



# Histone H3 Lysine 9 Methyltransferase DIM5 Is Required for the Development and Virulence of *Botrytis cinerea*

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Histone methylation is widely present in animals, plants and fungi, and the methylation modification of histone H3 has important biological functions. Methylation of Lys9 of histone H3 (H3K9) has been proven to regulate chromatin structure, gene silencing, transcriptional activation, plant metabolism, and other processes. In this work, we investigated the functions of a H3K9 methyltransferase gene *BcDIM5* in *Botrytis cinerea*, which contains a PreSET domain, a SET domain and a PostSET domain. Characterization of *BcDIM5* knockout transformants showed that the hyphal growth rate and production of conidiophores and sclerotia were significantly reduced, while complementary transformation of *BcDIM5* could restore the phenotypes to the levels of wild type. Pathogenicity assays revealed that *BcDIM5* was essential for full virulence of *B. cinerea*. *BcDIM5* knockout transformants exhibited decreased virulence, down-regulated expression of some pathogenic genes and drastically decreased H3K9 trimethylation level. However, knockout transformants of other two genes heterochromatin protein 1 (HP1) *BcHP1* and DNA methyltransferase (DIM2) *BcDIM2* did not exhibit significant change in the growth phenotype and virulence compared with the wild type. Our results indicate that H3K9 methyltransferase *BcDIM5* is required for H3K9 trimethylation to regulate the development and virulence of *B. cinerea*.

**Keywords:** *Botrytis cinerea*, histone H3 lysine 9 methyltransferase, H3K9 trimethylation, *BcDIM5*, virulence, development

## INTRODUCTION

Genetic information of eukaryotes is stored as chromatin, which consists of genomic DNA, histones, and a wide array of chromosomal proteins. In eukaryotes, DNA is wrapped around histones, which are subjected to a variety of covalent modifications, such as methylation, acetylation, phosphorylation, and ubiquitylation (Widom, 1998). Histone methylation mainly occurs on the side chains of lysines and arginines. Lysines may be mono-, di-, or tri-methylated, whereas arginines may be mono-, symmetrically, or asymmetrically di-methylated (Bedford and Clarke, 2009; Lan and Shi, 2009). Lysine methylation is highly selective, and K4 and K9 of histone H3 are the best characterized sites (Liu et al., 2010). In general, lysine 9 of histone H3 (H3K9) is associated with transcriptionally inactive heterochromatin, and is a well-conserved epigenetic mark

for heterochromatin formation and transcriptional silencing, while H3K4 methylation is associated with transcriptionally active euchromatin (Berger, 2007).

The first histone lysine methyltransferase to be identified was SUV39H1, which targets H3K9 (Rea et al., 2000). Strikingly, all of the histone lysine methyltransferases that methylate N-terminal lysines contain a so-called SET domain that harbors the enzymatic activity. *Neurospora crassa* DIM5 can specifically tri-methylate H3K9 (Xiao et al., 2003). Orthologs of SUV39H1, which are named as Clr4 in yeast (Nakayama et al., 2001), Su(var)3-9 in *Drosophila* (Tschiersch et al., 1994), and Suv39h1 in mice (O'Carroll et al., 2000), are the major heterochromatic H3K9 methyltransferases and play a dominant role in pericentric heterochromatin formation.

*Botrytis cinerea* (teleomorph: *Botryotinia fuckeliana*) causes severe loss in more than 200 crop species worldwide. It is most destructive on the mature or senescent tissues of dicotyledonous hosts and ornamentals (Williamson et al., 2007). *B. cinerea* is a typical necrotroph fungus, whose infection strategies include killing of the host cells and feeding on the dead tissues by secreting cell wall degrading enzymes, and toxic metabolites that induce cell death prior to the invasion of hyphae (Siewers et al., 2005; Choquer et al., 2007). Two polyketide synthases (*BcPKS6* and *BcPKS9*) are required for phytotoxin botcinic acid biosynthesis and virulence (Dalmais et al., 2011). Two velvet-like genes (*BcVeA* and *BcVELB*) are involved in the regulation of fungal development, oxidative stress response, and virulence in *B. cinerea* (Yang et al., 2013). Presilphiperfolan-8 beta-alpha synthase *BcBOT2* is responsible for the first step of botrydial synthesis (Wang et al., 2009), whereas *BcBOT1* is a P450 monooxygenase that acts in the later step of biosynthesis (Siewers et al., 2005). Methylation of histone H3 is required for the normal development of *N. crassa* (Adhvaryu et al., 2005), and methylation of lysine H3K9 is a mark that primes the formation of heterochromatin and a critical chromatin landmark for genome stability (Rivera et al., 2015). Here, to better understand the functions of histone H3 methylation in *B. cinerea*, we identified a K9 histone H3 methyltransferase gene *BcDIM5* in *B. cinerea* through Blastp searching the homologs of DIM5 in *N. crassa*. This protein contains a PreSET domain, a SET domain, and a PostSET domain, which is the typical structure of a H3K9 methyltransferase. We characterized the function of *BcDIM5*. The results indicate that *BcDIM5* is required for hyphal growth and the production of conidiophores and sclerotia. Furthermore, pathogenicity assays indicate that *BcDIM5* is essential for full virulence of *B. cinerea*. *BcDIM5* knockout transformants showed down-regulated expression of some pathogenic genes and H3K9 trimethylation was drastically decreased *in vivo*, but two other genes *BcHPI1* and *BcDIM2* were not required for the development and virulence of *B. cinerea*. Taken together, these results indicate that *BcDIM5* is important for the development and virulence of *B. cinerea*.

## MATERIALS AND METHODS

### Fungal Strains and Culture Conditions

*B. cinerea* wild-type strain B05.10 was used in this study. The fungus was grown on potato dextrose agar (200 g potato, 20 g

glucose per liter, 2% agar), and incubated for 7–30 days at 20°C. Knockout transformants were cultured on PDA amended with 75 µg/mL hygromycin B (Calbiochem, San Diego, CA, USA). Complementary transformants were cultured on PDA amended with 75 µg/mL hygromycin B and 50 µg/mL G418. *Escherichia coli* strain DH5α was used to propagate all of the plasmids, and *Agrobacterium tumefaciens* strain EHA105 was used for the transformation of fungi. Seedlings of *Arabidopsis thaliana* (ecotype Columbia-0) were grown in the greenhouse at 20 ± 2°C for 1 month under a 12 h light/dark cycle with 70% relative humidity.

### Gene Knockout and Complementation by Protoplast Transformation

*BcDIM5*, *BcDIM2*, and *BcHPI1* were knocked out in the WT strain B05.10 using the hygromycin B-resistance gene (*Hyg*) to replace the partial sequences of *BcDIM5*, *BcDIM2*, and *BcHPI1* (Figure S1). To construct the  $\Delta BcDIM5$  disruption vector, a 753-bp DNA fragment named 3U with the *EcoRI* restriction sites at 5'- and 3'-terminus and a 740-bp DNA fragment named 3D with the *EcoRI* restriction sites at 5'- and 3'-terminus were PCR-amplified from genomic DNA of strain B05.10. Using pKS1004 vector as the template, the 740-bp HY fragments were amplified by primers HYG-F and HYG-R, and the 1133-bp YG fragments were amplified by primers YG-F and YG-R. The 3U and HY fragments were fused with PCR to obtain a 1465-bp fragment named 3UHY, and 3D and YG fragments were fused with PCR to obtain a 1936-bp fragment named 3DYG. After digestion with enzymes *EcoRI*, the DNA fragments 3UHY and 3DYG were separately collected and inserted into the linearized plasmid pBluescriptII KS1004 for sequencing. 3UHY and 3DYG were individually linearized and transformed separately into the protoplasts of *B. cinerea* wild type strain B05.10 using the PEG-mediated protoplast transformation technique (Wei et al., 2013). For complementation assays, the 5.75-kb PCR product containing a 3-kb upstream sequence (which contained the endogenous promoter), a full-length *BcDIM5* gene coding region, and a 1-kb downstream sequence was amplified from the wild type strain B05.10 genomic DNA using primers DIM-5-CF-*Bam*HI and DIM-5-CR-*Bam*HI and cloned into the same sites of p3300neoIII to generate the *BcDIM5* complementary vector p3300neoIII-*BcDIM5*, which was then transformed into *BcDIM5* knockout transformants using the *A. tumefaciens*-mediated transformation technique.

### DNA Extraction and Southern Blot Analysis

The wild-type strain and knockout transformants were cultured on PDA plates covered with cellophane membranes, and mycelia were harvested at 3 days post inoculation (dpi). Genomic DNA was extracted using the CTAB method. Southern blot analysis was conducted following the method described by Wei et al. (2016). The genomic DNA of 15 µg was completely digested with *Hind*III, separated by electrophoresis on 0.8% agarose gel and transferred onto a Hybond N+ membrane (Amersham Pharmacia Biotech). The probe was amplified the specific fragment of hygromycin *hph* gene and be labeled by DIG (GE healthcare). The nylon membranes were autoradiographed and

analyzed using Bio-imaging analyser BAS-1800II (FUJIFILM, Tokyo, Japan).

### Pathogenicity Tests

Pathogenicity tests of transformed and wild-type *B. cinerea* strains were performed on Arabidopsis, tomato, soybean by the inoculation of detached leaves with young non-sporulating mycelium or conidial suspensions. Leaves were harvested from 4-week-old plants and placed in a transparent plastic box lined with tissue moistened with sterile water. Leaves were inoculated with 2-mm-diameter plugs of 3-day-old mycelium. Alternatively, conidia were collected from 10-day-old plates and suspended in water to a final concentration of  $5 \times 10^5$  conidia/mL. Droplets of 5  $\mu$ L were applied to the leaves. Storage boxes containing inoculated leaves were incubated in a growth cabinet at 20°C with 16 h of daylight. Disease development on leaves was recorded daily as the radial spread from the inoculation point to the lesion margin. Pathogenicity assays on leaves were repeated three times using at least three leaves per assay.

### Quantitative Real-Time PCR

The mRNA transcripts were measured using a SYBR Green I real-time PCR assay in a CFX96 real-time PCR detection systems (Applied Biosystems). The thermal cycling conditions were 95°C for 2 min for predegeneration, 40 cycles of 95°C for 20 s for denaturation, 60°C for 20 s for annealing and 72°C for 20 s for extension. All of the reactions were run in triplicate by monitoring the dissociation curve to control the dimers. The *B. cinerea*  $\beta$ -tubulin gene was used as reference gene. qRT-PCR was used to examine the expression of *B. cinerea* *Bcsod1* (BC1G\_00558), *BcSpl1* (BC1G\_02163), *BcVeA* (BC1G\_02976), *BcBOT2* (BC1G\_06357), *Bcmp3* (BC1G\_07144), *BcVELB* (BC1G\_11858), *BcPKS* (BC1G\_15837), *Bcbot1* (BC1G\_16381), *BcDIM2* (BC1G\_12419), *BcHP1* (BC1G\_06432), and *BcDIM5* (BC1G\_11188). The primers for qRT-PCR are listed in **Table S1**.

### Western Blot Analysis of H3K9 Trimethylates

Mycelia were harvested and ground in liquid nitrogen. Two hundred milligrams powders were resuspended in 400  $\mu$ L cell lysis buffer on ice (Beyotime, China). Total proteins of mycelium extract were centrifuged at 12,000 rpm at 4°C for 10 min, and the supernatant was used for western-blot analysis. Proteins were separated by 12% PAGE and electroblotted to a nitrocellulose membrane at 25 V for 40 min. The membrane was blocked with Tris-buffered saline plus Tween 20 containing 5% skim milk powder for 2 h at room temperature. After incubation with primary antibody and then with secondary antibody, the membrane was transferred for protein detection using a Thermo SuperSignal West Pico kit (Thermo Scientific). The sources and dilutions of antibodies were as follows: Histone H3 monoclonal antibody (Abmart, at 1: 2000), Histone H3 tri methyl K9 monoclonal antibody (Abmart, at 1: 2000), and goat anti-mouse horseradish peroxidase-conjugated antibody (Abmart, at 1: 5000).

## RESULTS

### *BC1G\_11188* Was Predicted as a Histone H3 Lysine 9 Methyltransferase

The *B. cinerea* *BC1G\_11188* gene (GenBank accession: XM\_001550366.1) is a single copy gene consisting of 4 exons and 3 introns and encoding a peptide of 357 amino acid residues. The protein contains the typical domain structures of H3K9 methyltransferase (**Figure 1A**), including a PreSET domain, a SET domain, and a PostSET domain (Rea et al., 2000; Tamaru and Selker, 2001; Adhvaryu et al., 2005). SET domain contains two conserved sequences of NHXCXPN and DY, which can form an AdoMet binding site and the catalytic activity site of methyl transferase, and participates in the formation of DIM5 hydrophobic structure (Zhang et al., 2002). PreSET domain contains nine conserved cysteine residues that can be combined with three zinc ions to form a zinc cluster. PostSET domain contains three conserved cysteine residues involved in the binding of DIM5 to AdoMet (**Figure 1B**). Similar to the histone lysine methyltransferases of NcDIM5, SpClr4, HsSUVH1, HsSUVH2, and AtSUVH4, *BC1G\_11188* includes cysteine-rich sequences that flank a SET domain (**Figure 1B**). The results of Bioinformatics Toolkit HHpred analysis indicate that the structure of *BC1G\_11188* is similar to the three-dimensional structure of histone lysine n-methyltransferase (Template: c4qoA, Confidence: 100%; **Figure 1C**). In summary, the protein coded by *BC1G\_11188* resembles histone H3 lysine 9 methyltransferase. Thus, we designated this gene which was derived from the homologs of *DIM5* in *N. crassa* as “*BcDIM5*.”

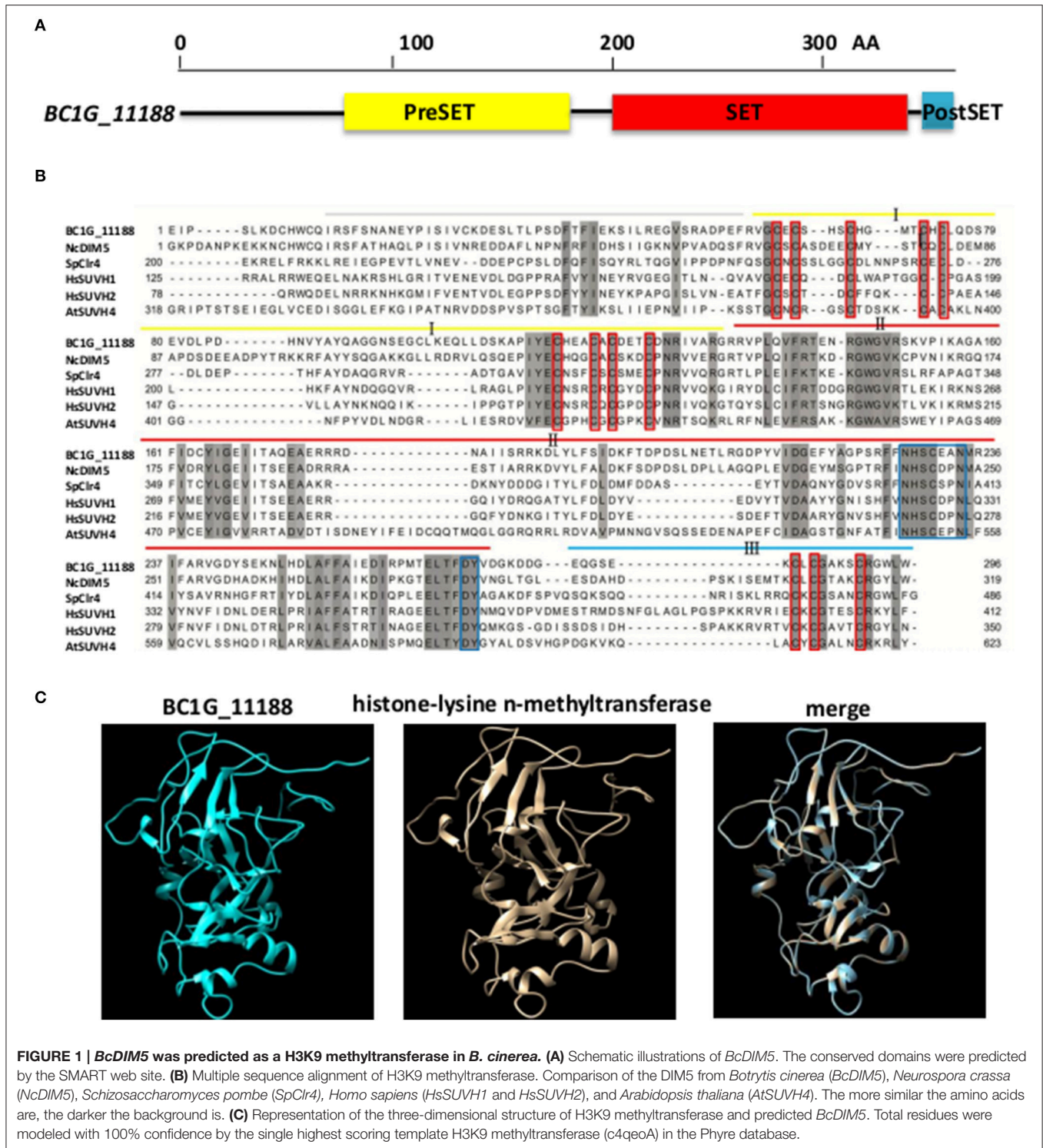
### Generation of *BcDIM5* Knockout and Complementary Transformants

To examine the function of *BcDIM5*, the *BcDIM5* knockout transformants were generated through homologous recombination of the *BcDIM5* open reading frame with a gene conferring hygromycin resistance (HYG) (**Figure S1A**). Positive knockout transformants were amplified with PCR (**Figure S1B**), and three *BcDIM5* knockout transformants (21, 24, and 25) were identified. Reverse transcription PCR was employed to detect the *BcDIM5* gene expression. The results showed that there were no transcripts in the three knockout transformants (**Figure S1C**). Two transformants ( $\Delta BcDIM5-21$  and  $\Delta BcDIM5-24$ ) were further confirmed by Southern blot analysis (**Figure S1D**). The mutant  $\Delta BcDIM5-21$  was used for phenotypic analysis and complemented with the full-length gene of wild type *BcDIM5*. After being verified by PCR, the complemented strain *BcDIM5-C1* was chosen for further study (**Figure S1E**).

### *BcDIM5* Played an Important Role in *B. cinerea* Growth and Development

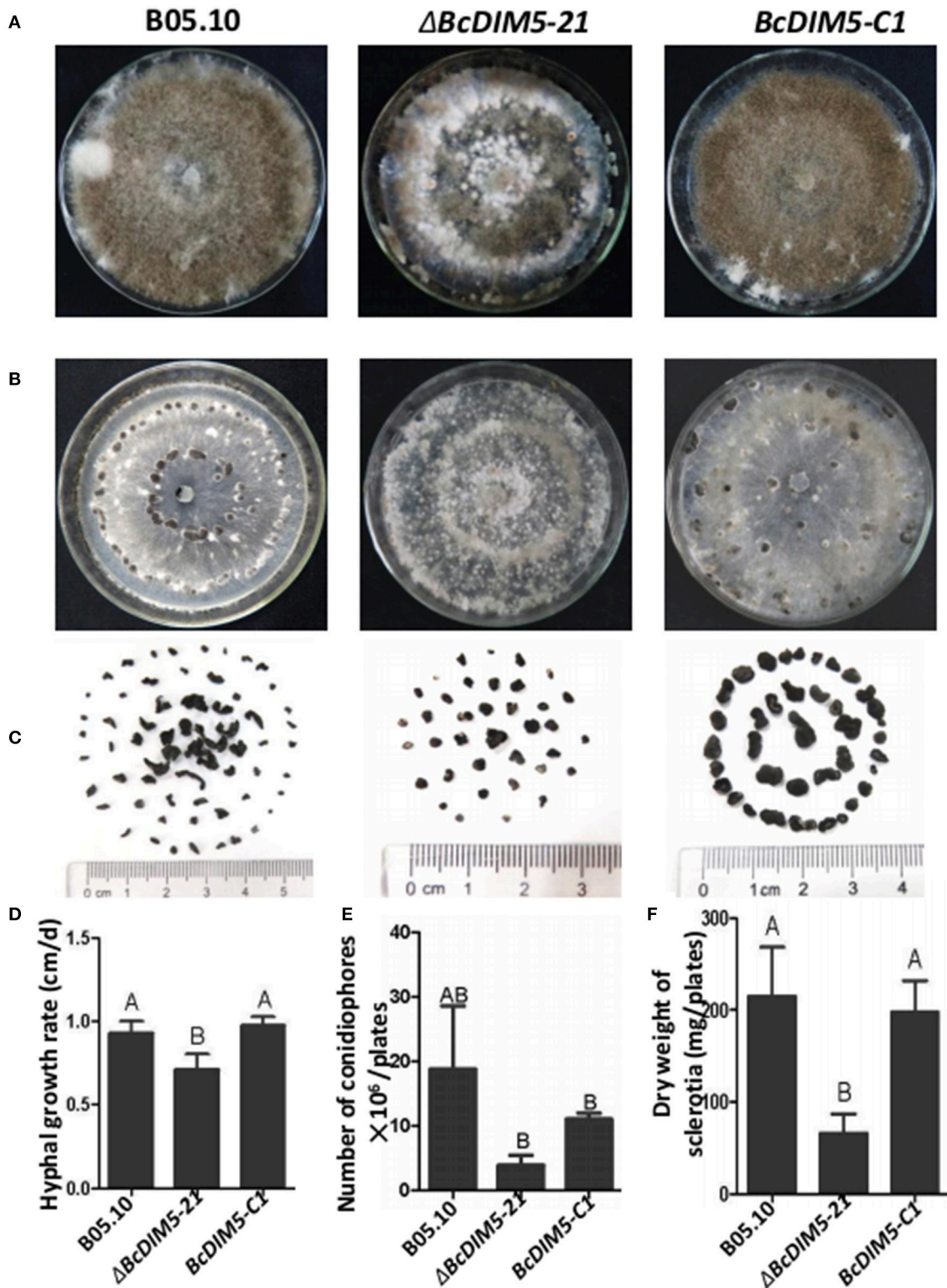
To determine the role of *BcDIM5* in the growth and development of *B. cinerea*, we compared the hyphal growth rate, the number of conidiophores and dry weight of sclerotia of the wild-type strain,  $\Delta BcDIM5-21$  strain and the *BcDIM5-C1* complemented strain. Although *BcDIM5* did not affect the morphology of tip hyphae (**Figure S2**), the hyphal growth rate was significantly





reduced in  $\Delta BcDIM5-21$  strains, while this phenotype was restored after complementation (Figure 2D). BcDIM5 affected the development of conidiophores and sclerotia, and the wild-type strain could averagely produce  $2 \times 10^7$  conidiophores per plate, while  $\Delta BcDIM5-21$  strains only produced  $3 \times 10^6$  conidiophores per plate, and the conidiophore production of

BcDIM5-C1 complemented strain was restored to  $1.1 \times 10^7$  conidiophores per plate (Figures 2A,E). Although  $\Delta BcDIM5-21$  strain was able to produce conidiophores, the time required for conidiophore initiation was about 3–4 days longer than that for the normal strain. The dry weight of sclerotia produced by wild-type *B. cinerea* was 217 mg per plate, while  $\Delta BcDIM5-21$



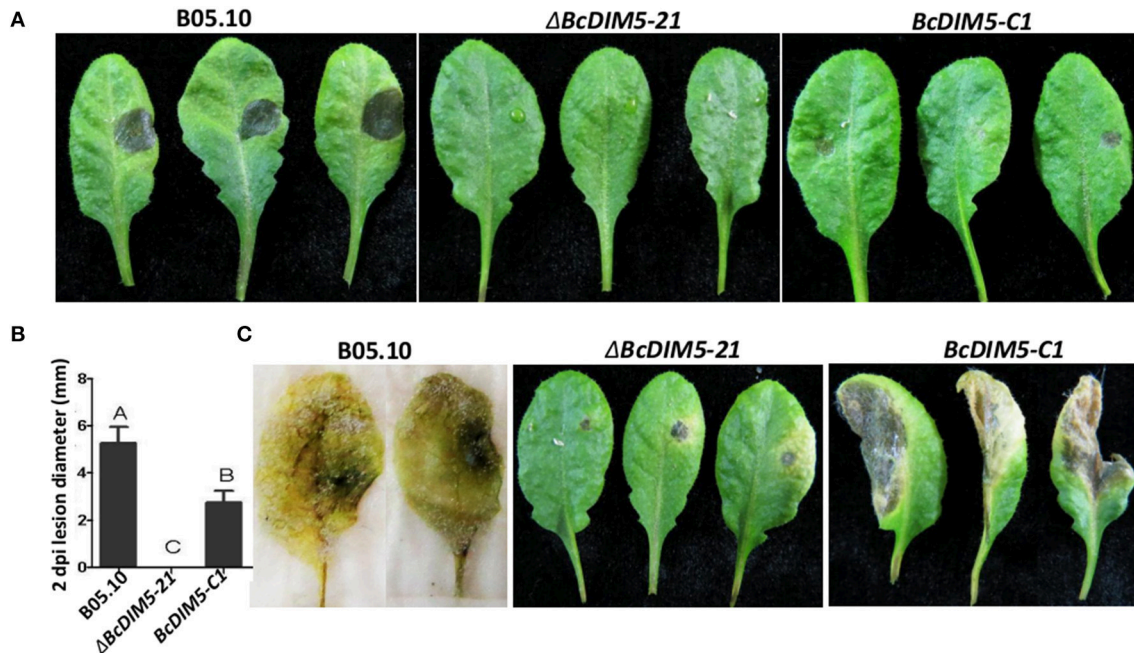
**FIGURE 2 | Biological characterization of the wild-type strain, *BcDIM5* knockout, and complemented transformants. (A)** Comparison of the phenotypes of the *BcDIM5* knockout and complemented strain. All the strains were grown on a PDA plate at 20°C for 15 days under a 12 h light/12 h dark photoperiod. **(B)** Comparison of the phenotypes of the *BcDIM5* knockout and complemented strain. All the strains were grown on a PDA plate at 20°C for 15 days under dark condition. **(C)** Sclerotia produced by strains on a PDA plate at 20°C for 30 days under dark condition. **(D)** Comparison of the hyphal growth rate of the *BcDIM5*

(Continued)



**FIGURE 2 | Continued**

knockout and complemented strains. Three independent replications were performed. Bars indicate the standard error. The values are presented as the mean  $\pm$  s.d. Differentiation was evaluated by *t*-test. Different letters on a graph indicate significant differences,  $P < 0.05$ . **(E)** Comparison of the number of conidiophores per plate of *BcDIM5* knockout and complemented strains. Three independent replications were performed,  $P < 0.05$ . **(F)** Comparison of dry weight of sclerotia produced by *BcDIM5* knockout and complemented strains. Three independent replications were performed,  $P < 0.05$ .



**FIGURE 3 | Pathogenicity assays of the  $\Delta BcDIM5-21$  strain of *B. cinerea* on detached Arabidopsis leaves. (A)** Four-week-old Arabidopsis detached leaves were inoculated by 5  $\mu$ L conidia (concentration is  $5 \times 10^5$  spores/mL) of wild type B05.10 (left), *BcDIM5* knockout  $\Delta BcDIM5-21$  strain (middle), *BcDIM5* complemented *BcDIM5-C1* strain (right). The representative symptoms were photographed at 2 dpi. The experiment was repeated three times. **(B)** Comparison of the lesion diameters of the wild-type B05.10 strain, *BcDIM5* knockout, and complemented strains. **(C)** Four-week-old Arabidopsis detached leaves were inoculated by 5  $\mu$ L conidia (concentration is  $5 \times 10^5$  spores/mL) of wild-type B05.10 strain (left), *BcDIM5* knockout  $\Delta BcDIM5-21$  strain (middle), *BcDIM5* complemented *BcDIM5-C1* strain (right). The representative symptoms were photographed at 5 dpi after inoculation.

strains only produced 57 mg sclerotia per plate, and the sclerotia was small and round. The sclerotia production of *BcDIM5-C1* complemented strain was restored to 200 mg per plate, which was similar to that of the wild type (Figures 2B,C,F). These data clearly suggest that *BcDIM5* knockout has a deleterious effect on hyphal growth and the development of conidiophores and sclerotia, meanwhile, the reduced hyphal growth rate of  $\Delta BcDIM5-21$  strains may delay conidiophore development and the sclerotia formation.

### *BcDIM5* Knockout Transformants Exhibited Decreased Virulence

To determine the role of *BcDIM5* in pathogenicity, detached Arabidopsis leaves were inoculated with conidia of the wild-type strain,  $\Delta BcDIM5-21$  strain and *BcDIM5-C1* complemented strain. At 2 dpi, the inoculation of the wild-type strain B05.10 caused the formation of necrotic lesions with 5–6 mm diameter, and the leaves completely rotted away at 5 dpi (Figure 3). However, the leaves inoculated with the  $\Delta BcDIM5-21$  strains exhibited no necrotic lesions at 2 dpi, and only slight necrotic

lesions in the infection site at 5 dpi. The pathogenicity of the *BcDIM5-C1* complemented strain was only partially restored, as the diameter of the necrotic lesions was half of that induced by wild-type strain (Figure 3). We further used mycelium pieces to inoculate soybean and tomato leaves. Similar results were observed in  $\Delta BcDIM5-21$  and  $\Delta BcDIM5-24$  mutants. The virulence of the *BcDIM5* knockout transformants was significantly reduced, and only small lesions were observed on the detached leaves of soybean and tomato (Figure S3), indicating that the virulence reduction is not host species-specific. These results indicate that *BcDIM5* plays crucial roles in the virulence.

### Pathogenic Genes Were Down-Regulated in *BcDIM5* Knockout Transformants

To determine whether *BcDIM5* regulates the expression of genes associated with pathogenesis, qRT-PCR was performed to analyze the expression of eight genes involved in the pathogenic process of *B. cinerea* based on previous studies. These eight genes included a Cu-Zn-superoxide dismutase gene *Bcsod1*, which is slightly increased by 0.5 mM  $H_2O_2$  and significantly increased

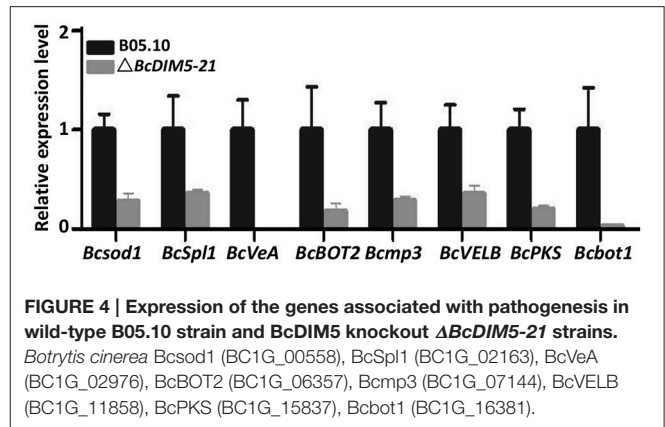
by 2.0 mM H<sub>2</sub>O<sub>2</sub> (Rolke et al., 2004); a cerato-platanin family protein gene *BcSpl1*, which was induced in tobacco systemic resistance to two plant pathogens (Frías et al., 2013); two velvet-like genes *BcVEA* and *BcVELB*, which act as negative regulators for conidiation and melanin biosynthesis in *B. cinerea* (Yang et al., 2013); a presilphiperfolan-8 beta-alpha synthase gene *BcBOT2*, which encode the sesquiterpene synthase that is responsible for the committed step in the biosynthesis of botrydial (Wang et al., 2009); a mitogen-activated protein kinase gene *Bcimp3*, which is required for normal saprotrophic growth, conidiation, plant surface sensing and host tissue colonization (Rui and Hahn, 2007), a polyketide synthase gene *BcPKS* (Dalmais et al., 2011) and a cytochrome P450 monooxygenase gene *Bcbot1* (Siewers et al., 2005). Expression analyses revealed that all of these eight genes were significantly down-regulated in the *BcDIM5* knockout transformants compared with in the wild type (Figure 4). These data indicate that many pathogenic genes may be regulated by *BcDIM5*.

### H3K9 Trimethylation Level Was Drastically Decreased in *BcDIM5* Knockout Transformants

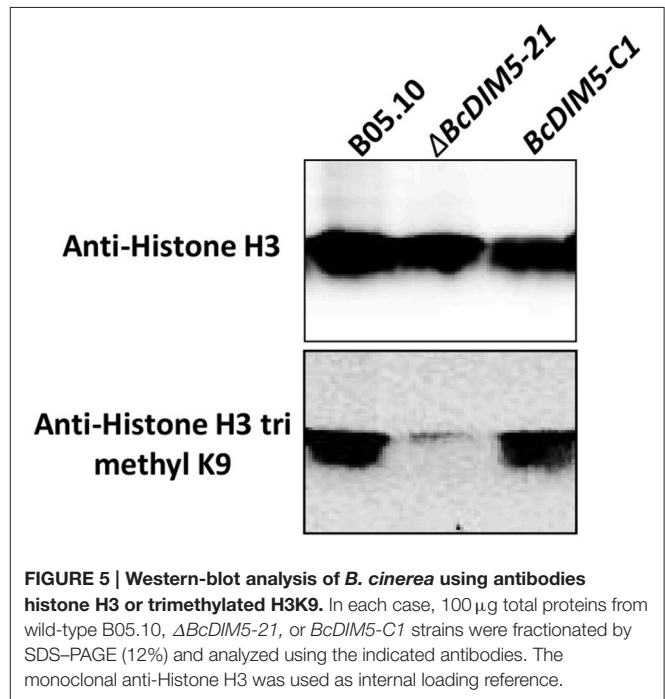
To investigate the possible effects of DIM5 on the trimethylation of H3K9, we carried out western-blot to analyze the histone methylation level of the wild-type strain,  $\Delta BcDIM5-21$  and *BcDIM5-C1* (Figure 5). All the strains presented equally robust signals when detected using the antibody histone H3. Notably, trimethylated H3K9 was detectable in bulk from the wild-type strain and *BcDIM5-C1*, while knockout transformant  $\Delta BcDIM5-21$  extinguished the trimethylated H3K9 signal, suggesting that *BcDIM5* is dominantly responsible for the trimethylation of H3K9, and DIM5 may generate trimethylated H3K9 from unmodified histone H3 *in vivo*.

### *BcDIM2* and *BcHP1* Were Not Required for Virulence in *B. cinerea*

In *N. crassa*, DIM5 trimethylates H3K9, and H3K9me3 directs DNA methylation through a complex containing heterochromatin protein 1 (HP1) and the DNA methyltransferase DIM2. We also investigated whether DIM2 and HP1 play important roles in *B. cinerea* development and virulence. Based on the homology to *N. crassa* DIM2 and HP1, we cloned *BcDIM2* (BC1G\_12419) and *BcHP1* (BC1G\_06432) from *B. cinerea*. *BcDIM2* contains typical domain structures of DNA methyltransferase and *BcHP1* contains typical domain structures of CD (chromo domain) and CSD (chromo shadow domain; Paro and Hogness, 1991; Aasland and Stewart, 1995). RT-PCR analysis showed that *BcDIM5*, *BcDIM2*, and *BcHP1* were all expressed in mycelium growth, sclerotial development and infection stages (Figure S4). *BcDIM5* was constitutively expressed in these three stages, and *BcDIM2* was significantly up-regulated during the infection stage, while *BcHP1* was significantly down-regulated during the sclerotial development. Through homologous recombination methods, we got two knockout transformants  $\Delta BcDIM2-16$  and  $\Delta BcHP1-7$ . There were no significant changes in the phenotype and number



**FIGURE 4 | Expression of the genes associated with pathogenesis in wild-type B05.10 strain and *BcDIM5* knockout  $\Delta BcDIM5-21$  strains.** *Botrytis cinerea* *Bcsod1* (BC1G\_00558), *BcSpl1* (BC1G\_02163), *BcVeA* (BC1G\_02976), *BcBOT2* (BC1G\_06357), *Bcimp3* (BC1G\_07144), *BcVELB* (BC1G\_11858), *BcPKS* (BC1G\_15837), *Bcbot1* (BC1G\_16381).

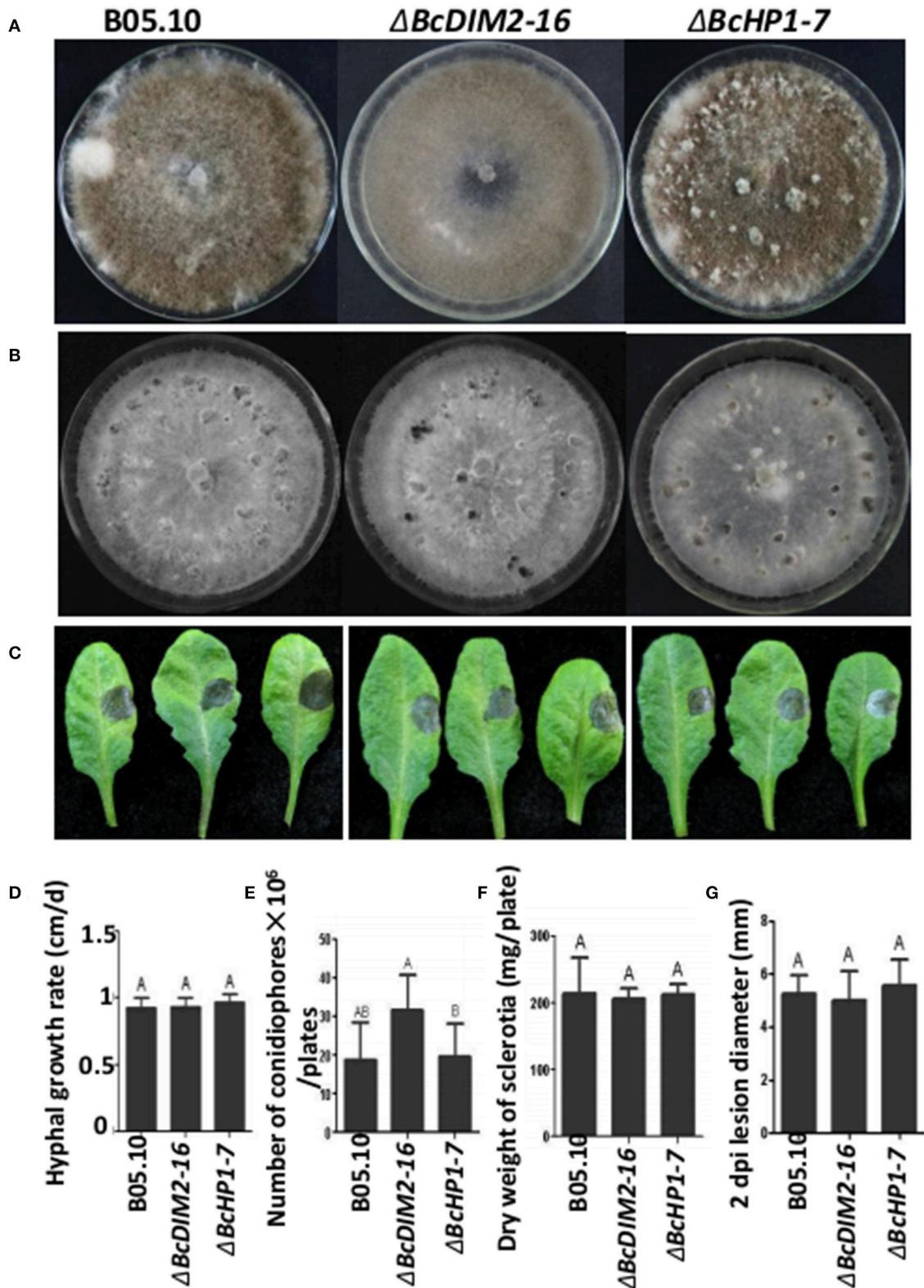


**FIGURE 5 | Western-blot analysis of *B. cinerea* using antibodies histone H3 or trimethylated H3K9.** In each case, 100  $\mu$ g total proteins from wild-type B05.10,  $\Delta BcDIM5-21$ , or *BcDIM5-C1* strains were fractionated by SDS-PAGE (12%) and analyzed using the indicated antibodies. The monoclonal anti-Histone H3 was used as internal loading reference.

of produced conidiophores as well as in hyphal growth rate, dry weight of sclerotia and virulence among the wild-type,  $\Delta BcDIM2-16$  and  $\Delta BcHP1-7$  strains (Figure 6). Therefore, it can be concluded that *BcDIM5* is required for the development and virulence whereas *BcDIM2* and *BcHP1* are not required for virulence in *B. cinerea*.

## DISCUSSION

Epigenetic alterations have important roles in certain biological processes such as cell phenotypic conversion, cell proliferation, and cell death by regulating transcriptional activity (Reik, 2007; Papp and Plath, 2011). The effect of epigenetic processes on transcription depends on various post-translational histone modifications (Wierda et al., 2015). The major histone modifications include acetylation, phosphorylation, methylation, and ubiquitylation (Arnaudo and Garcia, 2013).



**FIGURE 6 | Phenotypes of *BcDIM2* and *BcHP1* knockout transformants. (A)** Comparison of the phenotypes of the  $\Delta BcDIM2-16$  and  $\Delta BcHP1-7$  knockout transformants. All the strains were grown on a PDA plate at 20°C for 15 days under a 12 h light/12 h dark photoperiod. **(B)** Comparison of the phenotypes of the  
(Continued)



**FIGURE 6 | Continued**

$\Delta BcDIM2-16$  and  $\Delta BcHPI-7$  knockout transformants. All the strains were grown on a PDA plate at 20°C for 15 days under dark condition. **(C)** Pathogenicity assays of the  $\Delta BcDIM2-16$  and  $\Delta BcHPI-7$  knockout transformants of *B. cinerea* on detached Arabidopsis leaves. **(D)** Comparison of the hyphal growth rate of the  $\Delta BcDIM2-16$  and  $\Delta BcHPI-7$  knockout transformants. **(E)** Comparison of the number of conidiophores per plate of  $\Delta BcDIM2-16$  and  $\Delta BcHPI-7$  knockout mutants. **(F)** Comparison of dry weight of sclerotia produced by  $\Delta BcDIM2-16$  and  $\Delta BcHPI-7$  knockout transformants. **(G)** Comparison of the lesion diameters of the wild type,  $\Delta BcDIM2-16$  and  $\Delta BcHPI-7$  knockout transformants.

In this paper, we further described the extended role of H3K9 methyltransferase *BcDIM5* in *B. cinerea*. *BcDIM5* was predicted as a methyltransferase with a SET domain (**Figure 1**). Although there is no evidence to show that *BcDIM5* has methyltransferase activity *in vitro*, the structure-guided sequence alignment indicates that *BcDIM5* has all of the methyltransferase characteristics, since all the aligned sequences were verified to be active methyltransferases previously (Zhang et al., 2002). In addition, as shown in **Figure 5**, western blot analysis using dimethyl H3K9 antibodies demonstrates that trimethylated H3K9 in *BcDIM5* knockout transformants was drastically decreased and the phenotypes of the transformants were restored after complementation. These findings suggest that *BcDIM5* has methyltransferase activity *in vivo*.

Analyses of the  $\Delta BcDIM5$  and complemented  $\Delta BcDIM5$  strains in this study indicate that  $\Delta BcDIM5$  is involved in sclerotia production. The  $\Delta BcDIM5$  transformants exhibited declined hyphal growth rate and reduced production of conidiophores and sclerotia (**Figure 2**). Declined growth rate of  $\Delta BcDIM5$  strains may affect the conidiophores development and sclerotia formation. In order to avoid this, we delayed the statistical time for development of conidiophores and sclerotia for 15 or 30 days, respectively. The results suggest that *BcDIM5* is required for normal hyphal growth, conidiation, and sclerotia development. Additionally, in the pathogenicity assays,  $\Delta BcDIM5$  knockout transformants exhibited drastic reduction in virulence compared with wild type B05.10, while complemented  $\Delta BcDIM5$  strain could partially restore the phenotype (**Figure 3**). These results indicate that *BcDIM5* plays a crucial role in the development and pathogenicity of *B. cinerea*, which is consistent with the results of previous studies. In histone methyltransferase G9a knockout mice, H3K9 methylation was drastically reduced, resulting in severe growth retardation and early lethality, which indicates that G9a is essential for early mouse embryo development (Tachibana et al., 2002, 2005). We then identified that eight pathogenic genes expression in *BcDIM5* knockout transformants (**Figure 4**). *Bcsod1*, which is increased by H<sub>2</sub>O<sub>2</sub> accumulation, is drastically declined in  $\Delta BcDIM5$  strain. *Bcmp3*, which is required for normal saprotrophic growth, is drastically declined in  $\Delta BcDIM5$  strain. *BcSpl1*, which is induced in systemic resistance, is drastically declined in  $\Delta BcDIM5$  strain. *BcVeA* and *BcVELB* are involved in the regulation of fungal development, oxidative stress response and virulence in *B. cinerea*, are drastically declined in  $\Delta BcDIM5$  strain. *BcBOT1*, *BcBOT2*, and *BcPKS*, associated with P450, botrydial and phytotoxin botcinic acid biosynthesis, respectively, are drastically declined in  $\Delta BcDIM5$  strain. Down-regulated eight pathogenic genes may lead to the drastically declined pathogenicity of  $\Delta BcDIM5$  strain. High throughput sequencing can identify the suppressed genes in  $\Delta BcDIM5$  strain, and the transcriptionally

suppressive function of *BcDIM5* may depend on its histone methyltransferase activity, but the molecular mechanism needs further investigation.

In *N. crassa*, all DNA methylation requires the DNA methyltransferase DIM2 (Kouzminova and Selker, 2001). DNA methylation also requires the histone H3K9 methyltransferase DIM5 (Tamaru and Selker, 2001; Tamaru et al., 2003) and heterochromatin protein HP1 (Freitag et al., 2004). H3K9 trimethylation directs DNA methylation through a complex containing HP1 and the DNA methyltransferase DIM2 (Honda et al., 2012). Based on the homology to *N. crassa* DIM2 and HP1, we cloned *BcDIM2* and *BcHPI*. The  $\Delta BcDIM2-16$  and  $\Delta BcHPI-7$  knockout transformants showed no significant change in the development of conidiophores and sclerotia and virulence compared with wild type B05.10 (**Figure 6**). These results indicate that DNA methylation does not play important roles in this process. This growth phenotype is in agreement with the results of knockout of *N. crassa* DIM2 and *Aspergillus nidulans dmtA* (DNA methyltransferase homolog A), which did not affect the normal growth of the strain (Kouzminova and Selker, 2001; Lee et al., 2008). Although, DNA methylation is common in organisms such as bacteria, fungi, higher plants, and mammals, we did not use genomic DNA sequencing to detect the level of DNA methylation in *B. cinerea*, thus it is not known yet whether genomic DNA methylation is missing in  $\Delta BcDIM2-16$  knockout transformants. These data may be needed to explore the function of *BcDIM2* in *B. cinerea*. HP1 plays a key role in heterochromatin formation and gene silencing, and is a positive regulator of active transcription in euchromatin (Kwon and Workman, 2011; Yearim et al., 2015). A number of recent studies have linked HP1 proteins to the DNA damage pathway, and HP1 proteins are independent of H3K9 trimethylation (Ayoub et al., 2008; Luijsterburg et al., 2009). *Neurospora* HP1 is required for normal growth of fungi (Freitag et al., 2004). In *Schizosaccharomyces pombe*, the HP1 homologs swi6 and chp2/clo2 are important for normal growth but are not essential for viability (Halverson et al., 2000). In contrast,  $\Delta BcHPI-7$  knockout transformants in *B. cinerea* did not show noticeable growth defects. Most eukaryotes contain several HP1 homologs that display dramatic differences in both subcellular localizations and functions (Ryu et al., 2014). The function of *BcHPI* in *B. cinerea* needs further research. Unlike that of *BcDIM2* and *BcHPI*, knockout mutation of the histone methyltransferase gene *BcDIM5* causes growth abnormality, pathogenic gene silencing and virulence reduction in *B. cinerea*, suggesting that it is histone methylation rather than DNA methylation that is involved in the development and virulence of *B. cinerea*. The detailed mechanisms of *BcDIM5* to regulate the growth and pathogenic gene silencing need further investigation.

## AUTHOR CONTRIBUTIONS

XZ, XL contributed equally to the article, XZ, XL, JC, and TC designed the research and wrote the paper; XZ, XL, YZ, and TC executed the experiments. XZ, XL, YZ, JC, JX, YF, DJ, and TC performed the data and analyses. All authors read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.01289>

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**Figure S1 | Identification of *BcDIM5* knockout and complemented transformants. (A)** Principle of homologous recombination. **(B)** *BcDIM5* knockout transformants identified by PCR. **(C)** *BcDIM5* knockout transformants identified by RT-PCR. **(D)** *BcDIM5* knockout transformants identified by Southern blot using *HYG* labeled with DIG as probe. **(E)** *BcDIM5* complemented transformants identified by PCR.

**Figure S2 | Morphological comparison of the hyphal tips of *BcDIM5* knockout and complemented transformants.**

**Figure S3 | Virulence assay of *BcDIM5* knockout and complemented transformants on detached soybean and tomato leaves.** Virulence was evaluated based on the lesion diameter at 20°C for 48 h. **(A)** Virulence assay of *BcDIM5* knockout and complemented transformants on detached soybean leaves. **(B)** Virulence was evaluated based on the lesion diameter on soybean leaves. **(C)** Virulence assay of *BcDIM5* knockout and complemented transformants on tomato leaves. **(D)** Virulence was evaluated based on the lesion diameter on tomato leaves.

**Figure S4 | Expression level of genes in different phase.** Forty-eight hour indicates the wild type strain B05.10 grown in PDA for 48 h, which is the hyphal growth stage; 156 h indicates the wild-type strain B05.10 grown in PDA for 156 h, which is the sclerotial development stage. IN indicates the wild-type strain B05.10 inoculated on *A. thaliana* leaves for 48 h, which is the infection stage.

**Table S1 | Primers used in the study.**

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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