfa using homologous cloning technology, followed by conserved motif analysis and phylogenetic tree construction to establish its evolutionary position within the BBM clade. Subcellular localization of MsBBM was performed using a rice protoplast transient transformation system to confirm the nuclear targeting that is

essential for transcription factor function. Real-time quantitative PCR (qRT-PCR) was used to detect tissue-specific expression patterns and hormone-responsive dynamics of *MsBBM* in 'XinJiangDaYe' alfalfa, testing its putative role in parthenogenesis. Through integrated bioinformatics, subcellular localization, and expression pattern analysis, this study aims to lay a theoretical foundation for further exploring the potential of *MsBBM* to induce apomixis and create heterosis-fixing germplasm.

2. Materials and Methods

2.1. Plant Materials and Treatments

Seeds of 'XinJiangDaYe' alfalfa were provided by Xinjiang Agricultural University. Plump seeds were selected and planted in pots, then cultivated in a growth chamber. The pot soil mixture consisted of field soil and nutrient soil at a 5:1 mass ratio. Field soil (0–20 cm topsoil) was collected from the Forage Practical Training Base of Gansu Agricultural University, classified as loessal soil with uniform fertility. Vegetative organs (root tips, roots, stems, and leaves) were collected from mixed individual plants at 60 days of growth. Subsequently, the plants were transferred to field cultivation. Buds (green corolla tissue, Figure 1A), fully open flowers (keel petals sprung open and stamens fully exposed, indicated by arrow in Figure 1B), and green pods (green pods 10 days after full flowering, Figure 1C) were collected from mixed individual plants at the budding stage, flowering stage, and green pod stage, respectively. The corolla was removed, retaining only the gynoecium and carpels as the reproductive structure (Figure 1D). Additionally, leaves of 'XinJiangDaYe' alfalfa seedlings grown in pots for 40 days were sprayed with 10 $\mu mol \cdot L^{-1}$ solutions of NAA, GA_3, ABA, ETH, SA, or MeJA. Each treatment involved at least three plants. Leaves were collected from mixed individual plants at 0, 2, 4, 8, 12, and 24 h after hormone application. Based on the results of gene expression characterization, root tips were chosen for cDNA cloning. Gently shake off the potting soil to extract the entire seedling. Rinse twice with pre-chilled 0.1% DEPC-treated water, blot dry with filter paper, and immediately excise a 0.5 cm root tip segment. All collected samples were stored in cryovials, flash-frozen in liquid nitrogen, and kept at −80 °C.



Figure 1. Stages for alfalfa tissue sampling. (**A**) Green pod at budding stage; (**B**) flower at flowering stage (arrow indicates fully open flower); (**C**) green pod at green pod stage; (**D**) "Flower" after removal of perianth tissue.

2.2. Identification of Target Genes

Genome information for 'XinJiangDaYe', 'Zhongmu No.1', and 'Zhongmu No.4' alfalfa [28–30] was downloaded. Sequences were imported into BioEdit software (v7.7.1) to establish local genome databases. Coding sequences (CDS) and transcript sequences in Fasta format, obtained from the research group's previously published alfalfa transcriptome [31], were imported into BioEdit to establish local transcriptome databases for retrieving alfalfa BBM genes. Using the full-length cDNA of the *M. truncatula BBM* gene (NCBI accession: XM_003624164.4) as the query sequence, the "blastn" function in BioEdit

(expectation value = 1.0×10^{-10}) was used to search the local databases, yielding CDS sequences for alfalfa *BBM* genes. These CDS sequences were then used as queries to search the local alfalfa genome database, obtaining genomic information for the *BBM* genes. Exons and introns were identified based on the "GT-AG" rule.

2.3. Cloning of Target Genes

Total RNA was extracted from the alfalfa root tips using the Total RNA Kit (Tiangen Biochemical Technology Co., Ltd. Beijing, China). The RNA concentration and purity were measured using a Nanodrop 2010/2020 UV-Vis spectrophotometer, and the integrity was assessed by 1.0% agarose gel electrophoresis. cDNA was synthesized using the PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa). Cloning primers were designed based on the *BBM* gene sequence retrieved from the transcriptome database. All primers used in this experiment were designed using Oligo 7.0 software; specific sequences are listed in Table S1. PCR amplification was performed using Phanta® Max Super-Fidelity DNA Polymerase (Vazyme Biotech Co., Ltd., Nanjing). The reaction system (50 μL total) contained 1 μL cDNA, 2 μL MsBBM-F (10 μM), 2 μL MsBBM-R (10 μM), 25 μL 2× Phanta Max Master Mix (Dye Plus), and 20 μL ddH_2O. The PCR program was 95 °C for 3 min; 35 cycles of 95 °C for 15 s, 60 °C for 15 s, 72 °C for 2 min; and a final extension at 72 °C for 5 min. Amplified products were purified using the AxyPrep DNA Gel Extraction Kit and "A-tailed" using the DNA A-Tailing Kit (TaKaRa). The A-tailed PCR product was ligated into the pCR8/GW/TOPO vector (Mighty TA-cloning Kit, TaKaRa) and transformed into Escherichia coli DH5α competent cells. Positive clones were identified by PCR. Positive clones were sent to Beijing Liuhe BGI for sequencing using primers GW1, GW2, and MsBBM699-F. The assembled CDS was aligned and compared with the CDS obtained from the genome database using DNAMAN software.

2.4. Bioinformatics Analysis of MsBBM Protein

The promoter cis-acting elements of the retrieved alfalfa *BBM* genes were analyzed using the online software tools listed in Table 1. Conserved domains and phylogenetic tree analysis of the encoded protein from the cloned *MsBBM* gene were also performed.

Table 1. Bioinformatics analysis software.

Software Name	Analysis Project	Website
PlantCARE	Promoter analysis	https://bioinformatics.psb.ugent.be/webtools/plantcare/html/ (accessed on 10 August 2024)
MEME	Conservative structural domain analysis	https://meme-suite.org/meme/ (accessed on 11 August 2024)
MEGA 7.0	Phylogenetic analysis	https://www.megasoftware.net/ (accessed on 12 August 2024)
DNAMAN 8	Multiple Sequence Alignment	https://www.lynnon.com/ (accessed on 13 August 2024)

2.5. MsBBM Overexpression Vector Construction and Subcellular Localization

Using Golden Gate assembly technology, the target gene fragment was ligated into the pEGOEP35S-H-GFP vector (Figure S1A) to construct the pEGOEP35S-H-MsBBM-GFP (Figure S1B) overexpression vector. Using alfalfa leaf cDNA as a template, PCR amplification was performed with the specific primers MsBBM-F and MsBBM-R. The DNA was gel-purified, ligated to the pEGOEP35S-H-GFP vector, and transformed into *E. coli* DH5α. The pEGOEP35S-H-MsBBM-GFP recombinant plasmid was obtained using the DP103 Plasmid Miniprep Kit (Tiangen Biochemical Technology Co., Ltd. Beijing, China). Rice protoplasts were prepared and transfected with the pEGOEP35S-H-MsBBM-GFP fusion ex-

pression vector plasmid as described [32]. A plasmid expressing GFP alone was transfected as a control. After overnight incubation at room temperature in the dark, the subcellular localization of the GFP signal was observed using a confocal laser-scanning microscope (Nikon, A1 HD25).

2.6. Real-Time Fluorescence Quantitative PCR (qRT-PCR) Analysis

Based on the research group's previous findings [33], the *EF-1α* gene was the optimal reference gene for detecting expression in different tissues of 'XinJiangDaYe' alfalfa, and the Actin gene was optimal for detecting expression in leaves under different hormone treatments. qRT-PCR was performed using the TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) Kit (TaKaRa). The 20 μL reaction volume contained 2 μL cDNA, 6.4 μL ddH_2O, 0.8 μL forward primer (10 μM), 0.8 μL reverse primer (10 μM), and 10 μL TB Green Premix Ex Taq II (Tli RNaseH Plus) (2×). The thermal cycling was 95 °C for 30 s; 40 cycles of 95 °C for 5 s and 60 °C for 30 s, followed by a dissociation curve analysis: 95 °C for 1 s, 65 °C for 15 s, 95 °C for 1 s. Each sample had three biological replicates and four technical replicates. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. SPSS 22.0 and Origin 2019 software were used for statistical analysis and graphing.

3. Results

3.1. Identification and Cloning of Alfalfa BBM Genes

Three *BBM* gene homolog CDS sequences corresponding to the *M. truncatula BBM* gene were retrieved from the local alfalfa transcriptome database, exhibiting >98% sequence identity. Using these CDS sequences as queries against the local alfalfa genome database, three full-length *BBM* genes (*MS.gene024062*, *MS.gene033503*, and *MS.gene52603*) were identified on Chr7.1, Chr7.3, and Chr7.4, respectively, in 'Xinjiang Daye' with no corresponding allele detected on Chr7.2 (Table 2); 'Zhongmu No. 4' harbored three identical sequences to those in 'Xinjiang Daye'; while 'Zhongmu No. 1' possessed a single *BBM* sequence identical to *MS.gene033503*. BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (accessed on 1 August 2024) results in NCBI confirmed that all three genes were *BBM* genes with highly similar conserved regions. *MS.gene024062* is located between positions 23,989,481 and 23,993,325 on Chr7.1 (3845 bp), *MS.gene033503* at 27,043,751–27,047,733 on Chr7.3 (3983 bp), and *MS.gene52603* at 25,819,067–25,823,044 on Chr7.4 (3978 bp). The three CDS sequences identified from the transcriptome showed 100% identity with the exon sequences of the genomic genes. Following the "GT-AG" rule, exons and introns were identified in the full-length sequences of the three *BBM* genes.

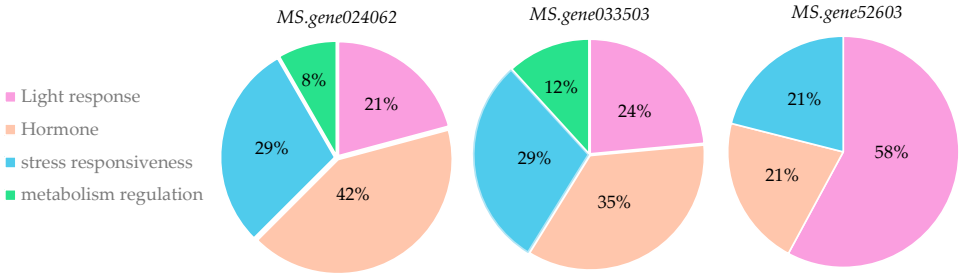
Table 2. Information on the *MsBBM* gene family.

Gene ID	Chr	ORF Length (bp)	Full-length Gene (bp)	AA Length	Number of Exons
MS.gene024062	Chr7.1	2085	3845	694	9
MS.gene033503	Chr7.3	2088	3983	695	9
MS.gene52603	Chr7.4	2091	3978	696	9

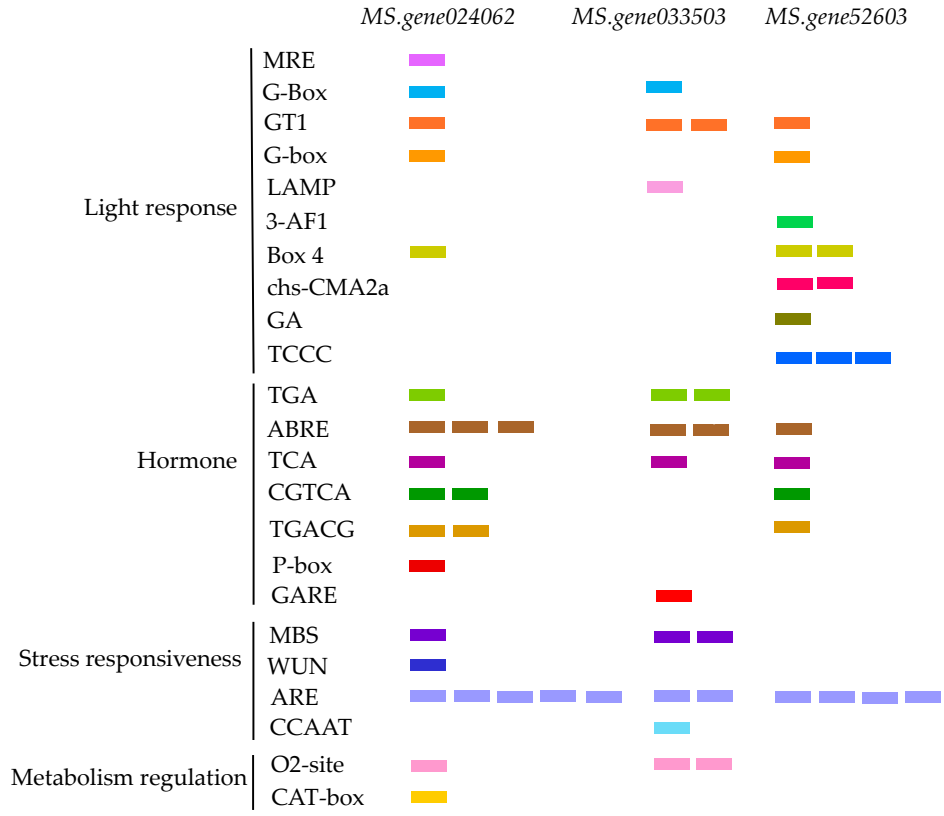
Using reverse-transcribed alfalfa cDNA as a template, PCR amplification with cloning primers yielded a band of approximately 2000 bp, consistent with the target size (Figure S2A). DNA sequencing confirmed identical sequences across multiple positive colonies. Colony Nos. 7 and 16 contained the complete *BBM* gene CDS sequence, 2082 bp in length, encoding 694 amino acids (Figure S2B). Alignment analysis using DNAMAN software showed 98.9% sequence similarity with *MS.gene52603* and *MS.gene033503* (Figure S3). BLAST analysis using NCBI online software confirmed it as a *BBM* gene, named *MsBBM.*

3.2. Analysis of Cis-Acting Elements in Alfalfa BBM Gene Promoters

The promoter sequences of *MS.gene024062*, *MS.gene033503*, and *MS.gene52603* (Figure S4) retrieved from the alfalfa genome database were analyzed for cis-acting elements. As shown in Figure 2, the 2000 bp upstream sequences of the three *MsBBM* genes contained various elements, including the core elements CAAT-box and TATA-box, light-responsive elements, hormone-responsive cis-acting elements, and cis-regulatory elements involved in stress responsiveness and metabolism regulation. Among them, hormone-responsive cis-acting elements were the most abundant in the promoter sequences of *MS.gene024062* and *MS.gene033503*. Light-responsive elements were predominant in the promoter sequence of *MS.gene52603*. The hormone response elements identified within the promoter regions of the three genes include those for GA, Auxin, ABA, MeJA, and SA. Among these, the ABA response elements and SA response elements were conserved regulatory elements present in all three promoters.



(**a**)



(**b**)

Figure 2. Analysis of cis-acting elements in the promoters of three *BBM* genes in the alfalfa genome. (**a**) Proportion of different cis-acting elements; (**b**) Classification and quantity of cis-acting elements.

3.3. Phylogenetic Tree Construction and Motif Analysis of Alfalfa MsBBM

A total of 19 BBM protein sequences from 15 species were obtained from NCBI (https://www.ncbi.nlm.nih.gov/) (accessed on 2 August 2024) and Phytozome v13 (https://phytozome-next.jgi.doe.gov/) (accessed on 2 August 2024). A phylogenetic analysis was performed with the protein sequence encoded by the cloned *MsBBM* gene. BBM/BBML protein sequences were from Fabaceae: *M. truncatula*, *M. sativa*, *Glycine max*, *Vigna angularis*, *Cicer arietinum*, *Pisum sativum*, *Trifolium pratense*; Brassicaceae: *Arabidopsis thaliana*, *Brassica napus*; Rosaceae: *Rosa canina*; Poaceae: *Oryza sativa*, *Zea mays*, *Triticum aestivum*, *Hordeum vulgare*; and apomictic plants: *Cenchrus ciliaris* (syn. *Pennisetum ciliare*) and *P. squamulatum*. The results showed that all BBM/BBML proteins clustered into six branches. MsBBM clustered with all Fabaceae plants in the first branch and exhibited the closest phylogenetic relationship with the *M. truncatula* BBM protein. Conserved motif analysis using MEME software identified 10 motifs among the 20 BBM protein sequences. Among these, the BBM proteins from seven leguminous species and two Rosaceae species exhibited the highest motif diversity, each possessing nine different motifs. BBM proteins from two Brassicaceae species, rice OsBBM4 and maize ZmBBM1, shared the same eight motifs. Apomictic *Cenchrus ciliaris* and *Pennisetum squamulatum* clustered with rice OsBBM1, possessing 6–8 motifs. Wheat and barley BBM proteins clustered together, possessing five motifs (Figure 3).

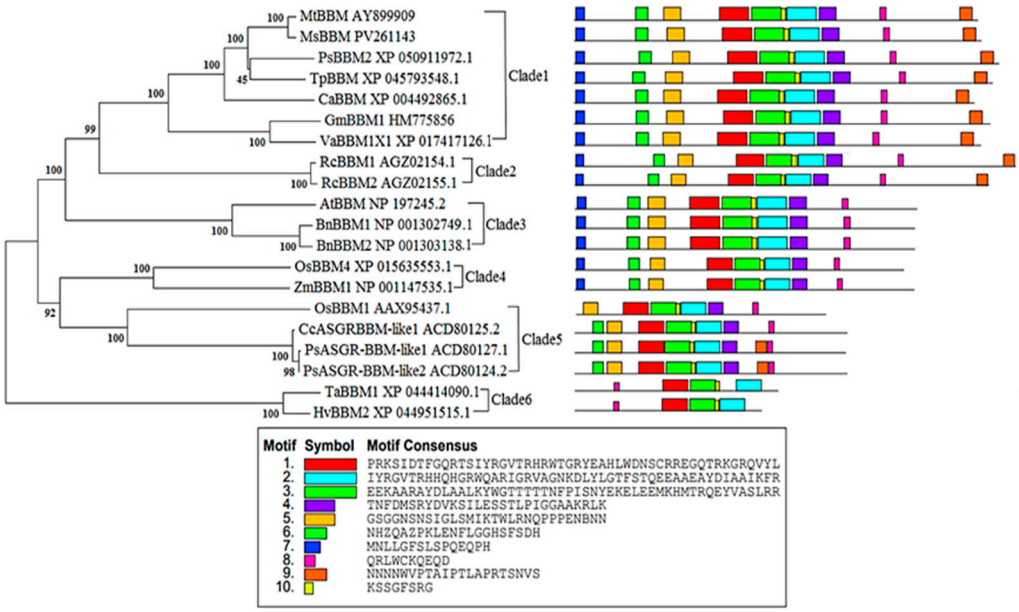


Figure 3. Motif structure and cluster analysis of MsBBM protein.

Based on the conserved motif analysis results, multiple sequence alignment of BBM proteins from seven Fabaceae plants was performed using DNAMAN 8 software. As shown in Figure 4, MsBBM contains two AP2 domains (AP2-R1 and AP2-R2) and the bbm-1 motif. The sequence of the bbm-1 motif was 100% identical among the seven Fabaceae plants. The sequence similarity of the two AP2 domains was 98.6% and 98.5%, respectively. Apart from a single amino acid residue change at position 60 in the AP2-R1 domain of PsBBM and position 61 in the AP2-R2 domain of CaBBML, the AP2 domains of the other five Fabaceae plants were highly conserved. Eleven motifs (euANT1–6 and bbm1–5) were identified among the seven Fabaceae BBM proteins. Among them, the euANT1 motif was inserted into AP2-R1 and was highly conserved. The conservation of the other ten motifs varied. The euANT2, euANT5, and bbm1 motifs were the most conserved, while the bbm-5

motif was the least conserved. Apart from the euANT4 motif, MsBBM shared identical motifs with MtBBM.

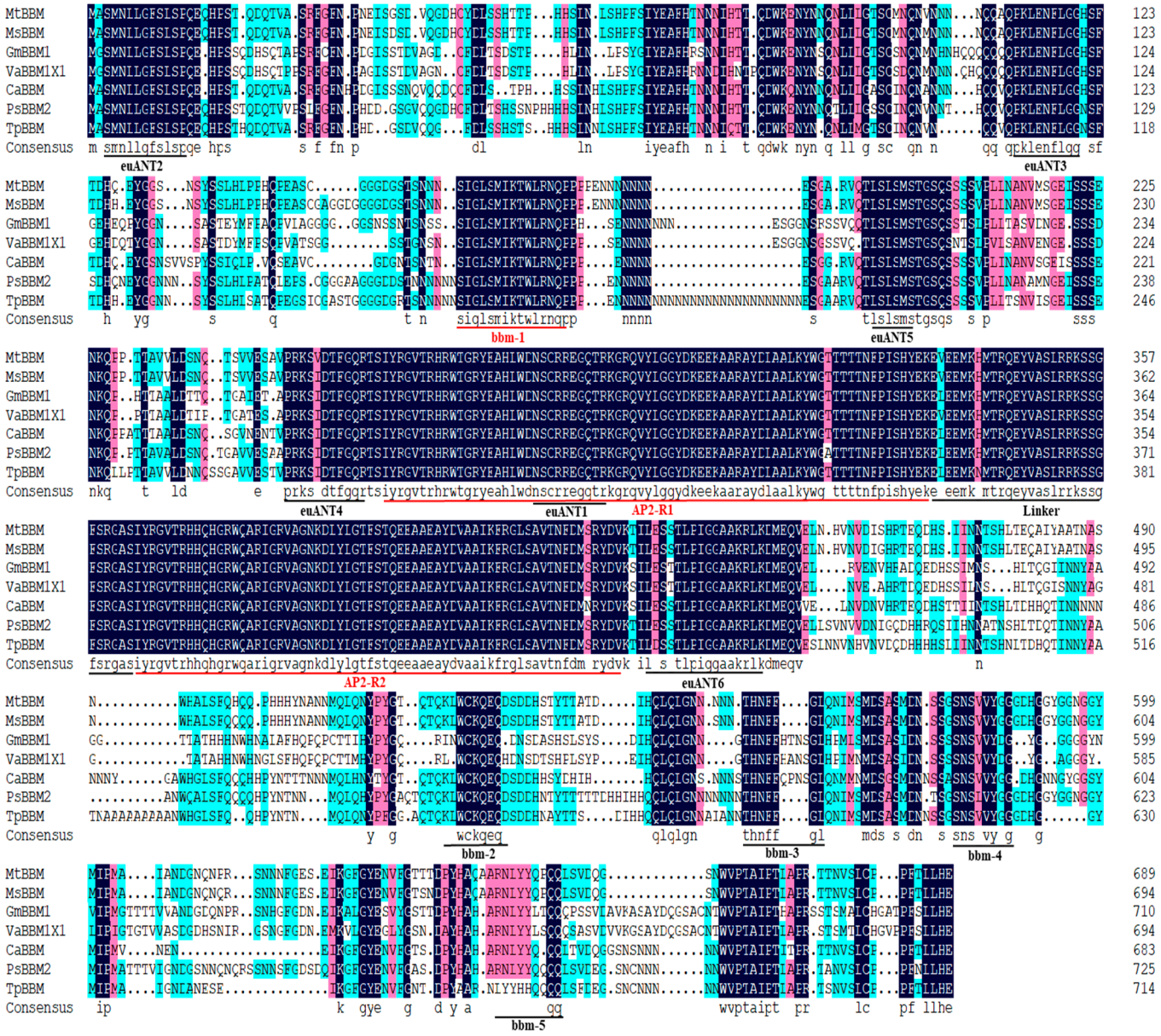


Figure 4. Sequence alignment analysis of MsBBM protein with BBM homologous proteins from different plants. Motif sequences were identified and underlined. The two AP2 domains are indicated as AP2-R1 and AP2-R2. Mt: *M. truncatula*; Ms: *M. sativa*; Gm: *Glycine max*; Va: *Vigna angularis*; Ca: *Cicer arietinum*; Ps: *Pisum sativum*; Tp: *Trifolium pratense*.

3.4. Subcellular Localization of Alfalfa MsBBM Protein

To determine the intracellular location of the protein encoded by the cloned MsBBM gene, the pEGOEP35S-H-MsBBM-GFP expression vector was constructed. Transient transformation experiments in rice protoplasts were conducted, and the distribution of the fluorescent signal was observed to determine MsBBM localization. As shown in Figure 5, the empty vector (GFP control) showed uniform and strong fluorescence signals distributed in both the cell membrane and nucleus, indicating no specific subcellular localization. In contrast, the strong fluorescence signal of MsBBM-GFP was primarily distributed within the nucleus, as expected for a transcription factor.

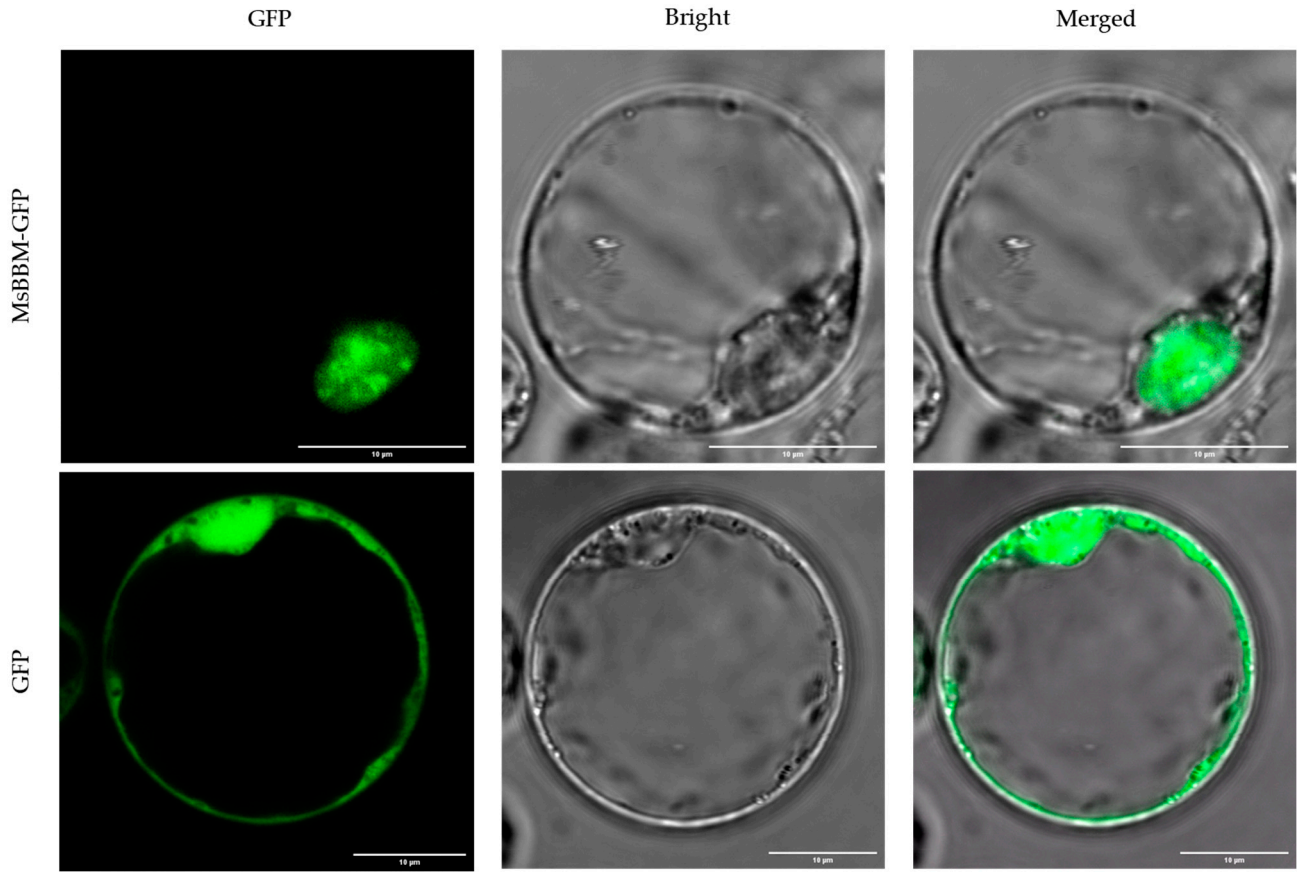


Figure 5. Localization of MsBBM-GFP fusion protein and GFP protein in rice protoplasts.

3.5. Analysis of MsBBM Gene Expression Pattern

MsBBM expression varied significantly among different tissues, displaying tissue specificity. The highest expression level was observed in roots, 63.67 times that in leaves, and significantly higher than in other tissues ($p < 0.05$). Expression was second highest in green pods and buds, 18.54 times and 12.44 times that in leaves, respectively, and significantly higher than in stems and flowers ($p < 0.05$). The lowest expression level was detected in leaves (Figure 6).

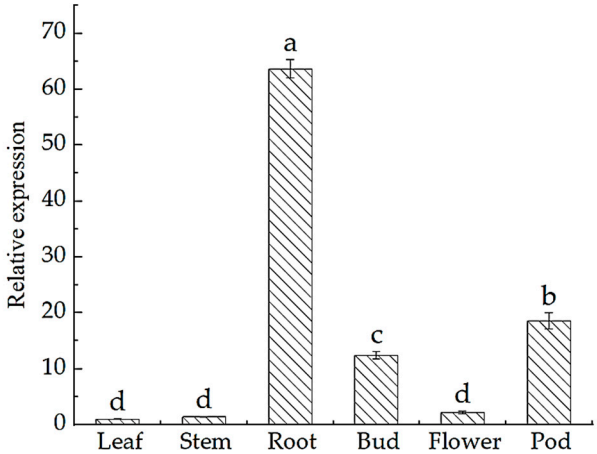


Figure 6. Expression analysis of *MsBBM* gene in different tissues and organs. Different lowercase letters indicate significant differences at the 0.05 level ($p < 0.05$).

To investigate the effects of exogenous hormones on *MsBBM* expression, qRT-PCR was performed on leaves of alfalfa sprayed with NAA, GA_3, ABA, ETH, MeJA, or SA. As shown in Figure 7, *MsBBM* expression peaked at 8 h after both NAA and MeJA treatments, reaching 1.11 times and 1.29 times the control (CK), respectively. Expression was lower than CK at other time points. Expression after the GA_3 (except at 4 h) and SA treatments was higher than CK. The GA_3 treatment induced the highest expression at 12 h, reaching 4.92 times CK ($p < 0.05$). The SA treatment induced the highest expression at 4 h, reaching 10.39 times CK, significantly higher than at other time points ($p < 0.05$). Expression after ABA treatment was consistently lower than CK, with significant differences ($p < 0.05$) at all time points except 24 h. ETH treatment induced the highest MsBBM expression at 2 h, reaching 2.8 times CK. The expression then decreased sharply, reaching its lowest level at 24 h.

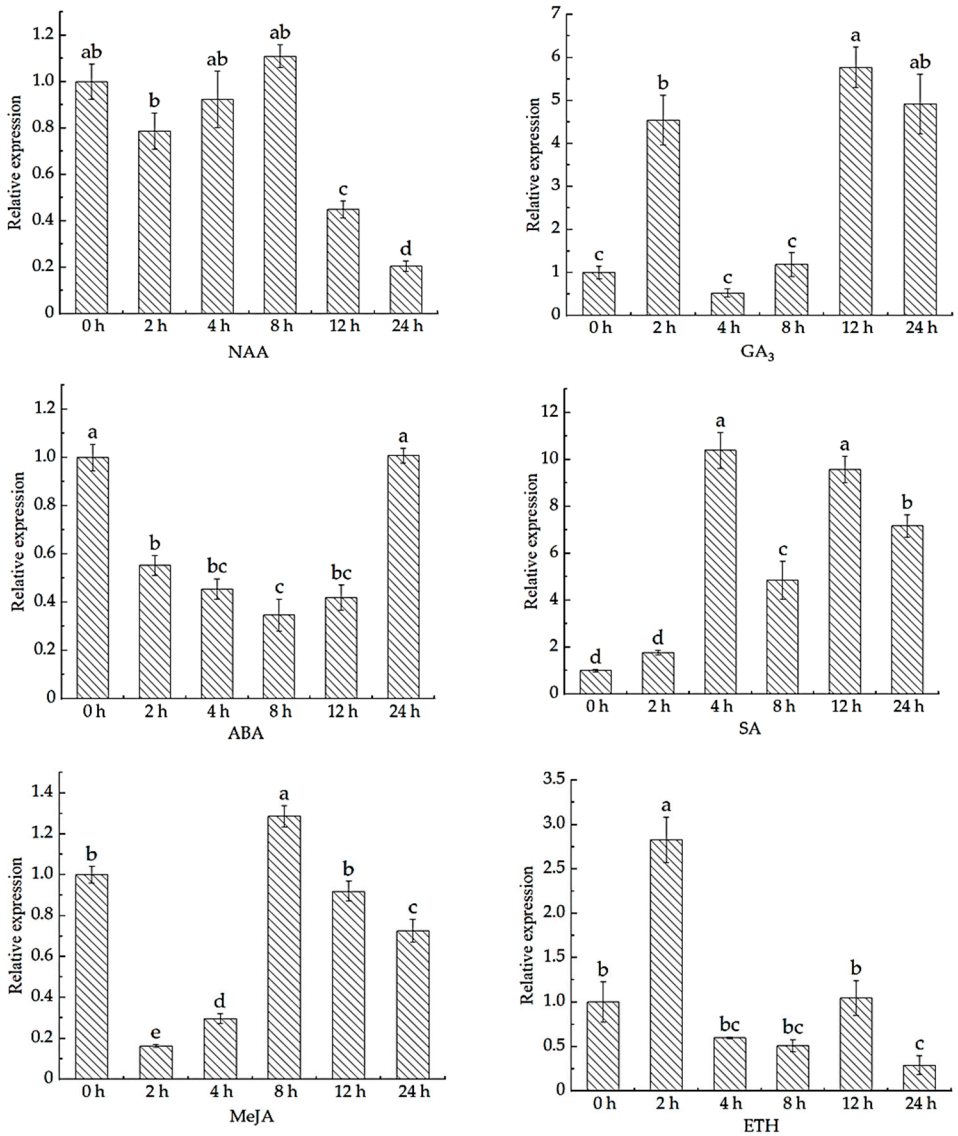


Figure 7. Expression analysis of *MsBBM* gene under hormone treatments. Different lowercase letters indicate significant differences at $p < 0.05$.

4. Discussion

4.1. Characteristics of Conserved Domains and Motifs in Alfalfa MsBBM Protein

The large plant AP2/ERF (APETALA2/ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR) transcription factor family has undergone extensive gene duplication and

domain shuffling during evolution [4,34,35]. DREB and ERF proteins within this family contain only one AP2/ERF domain. Studies show that the domain of Arabidopsis DREBs specifically binds the cis-acting element DRE/CRT (core element A/GCCGAC) involved in drought, cold, and salt stress-related genes in higher plants [36]. In contrast, the ERF protein domain specifically binds the GCC box (core element AGCCGCC) involved in ethylene signaling pathway-related genes [37]. The AP2-like family, also known as the AP2 family, plays key roles in cell proliferation, early primordium development, floral meristem determination, and embryogenesis [6,34,38]. A common feature of this family is the presence of two highly similar copies of the AP2/ERF domain [6,34,39], which are crucial for the specific transcriptional activity, protein interactions, and nuclear localization of different members [35].

In addition to the AP2/ERF domain, the AP2/ERF transcription factors contain 10 conserved motifs. Within the N-terminal sequence of BBM, Kim et al. [34] identified three euANT motifs (euANT2, euANT3, and euANT4). Quakfaoui et al. [6] identified five motifs. The first three were consistent with Kim's findings; the fourth was the bbm-1 motif, proven to be unique to all monocot and dicot *BBM* and *BBM*-like genes. Domain deletion and swap analyses indicated that the bbm-1 motif of BnBBM1, acting synergistically with the euANT2 motif, could induce somatic embryogenesis in Arabidopsis [6]. The fifth motif was euANT5, which is absent in some monocot *BBM*-like genes. In the C-terminal sequence of BBM, Quakfaoui also identified five motifs. The euANT6 motif immediately follows the second AP2/ERF domain; this motif contains lysine (Lys, K) and arginine (Arg, R) residues, suggesting potential involvement in nuclear localization signals [6,40]. The other four motifs in the C-terminal sequence (bbm-2, bbm-3, bbm-4, bbm-5) are relatively less conserved across species. This study found that the MsBBM protein encoded by the cloned *MsBBM* gene possesses two highly conserved AP2 domains and the BBM-specific bbm-1 motif, with amino acid residues 100% identical to the corresponding consensus sequences, confirming the reliability of the cloned *MsBBM* gene. Compared to previous studies, we found that the euANT5 motif, absent in Poaceae, is highly conserved in Fabaceae; the bbm-3 motif, absent in Brassicaceae, is relatively conserved in Fabaceae; and the bbm-2 and bbm-4 motifs are more conserved in Fabaceae than in Brassicaceae. The differential distribution of these motifs may underlie the lineage-specific adaptive divergence of AP2/ERF transcription factor functions. Differences in motifs within the N-terminal and C-terminal regions may contribute to variations in transcriptional activity, protein interactions, and nuclear localization among different genes [35], while shared conserved motifs provide the possibility for similar functions [41]. Domain deletion and swapping analyses will further determine whether cooperative interaction between the bbm-1 and euANT2 motifs in the MsBBM protein induces parthenogenesis—establishing a theoretical basis for obtaining dihaploid progenies via genetic manipulation of this gene.

4.2. Analysis of MsBBM Gene Expression Pattern in Alfalfa

BBM is expressed preferentially in developing embryos and seeds in *Arabidopsis* and *Brassica*, and the cells that are most competent to respond to an ectopic *BBM* signal appear to be the same cells that are normally competent to divide and differentiate [14]. The wheat *TaBBM* gene is expressed more highly in roots and grains than in leaves [42]. Two *RcBBM* genes in rose (*R. canina*) are expressed in young roots, callus, and protocorm-like bodies (PLBs), but not detected in the stems, leaves, or flowers [43]. The *SbBBM-like1* gene in sorghum (*S. bicolor*) shows the highest expression in young roots [44]. The *LkBBM2* gene in larch (*Larix kaempferi* × *L. olgensis*) is expressed significantly higher in adventitious roots than in the stems, leaves, and shoot tips [45]. Zhou et al. [46] analyzed the transcriptional regulatory cis-elements in the promoter region of the soybean *GmBBM* gene, finding

elements responsive to hormones such as ABA, GA, auxin, and MeJA. Overexpression of *GmBBM7* significantly increased the ABA and SA content in soybean hairy roots. Yavuz et al. [44] identified two *SbBBM* (*BBM-like*) genes in sorghum and found common cis-regulatory elements in their promoters: light-responsive elements (G-box, Sp1, TCCC) and GA (ABRE) and MeJA (CTTCA, TAGCG) response elements. Li [47] and Wang et al. [45] analyzed the promoter regions of larch *LkBBM1* and *LkBBM2* genes, respectively, identifying hormone response elements for auxin, CK, GA, ABA, SA, and ethylene. Wang et al. [48] found that *DfBBM* in *Dendrocalamus farinosus* responded to ABA, IAA, NAA, and MeJA treatments. Expression peaked at 2 h after ABA, IAA, and MeJA treatments, then declined, while expression significantly decreased after NAA treatment.

This study revealed that *MsBBM* expression was highest in roots, consistent with previous findings in wheat [42], sorghum [44], and soybean [46]. Furthermore, expression in the green pods was significantly higher than in the buds and flowers, indicating its role in early seed development. This observation is consistent with earlier reports in *Arabidopsis* and *Brassica* [14]. Therefore, our study confirms that *BBM* gene expression exhibits tissue specificity, with high levels in cells undergoing proliferation and morphogenesis [8]. Ectopic expression of the *BnBBM* gene can not only spontaneously induce somatic embryos in *B. napus* [5] but also promote apogamy without sugar supplement in a non-vascular plant, *Ceratopteris richardii* [49]. Recently, Boutilier's team reexamined *BBM* expression and function in *Arabidopsis* embryogenesis using reporter analysis and newly developed CRISPR mutants. They found that ectopic *BBM* expression in the egg cell of *Arabidopsis* and the dicot crops *B. napus* and *Solanum lycopersicon* is sufficient to bypass the fertilization requirement for embryo development [14]. Therefore, we hypothesize that *MsBBM*—which is predominantly expressed during early seed development in *M. sativa*—likely functions similarly to *AtBBM* and *BnBBM*.

We analyzed cis-regulatory elements in the promoter regions of three *MsBBM* genes, identifying the hormone-responsive elements associated with auxin, ABA, SA, GA, and MeJA. Consequently, the exogenous application of these hormones to alfalfa leaves revealed differential responses of *MsBBM* to all five phytohormones. *BBM* is a developmental regulatory gene involved in somatic embryogenesis and organogenesis via the auxin signaling pathway [5,8,50]. *BBM* induces somatic embryogenesis by transcriptionally activating auxin biosynthesis genes (*YUC*, *IAA30*, and *TAA1*) through the direct regulation of *LEAFY COTYLEDON1* (*LEC1*), *LEC2*, and *AGAMOUS-LIKE15* (*AGL15*), thereby promoting the auxin production that is essential for somatic embryo initiation and development [51]. Khanday et al. [24,50] reported that exogenous auxin-induced somatic embryogenesis in rice requires the presence of functional *OsBBM* genes, and the overexpression of *OsBBM1* promotes somatic embryogenesis without the use of exogenous auxins, suggesting that *OsBBM* overexpression increases endogenous auxin production. Our study observed the downregulation of *MsBBM* expression following exogenous auxin treatment, consistent with the findings for *DfBBM* in *D. farinosus*. This suppression may be attributed to the use of NAA as the auxin source. Notably, *DfBBM* expression increased upon IAA treatment. The mechanisms underlying these differential responses to distinct auxin types remain unclear. Horstman et al. [51] demonstrated that BBM transcriptionally controls *ABSCISIC ACID INSENSITIVE3* (*ABI3*), which functions as an essential positive regulator in BBM-mediated embryogenesis. ABI3 functions as a central regulator in the auxin-ABA crosstalk network [52,53]. However, ABA itself does not directly upregulate *ABI3*. Luo et al. [54] demonstrated that ABA directly represses expression of *YUCCA* auxin-biosynthesis genes and PIN efflux transporters via *ABI4*, thereby reducing endogenous auxin levels and suppressing primary root growth in *Arabidopsis*. The specific functions of ABA during somatic embryogenesis or apomixis remain unreported. This study observed *MsBBM* downregu-

lation following exogenous ABA treatment, whereas *DfBBM* expression increased under similar conditions. This differential response may reflect the lower ABA concentration applied here (approximately one-fourth of the dosage used in the *DfBBM* study). Studies report that *AGL15*, transcriptionally regulated by BBM during somatic embryogenesis, not only promotes auxin biosynthesis but also reduces endogenous GA levels through upregulating *GA2ox6* (encoding a GA catabolic enzyme) and downregulating *GA3ox2* (a GA biosynthetic gene), ultimately enhancing plant regeneration [55,56]. Hu et al. [57] found that *ABI3* modulated by BBM physically interacts with RGL3 (*RGA-LIKE* 3), a negative regulator of gibberellin (GA) accumulation. This study found that exogenous application of GA increased the expression level of *BBM*. Valencia-Lozano et al. [58] observed that *BBM* was upregulated in somatic embryos of *Coffea arabica* under osmotic stress. Therefore, BBM might play a role in abiotic stress tolerance mechanisms [59], possibly by directly regulating GA content, although this conclusion requires further validation. To better elucidate the impact of hormones on *MsBBM* gene expression during apomixis, subsequent experiments will involve application of hormones on alfalfa ovaries at pre- and post-fertilization stages. Expression levels of the *MsBBM* gene will be determined alongside in situ hybridization assays to unravel its spatiotemporal expression patterns. This integrated approach will establish direct evidence for understanding the parthenogenetic function of the *MsBBM* gene.

Expression of the *BBM* gene in the egg cells of both sexual and apomictic species is sufficient to bypass the fertilization requirement for embryo development. In monocots, *BBM* genes from both apomictic and sexual species can induce parthenogenesis [10,15,22,25,26]. In dicots, only two *BBM* genes have been successfully validated: *PsASGR-BBML* (*BBM-like*) from the apomictic *P. squamulatum* and the *BBM* gene from the sexual *A. thaliana*. The ectopic expression of these genes in the egg cell induced haploid embryo formation in tobacco (*Nicotiana tabacum*) [13], *Arabidopsis*, *Brassica napus*, and tomato (*S. lycopersicum*) [14]. Although the initiations of zygotic, apomictic, and in vitro embryogenesis are activated by different signals and often begin from different starting tissues, it is not unreasonable to assume that all three processes converge at a very early stage on the same signaling pathway. Both the transcription and translation of *OsBBM1* occur in the male gametophyte of rice prior to fertilization. The *OsBBM1* expressed in the early zygote originates exclusively from the paternal genome. Post-fertilization, its expression in the zygote triggers activation of the maternal allele, thereby initiating egg cell division. Subsequently, biallelic expression in the zygote critically regulates embryonic patterning and organogenesis [25,60]. Building on this principle, the ectopic expression of *OsBBM1* in rice MiMe mutants induced pre-fertilization division of egg cells, yielding clonal seeds retaining parental heterozygous genomes [25]. Thus, a differential spatiotemporal expression of *BBM* orthologs dictates whether egg cells undergo division before fertilization, ultimately determining the development of sexual or parthenogenetic embryos in higher plants. To identify the parthenogenesis function of the alfalfa *MsBBM* gene, the next step involves constructing overexpression vectors driven by the egg cell-specific *EC1* promoter [61,62]. Genetic transformation will be used to achieve an ectopic expression of *MsBBM* in the egg cell before fertilization. In vitro culture techniques will then be employed to obtain haploid embryos, laying the theoretical foundation for creating apomictic hybrid progenies in alfalfa and enabling an efficient fixation of heterosis.

5. Conclusions

This study deciphered the molecular function of the BABY BOOM (BBM) transcription factor in alfalfa (*M. sativa*), identifying three *BBM* genes with promoters enriched in light-responsive elements and hormone-signaling motifs (auxin, gibberellin, ABA, and SA). The

cloned *MsBBM* (2082 bp/694-aa) encodes a nuclear-localized protein carrying two AP2 domains and the BBM-specific bbm-1 motif, sharing the closest evolutionary relationship with *M. truncatula* MtBBM. Tissue-specific expression peaked in roots (>5-fold vs. leaves) and green pods, while hormonal responses revealed upregulation by GA_3/SA, downregulation by NAA/MeJA/ABA, and transient induction by ETH. These insights establish MsBBM as a key target for engineering synthetic apomixis, providing a transformative strategy to fix heterosis and accelerate high-efficiency hybrid seed production in alfalfa breeding.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy15081768/s1: Table S1: Primer sequences for alfalfa *BBM* gene cloning and qRT-PCR; Supplementary data: The promoter sequence of alfalfa *BBM* genes. Figure S1: (A): pEGOEP35S-H-GFP vector map; (B): pEGOEP35S-H-MsBBM-GFP vector map; Figure S2: PCR amplification and colony PCR verification of *MsBBM* gene; Figure S3: Sequence alignment of the coding sequence of three *BBM* genes found in the published genome 'XinJiangDaYe' and the cDNA sequenced in this work. Figure S4: Promoter sequence of three *BBM* genes found in the published genome 'XinJiangDaYe'.

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Abbreviations

The following abbreviations are used in this manuscript:

NAA	Naphthalene Acetic Acid
GA_3	Gibberellic Acid
ABA	Abscisic Acid
SA	Salicylic acid
MeJA	Methyl jasmonate
ETH	Ethylene

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