Phytopathology Research

RESEARCH

Open Access

# The $Zn(II)_2Cys_6$ transcription factor BcDIC affects the asexual reproduction of *Botrytis cinerea* by regulating pectinesterase genes

Shasha Lu[1,2†], Xiaokang Zhang[1,2†], Chang He[1,2], Guangjin Li[1,2], Tong Chen[1,2], Boqiang Li[1,2], Shiping Tian[1,2] and Zhanquan Zhang[1,2*]

## Abstract

*Botrytis cinerea* is one of the most destructive plant pathogens, causing great economic losses in a wide variety of crops. It is difficult to control the pathogen because of its flexible mode of infection, high reproduction, genetic plasticity and strong stress resistance. There are more than 400 genes encoding transcription factors in the genome of *B. cinerea*, among which $Zn(II)_2Cys_6$(C6) transcription factors constitute the largest family. However, the mechanisms of $Zn(II)_2Cys_6$(C6) transcription factors regulating the development and pathogenicity of *B. cinerea* are largely unknown. In this study, we identified an important C6 transcription factor BcDIC that regulates the asexual reproduction and pathogenicity of *B. cinerea*. The virulence of the knockout mutant *ΔBcDIC* was reduced on various hosts. Especially, *ΔBcDIC* completely lost the ability of conidiation. By analyzing the transcriptome data, we found that deletion of *ΔBcDIC* enhanced the expression of pectinesterase family genes in *B. cinerea*. Furthermore, the results of yeast-one-hybrid and dual-LUC reporter assay suggested that BcDIC could bind to the promoter of two pectinesterase genes and suppress their expression. In this work, we identified a C6 transcription factor involved in the pathogenesis and asexual reproduction of *B. cinerea*, and the results implied that the capacity of pectin degradation was closely related with the reproduction process.

**Keywords** *Botrytis cinerea*, BcDIC, Asexual production, Pathogenicity, Pectinesterase

## Background

*Botrytis cinerea*, the causal agent of grey mold disease, is one of the most destructive phytopathogenic fungi with a very broad of host range (Fillinger and Elad 2016). *B. cinerea* can infect more than 1400 plant species, including many important agricultural crops, such as grape, strawberry, kiwifruit, tomato, cucumber, rose, etc. The global economic losses caused by *B. cinerea* reach 10–100 billion dollars each year (Weiberg et al. 2013). *B. cinerea* ranks second among all plant pathogenic fungi due to its economic and scientific importance, and it has been used as a model organism to uncover the pathogenesis of necrotrophic pathogens (Dean et al. 2012). *B. cinerea* is difficult to control because of its flexible infection modes, high reproductive yield, strong stress tolerance and high genetic plasticity (Williamson et al. 2007; Cheung et al. 2020). Many pathogenic factors of *B. cinerea* have been characterized, including secreted proteins (Kars et al. 2005a; Brito et al. 2006; Zhu et al. 2017; Li et al. 2020), signal transduction components (Gronover et al. 2004; Yin et al. 2018), ROS generating systems (Segmüller et al. 2008; An et al. 2015, 2016; Li et al. 2016, 2019).

†Shasha Lu and Xiaokang Zhang contributed equally to this work.

*Correspondence:
Zhanquan Zhang
zhangzhanquan82@ibcas.ac.cn
[1] Key Laboratory of Plant Resources, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China
[2] University of Chinese Academy of Sciences, Beijing 100049, China

Transcriptional regulation is an important regulatory mechanism in eukaryotes. Transcription factors (TFs) are nucleus-located proteins, which interact specifically with cis-acting elements in the target gene promoter region, and regulate the gene transcription level in specific time and space. There are more than 400 genes encoding transcription factors in the genome of *B. cinerea* (Amselem et al. 2011), but transcriptional regulation mechanisms involved in the development and pathogenicity of *B. cinerea* are largely unknown. Up to now, only a few transcription factors have been identified that regulate the development or pathogenicity of *B. cinerea*. It has been reported that, knocking out of the STE family transcription factor Ste12, zinc finger transcription factor Crz1 and Reg1 significantly reduced the pathogenicity of *B. cinerea* (Schumacher et al. 2008; Schamber et al. 2010; Michielse et al. 2011). The GATA family transcription factor BcLTF1 of *B. cinerea* can be induced by light signal, and plays an important role in regulating sporulation, pathogenicity, reactive oxygen balance and secondary metabolism (Schumacher et al. 2014). In our previous work, we explored the function of MADS-box family transcription factor BcMADS1 in the pathogenesis and photomorphogenesis of *B. cinerea* (Zhang et al. 2016). Among the 410 transcription factors of *B. cinerea* (strain B05.10), there are 129 $Zn(II)_2Cys_6$ (C6) family transcription factors, which make up the largest transcription factor family in this fungus (Amselem et al. 2011). C6 transcription factors belong to zinc figure protein family, which contains six cysteine residues combined with two zinc atoms. C6 family transcription factors are unique and abundant in fungi (MacPherson et al. 2006). Gal4p in *Saccharomyces cerevisiae* is the well-studied C6 transcription factor (Marmorstein et al. 1992; MacPherson et al. 2006). Gal4p can activate the genes involved in galactose metabolism (Breunig 2000). Based on the conserved consensus amino acid sequence of $Zn(II)_2Cys_6$ motif, 55 C6 transcription factors were identified in *S. cerevisiae* (Akache et al. 2001; Tnrcotte et al. 2004; Todd and Andrianopoulos 1997), which were involved in various cellular processes, including sugar metabolism (Breunig 2000), gluconeogenesis and respiration (Vincent and Carlson 1998), amino acid metabolism (Iraqui et al. 1999), chromatin remodeling (Angus-Hill et al. 2001) and the stress response (Akache and Turcotte 2002; Hikkel et al. 2003; Larochelle et al. 2006). There are also a considerable number of C6 transcription factors in *Candida albicans*, an important human pathogenic fungus. The C6 transcription factor of *C. albicans* Upc2p is involved in the ergosterol biosynthesis (Silver et al. 2004; MacPherson et al. 2005). Recently, C6 transcription factors have been gradually characterized in filamentous fungi. In *Aspergillus nidulans*, the biosynthesis of aflatoxin is regulated by the C6 transcription factor AflR (Price et al. 2006). The melanin is required for the successful invasion in the plant pathogenic fungi *Colletotrichum lagenarium*. The C6 transcription factor Cmr1p can active the expression of melanin biosynthetic structural genes *SCD1* and *THR1* in *C. lagenarium* (Tsuji et al. 2000). In *Fusarium graminearum*, C6 transcription factor EBR1 affects virulence and apical dominance of the hyphae tip. Deletion of *EBR1* led to reduction of radial growth, conidiation, and virulence (Zhao et al. 2011). Tpc1 is a critical C6 transcription factor regulating polarized growth and virulence in *Magnaporthe oryzae*, which is the most important phytopathogenic fungus causing rice blast (Galhano et al. 2017). Tpc1 is involved in conidiogenesis, infection-associated autophagy, glycogen and lipid metabolism, and colonization in plant tissue (Galhano et al. 2017). In addition, two other C6 transcription factors, MoCOD1 and MoCOD2, have been characterized in *M. oryzae* (Chung et al. 2013). MoCOD1 is involved in appressorium formation and invasive growth within the host cells, and MoCOD2 is a critical regulator for conidiation and pathogenicity (Chung et al. 2013).

In this study, we explored the critical C6 transcription factor in regulating the development and pathogenesis of *B. cinerea*. Based on the expression pattern, we focused on the C6 transcription factors *BcDIC*, whose expression was significantly induced during the infection process of *B. cinerea*. The results suggested that *BcDIC* plays an important role in the pathogenesis and conidiation of *B. cinerea.* Furthermore, we found that BcDIC may regulate the conidiation process by modulating the expression of pectinesterase family genes. These results will deepen our understanding of the development and pathogenesis of *B. cinerea* at transcriptional level.

## Results

### C6 family transcription factors are involved in the infection process

The domain *Zn(2)-C6_fun-type_DNA-bd* (IPR001138) is the most prominent feature of C6 family transcription factor. This domain is usually located at the N-terminal region of protein and contains six cysteines (Cys), in which two zinc atoms are bound by six Cys residues. C6 transcription factors constitute the largest transcription factor family in *B. cinerea* (Amselem et al. 2011). In *B. cinerea* (B05.10), a total of 237 transcription factors contain the domain IPR001138, of which 141 proteins only contain this domain of transcription factor (Fig. 1a). Basing on the published transcriptome data of *B. cinerea*, we found that the expressions of six C6 transcript factor genes, *Bcin13g03120*, *Bcin02g08710*, *Bcin03g00940*, *Bcin12g04950*, *Bcin08g01810*, and *Bcin06g04550*, were strongly induced in the process of infecting grape fruit

(Kelloniemi et al. 2015), which are distributed in different subclasses (Fig. 1a). To further verify the expression patterns of these 6 C6 genes, we monitored their real-time expression upon *B. cinerea* infection on tomato fruit at the earlier stage. Similar to that in grape fruit, the expression of *Bcin13g03120*, *Bcin02g08710*, and *Bcin03g00940* were also significantly induced upon infection on tomato fruit (Fig. 1b). The expression level of *Bcin02g08710*, which was designated as *BcDIC* (**d**efective **i**n **c**onidiation) in this paper, was the most strongly induced gene in the early stage of infection. At 12 h post inoculation, the expression of *BcDIC* was 29 fold higher than that at the initial time point (Fig. 1b). However, the expressions of *Bcin12g04950*, *Bcin08g01810*, and *Bcin06g04550* were significantly inhibited, contrary to the data on grape fruit (Fig. 1b). These results implied that the 6 C6 transcription factors were involved in the pathogenesis of *B. cinerea*, and some of them showed different expression pattern in different hosts.

#### Generation of knockout mutant of *BcDIC*

As the expression of *BcDIC* was strongly induced during the infection process, it implies that it is actively involved in the development or pathogenesis of *B. cinerea*. Phylogenetic analysis using the whole protein sequence showed that BcDIC was classified into the subclass with the C6 protein Upc2 of *C. albicans* and AflR of *A. nidulans* (Additional file 1: Figure S1a). Alignment of the amino acid sequences of the $Zn(II)_2Cys_6$ motif regions suggested that BcDIC contain a typical $Zn(II)_2$-$Cys_6$ motif at N terminal (Additional file 1: Figure S1b). To further investigate the biological function of *BcDIC*, we generated the knockout mutant of *BcDIC* by homologous recombination of flanking sequence of target gene (Additional file 1: Figure S1a). The partial sequence of *BcDIC* (1939 bp) was replaced by hygromycin resistance cassette. Two independent transformants were obtained on screening medium supplemented with hygromycin B and were identified by flank-spanning PCR (Additional file 1: Figure S2a). To get homokaryon strains, one of the identified transformant was subjected to single-spore isolation (Additional file 1: Figure S2b), and then the homokaryon strain was subjected to Southern blot to exclude ectopic integration (Additional file 1: Figure S2c). The results indicated that we finally obtained the homokaryon mutant with single copy integration, which was named *ΔBcDIC*. In order to further confirm the biological function of *BcDIC*, we reintroduced the whole sequence of *BcDIC* into the knockout mutant, and the complementary strain was named $\Delta BcDIC^{com}$.

#### BcDIC is crucial for conidiation of *B. cinerea*

The knockout mutant *ΔBcDIC* showed significant lower growth rate than that in the wild-type (Fig. 2a, c). After being cultured for 4 days on PDA plate, the colony diameter of *ΔBcDIC* was 33% smaller than that of wild-type strain (Fig. 2c). Two weeks after inoculation, we collected and calculated the conidiation yield of each strain. The results showed that the conidia production of wild-type strain reached $5.25 \times 10^7$ per plate (Fig. 2a, d). *ΔBcDIC* was completely incapable of producing conidia (Fig. 2a, d). The branches at the hyphal tip of wild-type strain began to increase after culturing for 24 h, a small amount of conidia differentiated from the tip branches after 36 h, and a large number of mature conidia were formed after 48 h (Fig. 2b). In contrast, *ΔBcDIC* lacked the differentiation process of conidia, and maintained vegetative growth (Fig. 2b). The growth rate and conidiation yield of the complementary strain restored to the wild-type level (Fig. 2c, d). These results suggest that *BcDIC* is a critical gene regulating the asexual reproduction of *B. cinerea*.

#### BcDIC is involved in the pathogenicity of *B. cinerea*

We further evaluated the virulence of the *ΔBcDIC* on tomato and apple fruit. The results showed that the virulence of *ΔBcDIC* was significantly reduced compared to wild-type strain. The lesion diameter on tomato fruit caused by *ΔBcDIC* was reduced by 52% compared to wild-type at the 72 h after inoculation (Fig. 3a, b). The virulence of *ΔBcDIC* reduced by 32% compared with wild-type on apple fruit (Fig. 3c, d). After reintroducing the gene *BcDIC* to the knockout mutant, the pathogenicity of complementary strain completely restored to the level of the wild-type strain (Fig. 3a, c). These results indicated that the deletion of *BcDIC* leads to a significant decrease in the virulence of *B. cinerea*.

#### *BcDIC* arranges the gene expression of *B. cinerea* during the conidiation process

Because the most prominent phenotypic variation of *ΔBcDIC* is loss of conidiation, we attempt to explore the role of *ΔBcDIC* in the transition from vegetative

(See figure on next page.)

**Fig. 1** C6 transcription factors are involved in the infection process of *B. cinerea*. **a** Phylogenetic analysis of C6 transcription factors in *B. cinerea*. The alignment of C6 transcription factor sequences was conducted using Clustal W. The phylogenetic tree was constructed by MEGA 11.0 using the Neighbor-Joining method, and bootstrap test was carried out by 1000 replications. The gene ID highlighted in yellow are the genes for subsequent study. **b** The expression of six C6 transcription factor genes during the infection process of *B. cinerea*. Tomato fruits were inoculated with *B. cinerea* conidial suspension. The relative expressions of 6 C6 transcription factor genes were detected at 0, 8, 12, and 24 h post inoculation (hpi), respectively. Vertical bars represent standard errors of the means. Columns with different letters indicate significant differences ($P < 0.05$)

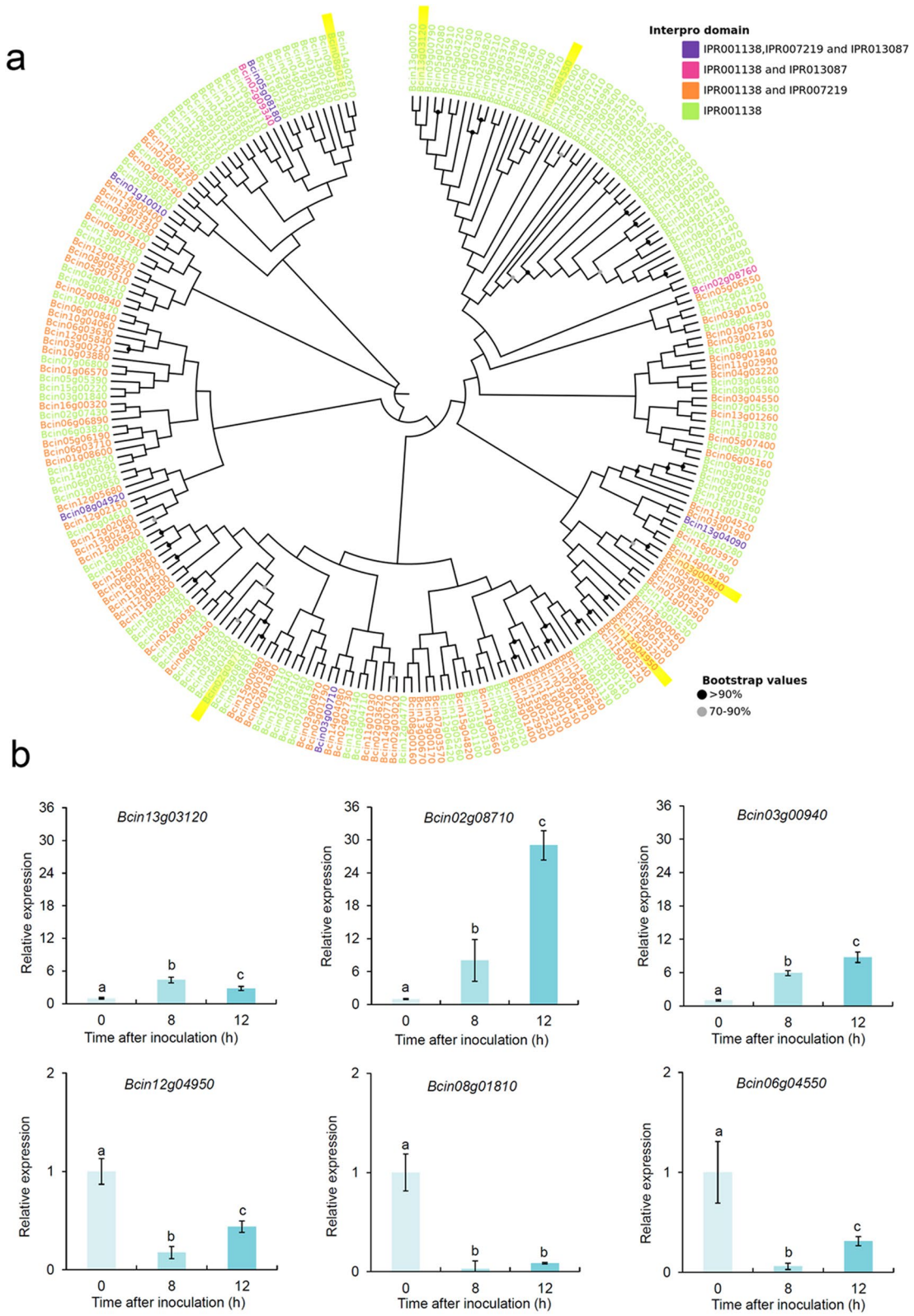


**Fig. 1** (See legend on previous page.)

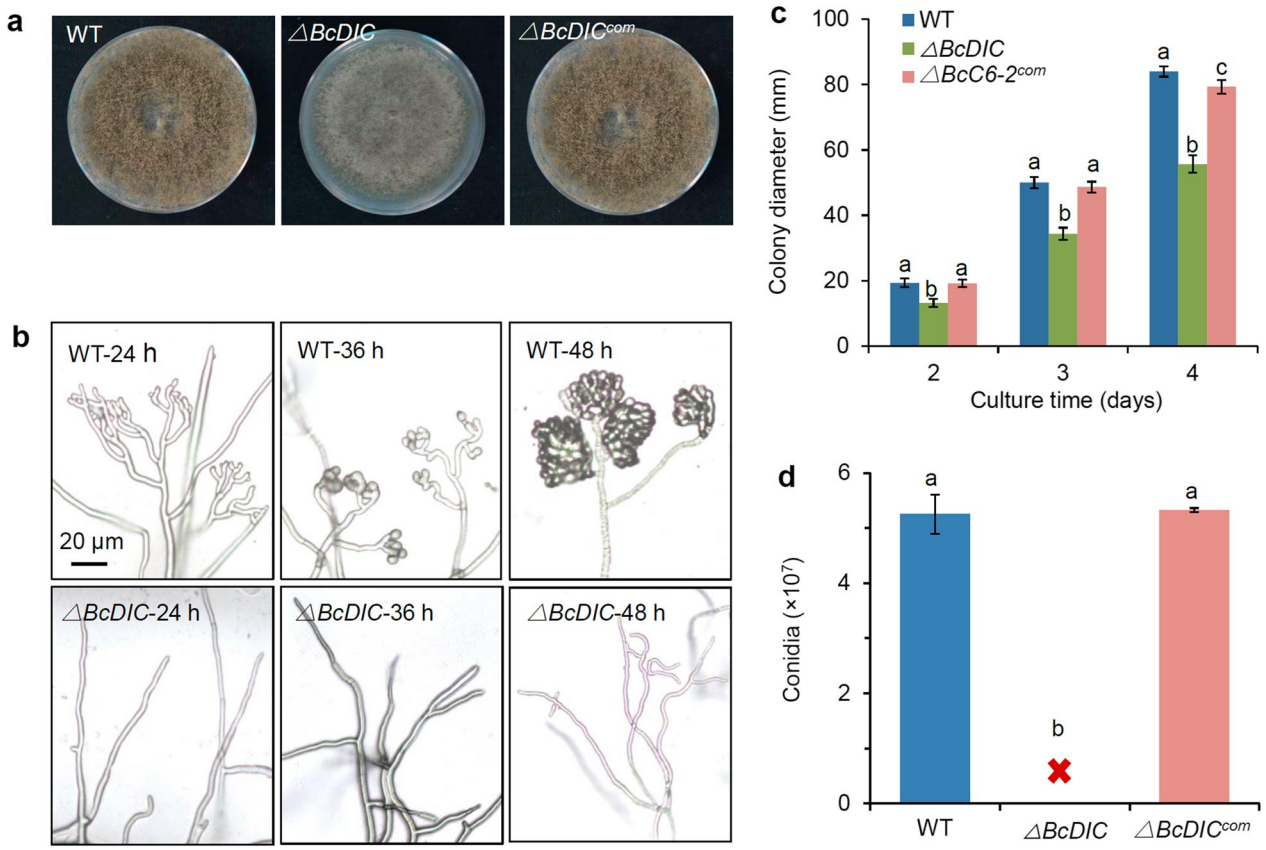


**Fig. 2** *BcDIC* affects the development of *B. cinerea*. **a** The colony phenotype of the knockout mutants after culturing for 2 weeks on PDA plates. **b** The conidiation structure of wild-type strain and *ΔBcDIC*. Wild-type strain and *ΔBcDIC* were first cultured on PDA plates for 2 days, and then the agar with mycelium were transferred to moist chamber slide culture for further culturing. Conidiogenesis were observed after 24, 36, and 48 hpi under optical microscope, respectively. **c** Growth rate of 6 C6 transcription factor gene mutants. **d** The conidial yield after culturing for 2 weeks on PDA plates. Vertical bars represent standard errors of the means. Columns with different letters indicate significant differences ($P < 0.01$)

growth to conidiation of *B. cinerea* through transcriptomics method. In the laboratory condition, the vegetative growth of *B. cinerea* is vigorous after culturing for 3 days on the plate, and conidia are formed after culturing for 5 days. Therefore, we performed the transcriptome analysis of wild-type strain and *ΔBcDIC* cultured on PDA plates for 3 days and 5 days, respectively (Fig. 4). The results showed that in the transition from vegetative growth to conidiation of wild-type strain, gene expression pattern was dramatically rearranged (Additional file 2: Table S1). In comparison of WT3 *vs* WT5, there were 1825 differential expression genes (DEGs), including 1156 up-regulated genes and 669 down-regulated genes (Fig. 5a and Additional file 3: Table S2). However, in the mutant *ΔBcDIC*, the number of DEGs in this transition was greatly reduced to 882 (C3 *vs* C5), including 608 up-regulated genes and 274 down-regulated genes (Fig. 4b). The number of genes with high fold change was also significantly higher in wild-type strain than in *ΔBcDIC* (Fig. 4b). At the initial stage of conidiation, there were 1089 genes differentially expressed (402 up-regulated and 687 down-regulated) between wild-type strain and *ΔBcDIC* (Fig. 4a). Nevertheless, the difference of gene expression between wild-type strain and *ΔBcDIC* in vegetative growth stage was much smaller (492 DEGs) (Fig. 4a). We further compared the expression of conidiation-associated genes between *ΔBcDIC* and wild-type strain. The results indicated that 10 of the 15 conidiation-associated genes were significantly induced during the conidiation process in wild-type strain. On the contrary, the expression of most of conidiation-associated genes in *BcDIC* mutant was suppressed (Additional file 1: Figure S3). These results suggested that *ΔBcDIC* plays an important role in the conidiation process of *B. cinerea*.

### Deletion of *BcDIC* increases the expression of pectinesterase genes in *B. cinerea*

We further analyzed the DEGs between the wild-type and *ΔBcDIC* at the initial state of conidiation (5 dpi). Among the 1089 DEGs between wild-type strain and

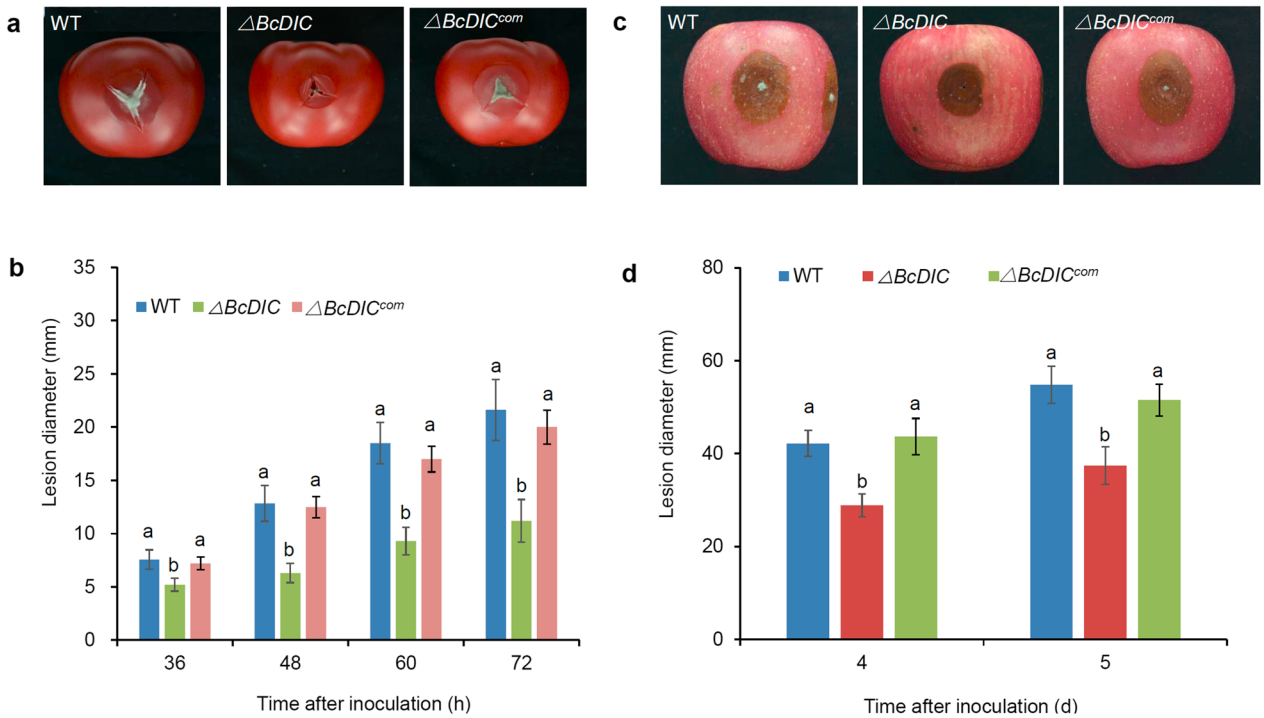


**Fig. 3** Virulence assay of mutants. **a** Disease symptoms on tomato fruits caused by wild-type strain and 6 mutants (72 hpi). **b** Lesion diameters on tomato fruits. **c** Disease symptoms on apple fruits caused by wild-type strain and *ΔBcDIC*. **d** Lesion diameters on apple fruits. Vertical bars represent standard errors of the means. Columns with different letters indicate significant differences ($P < 0.01$)

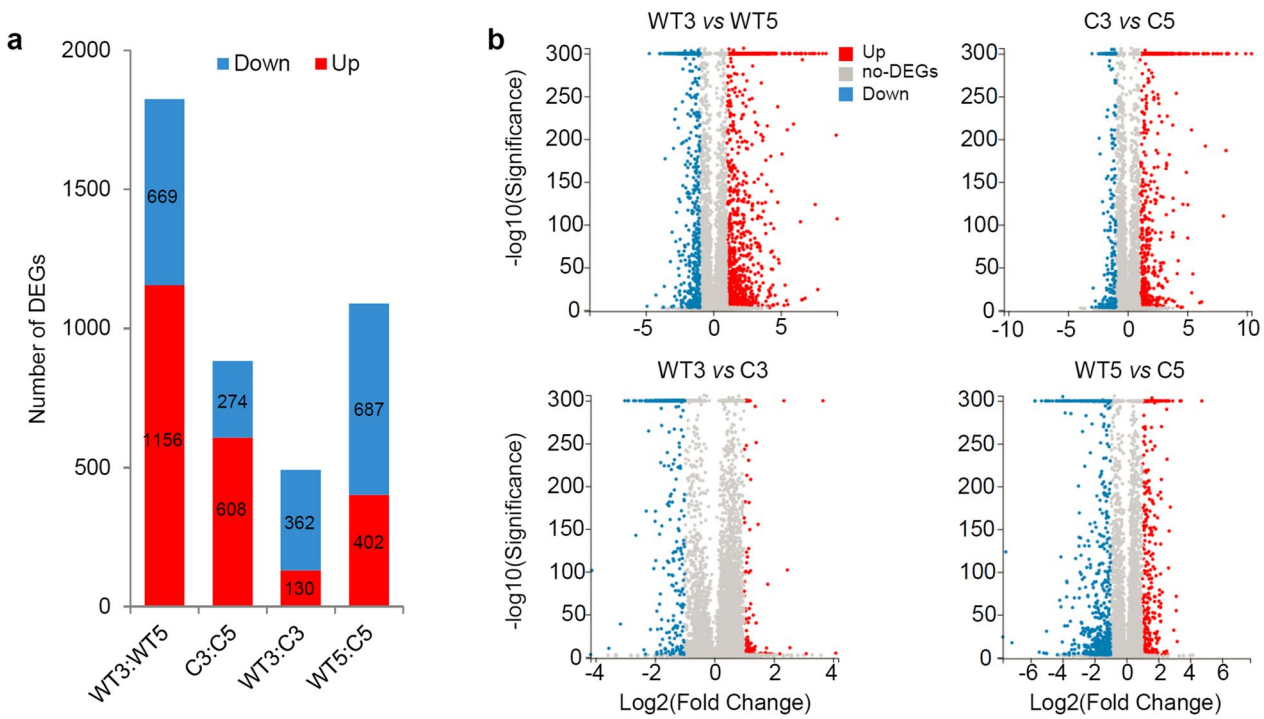


**Fig. 4** The number of differential expression genes in different comparable groups. **a** The number of up- and down-regulated genes in different comparable group. **b** Volcano maps show the distribution of differential expression genes in different comparison group. Each dot represents a gene. WT3: wild-type strain was cultured for 3 days under light condition; WT5: wild- type strain was cultured for 5 days under light condition; C3: *ΔBcDIC* was cultured for 3 days under light condition; C5: *ΔBcDIC* was cultured for 5 days under light condition

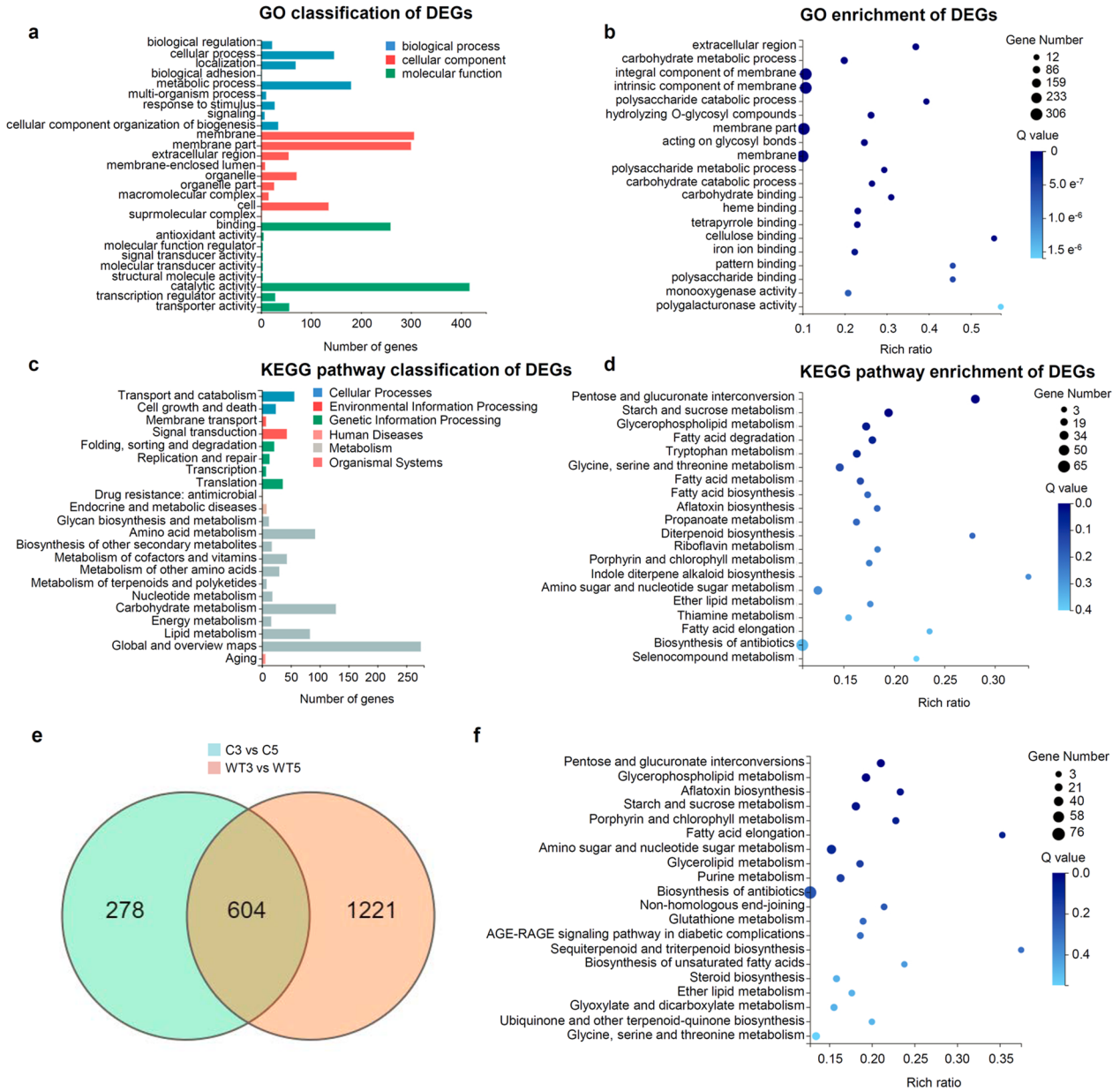


**Fig. 5** Analysis of the DEGs of wild-type *vs ΔBcDIC* at the initial state of conidiation. **a** Gene ontology classification of DEGs. **b** The top 20 of gene ontology enrichment of DEGs. **c** The Kyoto Encyclopedia of Genes and Genomes pathways classification of DGEs. **d** The top 20 Kyoto Encyclopedia of Genes and Genomes pathways enrichment of DGEs. **e** The venn diagram of DGEs in wild- type strain and *ΔBcDIC* during the transition from vegetative growth to conidiation. **f** The top 20 Kyoto Encyclopedia of Genes and Genomes pathways enrichment of DGEs unique in WT3 vs WT5

*ΔBcDIC*, 417 genes encode proteins with catalytic activity and 259 genes encode proteins with binding activity (Fig. 5a). The GO enrichment analysis of DEGs showed that the genes involved in polysaccharide metabolism, especially the polygalacturonase genes, had a high enrichment ratio (Fig. 5b). The results of KEGG pathway classification also showed that a high proportion of DEGs (128 genes) were involved in the carbohydrate metabolism pathway (Fig. 5c). There were 32 DEGs in the metabolic pathway of Pentose and Glucuronate Interconversions (ko00040), and the enrichment ratio reached 0.28 (Fig. 5d). This complex metabolic pathway begins with the decomposition of pectin, and various pectinases are responsible for this degradation process, including pectinesterase, pectinlyase, and polygalacturonase. Interestingly, knockout of *ΔBcDIC* resulted in significant

up-regulation of the expression of all 5 pectinesterase family genes of *B. cinerea* (Fig. 6a). Furthermore, we confirmed by qPCR that the expressions of the 5 pectinesterase genes in *ΔBcDIC* were significantly higher than that of wild-type strain at the initial conidation stage (Fig. 6b). Compared with *ΔBcDIC*, the wild-type strain had 1221 specific DEGs in the transition from vegetative growth to initial conidation stage (Fig. 5e). The 1221 specific DEGs were further subjected to KEGG pathway enrichment assay (Fig. 5f). The results further indicated that the transition of *B. cinerea* from vegetative growth to asexual reproduction was accompanied by rearrangement of gene expression involved in the pathway of 'Pentose and Glucuronate Interconversions', especially the down-regulation of pectin degrading enzyme genes (Fig. 5f). Pectinesterases are involved in step 1 of the subpathway of degrading pectin into 2-dehydro-3-deoxy-D-gluconate (Fig. 6c). This subpathway is part of the pathway pectin degradation, which is part of glycan metabolism. Furthermore, we compared the pectin degradation activity between *ΔBcDIC* and the wild-type strain by the method of ruthenium red staining. The results showed that the unstained halo produced by *ΔBcDIC* proteins was larger than that produced by wild-type strain proteins (Fig. 7a), which suggested that the pectin degradation ability of *ΔBcDIC* was higher than that of wild-type strain (Fig. 7b). It was consistent with the results of gene expression. These results implied that

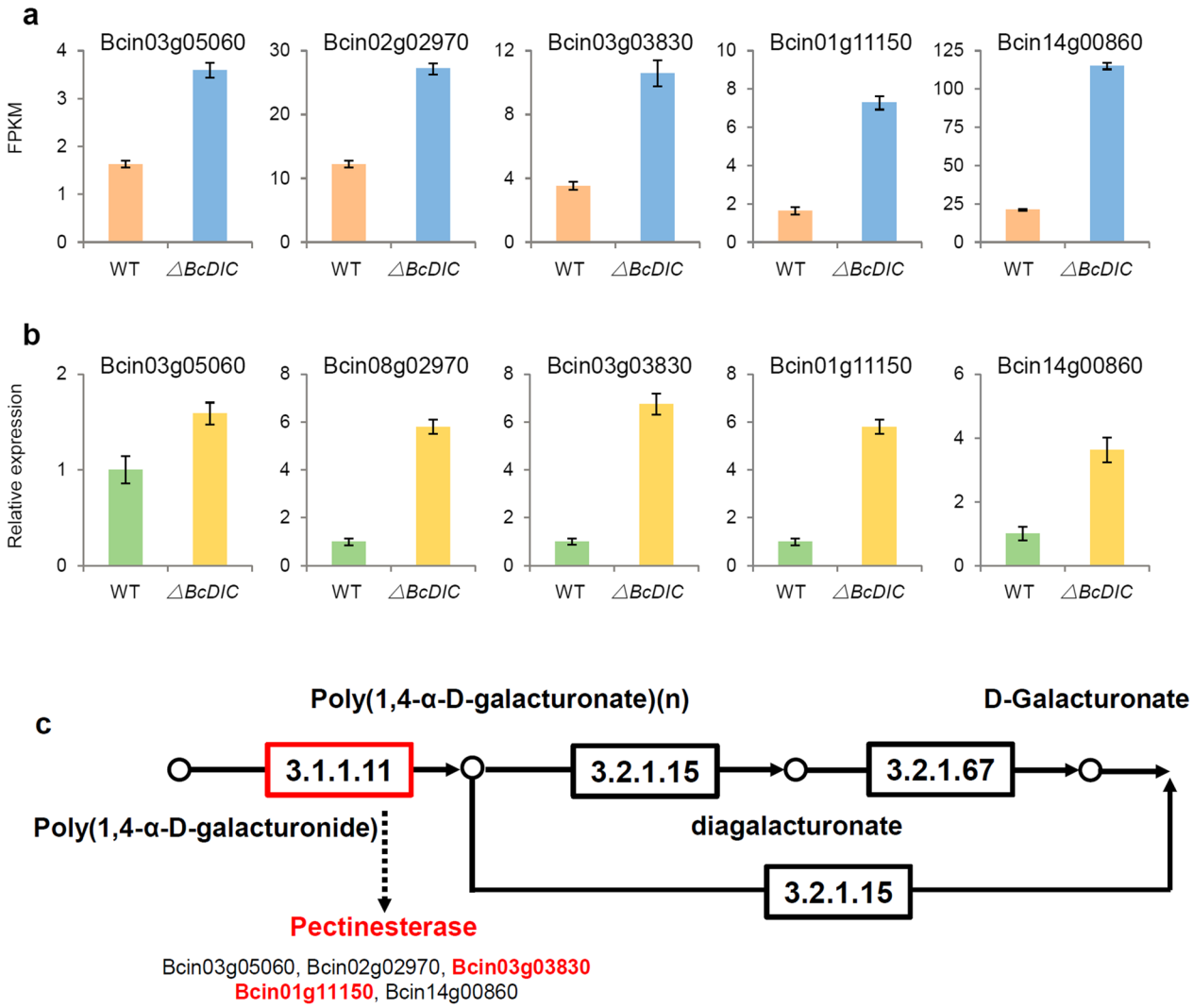


**Fig. 6** BcDIC influences the expression of pectinesterase genes. **a** The expression level (RNA-seq data) of all pectinesterase genes in wild-type strain and *ΔBcDIC* at the initial stage of conidiation. **b** Verification of of pectinesterase genes by qPCR. **c** Schematic representation of pectinesterases involved in pectin metabolism pathway. Vertical bars represent standard errors of the means

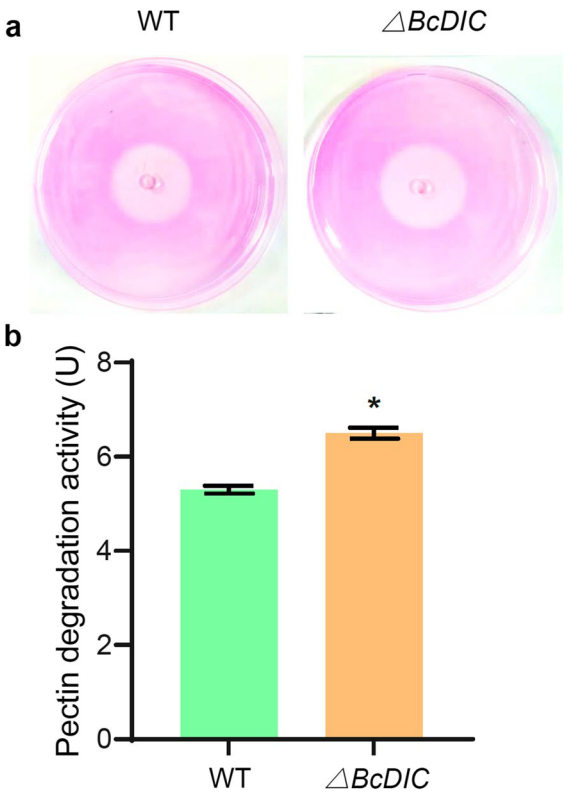


**Fig. 7** The pectin degradation activity of *ΔBcDIC* is higher than that of wild type strain. **a** The unstained halo produced by *ΔBcDIC* proteins is larger than that produced by wild type strain proteins. **b** Pectin degradation activity of proteins extract from *ΔBcDIC* and wild type strain

the degradation ability of pectin was closely related to the conidiation process of *B. cinerea*.

#### BcDIC can directly regulate pectinesterase genes

Yeast-one-hybrid (Y1H) was first used to evaluate whether BcDIC directly regulates the expression of pectinesterase genes (Fig. 8a). The coding sequence of BcDIC was cloned into the downstream position of the activation domain (AD) of the reporter gene LacZ in vector pB42AD. The 1000 bp sequences upstream of the start codons of the 5 pectinesterase genes were cloned as the promoter region, and ligated to the upstream position of the *LacZ* gene in vector pLacZi. The interaction between transcriptional factor ABI4 and the promoter of target gene AGO4 of *Arabidopsis* were used as positive control. Empty vectors pB42AD + pLacZi and pB42AD-BcDIC + pLacZi were used as negative controls. The results showed that BcDIC could directly bind the promoter sequence of two conserved pectinesterase genes, *Bcin03g03830* (*Bcpme2*) and *Bcin01g11150* (Fig. 8a). To explore whether BcDIC can regulate the activity of the promoters of these two pectinesterase genes, we conducted dual-luciferase reporter assay in *Nicotiana benthamiana* leaves by coexpressing a reporter construct of firefly luciferase (LUC) driven by the promoter of pectinesterase genes and a construct expressing the BcDIC protein (Fig. 8b). As shown in Fig. 8c, the LUC/REN ratio of BcDIC was significantly lower than that of the negative control (Fig. 8c). These results suggested that BcDIC could negatively regulate the expression of pectinesterase genes through both direct and indirect ways, and then affect the conidiation process of *B. cinerea*.

## Discussion

As one of the most destructive plant pathogens, the regulating mechanism of the pathogenicity and development of *B. cinerea* is an important scientific issue. Over the past few decades, various pathogenicity related factor have been identified in extracellular proteins of *B. cinerea* (Kars et al. 2005a; Brito et al. 2006; Zhu et al. 2017; Li et al. 2020), ROS systems (Segmüller et al. 2008; An et al. 2015, 2016; Li et al. 2016, 2019) and signal transduction components (Gronover et al. 2004; Yin et al. 2018). Among 11707 coding genes of *B. cinerea*, there are 410 genes encoding transcription factors (Amselem et al. 2011) However, the transcriptional mechanisms involved in the pathogenesis and asexual reproduction of *B. cinerea* are largely unclear. C6 transcription factors constitute the largest transcription factor family of *B. cinerea* (Amselem et al. 2011). Identifying critical C6 transcription factors involved in the pathogenesis or conidiation of *B. cinerea* and clarifying their regulatory network will help us better understand the pathogenic mechanism of *B. cinerea*. The previously published transcriptome data implied that a variety of C6 family transcription factors may be involved in the infection process of *B. cinerea* (Kelloniemi et al. 2015). Compared with the initial time point, Several C6 transcription factor genes showed a sustained up-regulation expression pattern after inoculation (16, 24, and 48 hpi) (Kelloniemi et al. 2015). Among them, the up-regulation multiples of 6 C6 transcription factors were remarkable. Especially, the expression of *BcDIC* at the three time point increased by 18, 21, and 19 times compared with the initial time point, respectively (Kelloniemi et al. 2015). We further examined the expression pattern of the 6 C6 transcription factors during the earlier infection course in tomato fruit (0, 8, and 12 hpi). The results showed that the expression of Bcin13g03120, *BcDIC*, and Bcin03g00940 were significantly up-regulated during infection, and the expression of Bcin12g04950, Bcin08g01810 and Bcin06g04550 were suppressed after inoculation (Fig. 1). The result indicated that these C6 family transcription factors were involved

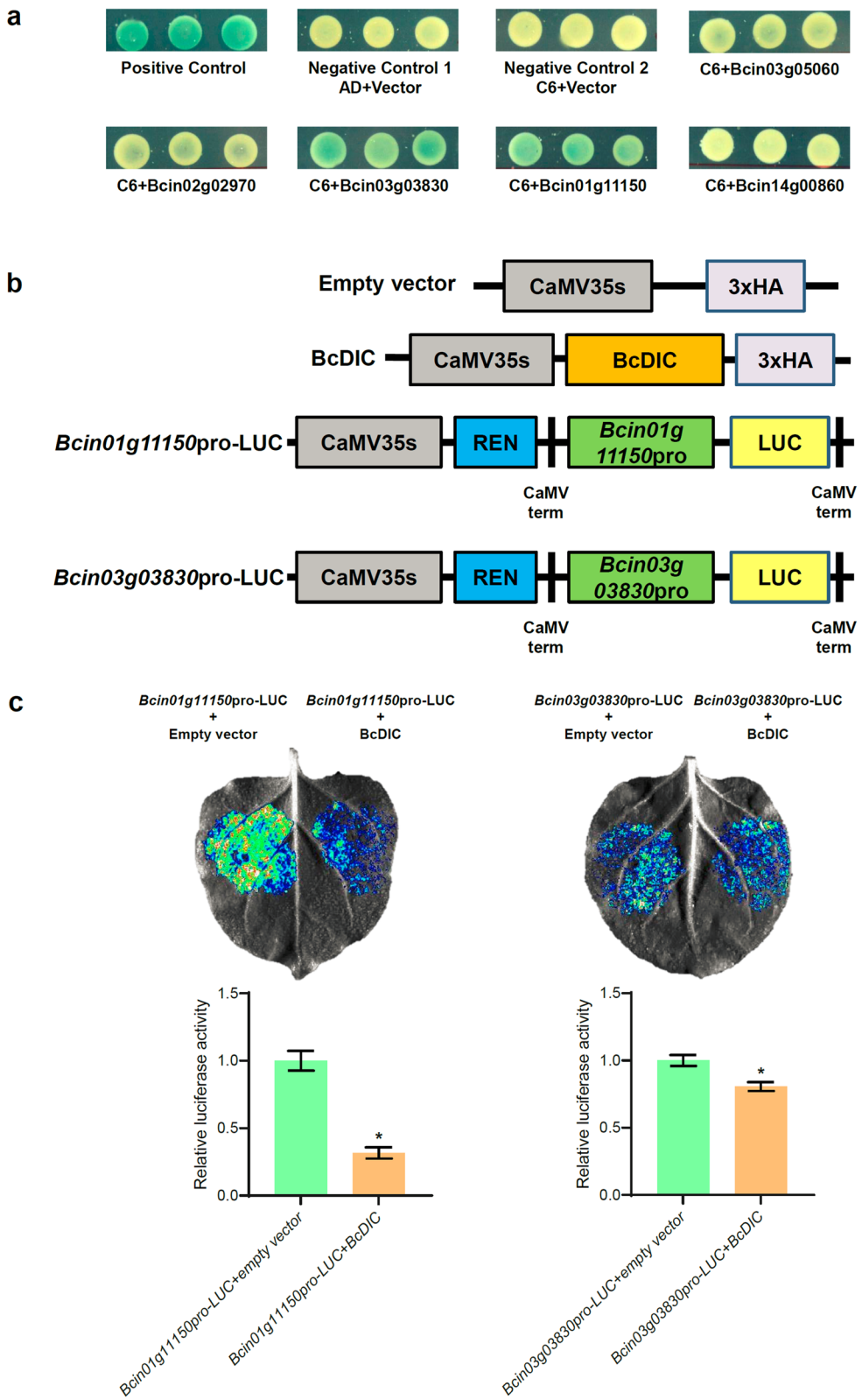


**Fig. 8** BcDIC bind to the promoter of two pectinesterase genes and suppress their transcription. **a** Binding analysis of BcDIC to promoter sequences of pectinesterase genes by the method of yeast-one-hybrid. **b** Schematic diagram of transient expression vector in dual-luciferase reporter assay. **c** The suppression of pectinesterase gene promoters by BcDIC, and the activity is shown by the LUC/REN ratio. The chemiluminescent images captured 48 h after infiltration

in the pathogenesis of *B. cinerea*, and their expression patterns were related to host type.

Considering that among the 6 detected C6 genes, *BcDIC* was the most strongly induced gene in the infectious process, we carried out further research on this gene. We first constructed the knockout mutants of *BcDIC*. Subsequent analysis showed that deletion of *BcDIC* caused significant phenotypic variation. The vegetative growth of the knockout mutant *ΔBcDIC* reduced by 33% compared with wild-type strain. The decline degree of the virulence of *ΔBcDIC* on hosts was comparable to its decrease in growth rate. This indicated that the virulence of *ΔBcDIC* decreased mainly due to its compromised vegetative growth. It is worth noting that *ΔBcDIC* completely lost the ability to produce conidia. In most cases, *B. cinerea* produces conidia through asexual reproduction in the field environment, and conidia serve as the main source of transmission and infection (Fillinger and Elad 2016). Therefore, the production of conidia directly influences the epidemic and severity of gray mold disease in field. In order to explore the underlying mechanism of this important phenotypic variation in *ΔBcDIC*, we compared the gene expression profile between *ΔBcDIC* and wild-type strain during the transition from vegetative growth to conidiation by using transcriptomic sequencing method. The transcriptome data showed that the gene expression of *B. cinerea* was significantly rearranged during the transition from vegetative growth to conidiation. During this transition in wild-type strain, a total of 1825 gene expression patterns were rearranged, accounting for 16% of the total genome genes. However, in the knockout mutant *ΔBcDIC*, the number of rearranged genes plummeted to 882 during this transition process, which was 52% lower than that of wild-type strain. At vegetative growth stage (3 dpi), there were 492 DEGs between wilt type strain and *ΔBcDIC*. However, at the initial stage of conidiation, the number of DEGs between wilt type strain and *ΔBcDIC* soared to 1089. The vegetative growth and conidiation are two different developmental stages of *B. cinerea*, and the change of the developmental stage is accompanied by a massive rearrangement of gene expression patterns. *BcDIC* play a critical role in the regulating gene expression during this developmental stage transition. Deletion of *BcDIC* inhibited the gene expression rearrangement required to initiate conidiation, resulting in the loss of conidiation process in *ΔBcDIC*. Interestingly, the GO and KEGG analysis of DEGs (WT vs *ΔBcDIC*) at the initial stage of conidiation showed that deletion of *BcDIC* led to the significant up-regulation of all five pectinesterase genes in the pectin degradation pathway in *B. cinerea*. We also found that with the transition of *B. cinerea* from vegetative growth to conidiation in the wild-type strain, the expressions of the five pectinesterase genes were all significantly inhibited. Pectin is one of the main components of the plant cell wall (Walton 1994). Pectinesterase catalyses the de-esterification of pectin into pectate and methanol, and facilitates the subsequent complete degradation of pectin by polygalacturoases and pectate lyases (Valette-Collet et al. 2003; Kars et al. 2005a). In fungal pathogens, pectinesterase is involved in the maceration of plant tissue. The results of Y1H and dual-LUC reporter assay suggested that BcDIC could directly regulate two pectin esterase genes Bcin05g03830 (*Bcpme2*) and Bcin01g11150 (Fig. 8a). Previous studies have shown that knocking out *Bcpme2* or knocking out *Bcpme1* and *Bcpme2* at the same time did not affect the growth and virulence of *B. cinerea* (Kars et al. 2005b). It implied that other pectin esterase genes in *B. cinerea* have functional redundancy with *Bcpme1* and *Bcpme2*. The results of this study showed that these five pectin esterase genes may play a role simultaneously. *B. cinerea* is a necrotrophic pathogen, which preferentially infects pectin-rich plant tissues. Galacturonic acid, the degradation product of pectin, is the most important carbon source for *B. cinerea*. These results implied that there may be a balance or antagonism between vegetative growth and asexual reproduction of *B. cinerea*. In the vegetative growth stage, *B. cinerea* needs to quickly absorb nutrients from environment, which requires high pectinase degradation activity. In the reproduction stage, the demand for *B. cinerea* to obtain nutrients from environment decreases, and the pectin degradation ability will decrease accordingly. The initiation of conidiation process of *B. cinerea* may need to inhibit vegetative growth. This inhibition is achieved by direct or indirect suppression of the pectin esterase genes by transcription factor BcDIC.

## Conclusions

In present study, we identified a critical C6 family transcription factor BcDIC that regulates conidiation and pathogenicity of *B. cinerea*. Especially, BcDIC plays a prominent role in regulating the transition from vegetative growth to conidiation in *B. cinerea*. Modulating the expression of pectinesterase family genes is an important mechanism for BcDIC regulating the asexual reproduction of *B. cinerea.*

## Methods

### Strains and culture conditions

*B. cinerea* strain B05.10 was used as the wild-type and recipient strain for gene replacement. Wild-type strain and all mutants were routinely cultured on potato dextrose agar (PDA) or in potato dextrose broth (PDB) at 22°C. Conidia of sporulation cultures were collected by sterile distilled water and filtrated through four layers of

sterile gauze. The concentrations of conidia were measured with hemacytometer.

#### RT-qPCR

To detect the expression pattern of C6 transcription factor genes during infection process, tomato fruits were inoculated with 10 μL conidial suspension ($10^7$ conidia/mL). Then, the pulps around the infection sites were collected at different time points (0, 8, 12, and 24 h post inoculation). Total RNA was extracted using Trizol reagent (Tiangen, Beijing). RNA samples were treated with DNase I (Takara, Dalian) to remove the contamination of genomic DNA. The first-stand cDNA was synthesized using M-MLV reverse transcriptase (Promega, Madison, WI, USA). RT-qPCR was performed using SYBR Premix Ex Taq (Takara, Dalian) on the Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The relative expression were expressed by the $2^{-\Delta\Delta Ct}$ analysis method. Actin gene (*Bcin16g02020*) of *B. cinerea* was used as endogenous control. Each sample contained 3 biological replicates.

#### Construction of knockout mutants and complemented strains

Knockout mutants were constructed by the principle of homologous recombination of gene flanking sequence. Approximately 1 kb flanking sequences upstream (Flank L) and downstream (Flank R) of target genes were amplified separately by high fidelity enzyme. The hygromycin resistance cassette was amplified from the vector pLOB7 by high fidelity enzyme. The reverse primer of Flank L and the forward primer of Flank R have 20 bp overlapping sequences with the forward primer and reverse primer of hygromycin resistance cassette, respectively. Then the fragments Flank L, hygromycin resistance cassette and Flank R were fused together by fusion PCR. The primers used for construction of mutants were list in Additional file 4: Table S3. The fusion fragment was used for protoplast transformation. Protoplast preparation and transformation were carried out according to our previous work (Zhang et al. 2014). The hygromycin resistant transformants were detected by flank-spanning PCR using the primer pair locating outside the flank L and inside the resistance cassette, respectively. To get homokaryotic transformants, the positive transformants were purified by one or several rounds single-spore isolation on hygromycin resistant plates. The homokaryotic mutant was subjected to Southern blot analysis to verify whether it was single copy insertion using the Flank L of target gene as probe.

To generate complementary strain, the target gene with 1000 bp upstream sequence and 500 bp downstream was amplified. The complementary fragment was cloned into the vector pNAN-OGG which contains the flanks of gene *bcniiA* (nitrite reductase) of *B. cinerea*, allowing the replacement of *bcniiA* by complementary fragment and nourseothricin resistance cassette.

#### Virulence assay

The virulence of different strains was evaluated on tomato fruit (*Lycopersicon esculentum* Mill cv Castlemart) and apple fruit (*Malus pumila* Mill cv. Fuji). The plugs with mycelium (1-mm-diameter) from the edge of 3-day-old culture on PDA plate were used for inoculation. The inoculated fruits were incubated in petri dishes at 25°C in enclosed plastic trays to maintain a high relative humidity (95%). Disease symptoms and lesion diameters were scored daily. The inoculation of each strain includes 5 fruits of leaves.

#### RNA extraction, cDNA library construction, and illumine sequencing

The wild-type stain strain and the mutant *ΔBcDIC* were cultured on the PDA plates covered with cellophane under white light. After culturing for 3 days (vegetative growth stage) and 5 days (starting sporulation), mycelium of wild-type and *ΔBcDIC* were collected to extract total RNA using Trizol reagent (Tiangen, Beijing). The process of constructing of cDNA library is as follow: 1) The mRNA with poly A tail was enriched with magnetic beads with oligo dT; 2) The enriched mRNA was fragmented into approximately 200 nt, and then, single-strand cDNA and double stand cDNA were synthesized sequentially; 3) The synthesized double stand cDNA was subjected to end-repair, dA-tailing, and adaptor ligation; 4) The modified cDNA were amplified by PCR with specific primers; 5) The PCR product was thermally denatured into single strand, and then the single strand cDNA was cyclized with bridge primer to get the single stand circular cDNA library. The library was sequenced using Illumina HiSeq 2500 platform (Huada BGI, Wuhan, China). Each sample contained 3 biological replicates.

#### RNA-seq data analysis

The raw reads containing adapter contamination and high content of unknown base N (> 10%) were removed before analysis. In addition, the low quality reads that more than 20% nucleotides had low Q-value was also screened out through SOAPnuke to get clean data. The clean sequences were aligned to the reference genome of *B. cinerea* B05.10 (http://fungi.ensembl.org/Botrytis_cinerea/Info/Index) using HISTA to obtain the uniquely mapping ratio. Use Bowtie2 to align clean reads to the reference sequence, and then use RSEM to calculate the expression of transcripts. Use the software DIAMOND to align the genes to PHI (Pathogen Host Interaction

Database) database for annotation. The gene expression level was evaluated by FPKM (Fragments Per Kilo bases per Million fragments) value. The analysis of differential expression genes (DGE) between different samples was carried out according to the method described by Wang et al. (2010). Genes with expression fold change ≥ 2 (adjusted *P* value ≤ 0.001) were considered as DGEs. DGEs were imported into Blast2GO program to classify the genes to specific gene ontology (GO) terms. Then, the analysis of the enrichment of DGEs in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was performed using the phyper function in the software R.

### Determination of pectin degradation activity

The pectin degradation activity assay was performed according to the method described by Lionetti (2015) and Sella et al. (2016) with some modifications. For the crude enzyme extraction, *B. cinerea* cultured for three days on cellophane-covered PDA medium was collected and ground in the extraction buffer (50 mM sodium acetate buffer, 100 mM NaCl, pH = 6.5) in an ice bath. The slurry was centrifuged for 10 min (15,871 *g*, 4°C), and the protein concentration of the supernatant was determined by nano-drop and adjusted to 10 μg/μL with the extraction buffer. The supernatant was used as the crude enzyme solution. 20 μL of the crude enzyme solution was added to PECTOPLATE and incubated at 30°C for 16 h. The plates were stained with 0.05% (w/v) ruthenium red for 30 min and subsequently rinsed several times with water. The inner unstained halo of the plate represents pectin degradation activity. A 0.5-cm diameter halo was defined as one pectin degradation activity unit.

### Yeast-one-hybrid

Yeast-one-hybrid assay was carried out to verify the regulation mode of BcDIC on pectinesterase family genes. The ORF of *BcDIC*was amplified and cloned into the bait vector pB42AD. The promoter region 1000 bp upstream of pectinesterase genes were amplified and cloned into the vector pLacZZ. The recombinant vectors were cotransformed into yeast EYG42 and screened on double dropout agar medium (SD/-Trp-Ura). The single colony was transferred to X-gal agar (SD/-Trp-Ura + X-gal) for chromogenic screening. One positive control (transcriptional factor ABI4 + promoter of AGO4) and two negative controls (empty vector pB42AD + empty vector pLacZZ; *BcDIC* + empty vector pLacZZ) were set.

### Dual-luciferase (LUC) reporter assay

The dual-luciferase reporter assays were performed to determine the effect of BcDIC on target genes (Ji et al. 2020). The promoters of the target genes (~ 1500 bp) were amplified from the *B. cinerea* genomic DNA and inserted into the pGreenII 0800-LUC vector. BcDIC was cloned into the pCAMBIA2300 vector, and the empty vector was used as a negative control. The constructed plasmids harboring BcDIC and the target gene promoter were transformed into *Agrobacterium tumefaciens* strain GV3101 and co-expressed transiently in *Nicotiana benthamiana*. After 48 h, the chemiluminescent images were captured with the Tanon 5200 Chemiluminescent Image system. The luciferase activities of LUC and REN were determined by the GloMax Luminometer (Promega). The ratios of LUC/REN of the empty vector and target gene promoter co-expression samples were used as a reference, and the relative fluorescence activities of BcDIC and target gene promoter co-expression samples were calculated.

### Statistical analysis

Statistical analysis of data was performed by the software SPSS 18.0. One-way analysis was used to evaluate the significance of the difference. Differences at $P < 0.01$ or $P < 0.05$ were both considered as statistically significant.

#### Abbreviations

| | |
|---|---|
| AD | Activation domain |
| *B. cinerea* | *Botrytis cinerea* |
| C6 | $Zn(II)_2Cys_6$ |
| Cys | Cysteines |
| DEGs | Differential expression genes |
| dpi | Days post inoculation |
| Flank L | Flanking sequences upstream of target genes |
| Flank R | Flanking sequences downstream target genes |
| FPKM | Fragments Per Kilo bases per Million fragments |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| PDA | Potato dextrose agar |
| PDB | Potato dextrose broth |
| SD/-Trp-Ura | Double dropout agar medium |
| TFs | Transcription factors |
| Y1H | Yeast-one-hybrid |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-023-00193-x.

**Additional file 1: Figure S1**. Phylogenetic analysis (**a**) and alignment (**b**) of BcDIC with C6 transcription factor of other fungi. **Figure S2**. Construction of knockout mutant *ΔBcDIC*. **Figure S3**. Expression patterns of conidiation-associated genes.

**Additional file 2: Table S1**. Total gene expression level.

**Additional file 3: Table S2**. Differential expression genes of WT5 vs C5.

**Additional file 4: Table S3**. Primers used in this study.

#### Acknowledgements

Not applicable.

#### Author contributions

ZZ conceived and designed the study. SL, XZ, CH, and GL performed the experiments. ZZ wrote the draft manuscript. ST, TC, and BL assisted with analyzing data and revised the manuscript. All authors read and approved the final manuscript.

**Funding**
This work was supported by the Natural Science Foundation of Beijing (6222048), the National Natural Science Foundation of China (32172642, 31972475), and the National Key Research and Development Program of China (2021YFD2100505).

**Availability of data and materials**
The RNA-seq data have been deposited in the NCBI Sequence Read Archive (BioProject accession number: PRJNA875246).

## Declarations

**Ethical approval and consent to participate**
Not applicable.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare that they have no competing interests.

Received: 14 January 2023 Accepted: 9 August 2023
Published online: 18 August 2023

**References**

Akache B, Turcotte B. New regulators of drug sensitivity in the family of yeast zinc cluster proteins. J Biol Chem. 2002;277:21254–60. https://doi.org/10.1074/jbc.M202566200.

Akache B, Wu K, Turcotte B. Phenotypic analysis of genes encoding yeast zinc cluster proteins. Nucleic Acids Res. 2001;29:2181–90. https://doi.org/10.1093/nar/29.10.2181.

Amselem J, Cuomo CA, van Kan JA, Viaud M, Benito EP, Couloux A, et al. Genomic analysis of the necrotrophic fungal pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea*. PLoS Genet. 2011;7:e1002230. https://doi.org/10.1371/journal.pgen.1002230.

An B, Li B, Qin G, Tian S. Function of small GTPase Rho3 in regulating growth, conidiation and virulence of *Botrytis cinerea*. Fungal Genet Biol. 2015;75:46–55. https://doi.org/10.1016/j.fgb.2015.01.007.

An B, Li B, Li H, Zhang Z, Qin G, Tian S. Aquaporin8 regulates cellular development and reactive oxygen species production, a critical component of virulence in *Botrytis cinerea*. New Phytol. 2016;209:1668–80. https://doi.org/10.1111/nph.13721.

Angus-Hill M, Schlichter A, Roberts D, Erdjument-Bromage H, Tempst P, Cairns BR. A Rsc3/Rsc30 zinc cluster dimer reveals novel roles for the chromatin remodeler RSC in gene expression and cell cycle control. Mol Cell. 2001;7:741–51. https://doi.org/10.1016/s1097-2765(01)00219-2.

Breunig K. Regulation of transcription activation by Gal4p. Food Technol Biotechnol. 2000;38:287–93.

Brito M, Espino J, González C. The endo-β-1,4-xylanase Xyn11A is required for virulence in *Botrytis cinerea*. Mol Plant-Microbe Interact. 2006;19:25–32. https://doi.org/10.1094/MPMI-19-0025.

Cheung N, Tian L, Liu X, Li X. The destructive fungal pathogen botrytis cinerea-insights from genes studied with mutant analysis. Pathogens. 2020. https://doi.org/10.3390/pathogens9110923.

Chung H, Choi J, Park SY, Jeon J, Lee YH. Two conidiation-related Zn(II)2Cys6 transcription factor genes in the rice blast fungus. Fungal Genet Biol. 2013;61:133–41. https://doi.org/10.1016/j.fgb.2013.10.004.

Dean R, Van Kan JA, Pretorius ZA, Hammond-Kosack KE, Di Pietro A, Spanu PD, et al. The Top 10 fungal pathogens in molecular plant pathology. Mol Plant Pathol. 2012;13:414–30. https://doi.org/10.1111/j.1364-3703.2011.00783.x.

Fillinger S, Elad Y. Botrytis—the fungus, the pathogen and its management in agricultural systems; 2016.

Galhano R, Illana A, Ryder LS, Rodriguez-Romero J, Demuez M, Badaruddin M, et al. Tpc1 is an important Zn(II)2Cys6 transcriptional regulator required for polarized growth and virulence in the rice blast fungus. PLoS Pathog. 2017;13:e1006516. https://doi.org/10.1371/journal.ppat.1006516.

Gronover C, Schorn C, Tudzynski B. Identification of Botrytis cinerea Genes Up-Regulated During Infection and Controlled by the Gα Subunit BCG1 Using Suppression Subtractive Hybridization (SSH). PLoS Pathog. 2004;17:537–46. https://doi.org/10.1094/MPMI.2004.17.5.537.

Hikkel I, Lucau-Danila A, Delaveau T, Marc P, Devaux F, Jacq C. A general strategy to uncover transcription factor properties identifies a new regulator of drug resistance in yeast. J Biol Chem. 2003;278:11427–32. https://doi.org/10.1074/jbc.M208549200.

Iraqui I, Vissers S, André B, Urrestarazu A. Transcriptional induction by aromatic amino acids in *Saccharomyces cerevisiae*. Mol Cell Biol. 1999;19:3360–71. https://doi.org/10.1128/MCB.19.5.3360.

Ji DC, Cui XM, Qin GZ, Chen T, Tian SP. SlFERL interacts with S-adenosylmethionine synthetase to regulate fruit ripening. Plant Physiol. 2020;184:2168–81. https://doi.org/10.1104/pp.20.01203.

Kars I, McCalman M, Wagemakers L, Vank JA. Functional analysis of *Botrytis cinerea* pectin methylesterase genes by PCR-based targeted mutagenesis: Bcpme1 and Bcpme2 are dispensable for virulence of strain B0510. Mol Plant Pathol. 2005;6:641–52. https://doi.org/10.1111/j.1364-3703.2005.00312.x.

Kars I, Krooshof GH, Wagemakers L, Joosten R, Benen JA, van Kan JA. Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in Pichia pastoris. Plant J. 2005;43:213–25. https://doi.org/10.1111/j.1365-313X.2005.02436.x.

Kelloniemi J, Trouvelot S, Heloir MC, Simon A, Dalmais B, Frettinger P, et al. Analysis of the molecular dialogue between Gray Mold (*Botrytis cinerea*) and grapevine (*Vitis vinifera*) reveals a clear shift in defense mechanisms during berry ripening. Mol Plant Microbe Interact. 2015;28:1167–80. https://doi.org/10.1094/MPMI-02-15-0039-R.

Larochelle M, Drouin S, Robert F, Turcotte B. Oxidative stress-activated zinc cluster protein Stb5 has dual activator/repressor functions required for pentose phosphate pathway regulation and NADPH production. Mol Cell Biol. 2006;26:6690–701. https://doi.org/10.1128/MCB.02450-05.

Li H, Zhang Z, He C, Qin G, Tian S. Comparative proteomics reveals the potential targets of BcNoxR, a putative regulatory subunit of NADPH oxidase of *Botrytis cinerea*. Mol Plant Microbe Interact. 2016;29:990–1003. https://doi.org/10.1094/MPMI-11-16-0227-R.

Li H, Tian S, Qin G. NADPH oxidase is crucial for the cellular redox homeostasis in fungal pathogen *Botrytis cinerea*. Mol Plant Microbe Interact. 2019;32:1508–16. https://doi.org/10.1094/MPMI-05-19-0124-R.

Li H, Zhang Z, Qin G, He C, Li B, Tian S. Actin is required for cellular development and virulence of *Botrytis cinerea* via the mediation of secretory proteins. mSystems. 2020. https://doi.org/10.1128/mSystems.00732-19.

Lionetti V. PECTOPLATE: the simultaneous phenotyping of pectin methylesterases, pectinases, and oligogalacturonides in plants during biotic stresses. Front Plant Sci. 2015;6:331. https://doi.org/10.3389/fpls.2015.00331.

MacPherson S, Akache B, Weber S, De Deken X, Raymond M, Turcotte B. *Candida albicans* zinc cluster protein Upc2p confers resistance to antifungal drugs and is an activator of ergosterol biosynthetic genes. Antimicrob Agents Chemother. 2005;49:1745–52. https://doi.org/10.1128/AAC.49.5.1745-1752.2005.

MacPherson S, Larochelle M, Turcotte B. A fungal family of transcriptional regulators: the zinc cluster proteins. Microbiol Mol Biol Rev. 2006;70:583–604. https://doi.org/10.1128/MMBR.00015-06.

Marmorstein R, Carey M, Ptashne M, Harrison S. DNA recognition by GAL4: structure of a protein-DNA complex. Nature. 1992;356:408–14. https://doi.org/10.1038/356408a0.

Michielse C, Becker M, Heller J, Moraga J, Collado I, Tudzynski P. The *Botrytis cinerea* Reg1 protein, a putative transcriptional regulator, is required for pathogenicity, conidiogenesis, and the production of secondary metabolites. Mol Plant Microbe Interact. 2011;24:1074–85. https://doi.org/10.1094/MPMI-01-11-0007.

Price MS, Yu J, Nierman WC, Kim HS, Pritchard B, Jacobus CA, et al. The aflatoxin pathway regulator AflR induces gene transcription inside and outside of the aflatoxin biosynthetic cluster. FEMS Microbiol Lett. 2006;255:275–9. https://doi.org/10.1111/j.1574-6968.2005.00084.x.

Schamber A, Leroch M, Diwo J, Mendgen K, Hahn M. The role of mitogen-activated protein (MAP) kinase signalling components and the Ste12 transcription factor in germination and pathogenicity of *Botrytis cinerea*. Mol Plant Pathol. 2010;11:105–19. https://doi.org/10.1111/j.1364-3703.2009.00579.x.

Schumacher J, de Larrinoa IF, Tudzynski B. Calcineurin-responsive zinc finger transcription factor CRZ1 of *Botrytis cinerea* is required for growth, development, and full virulence on bean plants. Eukaryot Cell. 2008;7:584–601. https://doi.org/10.1128/EC.00426-07.

Schumacher J, Simon A, Cohrs KC, Viaud M, Tudzynski P. The transcription factor BcLTF1 regulates virulence and light responses in the necrotrophic plant pathogen *Botrytis cinerea*. PLoS Genet. 2014;10:e1004040. https://doi.org/10.1371/journal.pgen.1004040.

Segmüller N, Kokkelink L, Giesbert S, Odinius D, van Kan J, Tudzynski P. NADPH oxidases are involved in differentiation and pathogenicity in *Botrytis cinerea*. Mol Plant Microbe Interact. 2008. https://doi.org/10.1094/MPMI-21-6-0808.

Sella L, Castiglioni C, Paccanaro MC, Janni M, Schafer W, D'Ovidio R, Favaron F. Involvement of fungal pectin methylesterase activity in the interaction between *Fusarium graminearum* and wheat. Mol Plant Microbe Interact. 2016;29:258–67. https://doi.org/10.1094/MPMI-07-15-0174-R.

Silver PM, Oliver BG, White TC. Role of *Candida albicans* transcription factor Upc2p in drug resistance and sterol metabolism. Eukaryot Cell. 2004;3:1391–7. https://doi.org/10.1128/EC.3.6.1391-1397.2004.

Tnrcotte B, Akache B, MacPherson S. The zinc cluster proteins: a yeast family of transcriptional regulators. In: Pandalai SG, editor. Recent developments in nucleic acids research. Trivandrum: Transworld Research Network; 2004. p. 233–49.

Todd R, Andrianopoulos A. Evolution of a fungal regulatory gene family: the Zn(II)2Cys6 binuclear cluster DNA binding motif. Fungal Genet Biol. 1997;21:388–405. https://doi.org/10.1006/fgbi.1997.0993.

Tsuji G, Kenmochi Y, Takano Y, Sweigard J, Farrall L, Furusawa I, et al. Novel fungal transcriptional activators, Cmr1p of *Colletotrichum lagenarium* and pig1p of *Magnaporthe grisea*, contain Cys2His2 zinc finger and Zn(II)2Cys6 binuclear cluster DNA-binding motifs and regulate transcription of melanin biosynthesis genes in a developmentally specific manner. Mol Microbiol. 2000;38:940–54. https://doi.org/10.1046/j.1365-2958.2000.02181.x.

Valette-Collet O, Cimerman A, Reignault P, Levis C, Boccara M. Disruption of *Botrytis cinerea* pectin methylesterase gene Bcpme1 reduces virulence on several host plants. Mol Plant Microbe Interact. 2003;16:360–7. https://doi.org/10.1094/MPMI.2003.16.4.360.

Vincent O, Carlson M. Sip4, a Snf1 kinase-dependent transcriptional activator, binds to the carbon source-responsive element of gluconeogenic genes. EMBO J. 1998;17:7002–8. https://doi.org/10.1093/emboj/17.23.7002.

Walton JD. Deconstructing the cell wall. Plant Physiol. 1994;104:1113–8. https://doi.org/10.1104/pp.104.4.1113.

Weiberg A, Wang M, Lin FM, Zhao H, Zhang Z, Kaloshian I, et al. Fungal small RNAs suppress plant immunity by hijacking host RNA interference pathways. Science. 2013;342:118–23. https://doi.org/10.1126/science.1239705.

Williamson B, Tudzynski B, Tudzynski P, van Kan JA. *Botrytis cinerea*: the cause of grey mould disease. Mol Plant Pathol. 2007;8:561–80. https://doi.org/10.1111/j.1364-3703.2007.00417.x.

Yin Y, Wu S, Chui C, Ma T, Jiang H, Hahn M, et al. The MAPK kinase BcMkk1 suppresses oxalic acid biosynthesis via impeding phosphorylation of BcRim15 by BcSch9 in *Botrytis cinerea*. PLoS Pathog. 2018;14:e1007285. https://doi.org/10.1371/journal.ppat.1007285.

Zhang Z, Qin G, Li B, Tian S. Knocking out Bcsas1 in *Botrytis cinerea* impacts growth, development, and secretion of extracellular proteins, which decreases virulence. Mol Plant Microbe Interact. 2014;27:590–600. https://doi.org/10.1094/MPMI-10-13-0314-R.

Zhang Z, Li H, Qin G, He C, Li B, Tian S. The MADS-Box transcription factor Bcmads1 is required for growth, sclerotia production and pathogenicity of *Botrytis cinerea*. Sci Rep. 2016;6:33901. https://doi.org/10.1038/srep33901.

Zhao C, Waalwijk C, Wit PJGM, Lee TV, Tang D. EBR1, a Novel Zn2Cys6 transcription factor, affects virulence and apical dominance of the hyphal tip in *Fusarium graminearum*. Mol Plant Microbe Interact. 2011;24:407–18. https://doi.org/10.1094/MPMI-06-11-0158.

Zhu W, Ronen M, Gur Y, Minz-Dub A, Masrati G, Ben-Tal N, et al. BcXYG1, a secreted xyloglucanase from *Botrytis cinerea*, triggers both cell death and plant immune responses. Plant Physiol. 2017;175:438–56. https://doi.org/10.1104/pp.17.00375.