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# Genome-wide identification and expression profiling reveal the diverse role of Methyl-CpG-binding domain proteins in tea plant *Camellia sinensis*

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## Abstract

Methyl-CpG-binding domain (MBD) proteins are important DNA methylation readers that recognise methylated CpG sites and recruit histone deacetylase (HDAC) complexes and chromatin remodelling factors, leading to chromatin compaction, gene transcription, and genome integrity. Currently, *MBD* genes have only been identified in a few plant species and their structure and function in tea plants (*Camellia sinensis*) are unknown. In this study, 16 *C. sinensis MBD* genes (*CsMBD*) were identified on a genome-wide level and classified into eight classes. The *CsMBD* genes were mapped on nine chromosomes in tea plants, and nine pairs of *CsMBD* genes existed. Based on conserved domain analysis, all of the identified CsMBD proteins contained at least one MBD domain. Expression analyses showed that *CsMBD* genes were expressed in tissue- and organ-specific patterns. We investigated the expression patterns of *CsMBD* genes in response to abiotic and biotic stresses and during different plant growth and development stages. Multiple pthytohormone and stress-related *cis*-acting was evaluated in their promoter region, such as GGTCA, TGACG, ABRE and LTR. Specific *CsMBD* genes were associated with environmental stresses and developmental stages, with little overlap. Overall, our findings reveal the diverse roles of *CsMBD* genes under different stress and developmental conditions, highlighting candidate genes for further functional studies on tea plants.

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## INTRODUCTION

As a conserved epigenetic mark, DNA methylation is dynamically regulated by DNA methylases and demethylases, thereby playing critical roles in gene regulation[1], transposon silencing[2], and chromosome interactions[3]. In plant genomes, DNA methylation is more likely to occur in three types of bases (CG, CHG and CHH) during development and reproduction[4]. In plants, symmetric CG and CHG methylation occurs during DNA replication, which is catalysed by DNA METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3), respectively[4]. In contrast, asymmetric CHH methylation is established through *de novo* methylation by DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2)[5]. However, DNA glycosylase/lyases can catalyse DNA demethylation, with REPRESSOR OF SILENCING 1 (ROS1) and DEMETER (DME) being typical representatives[4]. Methyl-CpG-binding domain (MBD) proteins recognise DNA methylation signals and bind methylated DNA to contribute to transcriptional repression, chromatin remodelling, and DNA demethylation[6−8]. The first plant MBD protein was isolated from pea (*Pisum sativum*)[9]. Subsequently, the MBD family members were identified in *Arabidopsis*, rice, maize, tomato, and wheat[10−12].

In *Arabidopsis thaliana*, 13 members from the MBD family were classified into eight groups according to the similar MBD motif[7]. The function of most members is unclear, but three (MBD5, MBD6, and MBD7) of them were demonstrated to have capacity for binding symmetrically methylated DNA[7]. For example, AtMBD6 is the interactor of RNA binding proteins and histone deacetylase (AtHDA6, histone deacetylase 6) which regulates the RNA-mediated gene silencing[13]. AtMBD7 can form the MBD7-IDM complex with IDM1/2/3 (histone acetyltransferase IDM1, alpha-crystallin domain proteins IDM2 and IDM3) and HDP1/2 to regulate the recruitment of ROS1 promoting DNA demethylation[14]. In maize, 14 *MBD* genes have been identified[12], including *ZmMBD101*, which were reported to have capacity for binding DNA and maintaining mutator elements of chromatin in an inhabited state[15,16]. Moreover, in maize, the ZmMBD11 also showed specific DNA-binding activity indicating its important role in reading cytosine methylation[12].

Rice contains 17 proteins with MBD domains, in which the function of partial genes has been reported. The deposit of cyclobutane pyrimidine dimers were reduced by OsMeCP (MBD protein) on the rice genome[17]. While in tomato, MBD proteins were found to participate in fruit ripening[11]. These

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studies in different plants indicate a significant role for MBD proteins in genomic and genetic regulation. However, to date, the potential role of each individual member of the MBD family has not been demonstrated in any perennial woody plants.

The tea plant (*Camellia sinensis* (L.) O. Kuntze) is a perennial woody cash crop with a wide distribution area. During its growth cycle, *C. sinensis* encounters biotic and abiotic stresses including cold, drought, plant diseases and pests. During cold acclimation, a change in the DNA methylation level on the tea plant genome was suggested to be involved in cold resistance in this species[18]. The relative transcription levels of genes encoding DNA methylase and demethylase showed significant change in response to abiotic stress[19]. Epigenetic regulation was identified as one of the major mechanisms involved in overwintering bud dormancy formation and release[20]. However, the specific factors in the epigenetic regulation system in tea plants is largely unknown, especially the response of DNA methylation to adverse environmental conditions, growth, and development. Therefore, we present an overall study of DNA methylation reader proteins in tea plants. Since the draft genome of 'Yunkang 10' was reported, a total of eight genomes of different tea plant species have been sequenced and released. Among them, the 'Longjing 43' (LJ43) genome was one of the high-quality chromosome-scale tea genome, which was characterized by a scaffold N50 value of 144 Mb, 88.36% gene completeness, and a base accuracy of 99.999%. It provides a rich genomic resource for tea researchers[21]. Thus, we identified and characterised 16 genes encoding MBD proteins within the LJ43 genome[21]. Comprehensive expression detections of these genes under environment stresses and different growth and development stages were performed to provide useful insights into the epigenetic regulation in tea plants.

## MATERIALS AND METHODS

### Identification and characterization of *CsMBD* family members

The Hidden Markov Model (HMM) profile of the MBD domain (PF01429; http://pfam.sanger.ac.uk) was used to scan predicted proteins encoded in the *MBD* genes in the LJ43 genome (https://bigd.big.ac.cn/search/?dbId=gwh&q=GWHACFB00000000[21]). All candidate proteins were determined using the SMART database (https://smart.embl-heidelberg.de). The potential members of the *CsMBD* gene family were confirmed by homology analysis using the BLAST algorithm (*p*-value < $10^{-5}$) combined with MBD domain identification. The newly identified genes were named according to the *Arabidopsis* gene locations on the phylogenetic tree. The WoLF PSORT server (https://wolfpsort.hgc.jp) was used to predict the subcellular localisations of these proteins. The physicochemical parameters of these gene encoding proteins were calculated using the ProtParam tool (https://www.expasy.org).

### Phylogenetic tree construction and analysis of sequence alignment, conserved domains, gene structures, chromosomal location, duplication analysis and regulatory elements

To further determine the phylogenetic relationship of MBD proteins, the conserved MBD sequences from four plants (*Oryza sativa*, *A. thaliana*, *Populus trichocarpa, C. sinensis*) were aligned using ClustalW[22]. The unrooted tree was created using MEGA7.0 (https://www.megasoftware.net) by the neighbour-joining (N J) method. The bootstrap value = 1,000. The conserved domains of these proteins were analysed using Tbtools software[23] and the chromosomal location of the genes was investigated and analysed by MG2C (http://mg2c.iask.in/mg2c%5Fv2.1) based on their starting positions on the chromosomes. Exon/intron structure of *CsMBDs* were predicted with the Gene Structure Display Server 2.0 (http://gsds.gao-lab.org)[24]. The genome sequences and gene annotation files were used to conduct the duplication by TB tools. The 2 kb long upstream regions were chosen to analyse *cis*-acting elements using the Plant CARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html).

### Plant materials and treatments

LJ43 cultured at the Tea Research Institute of the Chinese Academy of Agricultural Sciences (Hangzhou, Zhejiang Province, China) was selected. To examine tissue-specific gene expression patterns, the leaves, roots, buds, and stems of LJ43 were collected in August 2020. Dormant axillary buds in the middle of branches were sampled from October 2018 to March 2019. In this study, the paradormant axillary buds were collected on 25 October 2017. The endodormant axillary buds were collected on 14 January 2018 and the ecodormant axillary buds were collected on 13 March 2018. Cold acclimation leaf sampling included mature LJ43 leaves sampled on 1 November and 15 November 2017, 18 December 2017, 11 January 2018, 6 February 2018, and 27 March 2018. All experimental materials and treatment are shown in Supplemental Table S1.

Five-year-old LJ43 cutting seedlings were planted in a climate chamber at 25 °C in a 16/8 h light/dark cycle with 60% humidity. For drought treatment, water was provided every 3 days to the control group, while the experimental group was given no water. The second and third leaves of the control and drought treatment plants were sampled on 0, 3, 6, 9, 12, 15 and 18 d. For seeding germination assays, the seeds were stripped of the seed coat and grown on vermiculite before being uprooted after 2 weeks. For floral development analysis, flower buds, semi-open flowers, and fully open flowers were collected. For biotic stress treatment, healthy branches were inoculated with conidial suspensions of *Colletotrichum camelliae* ($10^6$ spores/mL) as previously described[25]. The inoculation method for *Ectropis oblique* and *Pseudopestalotiopsis camelliae-sinensis* was conducted as previously described[26], while the control groups were given a sterile water spray. The bud and first leaves were sampled at 12 and 24 h post-inoculation.

Three biological replicates were set for the above sampling and treatment. After sampling, the samples were immediately placed in liquid nitrogen and stored at −80 °C for later use.

### Gene expression level detection

Total RNA was isolated from the experimental samples using an RNAprep PurePlant Kit (Tiangen, Beijing, China) by following the manufacturer's instructions. All cDNA samples were synthesised using PrimeScript RT enzymes with the gDNA eraser (Takara, Dalian, China). The qRT-PCR reactions

were set up using LightCycler® 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany). All primer pairs for expression detection are shown in Supplemental Table S2 and *CsPTB1* was chosen as the internal reference gene[27]. The $2^{-\Delta Ct}$ method was used to calculate the expression levels of cold acclimation–related and tissue-specific genes, while the $2^{-\Delta\Delta Ct}$ method was employed in the relative expression analysis of the remaining experimental treatments[28]. Two technical replicates were performed for each biological replicate.

### Cloning for full-length open reading frames and promoter sequence validation

Total RNA of LJ43 buds was extracted as described previously. The synthesise of cDNA is performed using the DNase I Amplification Grade and SuperScript® III First-strand kit (Invitrogen, USA) following the manufacturer's instructions. Based on previous data analysis for the LJ43 tea genome[21], the full-length open reading frames (ORF) encoding the *MBD* genes in tea plants were amplified using the primers provided in Supplemental Table S3. PCR amplification was conducted in 50 μL reaction mixtures for KOD-plus-Neo (TOYOBO, Osaka, Japan). The PCR product was gel-purified and cloned into the pEASY-Blunt zero vector (TransGen Biotech, Beijing, China) for sequencing by the Ykang Biotechnology Company (Ykang, Hangzhou, Zhejiang). The obtained sequences were uploaded to the National Center for Biotechnology Information (NCBI).

The genomic DNA of the LJ43 buds was isolated using the Plant Genomic DNA Kit (Tiangen, China). The upstream regulatory elements present in the first 2,000 bp of the start codon were used as the gene promoter for cloning. The primers are listed in Supplemental Table S4. KOD-plus-Neo (TOYOBO, Osaka, Japan) was used in PCR amplification. The PCR products were gel-purified and cloned into the pEASY-Blunt zero vector (TransGen Biotech, Beijing, China) for sequencing by the Ykang Biotechnology Company (Ykang, Hangzhou, China). The *cis*-elements in the obtained sequences were identified and uploaded to the NCBI (accession numbers shown in Supplemental Table S4).

### Data statistical analysis and graphic production

TTEST function in Microsoft Excel XP was used for statistical differences analysis, where $p < 0.05$ was considered significant and $p < 0.01$ was considered highly significant. Values are expressed as mean ± standard deviation. Except for the heatmaps by TBtools[23], all graphs were generated using GraphPad Prism 8 (https://www.graphpad.com).

## RESULTS

### Identification and cloning validation of tea plant MBD protein family genes

In this study, 16 genes encoding MBD domain-containing proteins were identified from the LJ43 genome databases. The basic characteristic analysis showed that the ORF of 16 *CsMBD* genes varied in size. The protein length of CsMBDs varied from 128 to 1,317 residues, with an average length of approximately 359 amino acids. Among these proteins, the smallest MBD was 128 amino acids long (CsMBD6), while the largest protein was composed of 1,317 (CsMBD9). The predicted molecular weight for CsMBD proteins ranged between 14.31 and 142.71 kDa, with an average molecular weight of approximately 47.58 kDa. The theoretical PI was between 4.54 and 9.29, while the theoretical PI values of nine proteins were smaller than 7.0. With the exception of CsMBD8, the instability index for MBD proteins isolated from tea plants was more than 40, indicating that these predicted proteins were unstable. Additionally, grand average hydropathicity (GRAVY) analysis showed that all CsMBD proteins were hydrophilic and according to their subcellular localisation, they were all localised in the nucleus (Table 1).

For cloning validation, we cloned the ORF of *MBD* gene family, with the exception of *CsMBD7*, we only obtained its partial sequences from genome DNA. Moreover, all the obtained sequences have been uploaded to the NCBI (accession number as shown in Table 1).

### Analysis of tea plant MBD protein structures

The conserved domains of MBD proteins in tea plants and *Arabidopsis* were determined using the SMART database and

**Table 1.** Basic characteristic of *MBD* genes identified in tea plants.

| Gene name | Genome ID | Accession number | ORF (bp) | Amino acids (aa) | Molecular weight (kDa) | Theoretical pI | Instability index | GRAVY | Subcellular localization |
|---|---|---|---|---|---|---|---|---|---|
| *CsMBD1* | Cha13g006360 | MW587685 | 951 | 316 | 35.02 | 4.61 | 54.26 | −1.275 | Nucleus |
| *CsMBD2* | ChaUn9803.1 | MW587686 | 984 | 327 | 36.68 | 4.79 | 58.95 | −0.689 | Nucleus |
| *CsMBD3* | Cha11g011930 | MW587687 | 624 | 207 | 23.37 | 7.55 | 48.43 | −0.780 | Nucleus |
| *CsMBD4* | Cha13g000660 | MW587688 | 564 | 187 | 22.30 | 4.76 | 46.09 | −0.971 | Nucleus |
| *CsMBD5* | Cha11g002910 | MW587689 | 750 | 248 | 27.52 | 5.58 | 45.25 | −0.919 | Nucleus |
| *CsMBD6* | Cha11g006220 | MW587690 | 387 | 128 | 14.31 | 8.73 | 50.12 | −0.834 | Nucleus |
| *CsMBD7* | Cha12g008120 | OK020039 | 543 | 180 | 21.45 | 8.62 | 43.23 | −0.756 | Nucleus |
| *CsMBD8* | Cha08g002560 | MW587691 | 1,011 | 336 | 36.19 | 8.38 | 31.07 | −0.519 | Nucleus |
| *CsMBD9* | Cha01g024050 | MZ962382 | 3,957 | 1,317 | 142.71 | 5.53 | 43.59 | −0.169 | Nucleus |
| *CsMBD10* | Cha06g012040 | MW587692 | 873 | 290 | 32.03 | 5.00 | 45.76 | −1.272 | Nucleus |
| *CsMBD11* | Cha03g006980 | MW587693 | 1,206 | 401 | 44.33 | 4.54 | 50.83 | −1.286 | Nucleus |
| *CsMBD12* | Cha14g013440 | MW587694 | 1,248 | 415 | 45.48 | 5.42 | 47.54 | −1.130 | Nucleus |
| *CsMBD13* | Cha12g001750 | MW587695 | 2,535 | 844 | 93.89 | 9.29 | 47.82 | −0.891 | Nucleus |
| *CsMBD14* | Cha14g007750 | MW587696 | 3,174 | 1,057 | 118.88 | 5.94 | 51.92 | −0.427 | Nucleus |
| *CsMBD15* | Cha11g005780 | MW587697 | 906 | 301 | 33.79 | 8.69 | 50.54 | −0.603 | Nucleus |
| *CsMBD16* | Cha04g016420 | MW587698 | 900 | 299 | 33.45 | 7.73 | 42.19 | −0.430 | Nucleus |

full protein sequences. According to structural domain analysis, 14 different motifs were determined in CsMBD proteins, while all CsMBD proteins contained the MBD domain (PF01429). Sixteen MBD proteins in tea plants were divided into eight classes according to the differences in the MBD domain in the sequences (Fig. 1) . All MBD proteins from class II and III contained a zf-CW domain related to transcriptional *cis*-acting element binding. CsMBD9 was placed in class V, which includes proteins with the most motifs, including the PHD, MBD, DDT and HSP70 domains. Interestingly, AtMBD13 fell into class VIII, which includes proteins that lack some domains. However, CsMBD13 and CsMBD14 were placed in this class even though they contain several domains. Moreover, CsMBD15 was most similar to AtMBD7, because they have two MBD motifs that share a high sequence similarity, suggesting functional equivalence.

To study the structural characteristics of the MBD proteins, the conserved MBD sequences from three species (tea plant, human, *Arabidopsis*) were aligned (Fig. 2). The results showed a high homology of amino acid sequences between the arabidopsis MBD motif of AtMBD1–AtMBD13 and that of the tea plant CsMBD1–CsMBD16. Here, we focused on these residues in the methyl cytosine binding and guanine recognition sites. Five residues (Arg22, Arg30, Asp32, Tyr34 and Arg44) were reported to be important for MBD protein DNA binding[29]. As shown in Fig. 2, these five residues were largely confined to the MBD motif both in the *Arabidopsis* and *C. sinensis* proteins. Indeed, the MBD protein sequences in the same class were also highly similar. Moreover, MBD proteins consist of four $\beta$-sheets, an $\alpha$-helix, and two hairpin loops, which form the MBD–C$^m$DNA complex. The recognition for the methyl group depends on conserved residues within the $\beta$-sheets, while the other structures are in contact with the DNA backbone[29].

## Phylogenetic analysis and duplication analysis of *CsMBD* genes

To further understand the evolutionary relationships of MBD proteins, related MBD domain sequences from four species (*O. sativa*, *Populus*, *C. sinensis* and *A. thaliana*) were chosen to generate the phylogenetic tree (Fig. 3). The result showed that the MBD proteins of these four species can be classified into eight classes. Class I and VI contained the most MBD proteins from *C. sinensis*, while class IV and VIII were comprised of two *C. sinensis* proteins. The other classes contained one protein from the tea MBD family. Phylogenetic analysis revealed sister pairs of paralog genes; for example, one At-Cs sister pair (*AtMBD10* and *CsMBD10*), one At-Pt sister pair, and one Pt-Cs sister pair. However, sister pairs from orthologous genes were more common than those from paralogous genes, which indicated that the orthologous genes may have descended from a common ancestor.

To investigate the genomic distribution of the *MBD* gene family in *C. sinensis*, the physical chromosomal locations of *CsMBD* genes were revealed. Fifteen *MBD* genes were distributed unevenly across nine of the 15 chromosomes (Fig. 4a). Notably, one gene (*CsMBD2*) was not mapped within the chromosome while it was assembled to the scaffold. Chromosome 11 contained the maximum number of genes

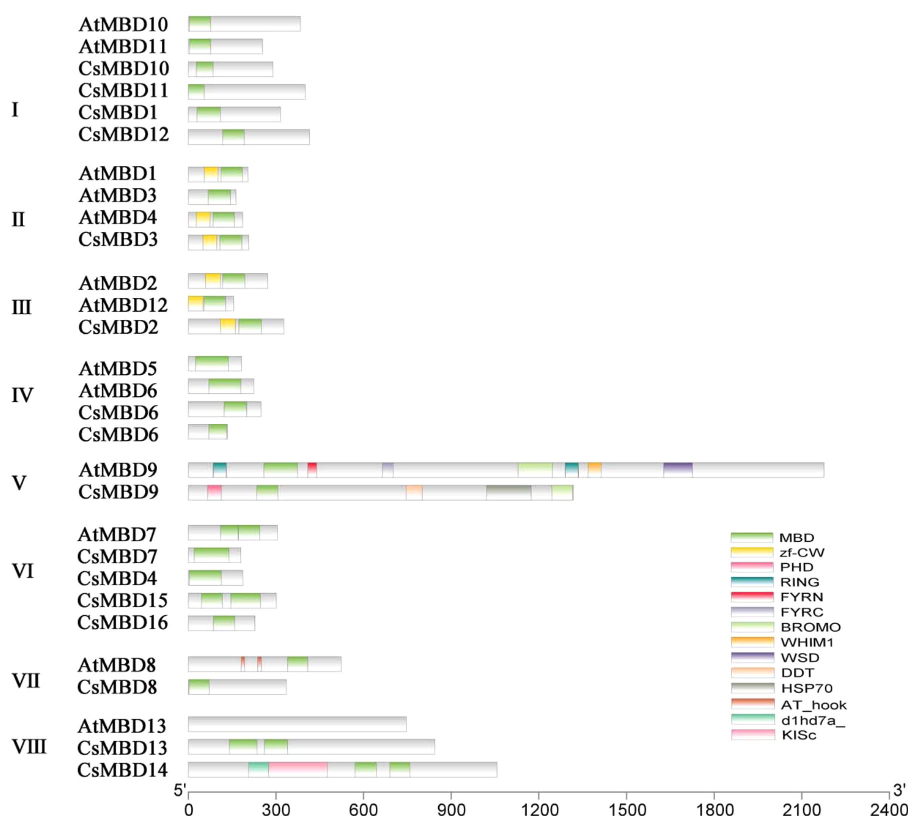


**Fig. 1** Schematic structures of MBD proteins; CsMBD1-16 from the tea plant and AtMBD1-13 from *Arabidopsis*. The conserved MBD domains are indicated with green rectangles. The length of the box represents the length of the domain. Different coloured rectangles represent different protein domains.

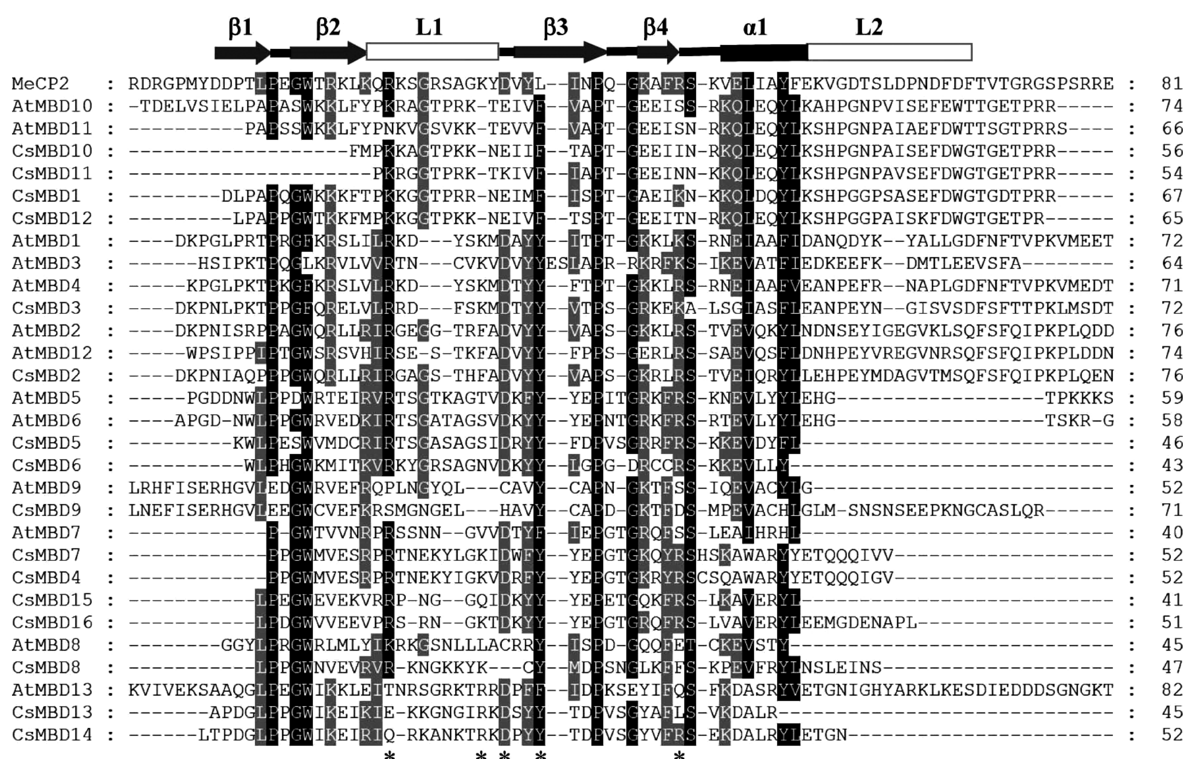


**Fig. 2** Alignment of MBD domains of the AtMBD1-13 from *Arabidopsis*, CsMBD1-16 from the tea plant and MeCP2 proteins from humans. The secondary structure for amino acid conservation is displayed at the top: four $\beta$-sheet strands ($\beta$1–4), one $\alpha$-helix, and two loop regions (L1–2). Five asterisks indicate the positions of residues that may be crucial for the binding of MBD proteins to methylated DNA.

(four), which were located in the middle of the chromosome. Chromosomes one, three, four, six, and eight only contained one *MBD* gene each, which were distributed randomly on the chromosomes. Chromosomes 12, 13, and 14 each had two *MBD* genes that were located in the middle and end of the chromosomes. Moreover, nine paralogous pairs were found in the *CsMBD* family genes (Fig. 4b). For example, *CsMBD11* and *CsMBD12* form a pair of orthologous pair. The syntenic analysis of *MBD* genes showed that 16 *CsMBD* genes were collinear with 8 *Arabidopsis* genes.

## *Cis*-acting element analysis of *CsMBD* genes

To study the potential biological functions of *CsMBD* genes, regulatory elements were identified in the promoter region with a length of 2,000 bp (Fig. 5). The *cis*-acting elements of *CsMBD* genes can be classified as light, phytohormone, stress responsiveness, and plant growth and development-related elements (Fig. 5). Among these four categories, the light responsiveness classification contained the most *cis*-acting elements, including an ACA-motif, ACE, and AE-box. With the exception of *CsMBD6*, *CsMBD8,* and *CsMBD16,* the other 13 genes contained four responsive classifications, among which *CsMBD11* had the largest number of stress-responsive elements. However, *CsMBD8* contained the most *cis*-acting elements without any regulatory elements for plant growth and development. In *CsMBD6,* we did not find any plant growth or development-related elements. In total, light and phytohormone responsive elements were the most common in all the *CsMBD* genes evaluated.

## Tissue-specific expression of *CsMBD* genes

To gain insight into their putative functions, we first analysed their expression patterns in different tissues (Fig. 6). The expression profiles of *CsMBD* genes in tissues or organs were mainly divided into three types according to cluster analysis. The first expression pattern included nine *CsMBD* genes, which had an extremely high expression in the buds and leaves. *CsMBD2/8/1* had the highest expression in the buds, and *CsMBD14/6* had the highest transcription in the leaves. The second type of expression pattern only included two genes (*CsMBD13/9*), whose expression was highest in the roots, followed by buds and leaves, and thus the lowest in the stems. *CsMBD4/10* belongs to the third expression pattern, and the expression level was the highest in the stems, followed by roots, buds, and leaves. Additionally, the expression of *CsMBD7* was not detected in the experimental samples. These results indicate that the *CsMBD* gene has three different expression patterns and obvious tissue specificity, and may play different biological roles in different tissues.

## Expression changes of *CsMBD* genes in response to biotic stresses

After *C. camelliae* infection, ten genes out of 15 *CsMBD* genes (*CsMBD1*, *CsMBD2*, *CsMBD4*, *CsMBD5*, *CsMBD6*, *CsMBD10*, *CsMBD11*, *CsMBD12*, *CsMBD15* and *CsMBD16*) were significantly upregulated after 12 h (Fig. 7) . The relative expression of other *CsMBD* genes showed no significant difference after 12 h. After *Ectropis obliqua* inoculation*,* the expression of nine genes (*CsMBD1*, *CsMBD2*, *CsMBD3*, *CsMBD5,*

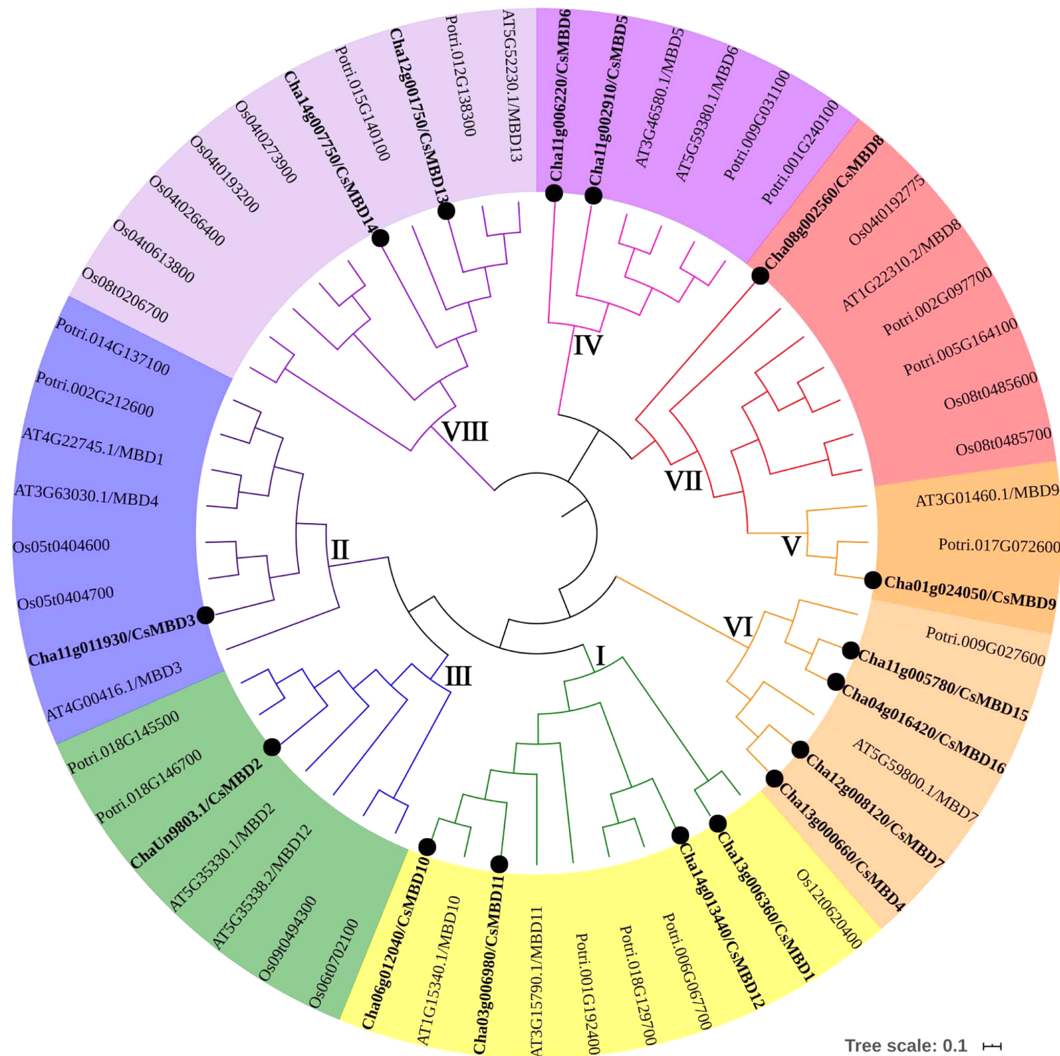


**Fig. 3** Phylogenetic tree of MBD proteins in tea plants (16), poplar (15), rice (14) and *Arabidopsis* (13). The unrooted tree was created using MEGA7.0 by the neighbour-joining method. The bootstrap value = 1,000.

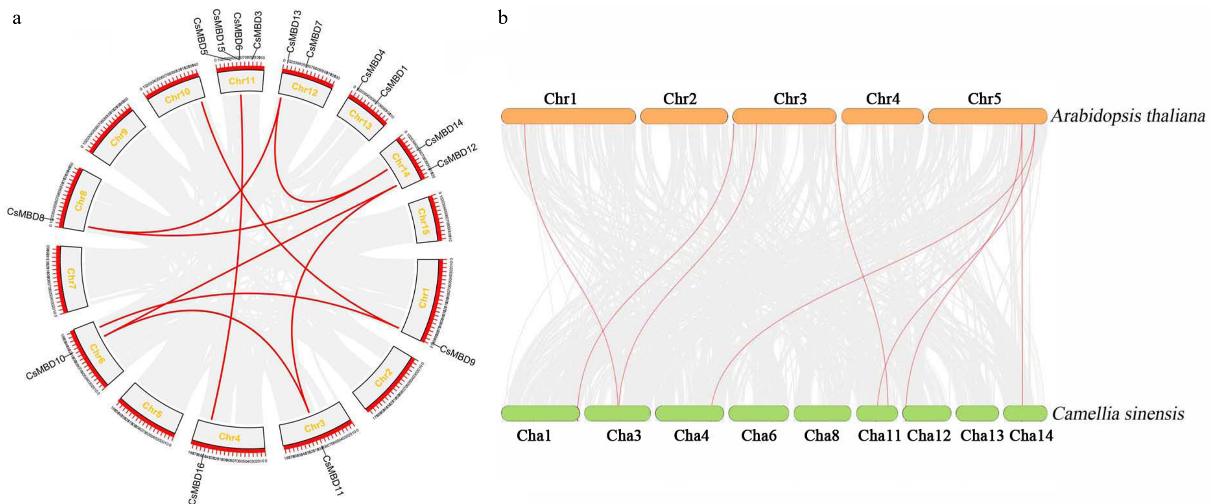


**Fig. 4** Duplication analysis of *MBD* genes in tea plants and *Arabidopsis*. (a) Synteny analysis of 16 *CsMBD* genes in tea plants. (b) Synteny analysis of *MBD* genes between tea plants and *Arabidopsis*. Gray lines in the background indicate the collinear blocks within the tea plant and other plant genomes, while the red lines highlight the syntenic *MBD* gene pairs.

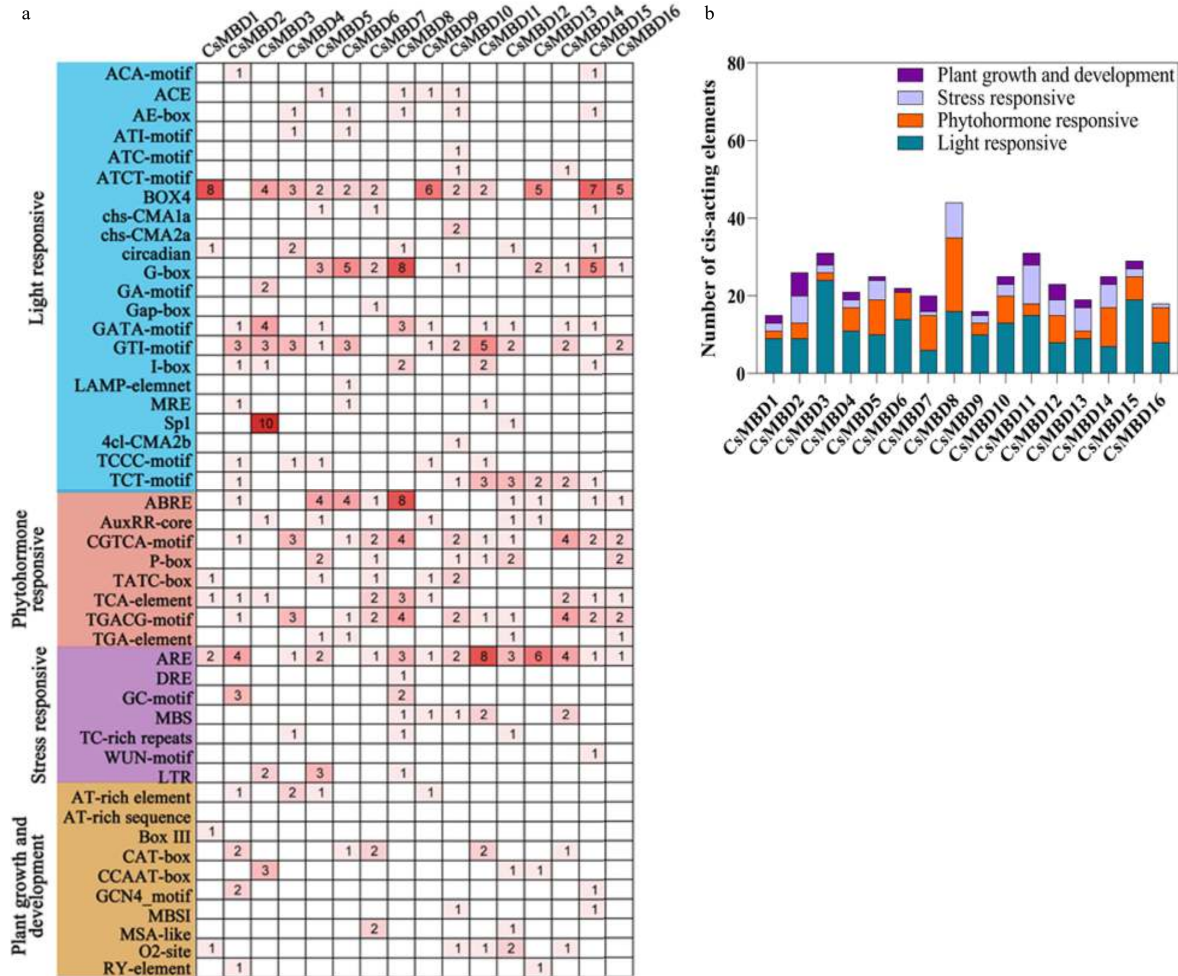

**Fig. 5** Analysis of *cis*-acting elements in the promoter region of 16 *CsMBD* genes. (a) The number of different *cis*-acting regulatory elements in the promoter region. (b) The histogram of *cis*-acting elements in four categories.

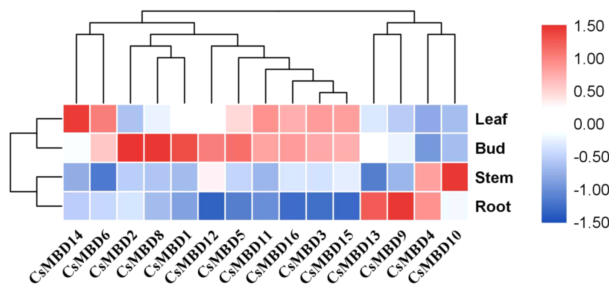


**Fig. 6** Tissue-specific expression profile clustering of 15 *CsMBD*s. The colour scale indicates $\log_2$ signal values (right); blue and red colours indicate low and high levels of transcript abundance, respectively. The different organs are noted on the right. A cluster dendrogram is shown above.

*CsMBD9, CsMBD10, CsMBD13*, *CsMBD14 and CsMBD16*) was significantly high after 12 h, while only three genes (*CsMBD3*, *CsMBD5* and *CsMBD9*) were significantly upregulated after 24 h. However, except for *CsMBD3*, *CsMBD4, CsMBD5, CsMBD8* and *CsMBD10*, we observed no significant changes in the transcript abundance of other genes between the infected and control plants after the *Pseudopestalotiopsis camelliae-sinensis* infection. Interestingly, the expression of *CsMBD3* was significantly induced by *Ectropis obliqua* and *Pseudopestalotiopsis camelliae-sinensis*, while the expression of *CsMBD8* was significantly induced by *Pseudopestalotiopsis camelliae-sinensis* infection.

## Expression profiles of *CsMBD* genes under abiotic stress conditions

Specifically, ten genes showed no significant changes in expression in response to cold stress, while five genes (*CsMBD1*, *CsMBD5*, *CsMBD11*, *CsMBD15 and CsMBD16*) were significantly induced (Fig. 8a). Under drought conditions, we observed that the genes of this family showed four expression patterns. The transcriptional abundance of six *CsMBD* genes (*CsMBD1*, *CsMBD5*, *CsMBD11*, *CsMBD15, CsMBD13 and CsMBD9*) reached its maximum after 18 d of drought (Fig. 8b). Moreover, the expression of five genes (*CsMBD14*, *CsMBD2*, and *CsMBD10*) were significantly upregulated after 15 d of drought. The transcript abundance of *CsMBD6* and *CsMBD12* were significantly upregulated after 12 d, while the transcript abundance of *CsMBD4* and *CsMBD8* showed no significant changes after being subject to drought conditions. Interestingly, *CsMBD9* expression was upregulated by drought treatment after 9 d, with a relative expression level that was approximately 80 times higher than that measured at 0 d, and

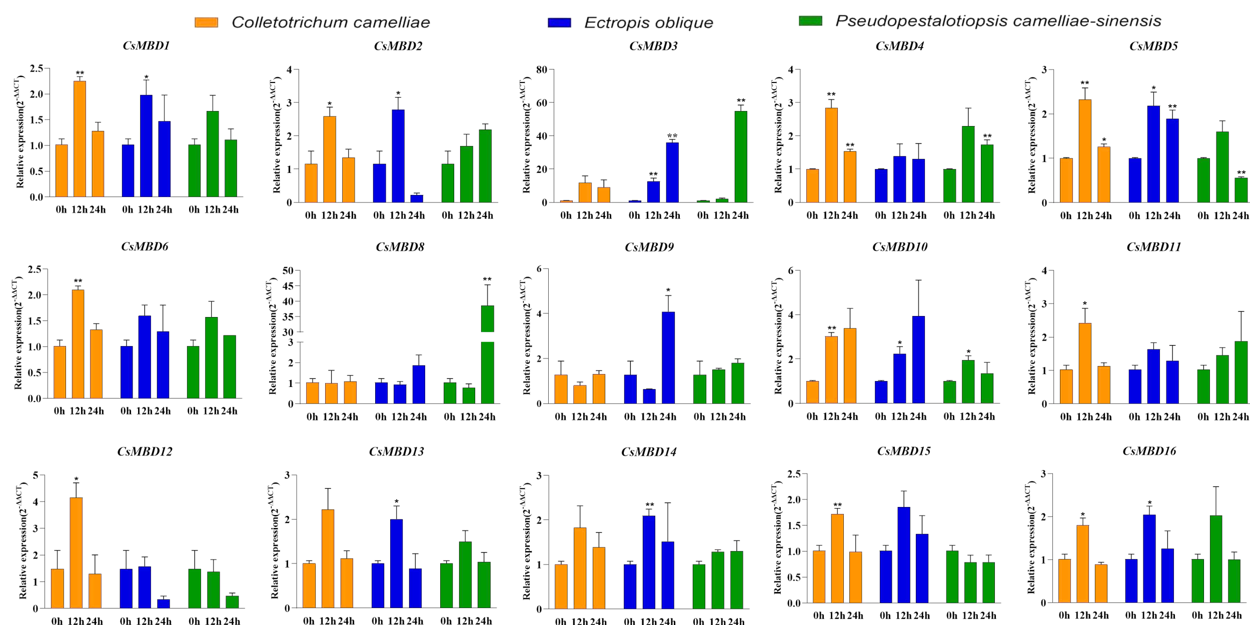


**Fig. 7** Expression profiles of 15 *CsMBD* genes under biotic stress treatments in tea plants. The different colours indicate different plant diseases and insect pests. The error bars indicate the standard error of the mean (SEM) from three biological replicates. * Significant differences ($p < 0.05$), ** highly significant differences ($p < 0.01$).

it remained high until 18 d. These findings revealed that most of *CsMBD* genes were induced by drought stress.

### Expression analysis of *CsMBD* genes related to growth and development

Among the 15 genes evaluated, the expression profiles of three genes (*CsMBD2*, *CsMBD3,* and *CsMBD4*) showed a significant increase in expression during seed germination, whereas the relative expression of *CsMBD10* decreased (Fig. 9). During floral development, the majority of *CsMBD* genes were downregulated, with only *CsMBD9* showing no obvious difference in expression during the different stages of flower development (Fig. 9). Furthermore, *CsMBD4* and *CsMBD12* expression increased, while the other 12 genes decreased in expression. These findings revealed that the *CsMBD* genes exhibit different expression patterns during floral development.

During winter dormancy, the expression of *CsMBD* genes in axillary buds exhibited two different patterns (Fig. 9). The results showed that 6 *CsMBD* genes (*CsMBD1/8/12/5/11/6*) were expressed at their lowest levels in axillary buds during the endodormancy stages, while their expression levels were significantly increased during the ecodormancy stages. This expression pattern is highly correlated with the physiological state of bud dormancy. These results indicated that *CsMBD* genes are involved in bud dormancy in tea plants, especially in the process of bud dormancy release.

## DISCUSSION

### Structural and evolutionary relationships of *CsMBD* genes

DNA methylation is essential in the response to environmental stress as well as to the developmental process. To better understand epigenetic regulation associated with environmental stimuli and plant growth and development, it is essential to study the methylation of MBD proteins. For now, only a limited number of *MBD* genes have been identified in plants, including 13 in *Arabidopsis*, 18 in tomato[11], 14 in maize[12], and 17 in rice[11]. In our study, a total of 16 *MBD* genes were identified and characterised in the tea plant genome, which possess typical characteristics of MBD proteins. These identified proteins were divided into eight subclasses based on phylogenetic analysis, and were consistent with *Arabidopsis* and populus. Moreover, genes belonging to the same subclass share similar structures. For example, class IV, V, and VII, *Arabidopsis* and tea plants owned equal numbers of *MBD* genes. However, subclass V included an MBD protein found in dicots but not monocots[10]. It is possible that either the *MBD* gene was inserted into the dicot genome, or lost from the monocot lineage. These results revealed that the evolution of *MBD* genes is related to the difference between monocots and dicots. In spite of the fact that plant genomes vary widely in size, such as the tea genome (approximately 3 Gb), rice (approximately 389 Mbp), and *Arabidopsis* (approximately 125 Mbp), the number of *MBD* genes were similar among these species, suggesting that MBD proteins are highly conserved throughout evolution. According to the synteny analysis of *MBD* genes between tea plants and *Arabidopsis*, we speculated some multicope genes and unicope genes during the evolution of tea plants. *AtMBD1* and *AtMBD3* were lost in the long process of evolution, which might be due to other DNA methylation reader genes taking over the biological functions of these two reader genes, or another alternative mechanism for DNA methylation. While four genes (*CsMBD1*, *CsMBD4, CsMBD8* and *CsMBD 10*) were novel genes, which suggests that these genes may have evolved new functions or a more complex mechanism.

*Cis*-acting element analysis defined four categories of elements in *CsMBDs*, including stress, phytohormone, light,

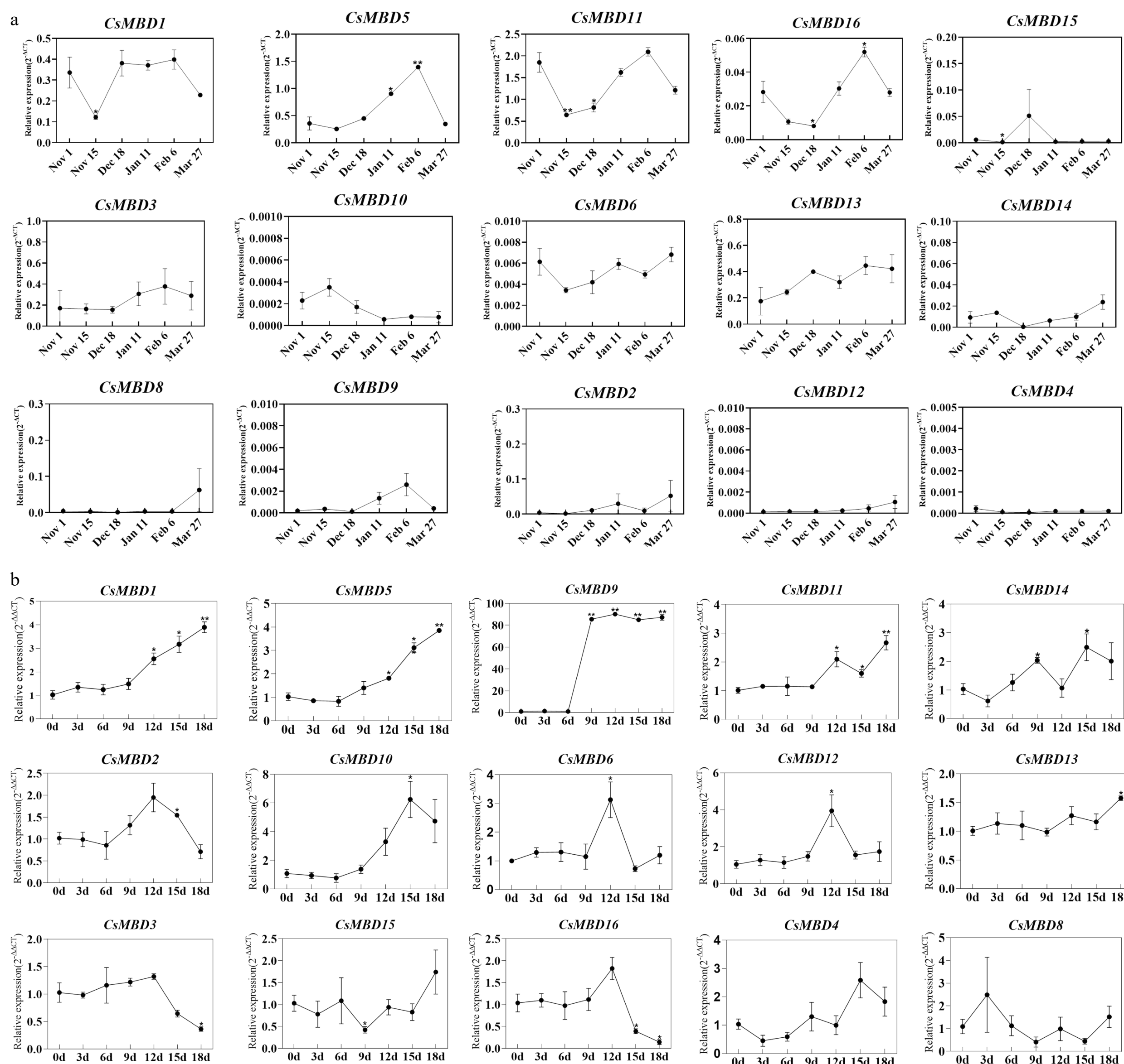


**Fig. 8** Relative expression of 15 *CsMBD* genes under abiotic stresses in tea plants. (a) Cold acclimation; (b) drought stress conditions. The y-axis represents the transcript abundance, and the x-axis represents the stress treatments (days or date). The error bars indicate the standard error of the mean (SEM) from three independent biological replicates. * Significant differences ($p < 0.05$), ** highly significant differences ($p < 0.01$).

and developmental response. These regulatory elements suggest that *MBD* genes have diverse roles in biological processes. Among these *cis*-acting elements, the TCA-element, involved in salicylic acid responsiveness, is commonly found in most *MBD* gene promoter regions and plays an indispensable role in response to biotic stress. In addition, RY-elements, related to seed-specific regulation, were found in *CsMBD2* and *CsMBD13* promoter regions, which indicates that these two genes are likely to be important in seed development. In tomato, the accumulation of *SlMBD* transcripts gradually decreased in wild types during fruit ripening and ethylene-responsive *cis*-elements were commonly found in these gene promoters, suggesting that these genes can be induced by exogenous ethylene[30]. Furthermore, the overexpression of *SlMBD5* caused pleotropic developmental changes, including darker green fruits due to the accumulation of plastids and elevated pigmentation, as well as an increased abundance of *SlGLK2*, which is essential in the regulation of plastid biogenesis[31]. In *Arabidopsis*, the overexpression of *AtMBD8* resulted in delayed flowering during both long and short days in the C24 background[32]. Moreover, the *atmbd9* mutant presented multiple phenotypes such as early flowering and the promotion of branches. These findings indicate that *MBD* genes participate in the regulation of plant growth, as well as in the responses to environmental stimuli.

## Genes encoding MBD proteins are involved in biotic stress responses

Plants showed genome-wide DNA methylation alterations when they were infected with pathogens and colonised by microorganisms. In *A. thaliana*, mild but widespread differential DNA methylation was observed after exposure to pathogens[33]. The DNA demethylase triple mutant

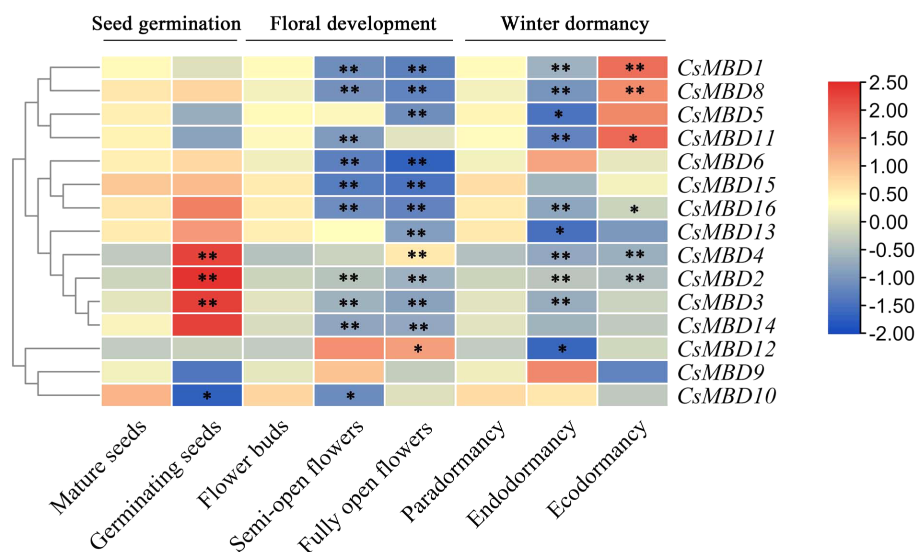


**Fig. 9** Expression patterns of 15 *CsMBD* genes during growth and development in tea plants. Including seed germination, floral development and different dormancy stages in tea plants; $p < 0.05$ was considered significant (*) and $p < 0.01$ was considered highly significant (**). The colour scale indicates the $\log_2$ signal values shown on the right. Red and blue indicate high and low levels of expression, respectively. A cluster dendrogram is shown on the left of the graph.

*ros1–dml2–dml3* was more susceptible to *Fusarium oxysporum* than the wild type *A. thaliana*[34]. Moreover, most genes of biotic stress responses are repressed in *ros1–dml2–dml3* plants, potentially because their promoters are enriched with small transposons[34], which suggests that DNA methylation plays an essential role in regulating the transcription of defence genes[5]. Among the biotic treatments tested, most *CsMBD* genes were significantly induced by *Colletotrichum camelliae* and were upregulated in the leaves 12 h after inoculation, indicating that these genes respond to *C. camelliae* infection by regulating DNA methylation. *CsMBD3* and *CsMBD8* are most significantly induced by a *P. camelliae-sinensis* infection; the promoter of *CsMBD8* contains eight *cis*-acting elements involved in methyl jasmonate-responsiveness (MeJA), such as the GGTCA and TGACG motifs. MeJA can enhance the defensive compounds synthesis and initiate transcription of pathogenesis-related genes for resistance[35]. Therefore, *CsMBD8* is a candidate gene for understanding the epigenetic regulation system that is involved in biotic stress in tea plants.

## Putative genes in the *MBD* family respond to abiotic stress

Numerous studies have shown that plants utilize epigenetic mechanisms to fine-tune their responses to abiotic stress[36]. Chromatin interaction, histone modification, and DNA methylation during cold and drought stress have been studied extensively. For example, in *A. thaliana*, the chromatin remodelling protein, PICKLE (PKL), is involved in the CBF-dependent cold stress response[37]. Moreover, the expression of two cold-responsive genes, *COR15A* and GALACTINOL SYNTHASE 3 (*GOLS3*), are accompanied by a decrease in H3K27me3 deposition in cold stress conditions[38]. Histone acetylation also plays an important role in the cold response; *HvDME* was found to be induced in response to drought treatment in barley[39]. We found that four genes (*CsMBD1*, *CsMBD5*, *CsMBD11*, and *CsMBD16*) were significantly induced by cold acclimation and most of the *CsMBD* genes were dynamic regulated between 0 and 18 d of drought stress, except for *CsMBD1*, *CsMBD5*, and *CsMBD11*. Interestingly, the transcript abundance of *CsMBD5* was upregulated in response to both cold and drought stress. *CsMBD5* contains three LTR and two ABRE *cis*-acting elements that are involved in low temperature responsiveness and abscisic acid responsiveness, respectively. Therefore, *CsMBD5* most likely participates in cold and drought responses. Moreover, the expression level of all DNA demethylase genes in tea plants significantly increased at certain times between 0 and 48 h of drought stress and the expression of genes encoding DNA methyltransferase was significantly inhibited[19]. These results demonstrate that DNA methylation may regulate the abiotic stress responses in tea plants. There is no doubt that DNA demethylase and genes encoding MBD proteins are differentially regulated. The inhibition of DNA methyltransferase promotes the demethylation of gene abiotic stress-related responses, induces their expression and promotes abiotic stress tolerance.

## *CsMBD* genes are involved in tea plant growth and development

DNA methylation is essential for regulation in plant growth, but how MBD proteins participate in these processes is complex and the mechanism is still unclear. In *Arabidopsis*, transposable elements cause significant accumulation and loss of CHH methylation during seed development and germination, respectively[40]. Those genes involved in RdDM and CMT2 pathways are highly expressed during seed development, and DNA demethylases are not expressed during germination[40]. In tea plants, three *CsMBD* genes (*CsMBD2*, *CsMBD3*, and *CsMBD4*) were upregulated during seed germination. Typically, *CsMBD2* is expressed only in buds and our promoter analysis revealed that the *CsMBD2* promotor contains *cis*-regulatory elements related to endosperm expression and seed-specific regulation, such as

the RY-element and the GCN4 motif. These results suggest that *CsMBD2* may regulate seed germination through participation in the histone acetylation pathway. During *Arabidopsis* flower development, the CG, CHG, and CHH sites were methylated and accounted for 17.5%, 13.7% and 5.2% of the 2,4035 genes, respectively[41]. Numerous cytosine sites were methylated during the meristem to the early flowering developmental stages[41]. Consistent with our study, the expression of most MBD protein-encoding genes in tea plants was suppressed in the flower bud to the semi-open flower bud stages.

Epigenetic processes play crucial roles in the regulation of the transition between bud set and burst[42,43]. Genomic DNA methylation patterns are associated with the state of shoot apical cells when poplar shifts to the dormant stage from the growing stage, with *PtaDML10* expression levels the highest during dormancy release[43]. In tea plants, five *CsMBD* genes were maximally expressed in the bud break stage. Among these genes, *CsMBD8* was specifically expressed only in the buds and was shown to contain eight ABRE *cis*-acting elements related to abscisic acid responsiveness (ABA). ABA is a widely reported exogenous hormone that regulates bud dormancy[44]. The results indicate that MBD proteins may be engaged in the regulation of the growth cycle from winter dormancy to bud growth in plants.

## CONCLUSIONS

The characterisation, features, and amino acid conservation of the *CsMBD* genes have provided new possibilities for improving light, phytohormone, stress, and plant growth and development responsiveness in tea plant crops. Comprehensive gene expression profiling of all *CsMBD* genes in tea plants was performed in different tissues and gene expression patterns were measured in leaves from plants grown under abiotic and biotic stress during different stages of development. We identified several *CsMBD* genes as candidates for further investigation due to their performance during plant stress responses and plant growth development. *CsMBD8* could be a candidate gene for investigating the epigenetic mechanism of abiotic stress and bud dormancy, while *CsMBD5* could serve as a model for understanding epigenetic mechanisms that are related to abiotic stress. In addition, *CsMBD2* could also be used to study the epigenetic mechanisms related to seed germination. Overall, these findings provide valuable clues for identifying the specific functions of this gene family and the genomic diversity among different tea plant genotypes (Supplemental Fig. S1).

### Data availability statement

The accession number of *CsMBD* genes sequences were obtained as shown in Supplemental Table S2, and accession number for cloning promoter sequences of *CsMBD* genes were exhibited in Supplemental Table S4.

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### Conflict of interest

The authors declare that they have no conflict of interest.

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