Genome-Wide Characterization of the Methyl CpG Binding Domain-Containing Proteins in Watermelon and Functional Analysis of Their Roles in Disease Resistance Through Ectopic Overexpression in Arabidopsis thaliana

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Methyl-CpG-Binding Domain (MBD) proteins play important roles in plant growth, development, and stress responses. The present study characterized the MBD families in watermelon and other cucurbit plants regarding the gene numbers and structures, phylogenetic and syntenic relationships, evolution events, and conserved domain organization of the MBD proteins. The watermelon ClMBD proteins were found to be localized in nucleus, and ClMBD2 and ClMBD3 interacted with ClIDM2 and ClIDM3. ClMBD2 bound to DNA harboring methylated CG sites but not to DNA with methylated CHG and CHH sites in vitro. The ClMBD genes exhibited distinct expression patterns in watermelon plants after SA and MeJA treatment and after infection by fungal pathogens Fusarium oxysporum f.sp. niveum and Didymella bryoniae. Overexpression of ClMBD2, ClMBD3, or ClMBD5 in Arabidopsis resulted in attenuated resistance against Botrytis cinerea, accompanied by down-regulated expression of AtPDF1.2 and increased accumulation of H$_2$O$_2$ upon B. cinerea infection. Overexpression of ClMBD1 and ClMBD2 led to down-regulated expression of AtPR1 and decreased resistance while overexpression of ClMBD5 resulted in up-regulated expression of AtPR1 and increased resistance against Pseudomonas syringae pv. tomato DC3000. Transcriptome analysis revealed that overexpression of ClMBD2 in Arabidopsis up-regulated the expression of a small set of genes that negatively regulate Arabidopsis immunity. These data suggest the importance of some ClMBD genes in plant immunity and provide the possibility to improve plant immunity through modification of specific ClMBD genes.

Keywords: watermelon (Citrullus lanatus L.), methyl-CpG-binding domain (MBD) protein, ClMBD2, disease resistance, DNA methylation, Arabidopsis
INTRODUCTION

As sessile organisms, plants have to face invasive attacks from diverse pathogenic microorganisms in the environment. To defend these pathogenic invasions, plants have evolved a complicated but fine-tuned innate immune system (Jones and Dangl, 2006; Yuan et al., 2021; Ngou et al., 2022). The first layer of the innate immunity, called PAMP-triggered immunity (PTI), is triggered by the recognition of microbial patterns via cell surface-localized pattern-recognition receptors, while the second layer, called effector-triggered immunity (ETI), is activated by the direct or indirect interaction between predominantly intracellularly localized nucleotide-binding leucine-rich repeat receptors and pathogen effectors. Activation of PTI and/or ETI is fine-tuned by a complicated molecular and genetic network, in which epigenetic regulation including DNA methylation/demethylation play critical roles (Huang and Jin, 2022).

Methylation of DNA, a conserved epigenetic mark, is one of the main mechanisms that play critical roles in epigenetic regulation of various biological processes including plant growth, development, and response to environmental cues (He et al., 2011; Arıkan et al., 2018; Zhang et al., 2018). Among DNA methylation, cytosine methylation (5-mC) is the most common epigenetic phenomenon that regulates the fate of gene expression (Grafi et al., 2007; Zhang et al., 2018). In higher plants, 5-mC occurs in CG dinucleotide regions, and CHG/CHH (H represents A, T, or C) trinucleotide regions (Gruenbaum et al., 1981). DNA methylation is a dynamic process that are achieved by different enzymes (Moore et al., 2013; Zhang et al., 2018) and is involved in many molecular processes, including genome stability, gene regulation, transposon silencing, and chromosome interactions (Zhang et al., 2006; Cokus et al., 2008; Lang et al., 2017). Extensive studies have indicated that DNA methylation plays important roles in plant growth and development, such as vegetable growth, pattern formation, flowering time, seed development, and fruit ripening (Gehring et al., 2009; He et al., 2011; Ibarra et al., 2012; Arıkan et al., 2018; Zhang et al., 2018) as well as in abiotic stress responses (Rambani et al., 2015; Xu et al., 2015; Yong-Villalobos et al., 2015; Hewezi et al., 2017). Importantly, DNA methylation, as one of the epigenetic regulation mechanisms, plays crucial roles in plant immunity (Huang and Jin, 2022); for example, Arabidopsis mutants with DNA hypomethylation are called effector-triggered immunity (ETI), is activated by the direct or indirect interaction between predominantly intracellularly localized nucleotide-binding leucine-rich repeat receptors and pathogen effectors. Activation of PTI and/or ETI is fine-tuned by a complicated molecular and genetic network, in which epigenetic regulation including DNA methylation/demethylation play critical roles (Huang and Jin, 2022).

Methylation of DNA, a conserved epigenetic mark, is one of the main mechanisms that play critical roles in epigenetic regulation of various biological processes including plant growth, development, and response to environmental cues (He et al., 2011; Arıkan et al., 2018; Zhang et al., 2018). Among DNA methylation, cytosine methylation (5-mC) is the most common epigenetic phenomenon that regulates the fate of gene expression (Grafi et al., 2007; Zhang et al., 2018). In higher plants, 5-mC occurs in CG dinucleotide regions, and CHG/CHH (H represents A, T, or C) trinucleotide regions (Gruenbaum et al., 1981). DNA methylation is a dynamic process that are achieved by different enzymes (Moore et al., 2013; Zhang et al., 2018) and is involved in many molecular processes, including genome stability, gene regulation, transposon silencing, and chromosome interactions (Zhang et al., 2006; Cokus et al., 2008; Lang et al., 2017). Extensive studies have indicated that DNA methylation plays important roles in plant growth and development, such as vegetable growth, pattern formation, flowering time, seed development, and fruit ripening (Gehring et al., 2009; He et al., 2011; Ibarra et al., 2012; Arıkan et al., 2018; Zhang et al., 2018) as well as in abiotic stress responses (Rambani et al., 2015; Xu et al., 2015; Yong-Villalobos et al., 2015; Hewezi et al., 2017). Importantly, DNA methylation, as one of the epigenetic regulation mechanisms, plays crucial roles in plant immunity (Huang and Jin, 2022); for example, Arabidopsis mutants with DNA hypomethylation are more resistant to Pseudomonas syringae pv. tomato (Pst) DC3000 and exhibit an elevated salicylic acid (SA)-dependent response (Dowen et al., 2012; Yu et al., 2013; Cambiagno et al., 2021).

In epigenetic model, proteins of the Methyl-CpG-binding domain (MBD) family are a group of key interpreters of DNA methylation and are generally associated with transcriptional silencing (Law and Jacobsen, 2010; He et al., 2011). The MBD proteins typically contain an MBD domain, with the ability to bind to 5-mC DNA (Ohki et al., 2001; Zemach and Grafi, 2003). Generally, the MBD proteins recognize 5-mC and recruit histone deacetylases, chromatin remodelers, and histone methyltransferases to repress transcription (Gigek et al., 2016). Genes coding for MBD proteins have been characterized in some plant species including Arabidopsis, rice, maize, poplar, potato, tomato, petunia, common bean, soybean, and rapeseed (Grafi et al., 2007; Parida et al., 2018; Coelho et al., 2022; Shi et al., 2022; Xiao et al., 2022); for example, 13 AtMBDs in Arabidopsis, 17 OsMBDs in rice, 14 ZmMBDs in maize, and 14 PtMBDs in poplar were identified (Grafi et al., 2007). The Arabidopsis AtMBD proteins can be divided into different subclasses (Zemach and Grafi, 2003; Springer and Kaeppler, 2005). AtMBD1, 2, 4, 8, 11 cannot specifically bind to 5-mC DNA, while AtMBD4 and AtMBD11 bind to methylated and unmethylated DNAs with or without 5-mC (Ito et al., 2003; Grafi et al., 2007). AtMBD5, 6, 7 show specific binding ability to mCG sites in vitro (Zemach and Grafi, 2003). Additionally, AtMBD5 also binds to mCHH sites while AtMBD6 binds to non-specific mCHH and mCHG sites (Ito et al., 2003). The binding activity and specificity have not been established for AtMBD3, 9, 10, 12, and 13 (Ito et al., 2003; Scebba et al., 2003; Grafi et al., 2007). Recently, it was demonstrated that AtMBD6 and AtMBD7 are actually readers for methylated DNA (Wu et al., 2022). AtMBD5 and AtMBD6, which are closely related and may have redundant functions (Berg et al., 2003), are recruited to chromatin by recognition of CG methylation to redundantly repress a subset of genes and transposons (Ichino et al., 2021), or participate in the formation of HDAC complexes to modulate the chromatin structure and gene transcription (Zemach et al., 2005). AtMBD6 also functions in RNA-mediated gene silencing (Parida et al., 2017). AtMBD7 interacts with the histone acetyltransferase Increased DNA Methylation 1 (IDM1) and its partners Increased DNA Methylation 2 (IDM2) and Increased DNA Methylation 3 (IDM3), and participates in DNA demethylation (Lang et al., 2015; Wang et al., 2015). Furthermore, AtMBD7 is also required for the H3K18 and H3K23 acetylation (Li Q., et al., 2015). AtMBD9 recognizes histone acetylation marks by IDM1 and functions in H2A.Z deposition (Nie et al., 2019). Furthermore, the biochemical activities of maize ZmMBD101 and tomato SbMBD5 have also been recently established (Li Y. et al., 2015; Questa et al., 2016).

The functions of MBD genes in plant growth, development, and response to abiotic stress have been explored. Mutation in AtMBD8 or knockdown of AtMBD11 led to a delay in flowering time, while the atmbd9 mutant showed a significantly earlier flowering time (Berg et al., 2003; Peng et al., 2006; Stangeland et al., 2009). Overexpression of Salix viminalis L. SvMBD5 led to an early flowering phenotype in transgenic Arabidopsis (Cheng et al., 2020). These observations indicate that the MBD proteins play critical roles in regulation of flowering in plants. The AtMBD11 knockdown mutant also displayed a variety of phenotypic effects, e.g., aerial rosettes, serrated leaves, abnormal position of flowers, and fertility problems (Berg et al., 2003), while the atmbd9 mutants produced more shoot branches (Peng et al., 2006). Overexpression of OsMBD707 leads to larger tiller angles and reduced photoperiod sensitivity in rice (Qu et al., 2021). The atmbd4 mutant exhibited altered root architecture and up-regulated expression of many phosphate transporters and transcription factors, indicating that AtMBD4 negatively regulates the phosphate starvation response (Parida et al., 2019). Some of the wheat TaMBD genes and most of the petunia PhMBD genes were highly induced by abiotic stress and hormones (Hu et al., 2011; Shi et al., 2016, 2022). However,
the possible involvement of the MBD genes in plant immunity remains elusive.

Watermelon (*Citrullus lanatus* L.) is one of important horticultural crops, providing favorite fresh fruits worldwide. Fusarium wilt, caused by *Fusarium oxysporum* fsp. *niveum* (*Fon*), and gummy stem blight, caused by *Didymella bryoniae* (*Db*), are two of the most devastating fungal diseases that lead to significant yield losses in watermelon industry (Michielse and Rep, 2009; Keinath, 2011). However, knowledge on the molecular mechanism of resistance in watermelon against *Fon* and *Db* is currently limited, which significantly impedes the breeding for watermelon cultivars with improved resistance against these two fungal diseases. The present study aimed to identify the watermelon CMBD family by characterization and expression analyses and explore the putative mechanism of the CMBD family in disease resistance. The transcript levels of the CMBD genes were changed after treatment with SA and methyl jasmonate (MeJA) and infection by *Fon* and *Db*. Functional analyses revealed that CMBD2, CMBD3, and CMBD5 negatively regulate resistance against *Botrytis cinerea* and that CMBD1 and CMBD2 negatively while CMBD5 positively regulate resistance against *Pst* DC3000 in Arabidopsis.

### MATERIALS AND METHODS

#### Plant Materials and Growth Conditions

Watermelon (*Citrullus lanatus*) cv. Zaojia was used for all experiments. *Nicotiana benthamiana* plants expressing a known nucleus-localized marker protein RFP-H2B (Chakrabarty et al., 2007) were used for subcellular localization and bimolecular fluorescence complementation (BiFC) assays. Plants were grown in a soil mix (clay : soil = 3:1) in a growth room under fluorescent light (200 µE m⁻² s⁻¹) at 22–24°C with 70% relative humidity (RH) and a 14 h light/10 h dark cycle. Arabidopsis seeds were surface sterilized in 75% ethanol for 5 min and 4% sodium hypochlorite for 10 min, rinsed with sterile water for three times, sowed on 1/2 MS plates and vernalized for 2 days at 4°C. Arabidopsis seedslings were grown on 1/2 MS plates at 22°C with 75% RH with a 16 h light/8 h dark cycle for 7 days and then transferred to a soil mix (clay : soil = 1:1) in a growth room at 22°C with 75% humidity under a 16 h light/8 h dark cycle for normal growth or under a 8 h light/16 h dark cycle for disease assays.

#### Hormone Treatment and Pathogen Inoculation for Gene Expression Analysis in Watermelon

For analysis of tissue-specific expression, leaf, stem and root samples of 4-week-old watermelon plants were collected and stored at −80°C till use. For SA and MeJA treatment, 4-week-old watermelon plants were treated by foliar spraying with 1 mM SA, 100 µM MeJA or an equal volume of solution containing only 0.1% ethanol and 0.02% Tween-20 as controls, and leaf samples were collected at different time points after treatment.

For analysis of gene expression in response to *Fon* infection, pathogen inoculation was performed according to a previously reported method (Song et al., 2015). Briefly, mycelial plugs from 6-day-old culture of *Fon* race 1 strain ZJ1 were transferred into 200 mL mung bean liquid broth (mung bean 20 g/L, boiled for 20 min, pH7.0) and incubated with shaking (250 rpm) at 26°C for 2 days. Spores were collected and spore suspension was adjusted to 1 × 10⁷ spores/mL for inoculation. Three-week-old watermelon plants were uprooted, washed in tap water, the main roots were cut up of one-third, and then dipped for 15 min in *Fon* spore suspension or in mung bean liquid broth as mock-inoculated controls. The inoculated plants were replanted in soil and allowed to grow in the same growth room as described above. Root samples were collected, frozen in liquid nitrogen and stored at −80°C until use.

*Db* strain DBTL4 was grown at 26°C on PNA (potato 200 g/L, NH₄H₂PO₄ 2 g/L, agar 15 g/L, pH7.0) for 6–7 days in dark and then treated with a 12 h UV light/12 h dark cycle for 5 days to induce spore production. After induction, the mycelial plugs were picked into distilled water, spores were collected and the spore suspension was adjusted to 2 × 10⁸ spores/mL. Five-week-old watermelon plants were foliar sprayed with *Db* spore suspension containing 0.05% Tween-20 or with an equal volume of 0.05% Tween-20 solution as mock controls. The inoculated plants were placed in a 22°C chamber with 100% RH for 48 h. Leaf samples were collected, frozen in liquid nitrogen, and stored at −80°C until use.

#### Identification of Watermelon CMBD Genes and Proteins

Arabidopsis AtMBD protein sequences were obtained from TAIR1 and were used as queries to search for putative MBD genes and proteins in watermelon, melon, cucumber, pumpkin, and zucchini genomes at Cucurbit Genomics Databases. The obtained nucleotide and protein sequences were examined by domain analysis programs PFAM1 (PF01429) and SMART4 with the default cutoff parameters. The isoelectric points and molecular weights were predicted on the ExPASy Proteomics Server. Sequence alignment was carried out by the ClustalX program. Phylogenetic trees were constructed using the neighbor-joining method of the MEGA7 program with the p-distance and complete deletion option parameters.

#### Synteny Analysis of the CMBD Genes

The reliability of the obtained trees was tested using a bootstrapping method with 1,000 replicates. The MCScanX algorithm with default parameters (Wang et al., 2012) was used to scan orthologous regions containing the watermelon CMBD genes. The corresponding plot was created by Dual Synteny Plot for MCscanX in TBtools software (Chen et al., 2020). The chromosomal localization of CMBDs in the *C. lanatus* genome

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1https://www.arabidopsis.org
2http://www.icugi.org/
3http://pfam.xfam.org
4http://smart.embl-heidelberg.de
5http://expasy.org/
was obtained by TBtools software (Chen et al., 2020) according to the annotation data of the *C. lanatus* genome. The genomic and annotation data of melon, cucumber, zucchini, and pumpkin were downloaded from the Cucurbit Genomics Database (see text footnote 2), and those of Arabidopsis were downloaded from TAIR (see text footnote 1). The syntenic relationship of the orthologous *MBD* genes obtained between watermelon and other selected species was visualized by the Advanced Circos package of TBtools (Chen et al., 2020). Dnasp software was used to calculate the non-synonymous (Ka)/synonymous (Ks) values of the duplicated *CIMBD* gene pairs (Librado and Rozas, 2009).

### Cloning of the CIMBD Genes

Total RNA was extracted using RNA Isolater reagent (Vazyme, Nanjing, China) according to the manufacturer's instructions. RNA was treated with RNase-free DNase and then reverse-transcribed into cDNA using the HiScript QRT SuperMix kit (Vazyme, Nanjing, China). The obtained cDNAs were used for cloning, semi-RT-PCR and qRT-PCR. The coding sequences (CDs) of *CIMBDs* were amplified using gene-specific primers (Supplementary Table 1) and cloned into pCAMBIA1300s vector, yielding pCAMBIA1300s-CIMBDs-GFP. After confirmation by sequencing, these pCAMBIA1300s-CIMBDs-GFP plasmids were used as templates to amplify the target genes for further experiments.

### Subcellular Localization Assays

The recombinant pCAMBIA1300s-CIMBDs-GFP plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101. Agrobacteria carrying pCAMBIA1300s-CIMBDs-GFP or pCAMBIA1300s-CIMBDs-GFP or pCAMBIA1300s-CIMBDs-GFP were separately infiltrated into leaves of *Nicotiana benthamiana* plants expressing a known nucleus-localized marker protein RFP-H2B (Chakrabarty et al., 2007). At 48 h after agroinfiltration, GFP fluorescence signals were excited at 488 nm and detected under a Zeiss LSM780 confocal laser scanning microscope (Zeiss, Oberkochen, Germany) using a 500–530 nm emission filter.

### Yeast Two-Hybrid Assays

Putative interactions between *CIMBDs* and ClIDM2 or ClIDM3 were examined using the yeast two-hybrid (Y2H) System according to the manufacturer's instructions (Clontech, Mountain View, CA, United States). The CDs of *CIMBDs* were amplified using gene-specific primers (Supplementary Table 1) from pCAMBIA1300s-CIMBDs-GFP and cloned into pGBK7 vector, yielding pGBK7-CIMBDs. ClIDM2 and ClIDM3 were obtained by homologous searching using Arabidopsis AtIDM2 and AtIDM3 as queries and the CDs of ClIDM2 and ClIDM3 were amplified with gene-specific primers (Supplementary Table 1) and cloned into pGADT7 vector, generating pGADT7-ClIDM2 and pGADT7-ClIDM3. The resultant pGBK7-CIMBD plasmids were transformed with or without pGADT7-ClIDM2 or pGADT7-ClIDM3 into yeast strain Y2HGold by the LiAc/SS method and confirmed by colony PCR. The transformed yeasts were cultivated on DDO (SD/-Leu/-Trp) medium (Clontech, Mountain View, CA, United States) at 30°C for 3 days, followed by screening on QDO medium containing 40 µg/mL X-α-Gal (Clontech, Mountain View, CA, United States) and 125 ng/mL Aureobasidin A (Clontech, Mountain View, CA, United States). Interactions between CIMBDs and ClIDM2/3 were evaluated according to the growth performance of the transformed yeast cells on QDO and the production of blue pigments after the addition of X-α-Gal. Co-transformation of pGBK7-T53 or pGBK7-Lam and pGADT7-T were used as positive and negative controls, respectively.

### Bimolecular Fluorescence Complementation Assays

The CDs of *CIMBD2* and *CIMBD3* were amplified using gene-specific primers (Supplementary Table 1) and inserted into p2YN vector, yielding p2YN-CIMBD2 and p2YN-CIMBD3. Similarly, the CDs of ClIDM2 and ClIDM3 were inserted into p2YC vector, yielding p2YC-ClIDM2 and p2YC-ClIDM3. Agrobacteria harboring different indicated pairs of plasmids were infiltrated into leaves of *N. benthamiana* plants expressing a red nuclear marker protein RFP-H2B (Chakrabarty et al., 2007). At 48 h after agroinfiltration, YFP and RFP signals were detected and photographed under a Zeiss LSM780 confocal laser scanning microscope (Zeiss, Oberkochen, Germany).

### Electrophoretic Mobility Shift Assays

The CDs of the *CIMBD* genes were amplified using gene-specific primers (Supplementary Table 1) and inserted into pGEX-4T-3 vector, generating pGEX-4T-3-GST-CIMBDs, followed by transforming into *Escherichia coli* strain BL21 (DE3), a widely used non-T7 expression strain that is suitable for transformation and protein expression (New England BioLabs, Beverly, MA, United States). To induce the expression of GST-CIMBD proteins, isopropyl-D-thiogalactoside was added to the bacterial cultures to a final concentration of 1 mM and incubated at 18°C for 20 h. The recombinant GST-CIMBD fusion proteins were purified using glutathione resin columns (Genscript, Shanghai, China) according to the manufacturer's protocol. The following double-stranded DNA probes were synthesized and used in EMSA assay: 5mCG (GTCCTGAGCTAAAGCTGCAGTCTGTGACATAGGCCATGGGTACACTGACTGACTGATCG) (methylated nucleotides underlined) and its complementary strand with m5C at symmetrical positions, 5mCHG (GTCCTGAGCTAAAGCTGCAGTCTGTGACATAGGGCCATGGGTACACTGACTGACCTGACTGATCG) (methylated nucleotides underlined) and its complementary strand with m5C at symmetrical positions, 5mCHH (GTCCTGAGCTAAAGCTGCAGTCTGTGACATAGGGCCATGGGTACACTGACTGATCG) (methylated nucleotides underlined) and its complementary strand with m5C at symmetrical positions, 5mCHH (GTCCTGAGCTAAAGCTGCAGTCTGTGACATAGGGCCATGGGTACACTGACTGATCG) (methylated nucleotides underlined) and its complementary strand with m5C at symmetrical positions, 5mCHH (GTCCTGAGCTAAAGCTGCAGTCTGTGACATAGGGCCATGGGTACACTGACTGATCG) (methylated nucleotides underlined) and its complementary strand with m5C at symmetrical positions, 5mCHH (GTCCTGAGCTAAAGCTGCAGTCTGTGACATAGGGCCATGGGTACACTGACTGATCG) (methylated nucleotides underlined) and its complementary strand with m5C at symmetrical positions, 5mCHH (GTCCTGAGCTAAAGCTGCAGTCTGTGACATAGGGCCATGGGTACACTGACTGATCG) (methylated nucleotides underlined) and its complementary strand with m5C at symmetrical positions, 5mCHH (GTCCTGAGCTAAAGCTGCAGTCTGTGACATAGGGCCATGGGTACACTGACTGATCG) (methylated nucleotides underlined) and its complementary strand with m5C at symmetrical positions. Equal volumes of single-stranded DNAs were mixed in annealing buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH7.5) and incubated at 85°C for 5 min to form double-stranded DNAs. EMSA was performed as previously described (Yuan et al., 2019) using LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific, Waltham, MA, United States). In brief, binding reactions (10 µL) contained 1 µL 10 × binding...
buffer, 2 μg GST-CIMBD protein or GST protein (as a negative control) and 1 μL biotin-labeled 5mCG, 5mCHG, or 5mCHH probe. In the competitive reactions, unlabeled 5mCG probe was added in excess of 500 times. The binding reactions were incubated at 28°C for 20 min and separated on 6% native PAGE gels. After electrophoresis, the gels were transferred onto Amersham Hybond-N+ nylon membrane (GE Healthcare, Buckinghamshire, United Kingdom), and signals from the biotin-labeled probes were detected using a Chemiluminescent Biotin-labeled Nucleic Acid Detection Kit (Beyotime Biotechnology, Haimen, China) according to the manufacturer’s recommendations.

**Generation and Characterization of CIMBDs-Overexpressing Transgenic Arabidopsis Lines**

Arabidopsis transformation was performed using the floral dip method as previously described (Clough and Bent, 1998). In brief, flowers of 5-week-old Arabidopsis plants were dipped in a suspension of agrobacteria carrying pCAMBIA1300s-CIMBD-GFP plasmids for 1 min. The infected plants were placed in dark for 12 h under 100% RH, returned to the growth room with normal conditions and allowed to grow until the silique maturation. T0 seeds were surface sterilized and then plated on 1/2 MS plates containing 50 μg/mL hygromycin. After treatment at 4°C for 2 days, the plates were transferred to 22°C under a 16 h light/8 h dark cycle, seedlings showing hygromycin resistance, regarded as positive transgenic plants, were transferred in the mixed nutrient soil and allowed for growth for 5–6 weeks to collect seeds. Putative single-copy transgenic lines and homozygous lines were obtained by screening for a 3:1 segregation ratio of hygromycin-resistant (HgrR) character and 100% HgrR phenotype in T2 and T3 generations on 1/2 MS medium supplemented with 50 μg/mL hygromycin, respectively. The transcript levels of the CIMBD genes in the transgenic Arabidopsis lines were analyzed by semi-PCR and qRT-PCR. Two homozygous transgenic Arabidopsis lines with single-copy for each of the CIMBD genes (T3 generation) and similar expression levels of the transgenes were chosen for further experiments.

**Disease Assays on Transgenic Arabidopsis Plants and Measurement of in planta Pathogen Growth**

Disease assays with *B. cinerea* were performed as previously described (Wang et al., 2009). Briefly, spores were collected from 8–10-day-old culture of *B. cinerea* strain BO5.10 grown on 2 × V8 plates and resuspended in 4% maltose and 1% peptone buffer to a final concentration of 2 × 10⁵ spores/mL. Four-week-old Arabidopsis plants were inoculated by hand infiltration using the spore suspension containing 0.05% Tween-20 or with an equal volume of 0.05% Tween-20 solution as mock controls. The inoculated plants were placed in a 22°C chamber with 100% RH for 48 h, and disease development was continuously observed. Measurement of in planta fungal growth was performed by analyzing the transcript level of *B. cinerea* BcActin gene and comparing with the transcript level of an Arabidopsis Actin gene as an internal control according to a previously reported protocol (Wang et al., 2009).

Disease assays with *Pst* DC3000 were carried out as previously described (Zhang et al., 2016). *Pst* DC3000 was grown on King’s B (KB) broth and bacteria were collected and resuspended in 10 mM MgCl₂ solution to OD₆₀₀ = 0.002. The bacterial inoculation was performed by hand infiltration using 1-mL syringes without needle into rosette leaves of 4-week-old Arabidopsis plants and the inoculated plants were kept in sealed containers 22°C for 72 h. For quantification of in planta bacterial growth, leaf discs from inoculated leaves were collected and homogenized in 10 mM MgCl₂. After a series of gradient dilutions, the homogenate was plated on KB plates supplemented with 25 μg/mL rifampicin and bacterial colonies were counted at 3 days after incubation at 28°C.

**In situ Detection of H₂O₂ Accumulation**

Detection of H₂O₂ was performed using the DAB staining method (Thordal-Christensen et al., 1997). Leaf samples were collected from Arabidopsis plants with or without infection of *B. cinerea* at 24 h post inoculation (hpi) and dipped into DAB solution (1 mg/mL) in 10 mM Na₂HPO₄ (pH7.0). After incubation for 5 h in dark with shaking (80 rpm) at room temperature, the DAB-treated leaves were transferred into acetic acid/glycerol/ethanol (1:1:1, vol/vol/vol) and boiled for 5 min, followed by several washes with the same solution. The DAB-stained leaves were photographed using a digital camera.

**RNA-Seq Analyses**

Leaf samples were collected from 4-week-old Col-0 and CIMBD2-OE2 Arabidopsis plants, frozen in liquid nitrogen and stored at −80°C. RNA-seq was performed by BioMarker Technologies (Beijing, China) on Hiseq 2500 platform (Illumina). Raw data were filtered to get clean data, sequence comparison with the NCBI database was performed with Blast alignment tool. reads were aligned to the Arabidopsis genome (TAIR10), and expression levels of unique gene models were normalized to FPKM (fragments per kilobases of transcript per million fragments mapped). Gene Ontology (GO) enrichment analysis was used to investigate the functional enrichment of DEGs. GO enrichment analysis of DEGs was implemented by the GOseq R packages based on Wallenius non-central hyper-geometric distribution (Young et al., 2010). KOA (Mao et al., 2005) software were used to test the statistical enrichment of differential expression genes in KEGG pathways (Kanehisa et al., 2008).

**Semiquantitative RT-PCR and qRT-PCR Analyses**

Extraction and treatment of total RNA were performed as mentioned above. Semiquantitative RT-PCR reactions contained 0.5 μL Phanta Max Super-Fidelity DNA Polymerase (Vazyme, Nanjing, China), 0.5 μL dNTP Mix, 12.5 μL 2 × Phanta Max Buffer, 0.1 μg cDNA, 7.5 pmol of each of gene-specific primers (Supplementary Table 1), and 8.5 μL ddH₂O in a final volume of 25 μL. Arabidopsis AtActin was used as the control. Each qPCR reaction contained 10 μL 2 × AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China), 0.1 μg cDNA and 7.5 pmol...
of each of gene-specific primers (Supplementary Table 1) in a final volume of 20 mL, and had two independent biological replicates. The qPCR was performed in a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, United States). Watermelon ClGAPDH or Arabidopsis AtActin were used as internal controls to normalize the data. Relative gene expression level was calculated using $2^{-\Delta\Delta CT}$ method as described.

### Statistical Analysis

All experiments were independently repeated three times and the obtained data were subjected to statistical analysis according to the Student's t-test. The probability values of $p < 0.05$ were considered as significant difference between the treatments and corresponding controls.

## RESULTS

### Identification and Characterization of the Watermelon ClMBD Family

To identify putative ClMBD genes in watermelon, BLASTp searches were performed against the watermelon genome database using the Arabidopsis AtMBDs as queries and 10 non-redundant sequences that are putative ClMBD genes were identified (Table 1). For convenience, unique identities to each of the identified ClMBD genes were assigned as ClMBD1–10 according to their chromosomal locations (Table 1). The CDs of ClMBD1–10 were confirmed by cloning of the full-length cDNAs using primers designed according to their predicted cDNA sequences. The sizes of the ClMBD open reading frames (ORF) ranged from 798 bp (ClMBD2) to 6,636 bp (ClMBD8) and the sizes of the encoded proteins varied from 265 amino acids (ClMBD2) to 2,211 amino acids (ClMBD8), with molecular weight of 23.34–244.99 kDa and pi of 4.80–9.49 (Table 1). Similarly, the MBD families in other cucurbit plants were also characterized and 9, 10, 15, and 16 MBD genes in melon, cucumber, zucchini, and pumpkin, respectively, were identified (Supplementary Table 2).

### Structure of ClMBD Genes and Organization of Conserved Domains in ClMBD Proteins

The 10 ClMBD genes are unevenly distributed on eight chromosomes in the watermelon genome and chromosomes 2, 4, and 8 do not host any ClMBD gene (Table 1 and Supplementary Figure 1). Chromosomes 9 and 11 harbor two ClMBD genes while each of the other chromosomes 1, 3, 5, 6, 7, and 10 carry one ClMBD gene (Table 1 and Supplementary Figure 1). Phylogenetic tree analysis revealed that the watermelon ClMBD proteins were divided into two clades: Clade I contained six ClMBD proteins (ClMBD1, 3, 4, 5, 6, and 9) while Clade II contained four ClMBD proteins (ClMBD2, 7, 8, and 10) (Figure 1A). Phylogenetic tree analysis of the MBD proteins from cucurbit plants showed that the MBD proteins from watermelon, melon, cucumber, zucchini, and pumpkin, and zucchini have a high level of similarity in the amino acid sequences (Supplementary Figure 2).

### Evolution and Interspecific Synteny of the Watermelon ClMBD Family

Gene duplication events in ClMBDs in the watermelon genome were detected and seven gene pairs, ClMBD1/3, ClMBD4/5, ClMBD4/6, ClMBD4/9, ClMBD5/6, ClMBD5/9, and ClMBD6/9, were localized in duplicated genomic regions (Figure 1D), implying the occurrence of gene duplication during the evolution of the ClMBD gene family in watermelon. The Ka/Ks ratios of ClMBD1/3, ClMBD4/6, ClMBD4/9, ClMBD5/6, ClMBD5/9, and ClMBD6/9 were less than 1 (Supplementary Table 3), indicating that these gene pairs evolved through purifying selection. Interspecific collinearity analyses identified 9, 15, 20, 19, and 26 collinear gene pairs between watermelon and other tested plant species Arabidopsis, melon, cucumber, zucchini, and pumpkin, respectively (Figure 2 and Supplementary Table 4). Some ClMBD genes, e.g., ClMBD4 and ClMBD6, were found to be associated with at least 15 collinear gene pairs identified between watermelon and other tested plant species (Supplementary Table 4), indicating that ClMBD4 and ClMBD6 may play essential roles during the evolution of the ClMBD genes. ClMBD2, 4, 6,
Figure 3A localization (ubiquitously throughout the cell without specific compartmental which was co-localized with the known nucleus marker RFP-ClMBD1–10-GFP protein was solely localized to the nucleus, marker RFP-H2B protein (Chakrabarty et al., 2007). The Supplementary Figure 6 interacted with ClIDM2 and ClIDM3, but the remaining ClIDM3 were examined. In Y2H assays, ClMBD2 and ClMBD3 properties, the interactions of ClMBDs with ClIDM2 and 2015). To examine whether the ClMBD proteins have similar Interactions Between ClMBDs and ClIDM2/3

ClMBDs Are Nucleus-Localized Proteins

To explore the subcellular localization of the ClMBD proteins, agrobacteria carrying ClMBD1–10-GFP or GFP was infiltrated into leaves of N. benthamiana plants expressing a red nuclear marker RFP-H2B protein (Chakrabarty et al., 2007). The ClMBD1–10-GFP protein was solely localized to the nucleus, which was co-localized with the known nucleus marker RFP-H2B protein (Figure 3A). By contrast, GFP alone distributed ubiquitously throughout the cell without specific compartmental localization (Figure 3A). These results indicate that ClMBD1-CIMBD10 are nucleus-localized proteins.

Interactions Between ClMBDs and CIIDM2/3

The Arabidopsis AtMBD7 interacts with AtIDM2 and AtIDM3 to activate ROS1 to function in demethylation (Lang et al., 2015). To examine whether the ClMBD proteins have similar properties, the interactions of ClMBDs with CIIDM2 and CIIDM3 were examined. In Y2H assays, ClMBD2 and ClMBD3 interacted with CIIDM2 and CIIDM3, but the remaining ClMBDs did not (Supplementary Figure 6). Due to self-activation of ClMBD2 and CIIDM3 in Y2H, the interactions of ClMBD2 and ClMBD3 with CIIDM2 and CIIDM3 were further confirmed using the BiFC assays. YFP signal was not detected in N. benthamiana leaves co-infiltrated with agrobacteria harboring p2YN-CIMBD2 + p2YC, p2YN + p2YCIIDM2, p2YN-CIMBD3 + p2YC, or p2YN + p2YCIIDM3; by contrast, like that in the positive control, significant YFP fluorescence was clearly observed in leaves co-infiltrated with agrobacteria carrying p2YN-CIMBD2 + p2YC-CIIDM2, p2YN-CIMBD3 + p2YC-CIIDM2, p2YN-CIMBD2 + p2YC-CIIDM3, or p2YN-CIMBD3 + p2YC-CIIDM3 (Figure 3B). These results confirmed the interactions of ClMBD2 and ClMBD3 with CIIDM2 and CIIDM3.

CIMBD2 Specifically Binds to Methylated CG DNA

It is well known that MBD proteins have the capability to bind methylated DNA (Ito et al., 2003; Zemach and Grafi, 2003; Grafi et al., 2007). To explore the biochemical activity of the watermelon ClMBDs, recombinant GST-tagged ClMBD1–7, 9, 10 proteins were purified (Supplementary Figure 7) and their binding activity to methylated CG DNA was examined by EMSA. Two complementary single-stranded DNA probes with 5 methylated CG sites (5mCG) were synthesized and the double-stranded 5mCG DNA was generated (Figure 4A). In repeated EMSA, only ClMBD2 bound to labeled double-stranded 5mCG DNA, and the binding of ClMBD2 to labeled double-stranded 5mCG DNAs was specific as this binding was completely suppressed by the excessive unlabeled double-stranded 5mCG DNA in the competition binding assay (Figure 4A). The remaining ClMBDs did not show binding activity to the labeled double-stranded 5mCG DNA (Figure 4A). However, ClMBD2 did not bind to DNA harboring mCHG or mCHH sites (Figure 4B). These results indicate that ClMBD2 specifically binds to mCG DNA, but not to mCHG DNA or mCHH DNA.

CIMBDs Have Similar Expression Patterns in Root, Stem, and Leaf Tissues

The expression patterns of the ClMBD gene in root, stem and leaf tissues of 4-week-old watermelon plants were analyzed and the qRT-PCR results showed that the ClMBD genes have similar expression patterns: highest expression in leaves, moderate in stems, and lowest in root (Supplementary Figure 8).
CIMBDs Are Responsive to Defense Hormones Salicylic Acid and Methyl Jasmonate

To explore the possible involvement of the CIMBD genes in disease resistance, expression changes of the CIMBD genes were analyzed in watermelon plants after treatment with SA and MeJA. After foliar spraying with 1 mM SA, the expression of CINPR1 and CIPIP1, the marker genes of SA signaling pathway, was significantly up-regulated. Particularly, the expression of CIPIP1 significantly up-regulated at 6 h after treatment and peaked at 12 h, showing a > 59-fold increase, as compared with that in mock control (Figure 5A). After SA treatment, the expression of most of the CIMBD genes were up-regulated with distinct patterns: CIMBD2, 3, 6, 7, 8, 9, and 10 were up-regulated at 6 h;
FIGURE 2 | Syntenic relationships of the watermelon ClMBD genes with other MBD genes in representative plant species. Gray lines in the background indicate the collinear blocks within the watermelon and other plant genomes, while the red lines highlight the syntenic MBD gene pairs between watermelon and other plant species.

ClMBD1, 4, 5, 7, and 10 were up-regulated at 12 h; while ClMBD1, 6, and 8 were markedly up-regulated at 24 h, as compared with those in the mock controls (Figure 5A). Notably, ClMBD2, 3, 9, and 10 showed similar expression patterns after SA treatment, implying that these ClMBD genes may have similar functions. These data indicate that the ClMBD genes can respond to SA and thus may be involved in disease resistance in watermelon.

After foliar spraying with 100 µM MeJA, the expression of ClJAZ1 and ClPDF1.2, the marker genes of the JA signaling pathway (Yang et al., 2019), was significantly up-regulated and peaked at 24 h (Figure 5B). After MeJA treatment, ClMBD2, 6, 7, 8, and 10 were highly up-regulated, while ClMBD5 and ClMBD9 were significantly down-regulated, as compared with those in the mock controls (Figure 5B). ClMBD4 was up-regulated at 12 h but down-regulated at 24 h, as compared with those in the mock controls (Figure 5B). Notably, ClMBD8/ClMBD10 and ClMBD1/ClMBD4 exhibited similar expression patterns in response to exogenous MeJA, indicating these two pairs of the ClMBD genes may have similar biological functions. These data indicate that the ClMBD genes differentially respond to MeJA and thus may play different roles in disease resistance in watermelon.

ClMBDs Differentially Respond to Fungal Pathogens

To explore the possible involvement of ClMBDs in watermelon disease resistance, the expression changes of the ClMBD genes in watermelon plants after infection with different fungal pathogens were analyzed. Fon is the most important soilborne vascular pathogen that causes Fusarium wilt, one of the most devastating fungal diseases in watermelon (Michielse and Rep, 2009). Fon infects watermelon plants through root system and proliferation within the xylem vessels (Michielse and Rep, 2009). Therefore, the expression changes of the ClMBD genes in root tissues of watermelon plants after Fon infection were analyzed. The qRT-PCR results showed that the expression level of ClPR1 started to increase at 3 days post inoculation (dpi), peaked at 6 days, leading to 65.5-fold higher over that in mock-inoculated plants, and then decreased (Figure 5C). The expression of ClMBD1 and ClMBD10 in root tissues of the Fon-infected watermelon plants were up-regulated, as compared with those in the mock-inoculated plants, at 3 dpi (Figure 5C). As compared with those in the mock-inoculated plants, the expression of ClMBD2, 6, and...
FIGURE 3 | Subcellular localization of the ClMBD proteins and the interactions between ClMBD2/3 and ClIDM2/3. (A) ClMBDs are nucleus-localized proteins. Agrobacteria carrying pCAMBIA1300-ClMBDs-GFP or pCAMBIA1300-GFP were infiltrated into leaves of N. benthamiana plants expressing a known nucleus-localized marker protein RFP-H2B. At 48 h after agroinfiltration, GFP signals were visualized under a confocal laser scanning microscope in dark field for green fluorescence (left), red fluorescence (middle left), white field for cell morphology (middle right) and in combination (right), respectively. (B) Interactions of ClMBD2 and ClMBD3 with ClIDM2 and ClIDM3. Agrobacteria carrying indicated pairs of p2YC and p2YN plasmids were infiltrated into leaves of N. benthamiana plants expressing a red nuclear marker RFP-H2B protein, and YFP and RFP signals were observed at 48 h after infiltration. Images were taken in dark field for green fluorescence (left) and red fluorescence (middle right), white field for cell morphology (middle left) and in combination (right), respectively. Experiments in (A,B) were repeated for three times with similar results.

9 was up-regulated at 6 dpi, while the expression of the ClMBD genes, except for ClMBD3, 5, and 10, was up-regulated at 9 dpi (Figure 5C). Overall, the expression changes of the ClMBD genes exhibited similar significant up-regulation patterns in root tissue at 3, 6, or 9 dpi; for example, the expression level of ClMBD7 in root tissue was markedly up-regulated with a > 639-fold increase.
than that in mock-inoculated plants at the 9 dpi (Figure 5C). Notably, the expression changes of ClMBD8, 9, and 10, and of ClMBD1, 2, 4, 5, 6, and 7 showed similar patterns in response to Fon, implying that these two groups of the ClMBD genes may be involved in resistance with similar functions. The results suggest that the ClMBD genes are responsive to Fon infection during the relative late stage of the pathogenesis and thus may play roles in the process of regulating watermelon resistance to vascular Fusarium wilt disease.

Db is another devastating fungal pathogen that infects leaf and stem tissues and causes gummy stem blight, which is a very common fungal disease on cucurbitaceous crops including watermelon (Keinath, 2011). The responsiveness of the ClMBD genes to Db infection was also analyzed in leaf tissues of watermelon plants after foliar spraying with a fungal spore suspension. After Db inoculation, the expression of ClPR1 in leaf tissues started to increase at 12 hpi, gradually increased and peaked at 48 h, leading to a 55-fold increase over that in mock-inoculated plants (Figure 5D). The expression of the ClMBD genes in leaf tissues exhibited distinct patterns in response to Db infection. The expression levels of ClMBD3, 4, 7, and 9 in Db-infected leaves were significantly up-regulated at 12, 24, or 48 hpi, as compared with those in mock-inoculated leaves (Figure 5D). By contrast, the expression levels of ClMBD2, 6, 8, and 10 in Db-infected leaves were highly down-regulated at 12, 24, or 48 hpi, as compared with those in mock-inoculated leaves (Figure 5D). The expression of ClMBD1 and ClMBD5 in leaf tissues was not significantly affected by Db infection during a 48 h period of the experiments (Figure 5D). Notably, the expression changes of ClMBD3, 5, and 7 exhibited similar patterns in response to Db, indicating similar involvement for these three ClMBD genes in Db resistance. These results indicate that the expression of

![FIGURE 4](image-url) Binding activity of ClMBD2 to methylated CG DNA. (A) Binding of ClMBD proteins to the methylated CG DNA. Biotin-labeled mCG DNA (for binding assays) or biotin-labeled mCG DNA in combination with excessive unlabeled mCG DNA (for competitive assays) were incubated with GST-ClMBD or GST (as a negative control). (B) Binding activity of ClMBD2 to methylated CG DNA, methylated CHG DNA and methylated CHH DNA. Biotin-labeled mCG DNA, biotin-labeled mCHG DNA and biotin-labeled mCHH DNA (for binding assays) or biotin-labeled mCG DNA in combination with excessive unlabeled mCG DNA (for competitive assays) were incubated with GST-ClMBD or GST (as a negative control). The mCG, mCHG, and mCHH DNA sequences are shown and the methylated sites are indicated in red color. Experiments in (A,B) were repeated for three times with similar results.
FIGURE 5 | Expression changes of the watermelon ClMBD genes in response to defense hormones and fungal pathogens. (A,B) Expression changes of the ClMBD genes to SA (A) or MeJA (B). Four-week-old watermelon plants were treated by foliar spraying with 1 mM SA, 100 µM MeJA or similar volume of solution (as mock controls) and leaf samples were collected at indicated time points after treatment. (C) Expression changes of the ClMBD genes to Fusarium oxysporum f.sp. niveum. Three-week-old plants were inoculated by dipping the roots in spore suspension (1 × 10⁷ spores/mL) of F. oxysporum f.sp. niveum or in mung bean liquid broth as mock-inoculated controls, and root samples were collected at indicated time points after inoculation. (D) Expression changes of the ClMBD genes to Didymella bryoniae. Five-week-old watermelon plants were inoculated by foliar spraying with D. bryoniae spore suspension (2 × 10⁶ spores/mL) or similar volume of solution as mock controls, and leaf samples were collected at indicated time points after inoculation. qRT-PCR was performed using the watermelon ClGAPDH gene as an internal control. Relative expression was calculated using the 2^−ΔΔCT method. Experiments were repeated for three times and the data presented are the means ± SE from three independent experiments. * or ** above the columns indicate significant difference at p < 0.05 or p < 0.01 levels (Student's t-test), respectively, between treatment/inoculation and mock controls at the same time point.
the ClMBD genes in leaf tissues exhibited distinct patterns in response to Db infection and therefore may play different roles in the process of regulating watermelon resistance against Db.

**Generation and Characterization of ClMBD-Overexpressing Arabidopsis Lines**

To investigate the functions of the ClMBD genes, transgenic Arabidopsis lines with overexpression of an individual ClMBD gene were generated. The ClMBD genes were transcribed normally in their own transgenic Arabidopsis lines (Supplementary Figures 9A,B). The ClMBD-overexpressing Arabidopsis plants showed no significant defect in growth and development, including plant height and size, in comparison to WT plants, when grown in a greenhouse (Supplementary Figure 10).

**ClMBD2, ClMBD3, and ClMBD5 Negatively Regulate Arabidopsis Immunity Against Botrytis cinerea**

To explore the possible functions of the ClMBD genes in plant immunity, disease resistance phenotype of the ClMBD-overexpressing Arabidopsis lines and the wild-type (WT) Col-0 plants after infection with *B. cinerea*, a necrotrophic fungus causing grey mold disease, was assessed. In repeated detached leaf punch inoculation assays, *B. cinerea* causing grey mold disease, was assessed. In repeated detached leaves from the ClMBD2-OE, ClMBD3-OE, and ClMBD5-OE plants were significantly larger than those on leaves of WT plants, resulting in increases of approximately 88.9, 55.6, and 66.7% in lesion length, respectively, as compared with those on WT leaves (Supplementary Figure 11). By contrast, *B. cinerea*-caused necrotic lesions on leaves detached from the ClMBD2-OE, ClMBD3-OE, and ClMBD5-OE plants were significantly smaller than those on leaves of WT plants, resulting in decreases of 73–81, 80–84, and 59–63%, respectively, as compared with those on WT leaves (Supplementary Figure 11). To confirm these results, the ClMBD-overexpressing plants were inoculated by foliar spraying with *B. cinerea* spore suspension and disease severity and fungal growth were compared with those in WT plants. After infection, typical *B. cinerea*-caused disease symptom was seen at 3 dpi. Much severe diseases were observed on leaves of the ClMBD2-OE, ClMBD3-OE, and ClMBD5-OE plants, especially the *B. cinerea*-infected ClMBD2-OE plants decayed and died at 3 dpi (Figure 6A). By contrast, disease severity on leaves of the *B. cinerea*-infected ClMBD1-OE, ClMBD4-OE, ClMBD6-OE, ClMBD7-OE, ClMBD8-OE, ClMBD9-OE, and ClMBD10-OE plants were similar to that in WT plants (Supplementary Figure 12A). Accordingly, the ClMBD2-OE, ClMBD3-OE, and ClMBD5-OE plants supported more in planta fungal growth, leading to increases of 57.5–851.3% over that in WT plants (Figure 6B), while the ClMBD1-OE, ClMBD4-OE, ClMBD6-OE, ClMBD7-OE, ClMBD8-OE, ClMBD9-OE, and ClMBD10-OE plants supported similar amounts of in planta fungal growth (Supplementary Figure 12B). These data from detached leaf punch inoculation and whole plant inoculation assays indicate that overexpression of ClMBD2, 3, and 5 attenuates the resistance of transgenic Arabidopsis plants against *B. cinerea*, while overexpression of each of the remaining ClMBD genes does not affect the resistance against *B. cinerea*.

To get insights in the possible mechanism of the attenuated *B. cinerea* resistance, the expression of a marker defense gene AtPDF1.2 and accumulation of reactive oxygen species (ROS) were analyzed and compared between the ClMBD2-OE, ClMBD3-OE, and ClMBD5-OE plants and WT plants after infection by *B. cinerea*. In mock-inoculated plants, the expression level of AtPDF1.2 in the ClMBD2-OE, ClMBD3-OE, and ClMBD5-OE plants was similar to that in WT plants (Figure 6C). At 24 hpi with *B. cinerea*, the expression level of AtPDF1.2 in the ClMBD2-OE, ClMBD3-OE, and ClMBD5-OE plants and WT plants were markedly up-regulated, as compared with those in the mock-inoculated plants; however, the expression level of AtPDF1.2 in the ClMBD2-OE, ClMBD3-OE, and ClMBD5-OE plants were significantly suppressed, resulting in a decrease of 97–100, 94–96, and 89–91%, respectively, as compared with those in Col-0 plants (Figure 6C). Similarly, no difference in accumulation of H$_2$O$_2$ as revealed by in situ DAB staining, was observed among the WT, ClMBD2-OE, ClMBD3-OE, and ClMBD5-OE plants without *B. cinerea* challenge (Figure 6D). At 24 hpi with *B. cinerea*, accumulation of H$_2$O$_2$ increased markedly in *B. cinerea*-infected leaves of the WT, ClMBD2-OE, ClMBD3-OE, and ClMBD5-OE plants, as compared with those in mock-inoculated controls (Figure 6D). However, more staining for H$_2$O$_2$ in *B. cinerea*-infected leaves of the ClMBD2-OE, ClMBD3-OE, and ClMBD5-OE plants was detected, as compared to that in WT plants (Figure 6D). These data indicate that overexpression of ClMBD2, 3, and 5 in transgenic Arabidopsis plants attenuates the *B. cinerea*-induced expression of defense genes but promotes the *B. cinerea*-induced ROS accumulation.

**ClMBD1 and ClMBD2 Negatively but ClMBD5 Positively Regulate Arabidopsis Immunity Against Pseudomonas syringae pv. tomato DC3000**

The possible involvement of the ClMBD genes in resistance against *Pst* DC3000, a hemibiotrophic pathogen that causes bacterial spot disease, was also investigated. At 3 dpi, typical *Pst* DC3000 provoked symptom with chlorotic lesions was seen in WT plants and the ClMBD-overexpressing plants (Figure 7A and Supplementary Figure 13A). The ClMBD1-OE and ClMBD2-OE plants displayed much severe disease with extensive chlorotic lesion while the ClMBD5-OE plants showed less severe disease (Figure 7A). Accordingly, the bacterial growth in the ClMBD1-OE and ClMBD2-OE plants was 0.43–0.69 order of magnitude higher while the growth in the ClMBD5-OE plants was ~1.0 order of magnitude lower, as compared to that in WT at 3 dpi (Figure 7B). Disease severity and bacterial growth in the ClMBD3-OE, ClMBD4-OE, ClMBD6-OE, ClMBD7-OE, ClMBD8-OE, ClMBD9-OE, and ClMBD10-OE plants were indistinguishable to those in WT plants (Supplementary Figure 13B). These data indicate that overexpression of ClMBD1 and ClMBD2 leads to attenuated...
FIGURE 6 | ClMBD2, ClMBD3, and ClMBD5 negatively regulate resistance of the transgenic Arabidopsis plants against Botrytis cinerea. (A) Typical B. cinerea-caused disease on WT, ClMBD2-OE, ClMBD3-OE, and ClMBD5-OE plants. Four-week-old plants were inoculated by foliar spraying with B. cinerea spore suspension (2 $\times$ 10^5 spores/mL) and photographed at 3 dpi. (B) In planta fungal growth in inoculated plants. Fungal growth was shown as ratios of the transcript level of B. cinerea BcActin to that of the Arabidopsis AtActin. (C) Relative expression of AtPDF1.2 in the mock- and B. cinerea-inoculated plants. qRT-PCR was performed using AtActin as an internal control to analyze the expression level of AtPDF1.2. (D) Accumulation of H$_2$O$_2$ in leaves of the in mock- and B. cinerea-inoculated plants, as revealed by DAB staining at 24 hpi. Experiments in (A,D) were repeated for three times with similar results, and results from one representative experiment are shown. Data presented in (B,C) are the means ± SE from three independent experiments and * or ** above the columns indicate significant differences at $p$ < 0.05 or $p$ < 0.01 levels (Student's t-test), respectively, between the ClMBD2/3/5-OE plants and WT plants at the same time point.
resistance while overexpression of ClMBD5 results in increased resistance against Pst DC3000.

To gain insights in the possible mechanism of the altered Pst DC3000 resistance, the expression of a marker defense gene AtPR1 was analyzed and compared between the ClMBD1-OE, ClMBD2-OE, and ClMBD5-OE plants and WT plants after infection by Pst DC3000. In mock-inoculated plants, the expression level of AtPR1 in the ClMBD1-OE, ClMBD2-OE, and ClMBD5-OE plants was not significantly affected, as compared with that in WT plants (Figure 7C). At 24 hpi with Pst DC3000, the expression level of AtPR1 in the ClMBD1-OE, ClMBD2-OE, and ClMBD5-OE plants was significantly suppressed, resulting in a decrease of 51–55 and 39–47%, respectively, while the Pst DC3000-induced expression of AtPR1 in the ClMBD5-OE plants was markedly increased by ~21-folds (Figure 7C). These data indicate that overexpression of ClMBD1 and ClMBD2 in transgenic Arabidopsis plants attenuates while overexpression of ClMBD5 strengthens the Pst DC3000-induced expression of defense genes.

Identification of Differentially Expressed Genes in ClMBD2-OE Plants

Considering that overexpression of ClMBD2 led to attenuated resistance against B. cinerea and Pst DC3000 (Figures 6, 7), transcriptome profiling of the ClMBD2-OE2 and WT plants grown under normal growth conditions was performed to gain
insights into the possible molecular mechanisms of ClMBD2 in regulating resistance against the two pathogens. With criteria of expression change $>1.5$-folds and $P < 0.05$, a total of 70 genes (52 up-regulated and 18 down-regulated) were found to be differentially expressed genes (DEGs) in the ClMBD2-OE2 plants as compared with WT plants (Supplementary Tables 5, 6). The DEGs in the ClMBD2-OE2 plants grown under normal condition were categorized into functional groups based on Gene Ontology (GO). DEGs that were up-regulated (Supplementary Table 5) or down-regulated (Supplementary Table 6) in the ClMBD2-OE2 plants were clustered into 31 (Supplementary Table 7) and 22 categories (Supplementary Table 8), respectively. Some overrepresented categories include genes involved in DNA binding, molecular transducer activity, and transcriptional factor activity in molecular function category, and response to stimulus, immune system process, signaling, and biological regulation in biological processes category (Figures 8A,B), implying that overexpression of ClMBD2 in transgenic Arabidopsis plants may affect the immune signaling and response. Among the DEGs (Supplementary Tables 5, 6), some genes have been previously reported to be involved in Arabidopsis immunity, including AtWRKY18 (Xu et al., 2006), AtWRKY30 (Zou et al., 2019), AtWRKY54 (Chen et al., 2021), AtANAC019 (Zheng et al., 2012), AtMLD6 (Acedo-Garcia et al., 2017; Kuhn et al., 2017), and AtNATA1 (Lou et al., 2016). The expression patterns of 10 selected genes were further validated by qRT-PCR in ClMBD2-OE and WT plants before and after the infection of B. cinerea and Pst DC3000. In the ClMBD2-OE plants without pathogen infection, the expression levels of AtWRKY18, AtWRKY30, AtANAC019, AtARCK1, AtMLD6, and AtERF54 were significantly up-regulated while the expression levels of AtMAF5, AtBEE1, AbZIP34, and AtT5G52190 were markedly down-regulated (Figure 8C). After infection of B. cinerea, the expression of AtbZIP34 was down-regulated, while the expression of other genes was up-regulated in ClMBD2-OE and WT plants (Figure 8C). The expression levels of AtWRKY18 and AtbZIP34 were significantly increased, while the expression levels of AtANAC019 and AtBEE1 were significantly suppressed in ClMBD2-OE plants after infection of B. cinerea, as compared with those in WT plants (Figure 8C). After infection of Pst DC3000, the expression of AtT5G52190, AtANAC019, AtARCK1, AtERF54, AtMLD6, and AtMAF5 was up-regulated, but the expression of AtBEE1, AT5G52190, and AtbZIP34 was down-regulated in ClMBD2-OE and WT plants (Figure 8C). The expression levels of AtWRKY18, AtERF54, AtMAF5, AT5G52190, and AtBEE1 were significantly decreased in ClMBD2-OE plants after infection of Pst DC3000, as compared with those in the WT plants (Figure 8C). These data consistently confirmed the results from RNA-seq analysis and indicate that ClMBD2 regulates a small set of defense and signaling genes that are involved in Arabidopsis immunity.

**DISCUSSION**

It has been documented that the MBD proteins play important roles in plant growth, development, and abiotic stress response; however, the involvement of the MBD proteins in plant immunity has not been established. The present study characterized the MBD families in watermelon and other cucurbit plants, examined the subcellular localization and binding activity of ClMBDs to 5-mC DNAs, analyzed the expression patterns of ClMBDs in response to defense hormones and pathogens, and explored the functions of ClMBDs in disease resistance. The functional analysis in transgenic Arabidopsis revealed that ClMBD1, 2, 3, and 5 play roles in immunity against B. cinerea and Pst DC3000, providing novel insights into the function of the MBD genes in plant immunity and a possibility to improve plant disease resistance through genetic manipulation of specific MBD genes.

The present study identified 10 watermelon ClMBD genes and 9, 10, 15, and 16 MBD genes in melon, cucumber, zucchini, and pumpkin, respectively (Table 1 and Supplementary Table 2). The numbers of ClMBDs in watermelon and MBD genes in other cucurbit plants are comparable to those of Arabidopsis (13), rice (17), maize (14), poplar (14), potato (15), tomato (18), and petunia (11) (Grafi et al., 2007; Parida et al., 2018; Shi et al., 2022). The presence of gene pairs in duplicated genomic regions of the watermelon genome and syntenic collinearity gene pairs between watermelon and other cucurbit plant species (Figures 1, 2 and Supplementary Tables 3, 4) suggests that gene duplication events occurred during the evolution of the ClMBD family. In addition to the typical MBD domain, other conserved domains such as zf-CW domain, SPARK domain, PKINase domain, and Bromo domain were also identified in some of the ClMBD proteins (Figure 1). Similar conserved domains are also present in Arabidopsis AtMBDs, tomato SlMBDs, and potato StMBDs (Grafi et al., 2007; Parida et al., 2018). It is thus likely that some watermelon ClMBD proteins may exert their functions in affecting transcription of target genes through different biochemical mechanisms including protein-protein interactions. Subcellular localization observations revealed that the ClMBD proteins were localized in nucleus when transiently expressed in N. benthamiana (Figure 3). This is consistent with the previous observations that most of the Arabidopsis AtMBD proteins displayed clear localization within the nucleus in onion cells (Berg et al., 2003; Ito et al., 2003) and that ZmMBD101 localized to nucleoplasmic foci (Questa et al., 2016). Another, ClMBD2 and ClMBD3 interacted with CIIDM2 and CIIDM3, and the interactions occurred in nucleus in BiFC assays (Figure 3). This feature is similar to the interaction of the Arabidopsis AtMBD7 with AtIDM2 and AtIDM3 (Lang et al., 2015), and further confirmed the nuclear localization of the ClMBD2 and ClMBD3. ClMBD2 is phylogenetically related to AtMBD5 and AtMBD6 and also shows an evolutionary syntenic relationship with AtMBD5 (At3G46580) and AtMBD6 (At5G9380) (Supplementary Table 4), implying that ClMBD2 may have a similar biochemical activity to AtMBD5 and AtMBD6. In the present study, ClMBD2 showed the ability to bind to mCG DNA (Figure 4), similar to AtMBD5 and AtMBD6, maize ZmMBD101, and tomato SIMBD5, which have the binding ability to mCG DNA (Ito et al., 2003; Scebb et al., 2003; Grafi et al., 2007; Li Y. et al., 2015; Questa et al., 2016). However, ClMBD2 did not bind to mCHH and mCHG DNA (Figure 4), different from AtMBD5 and AtMBD6, which also have the ability
FIGURE 8 | Differentially expressed genes in ClMBD2-OE2 plants. (A,B) Enriched GO terms of up-regulated (A) and down-regulated (B) genes in ClMBD2-OE2 plants, in comparison to WT plants. (C) qRT-PCR validation of expression patterns of selected differentially expressed genes in ClMBD2-OE and WT plants with or without infection of B. cinerea or Pst DC3000 at 24 hpi. Relative expression of the selected genes was analyzed by qRT-PCR using AtActin as an internal control. Data presented in (C) are the means ± SE from three independent experiments and * or ** above the columns indicate significant differences at $p < 0.05$ or $p < 0.01$ levels (Student's t-test), respectively, between the ClMBD2-OE plants and WT plants.
to bind to mCHH DNA (Ito et al., 2003; Scebba et al., 2003; Grafi et al., 2007). Surprisingly, the binding activity of the other ClMBD proteins to mCG DNA was not detected in the present study (Figure 4), implying that the ClMBD proteins may have different biochemical activities in recognizing methylated or unmethylated DNA and thus confer specific biological functions.

It was previously observed that the expression of some tomato SlMBD, wheat TaMBD and petunia PhMBD genes were affected by abscisic acid and abiotic stress, e.g., drought, salt, and cold stress (Li et al., 2008; Hu et al., 2011; Parida et al., 2018; Shi et al., 2022). The expression of most of the watermelon ClMBD genes was up-regulated after SA or MeJA treatment, except that ClMBD5 and ClMBD9 were down-regulated by MeJA and that the expression of ClMBD1 and ClMBD3 was not affected by MeJA (Figure 5). In response to Fon, the expression of almost all of the ClMBD genes in root tissues was up-regulated (Figure 5). By contrast, the expression of ClMBD3, 4, 7, and 9 was up-regulated, while the expression of ClMBD2, 6, 8, and 10 was down-regulated in response to Db (Figure 5). Notably, the expression changes of the ClMBD genes exhibited differential but inconsistent patterns in leaf and root tissues of watermelon plants in response to treatment of SA and MeJA and to infection of Fon and Db. However, the expression changes induced by the two defense hormones and the two fungal pathogens imply the involvement of the watermelon ClMBD genes in disease resistance, probably through affecting transcription of a set of genes including those involved in defense response.

The responsiveness of the watermelon ClMBD genes to exogenous SA and JA, two hormones that mediated defense response against (hemi)biotrophic pathogens such as Pst DC3000 and necrotrophic fungi like B. cinerea (Glazebrook, 2005; Grant and Jones, 2009) led to evaluate the disease resistance phenotype of the ClMBD-OE Arabidopsis lines against Pst DC3000 and B. cinerea. In the present study, ectopic overexpression of ClMBD2, 3, and 5 in transgenic Arabidopsis led to increased susceptibility to B. cinerea (Figure 7 and Supplementary Figure 12), suggesting that ClMBD2, 3, and 5 are negative regulators of defense response against B. cinerea. This is further supported by the suppression of pathogen-induced expression of defense gene AtPDF1.2, an indicator gene of defense response against necrotrophic fungal pathogens, and overaccumulation of ROS in ClMBD2-OE, ClMBD3-OE, and ClMBD5-OE plants (Figure 6). This is consistent with the general concept that excessive ROS accumulation during early stage often benefits the infection by the necrotrophic fungi like B. cinerea, but is different from the phenomenon that early ROS accumulation is critical to the activation of immune response against (hemi)biotrophic pathogens (Mengiste, 2012). On the other hand, overexpression of ClMBD1 and ClMBD2 in transgenic Arabidopsis attenuated while overexpression of ClMBD5 strengthened resistance to Pst DC3000 (Figure 7), indicating that ClMBD1 and ClMBD2 are negative regulators while ClMBD5 is a positive regulator of immunity against this bacterial pathogen. This is consistent with the suppression of expression of AtPR1 in ClMBD1-OE and ClMBD2-OE plants but elevation of expression of AtPR1 in ClMBD5-OE plants after infection of Pst DC3000 (Figure 7). This is also indirectly supported by the down-regulated expression of ClMBD2 in leaf tissues of watermelon plants after infection of Db, a heminecrotrophic fungal pathogen (Figure 5). Notably, overexpression of ClMBD2 in transgenic Arabidopsis resulted in attenuated immunity against both B. cinerea and Pst DC3000; however, overexpression of ClMBD5 led to opposite functions in immunity against these two pathogens, e.g., attenuated immunity against B. cinerea but strengthened immunity against Pst DC3000 (Figures 6, 7). It is generally accepted that immune response against (hemi)biotrophic pathogens such as Pst DC3000 is modulated through the SA signaling while the defense response against necrotrophic pathogens like B. cinerea is regulated by the JA/ET signaling (Glazebrook, 2005; Grant and Jones, 2009). Both antagonistic interaction and synergistic cross-talks between the SA and JA/ET signaling pathways occur and allow plants to mount appropriate immune responses against different invading pathogens (Glazebrook, 2005; Koornneef and Pieterse, 2008; Verhage et al., 2010). It is therefore likely that ClMBD2 and ClMBD5 function in immunity through regulating different mechanisms.

Transcriptome profiling identified a limited number of DEGs in the ClMBD2-OE2 plants grown under normal growth conditions (Supplementary Tables 5, 6). The fact that genes involved in DNA binding, transcriptional factor activity, response to stimulus, and immune system process were overrepresented in DEGs in ClMBD2-OE2 plants (Figures 8A,B) further confirms the function of ClMBD2 in immunity of the transgenic Arabidopsis plants against B. cinerea and Pst DC3000. Generally, the MBD proteins recognize the methylated CG sites and recruit chromatin remodelers and histone deacetylases to repress transcription of target genes (Lang et al., 2015). Considering the attenuated immunity against B. cinerea and Pst DC3000 (Figures 6, 7), it is speculated that overexpression of ClMBD2 should lead to down-regulation of a set of genes that are involved in Arabidopsis immunity. Surprisingly, only 18 genes were identified as down-regulated genes (expression change $>1.5$-folds and $P$-value < 0.05) in the ClMBD2-OE2 plants (Supplementary Table 6). Among these down-regulated genes, AtWRKY54 was previously reported to function as a positive regulator of SARD1 and CBP60g expression in immunity against P. syringae pv. maculicola (Chen et al., 2021; Figure 8). No other gene with known function in Arabidopsis immunity was identified in the down-regulated genes in ClMBD2-OE2 plants (Supplementary Table 6). This might be due to the fact that samples from healthy ClMBD2-OE2 plants without pathogen infection were used for RNA-seq analysis. Indeed, the expression of defense genes such as AtPR1 and AtPDF1.2 was suppressed significantly in the ClMBD2-OE plants upon infection with Pst DC3000 and B. cinerea, respectively (Figures 6, 7). If it is the case that ClMBD2, like its closely related AtMBD5 and AtMBD6, acts to repress transcription of target genes, this may imply that the function of ClMBD2 in suppression of transcription of defense genes in transgenic Arabidopsis plants occurs upon pathogen infection. By contrast, some genes that negatively regulate Arabidopsis immunity were found to be up-regulated in the ClMBD2-OE2 plants (Figure 8 and Supplementary Table 5). For example, AtWRKY18 negatively regulates resistance against Pst DC3000 but positively modulates resistance to B. cinerea.
B. cinerea immunity against performed by ectopic overexpression in Arabidopsis may and watermelon, the functional analysis in the present study divergence of gene functions in immunity between Arabidopsis.

ClMBD2 Berg, A., Meza, T. J., Mahiæ, M., Thorstensen, T., Kristiansen, K., and Aalen, watermelon disease resistance. Therefore, further investigations wide binding sites in the DNA methylation levels and characterizing the genome- that are involved in Arabidopsis immunity. Further analyzing the DNA methylation levels and characterizing the genome-wide binding sites in the ClMBD2-OE and ClMBD5-OE transgenic Arabidopsis plants will definitely provide detailed molecular mechanisms by which ClMBD2 and ClMBD5 regulate immunity against B. cinerea and Pst DC3000. Transcriptome analysis showed that overexpression of ClMBD2 in transgenic Arabidopsis affected the expression of a small set of genes that are involved in Arabidopsis immunity. Further analyzing in watermelon through overexpression and CRISPR/Cas9-based knockout approaches will be critical to elucidate the functions and molecular mechanisms of the ClMBD genes, especially the ClMBD1, 2, 3, and 5 in disease resistance against Fon, Db, and other pathogens.

DATA AVAILABILITY STATEMENT
The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA803007.

AUTHOR CONTRIBUTIONS
FS and DL conceived the project and designed the experiments. JL generated all material used in this study (cloning, vector, transformations, transgenic plants). JL, XL, YW, and XW performed the experiments and collected the data. FS, JL, and DL analyzed the data. FS and JL drafted the manuscript. All authors commented on the manuscript.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.886965/full#supplementary-material

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