Genome Sequencing of *Ciboria shiraiana* Provides Insights into the Pathogenic Mechanisms of Hypertrophy Sclerosis Scleroteniosis

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Ciboria shiraiana causes hypertrophy sclerosis scleroteniosis in mulberry trees, resulting in huge economic losses, and exploring its pathogenic mechanism at a genomic level is important for developing new control methods. Here, genome sequencing of *C. shiraiana* based on PacBio RSII and Illumina HiSeq 2500 platform as well as manual gap filling was performed. Synteny analysis with *Sclerotinia sclerotiorum* revealed 16 putative chromosomes corresponding to 16 chromosomes of *C. shiraiana*. Screening of rapid-evolution genes revealed that 97 and 2.4% of genes had undergone purifying selection and positive selection, respectively. When compared with *S. sclerotiorum*, fewer secreted effector proteins were found in *C. shiraiana*. The number of genes involved in pathogenicity, including secondary metabolites, carbohydrate active enzymes, and P450s, in the *C. shiraiana* genome was comparable with that of other necrotrophs but higher than that of biotrophs and saprotrophs. The growth-related genes and plant cell-wall-degradation-related genes in *C. shiraiana* were expressed in different developmental and infection stages, and may be potential targets for prevention and control of this pathogen. These results provide new insights into *C. shiraiana* pathogenic mechanisms, especially host range and necrotrophy features, and lay the foundation for further study of the underlying molecular mechanisms.

Keywords: *Ciboria shiraiana*, fungal development, genome, genomics, mechanisms of pathogenicity, metabolomics, mulberry, pathogenicity, plant cell wall-degrading enzymes, proteomics

Ciboria shiraiana is a plant-pathogenic fungus belonging to the genus *Ciboria* of the Sclerotiniaceae family. Owing to their wide host range, pathogens from the Sclerotiniaceae family cause devastating plant diseases worldwide, leading to huge economic losses each year. For instance, *Botrytis cinerea* invades more than 200 crop hosts worldwide and causes massive losses in crops prior to harvest or even at the seedling stage of some hosts (Williamson et al. 2007). The annual losses caused by *Sclerotinia sclerotiorum* in the United States have exceeded \$200 million (Bolton et al. 2006). However, to date, other than chemical fungicides, there are no efficient methods to control these plant pathogens. Analysis of the pathogenic mechanism of these fungi at the genomic level is crucial to develop novel control strategies. In 2011, comparative genomic analysis of two necrotrophic fungi (*B. cinerea* and *S. sclerotiorum*) shed light on the evolutionary and mechanistic bases of genetically complex traits of necrotrophic pathogenicity (Amselem et al. 2011). Genome sequencing of these two pathogenic fungi helped researchers gain insights into genes involved in fungal development, including 245 sclerotia production-associated orthologs and 15 conidiation-related orthologous gene pairs shared by *S. sclerotiorum* and *B. cinerea*, which could be used as antifungal drug targets. Necrotrophs are defined as pathogens that first kill host plant cells and then derive nutrients from killed host cells (Pieterse et al. 2009).

To acquire nutrition from the host, necrotrophic pathogens break through cuticles and plant cell walls and secrete cell-wall-degrading enzymes (CWDEs) and secondary metabolites (SMs) such as toxins to promote decomposition of the cuticle and plant cell wall (Blanc et al. 2018; Kars et al. 2005; Thomma 2003; Toth et al. 2003). When pathogens break through the cuticle and plant cell wall, cuticular and plant cell wall fragments induce danger-associated molecular patterns, and pathogen-associated molecular patterns (PAMPs) are released and recognized by pattern recognition receptors (Boller and Felix 2009). This is the first line of defense in plants and is named PAMP-triggered immunity (PTI), and the effectors secreted by the pathogens are recognized by intracellular receptors and induce effector-triggered immunity (ETI) (Dodds and Rathjen 2010). In general, PTI acts against nonadapted

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pathogens, whereas ETI has been shown to increase pathogen toxicity or inhibit host immune responses (Bos et al. 2006; Dou et al. 2008; Ridout et al. 2006; Sohn et al. 2007).

Mulberry fruit sclerotienosis is a fungal disease and was first reported by Siegler and Jenkins (1922). Three types of sclerotienosis have been identified; namely, hypertrophy sorosis sclerotienosis, reduced sorosis sclerotienosis, and pellet sorosis sclerotienosis, caused by *C. shiraiana*, *Scleromitula shiraiana*, and *C. carunculoidea*, respectively (Kishi 1998; Kohn and Nagasawa 1984; Whetzel and Wolf 1945). In China, *C. shiraiana* causes the most serious sclerotienosis as a typical epidemic disease during mulberry fruit growth. Three factors have been found to cause hypertrophy sorosis sclerotienosis, leading to severe damage, seriously threatening the production of mulberry fruit, and, thus, restricting the development of the mulberry industry. First, the germination time of pathogenic sclerotia is from January to April, which exactly covers the flowering period of the existing mulberry fruit varieties. Second, the ascospores of *C. shiraiana* infect female mulberry flowers, causing an initial infection, and the conidiospores spread from diseased fruit to healthy fruit, causing secondary infection because mulberry is an aggregate fruit (Lü et al. 2019).

Although *C. shiraiana* has been reported in the fields many times in China, studies of its molecular pathogenesis are still very limited (Kuai and Wu 2012; Wang 2003, 2009). Furthermore, owing to the lack of genomic information, it is extremely difficult to conduct molecular studies and functional characterization of genes in *C. shiraiana*. In this study, we determined the genome of the pathogenic fungus *C. shiraiana* by sequencing, assembling, and annotating. To investigate the pathogenic mechanisms of *C. shiraiana*, a comparative genomics study with two related necrotrophic fungi, one biotrophic fungus, and two saprobic fungi was also conducted. This study is the first to conduct high-quality genome sequencing of *C. shiraiana* and is also the first to report on the genome of the pathogen causing mulberry fruit sclerotienosis. The findings of this study may serve as a model for investigating the pathogenic mechanisms in mulberry fruit, and also complement and validate the pathogenesis of necrotrophic pathogens. In addition, the present study will provide knowledge for improving mulberry fruit yield in sericulture and also offer a scientific basis for research and development of antifungal drug targets and biological control methods.

RESULTS

General genomic features.

In total, 6.7 Gb of raw sequences were generated from the Illumina HiSeq 2500 platform, representing 180× high coverage, and 4.7 Gb of raw sequences were generated from PacBio RSII platform (Table 1). The long reads of PacBio were assembled de novo and the short reads of Illumina were mapped to the assembled genome sequences to create a consensus sequence. To generate a more complete and accurate genome for *C. shiraiana*, we used the first-generation sequencing Sanger method to repair the gap between the short scaffolds (Supplementary Table S2).

The final assembly size of the *C. shiraiana* genome was noted to be 39.2 Mb, which was assembled into 23 scaffolds. The genome size of *C. shiraiana* was found to be similar to its closely related fungi *Sclerotinia sclerotiorum* (38.3 Mb) and *B. cinerea* (39.5 Mb), indicating that genome size is conserved among these three fungi (Amselem et al. 2011). The GC content of the *C. shiraiana* genome sequence (41.6%) was comparable with that of *S. sclerotiorum* (41.8%) but significantly lower than that of *Pyronema omphalodes* (47.8%,

Pezizomycotina). Unlike most of the other members of class Pezizomycetes (50 to 52%), class Leotiomycetes has been reported to have a relatively low GC content, which may affect the chromosome organization and stable transcription level (Leroch et al. 2011). In total, 14,439 protein-coding genes were predicted in this study, and the other assembly statistics of the genome are summarized in Table 1 and Supplementary Table S3.

Improvement in assembly level via synteny analysis with *S. sclerotiorum*.

To assemble the genome of *C. shiraiana* at the chromosome level, we used the 16 chromosome sequences of *S. sclerotiorum* as the reference sequence and aligned the 23 scaffolds of *C. shiraiana* to the reference sequence for synteny analysis (Fig. 1A; Supplementary Fig. S1). In total, 97 and 96% of the *C. shiraiana* genome could be aligned to the *S. sclerotiorum* genome at the nucleotide level and protein level, respectively. The large synteny regions shared by these two fungi indicate that *C. shiraiana* adequately retained the progenitor genomic features before divergence from *S. sclerotiorum*. Meanwhile, some rearrangement events were also noted in the *C. shiraiana* genome. The synteny analysis consisted of 16 putative chromosomes, corresponding to the 16 chromosomes of *C. shiraiana*, as observed under the microscope (Fig. 1B and C). Among the 16 putative chromosomes, 8 scaffolds were found to correspond to one chromosome, while the rest exhibited one-to-one correspondence (Supplementary Table S4). These eight scaffolds failed to accomplish gap filling via Sanger assembly owing to repeat sequences.

Genome annotation.

The average gene length was 1,211 bp, and 368 genes were spaced at every 1 Mb of the genome, which indicated that the gene density of *C. shiraiana* is similar to that of many other fungi. Only 5,956 (41.25%), 7,480 (51.80%), 10,817 (74.92%), and 14,135 (97.89%) of the predicted genes presented homologies with known functions in the Cluster of Orthologous Groups of proteins, SwissProt, Kyoto Encyclopedia of Genes and Genomes, and nonredundant protein (nr) databases, respectively (Supplementary Table S3). With regard to noncoding genes, we identified 213 transfer RNA (tRNA) and 99 ribosomal RNA (rRNA) from the assembly (Supplementary Table S5). In the present study, repetitive sequences were identified by RepeatMasker, which revealed 12.54% of the *C. shiraiana* genome as repeats, with almost all the repeats being transposable elements (TEs). The TEs

Table 1. Assembly statistics of *Ciboria shiraiana* genome^a

Parameters	Illumina HiSeq 2500	PacBio RSII	Integration statistics ^b
Scaffold (N)	1,531	29	23
Total length (Mb)	37	39.2	39.2
Gap (bp)	497,283	0	NA
N ₅₀ length (bp)	59,408	2,416,698	NA
N ₉₀ length (bp)	14,564	1,835,576	NA
Coverage	180×	120×	NA
GC content (%)	41.73	41.63	NA
Gene number (N)	NA	NA	14,439
Average gene length (bp)	NA	NA	1,211
Gene GC content (%)	NA	NA	45.67

^a NA denotes not available.

^b Integration statistics represents the result of the integration of Illumina HiSeq 2500, PacBio RSII, and Sanger assembly statistics.

identified in the *C. shiraiana* genome were grouped into families and annotated as class I (LTR and LINE) and unknown TEs, whereas class II TEs (MITE and TIR) were absent. Among them, unknown TEs accounted for 5.36% of the *C. shiraiana* genome. The proportion of TEs in the *C. shiraiana* genome was found to be comparable with that in the *S. sclerotiorum* genome (10%) (Amselem et al. 2011) but significantly lower than that in the *Blumeria graminis* genome (Supplementary Table S6).

Analysis of the gene family.

To identify the gene family of the six fungi, 36,819 genes were grouped into 8,965 clusters. Among these clusters, 1,196 gene families were identified as single-copy gene families (data not shown). In total, 3,083 common gene families were noted among the five fungi (Fig. 2). In addition, *C. shiraiana* and *S. sclerotiorum* were also found to share the most common gene families when compared with other fungal species, which further supported the results of phylogenetic tree analysis.

Fig. 1. Synteny analysis with *Sclerotinia sclerotiorum* and karyotype of *Ciboria shiraiana*. **A**, Synteny analysis with *S. sclerotiorum*. The *S. sclerotiorum* chromosome as reference sequence is shown on the left (black line), scaffold of *C. shiraiana* is shown on the right (black line), and syntenic regions are shown in red. **B**, Shape and size of each chromosome. **C**, Number of chromosomes observed under the microscope. Bar = 5 μm.

C. shiraiana contains 74 specific gene families, including 189 genes, of which 99 genes were enriched in the cellular process and 15 genes were enriched in metabolic pathways, and these unique genes can be explored as a potential target to affect the metabolism and pathogenicity of *C. shiraiana* (Supplementary Fig. S2).

The gain and loss of genes is one of the major contributors to functional changes (Li et al. 2010). In total, 435 gene families consisting of 2,460 genes were found to be expanded (more than one copy of the gene) in the *C. shiraiana* genome (Supplementary Fig. S3). Among these expanded gene families, 138 genes were enriched in binding and seven genes were enriched in metabolic pathways (Supplementary Fig. S4). Meanwhile, 1,045 gene families were noted to be contracted, and their proportion was higher than gene expansion in the six fungal genomes examined, indicating that loss-of-function might play an important role in functional evolution.

Pairwise gene nonsynonymous/synonymous sites.

The diverse environments and hosts in which various fungi exist may reflect different selections on orthologous genes. A high pairwise nonsynonymous/synonymous (Ka/Ks) value suggests that the protein rapidly evolved in the species. The evolution rate can be revealed by the footprints of past positive selection, which is measured as the rate of nonsynonymous nucleotide substitution per nonsynonymous site (Ka) relative to the underlying neutral mutation rate, given by the rate of synonymous nucleotide substitution per synonymous site (Ks) (Hurst 2002; Navarro and Barton 2003). To analyze the Ka/Ks nucleotide substitution rate ratio, we estimated that 1,976, 126, 22, 48, and 47 gene pairs have significantly higher neutral Ka/Ks nucleotide substitution rate ratios (Ka/Ks = 1), indicating positive selections for *C. shiraiana*–*S. sclerotiorum*, *C. shiraiana*–*S. borealis*, *C. shiraiana*–*B. graminis*, *C. shiraiana*–*P. omphalodes*, and *C. shiraiana*–*Saccharomyces cerevisiae*, respectively (data not shown). In total, 119,218 gene pairs were used

to identify nucleotide substitution events. Among these gene pairs, 97% were considered to have undergone purifying selections, while only 2.4% were presumed to have undergone positive selections.

Secreted effector proteins.

In the present study, we first filtered the prediction of secreted proteins using SignalP to identify 738 proteins with a secretion signal. Next, 738 proteins were predicted by TMHMM to harbor transmembrane helices, and 149 proteins were identified using TargetP v1.1 to remove predicted signal peptide related to organelles. Finally, 120 proteins were predicted by PredGPI to harbor glycosylphosphatidylinositol anchor motifs, which mostly represent membrane proteins rather than secreted effectors. In addition, we predicted six candidates of small secreted effector proteins, which were small (76 to 300 amino acids), secreted (signal peptide), and rich in cysteine (3%) when compared with other genes encoding proteins from the genome of *C. shiraiana*. One of these proteins was noted to contain a cell wall stress-responsive component domain, while the rest of the predicted small secreted effector proteins did not contain any known PFAM domains (data not shown).

Carbohydrate active enzymes annotation.

The ability to decompose plant carbohydrates is an important characteristic of necrotrophic fungal lifestyle. Plant cell wall carbohydrates are composed of a complex network of different polysaccharides that are segmented into three categories: cellulose, hemicellulose, and pectin. Carbohydrate active enzymes (CAZymes) are proteins involved in the degradation of plant biomass and conversion to fungal mass (van Kan 2006). In total, 400 CAZyme-coding gene homologs were determined using the domain-based annotation dbCAN in combination with CAT in the genome of *C. shiraiana*, which comprised 244 glycoside hydrolases (GHs), 7 polysaccharide lyases (PLs), 35 carbohydrate esterases (CEs), 99 glycosyl transferases (GTs), and 15 carbohydrate-binding modules (CBMs) (Table 2). The former three types of CAZymes are often known as CWDEs because they play a central role in the decomposition of plant cell walls by fungi. GHs are the most abundant CAZymes and the majority of them are related to hemicellulose or pectin decomposition (Yang et al. 2016). In the present study, the CAZymes of the other five fungi were also examined, and the number of CAZymes in three necrotrophic fungi was noted to be significantly higher than that in the biotrophic fungus *B. graminis* as well as the two nonpathogenic fungi *P. omphalodes* and *S. cerevisiae* (Table 2).

P450 annotation.

Cytochrome P450 enzymes belong to a group of structurally conserved but functionally diverse heme-containing mixed-function oxidases distributed in different life forms, including

Fig. 2. Common and specific gene families among the five fungi. The Venn diagram was used to compare the common gene families and specific gene families of the five fungi. The five colors indicate five species (red = *Ciboria shiraiana*, purple = *Pyronema omphalodes*, yellow = *Sclerotinia borealis*, green = *S. sclerotiorum*, and blue = *Blumeria graminis*), and the overlap parts have overlapping color. Numbers in the overlap parts indicate common gene families and nonoverlap parts denote specific gene families in each comparison.

Table 2. Comparison of carbohydrate active enzymes between *Ciboria shiraiana* and other five Ascomycetes with different lifestyles^a

Fungal species	Lifestyle	GHs	CBMs	CEs	GTs	PLs	Total
<i>C. shiraiana</i>	Necrotroph	244	15	35	99	7	400
<i>Sclerotinia sclerotiorum</i>	Necrotroph	241	16	41	102	5	405
<i>S. borealis</i>	Necrotroph	217	17	33	100	8	375
<i>Botrytis graminis</i>	Biotroph	75	5	15	63	0	158
<i>P. omphalodes</i>	Saprotroph	129	15	28	67	5	244
<i>S. cerevisiae</i>	Saprotroph	52	9	4	71	0	136

^a Values reflect the total number of members in the families. GHs = glycoside hydrolases, CBMs = carbohydrate-binding modules, CE = carbohydrate esterases, GTs = glycosyl transferases, and PLs = polysaccharide lyases.

prokaryotes and eukaryotes (van den Brink et al. 1998). In the present study, based on online database and multiple rigorous screening, 77 genes were identified as P450s in the *C. shiraiana* genome, which is similar to those in the other two fungi that are from the family Sclerotiniaceae; namely, *Sclerotinia sclerotiorum* (87 genes) and *S. borealis* (78 genes), slightly higher than those in *B. graminis* (52 genes) and *P. omphalodes* (51 genes), and significantly higher than those in *Saccharomyces cerevisiae* (5 genes) (Fig. 3).

Secondary metabolism.

Necrotrophic pathogens kill the host cells by producing a diverse array of SMs, including peptides, polyketides, terpenes, and alkaloids, which are synthesized by NRPS, PKS, terpene synthase, and dimethylallyl tryptophan synthases, respectively. In most fungi, genes involved in the biosynthesis of SMs are generally clustered together, and a classical gene cluster contains genes encoding the signature enzymes that synthesize the skeletons of different classes of SMs as well as genes encoding the tailoring enzymes that modify the skeletons. In the present study, *C. shiraiana* genome was found to be enriched with NRPS, PKS, and diterpene cyclase. In total, 23 SM clusters, including 25 key SM enzyme genes, were identified using the antiSMASH software, and these clusters were observed to be evenly distributed across the *C. shiraiana* genome (Table 3). The number of key enzyme genes in *C. shiraiana* was determined to be similar to that in the other two necrotrophic fungi examined but was significantly higher than that in the Leotiomycete *B. graminis*, whose obligate biotrophic lifestyle caused numerous gene losses (Spanu et al. 2010; Wicker et al. 2013).

Fig. 3. Distribution of P450 gene in the six fungi examined. The x axis shows the name of six fungi (Sc = *Sclerotinia cerevisiae*, Ss = *S. sclerotiorum*, Cs = *Ciboria shiraiana*, Sb = *Sclerotinia borealis*, Bg = *Blumeria graminis*, and Po = *Pyrenopeziza omphalodes*). The y axis shows the number of P450 genes.

Table 3. Number of secondary metabolism biosynthetic genes in four fungi^a

Species	PKS	NRPS	CHS	DMATS	STC	DTC	NRPS-PKS
<i>Ciboria shiraiana</i>	9	10	1	1	0	3	1
<i>Botrytis cinerea</i>	16	9	1	1	6	3	5
<i>Sclerotinia sclerotiorum</i>	15	6	1	1	0	1	1
<i>B. graminis</i>	1	1	1	0	0	0	0

^a PKS = polyketide synthase, NRPS = nonribosomal peptide synthetase, CHS = chalcone synthase, DMATS = dimethylallyl tryptophan synthase, STC = sesquiterpene cyclase, DTC = diterpene cyclase, and NRPS-PKS = polyketide synthase–nonribosomal peptide synthase hybrid.

Transcription factors.

Transcription factors (TFs) play a vital role in regulatory networks by acting as a linker between signal flow and target gene expression. Approximately 290 protein sequences (2% of total genes) were found to encode five main different classes of TFs in *C. shiraiana*. Among the five classes of TFs, Cys6 (124 genes) was found to be predominantly distributed, followed by C₂H₂ (61 genes), bZIP (15 genes), GATA (10 genes), and bHLH (6 genes) (Table 4). The number of Cys6 TFs was determined to be comparable with that in other Ascomycetes (*Sclerotinia sclerotiorum*, *n* = 155, and *Botrytis cinerea*, *n* = 222) because Cys6 TFs participate in many biological functions such as sugar and amino acid metabolism, gluconeogenesis, and respiration. Another major class of TFs in *C. shiraiana* is C₂H₂ (approximately 61 genes), which is known to be involved in growth, development, and virulence of the plant pathogen (Kim et al. 2009; Schumacher et al. 2008). In addition, 15 genes encoding bZIP TFs were detected in *C. shiraiana* (Table 4). In previous studies on *Magnaporthe oryzae*, deletion mutants of bZIP TFs *Moatf1* and *MoAP1* were reported to show H₂O₂ sensitivity, various defects in aerial hyphal growth, and conidia formation, suggesting that this class of TFs is necessary for complete virulence of *M. oryzae* (Guo et al. 2010, 2011). Therefore, it would be of interest to characterize the regulatory networks controlled by these TFs in *C. shiraiana*.

Analysis of growth-related genes.

Sclerotia are dormant structures that could remain viable for up to 8 years in soil (Young and Ashford 1992). In southwest China, sclerotia revive from dormancy from January until April, which coincides with the entire blossom period of mulberry trees. In fungi, the *MADS-box* gene has been found to be involved in growth, sclerotia production, and pathogenicity of *B. cinerea* (Zhang et al. 2016). In the present study, the predicted gene *Cs11201* was observed to be orthologous to *SsMADS*, with 99% identity at the nucleotide level (Supplementary Table S7). Detection of the transcript levels of *Cs11201* via quantitative real-time reverse-transcription PCR (qRT-PCR) showed that *Cs11201* is transcriptionally induced during sclerotial development stages, implying that *Cs11201* may be involved in sclerotia development (Fig. 4A).

When sclerotia break dormancy, apothecia germinate. One sclerotium could contain 1 to 15 germinating apothecia, and the number of ascospores in a 1.5-cm-diameter apothecia can reach up to 5.6×10^7 to 6.3×10^7 , which is a significantly high inoculum potential (Lü et al. 2019). Genes involved in the formation of apothecia in other fungi include genes encoding the Forkhead-box TF *Ss-FoxE2* (Wang et al. 2016), tactin-bundling protein *FgFim* (Zheng et al. 2014), GATA-type IVb zinc-finger TF *SsNsd1* (Li et al. 2018), and endo- β -1,4-xylanase *SsXyl-1* (Yu et al. 2016). In the present study, orthologs of these apothecia formation-associated genes were detected in the *C. shiraiana* genome. The qRT-PCR results showed that *Cs13232* and *Cs04366* exhibited an increased transcription level in

Table 4. Number of transcription factors (TFs) in *Ciboria shiraiana* and other two Ascomycetes

TF types	<i>Sclerotinia sclerotiorum</i>	<i>Botrytis cinerea</i>	<i>C. shiraiana</i>
Cys6	155	222	124
C ₂ H ₂	98	116	61
bHLH	9	9	6
bZIP	25	25	15
GATA	9	8	10
Total	296	380	216

sclerotia3, while *Cs04365* and *Cs02399* presented a higher transcription level in conidia (Fig. 4B to E).

A number of genes are crucial for the virulence of pathogenic fungi, including *Bc-Mtg2* (Shao et al. 2016), *Ss-Ggt1* (Li et al.

2012), *Fg-Fim* (Zheng et al. 2014), *Ss-Nsd1* (Li et al. 2018), and *Ss-oah1* (Liang et al. 2015), and the orthologs of these pathogenicity-associated genes were found in the genome of *C. shiraiana* (Supplementary Table S7). Detection of the

Fig. 4. Detection of the relative expression of predicted growth-related genes in six tissues (hyphae, sclerotia1 = initial sclerotia, sclerotia2 = developmental sclerotia, sclerotia3 = mature sclerotia, apothecia, and conidia) of *Ciboria shiraiana* by quantitative real-time reverse-transcription PCR. **A**, Relative expression of sclerotia development-related gene is shown in gray. **B** to **E**, Relative expressions of apothecia formation-related genes are shown in orange. **F** to **H**, Relative expression of infection of mulberry fruit-related genes is indicated in blue. **I** to **L**, Relative expressions of sclerotia formation-related genes are denoted in green. Data presented represent mean $\pm$ standard deviation of triplicate measurements.

expression of *Cs09614*, *Cs13590*, and *Cs11302* during the six development stages of *C. shiraiana* implied that the infection-associated genes are also related to development (Fig. 4F to H). The expression of these three genes was detected at different periods of infection on detached tomato leaves, and the qRT-PCR results revealed that the transcription of *Cs09614* and *Cs13590* increased in the late stage of infection (72 to 96 h), while that of *Cs11302*, *Cs04366*, and *Cs12286* increased during all the stages of infection, when compared with the noninfected group (Fig. 5).

In many fungi, sclerotia formation is considered to play an important role in protection from adverse conditions. Genes associated with sclerotia formation include sclerotium-specific protein *Ssp1* (Li and Rollins 2010), microbial opsin homolog *Sop1* (Lyu et al. 2016a), oxaloacetate acetylhydrolase gene *Ss-oh1* (Liang et al. 2015), and so on. These genes were also detected in the *C. shiraiana* genome (Supplementary Table S7), and the qRT-PCR results revealed that the expression of *Cs08397*, *Cs12804*, *Cs00985*, and *Cs12286* increased during sclerotia development stages, implying that these genes are associated with sclerotia formation (Fig. 4I to L).

Thus, genes involved in sclerotia dormancy, apothecia formation, infection of mulberry fruit, and sclerotia formation play vital roles in *C. shiraiana* infection and lifecycle. Because ascospores from the apothecia of *C. shiraiana* might play a key role in infection of mulberry fruits owing to its significantly large pathogen base, our future research will focus on development of strategies to block apothecia formation.

Expression analysis of plant cell-wall-degradation genes during infection.

Plant cell wall carbohydrates are mainly composed of cellulose and pectin. Previous studies have reported that cellulase and pectinase expressed in vitro could enzymatically hydrolyze the cell wall of mulberry plant (Lü et al. 2015a), indicating that these enzymes play important roles in the infection process. The process of infection is very fast; therefore, in the present study, the infected leaves were collected at 0, 1, 3, 6, 12, and 24 h postinoculation to analyze the expression patterns of cellulase- and pectinase-coding genes. The RT-PCR results

showed that all 14 cellulase genes were expressed during infection, among which 8 genes showed an increased expression pattern in the initial stage of infection (1, 3, or 6 h after inoculation) (Fig. 6). The expression levels of all of the cellulase genes, except *Cs00913* and *Cs01714*, were increased at 24 h postinoculation, when compared with those after 12 h of inoculation. It must be noted that the expression of *Cs07879*, *Cs12956*, and *Cs07604* gradually increased along with infection duration and peaked at 24 h (Fig. 6).

Previous studies have shown that the GH28 family plays an important role in pectin degradation in pathogens (Zhao et al. 2013). In the present study, all 16 genes within the pectinase gene family were expressed during the process of infection, among which the expression pattern of 12 genes showed a peak at 6 h, suggesting that these genes may play a significant role during the initiation of infection (Fig. 7). In addition, the expression of *Cs05107* gradually increased along with infection duration, indicating that this gene may be crucial for the pathogenicity of *C. shiraiana*.

DISCUSSION

Mulberry fruit sclerotienosis is one of the most common and destructive diseases of mulberry in China and around the world; however, studies of its pathogenic mechanism are still limited. It has been reported that repeat sequences can result in significant differences in the entire architecture of the genome and cause different genetic variations so that organisms can better adapt to environmental changes and occupy novel niches (Faino et al. 2016; Lisch 2013). Most of the pathogenic ascomycetes fungi have a slightly higher percentage of repeat elements than nonpathogenic fungi (Hittalmani et al. 2016), such as *S. sclerotiorum*, which has a genome size of 38.3 Mb and TE content of 7% (Amselem et al. 2011). Although repeat sequences may play a beneficial role in improving genetic diversity, some of them are also detrimental, particularly with respect to genome stability (Galagan et al. 2005). The low percentage of repeats can also be attributed to the function of repeat-induced point (RIP) mutations in the genome (Galagan and Selker 2004). The genomes of other Ascomycetes fungi

Fig. 5. Expression level of infection-related genes at different periods on detached tomato leaves. Data presented represent mean $\pm$ standard deviation of triplicate measurements.

such as *S. sclerotiorum* and *B. cinerea* have been observed to exhibit higher repeat elements content along with the RIP mechanism (Amselem et al. 2011). Thus, these fungal species may have a close relationship with *C. shiraiana*, probably sharing similar phenomenon such as RIP in their genomes (Galagan and Selker 2004).

CAZymes play an important role in the degradation of cell wall components during the infection process (Xu et al. 2016). *Peltaster fruticola*, a pathogen that cannot penetrate host cell walls, contains only 38 putative plant cell wall-degrading enzymes (PCWDEs); this number is slightly higher than the number of PCWDEs in saprophytic *Saccharomyces cerevisiae* and symbiotic *Rhizophagus irregularis* but is significantly lower than the number of PCWDEs in most of the plant-pathogenic fungi, indicating that the number of CAZymes has a positive effect on pathogenesis (Xu et al. 2016). In most of the obligate biotrophs such as *Oidium heveae*, *Ustilago maydis*, and *Sporisorium scitamineum*, the number of CAZymes has been observed to be significantly lower than that in hemi-biotrophic and necrotrophic phytopathogens; as a result, these obligate biotrophs cause minimum damage to the host cell and do not trigger plant defense responses (Kämper et al. 2006; Liang et al. 2018; Que et al. 2014). The decrease in the number of CAZymes in obligate biotrophs suggests the reduction in the requirement of these fungi to attack their hosts, which seems to perfectly match their biotrophic lifestyle (Que et al. 2014). In the present study, *C. shiraiana* was found to exhibit the

maximum number of CAZymes among the three necrotrophic fungi, which is in line with its necrotrophic lifestyle and ability to cause infection similar to *Sclerotinia sclerotiorum* and *Botrytis cinerea* (Table 2).

Tomato is one of the most important vegetables around the world, with important economic value. It is widely used as a model plant in research on fruit development and biotic stress owing to its short growth period. A cross-infection study on *C. shiraiana* and *S. sclerotiorum* has revealed that the ascospores of *C. shiraiana* could infect oilseed rape, while those of *S. sclerotiorum* could similarly infect sorosis (Lü et al. 2015b). In the present study, preliminary experiments showed that *C. shiraiana* could infect tomato leaves in the short term and can serve as a model for performing infection experiments. The RT-PCR results indicated that all 14 cellulase genes were expressed during infection, among which 8 genes showed increased expression patterns in the initial stage of infection (Fig. 6). Similarly, all 15 genes within the pectinase gene family were expressed in the process of infection, among which the expression pattern of 11 genes presented a peak at 6 h postinfection, implying that this time point may be an important stage during infection (Fig. 7). Because only a few CWDEs have been reported thus far to have an important role in fungal pathogenicity (Baroncelli et al. 2016), the results of the present study will lay the foundation for a better understanding of the role of CWDEs in fungal pathogenicity.

Fig. 6. A, Expression pattern of predicted genes encoding cellulose-degrading enzymes in *Ciboria shiraiana* at six stages (0, 1, 3, 6, 12, and 24 h after inoculation) of the infection process. B, Diseased tomato leaves at the indicated times after inoculation.

To successfully infect hosts, pathogenic fungi must inhibit or avoid activation of plant defense responses. Accordingly, pathogens secrete effectors that play important roles within or outside of the plant cells, interacting with various host targets to suppress PTI and promote colonization (Guyon et al. 2014). The secreted effector proteins have been found to participate in the interactions between biotrophic or hemibiotrophic pathogens and their hosts such as biotrophic powdery mildews (Spanu et al. 2010), smuts, and rusts (Ravensdale et al. 2011; Schirawski et al. 2010), as well as hemibiotroph *M. oryzae* (Khang et al. 2010), in a gene-for-gene manner (De-Wit et al. 2009). In addition, secreted enzymes can act as virulence factors to improve the catalytic activity of pathogens (Lanver et al. 2017; Noda et al. 2010). The number of effectors may vary among different pathogens and are related to the pathogen's lifestyle (Lowe and Howlett 2012); in particular, the interactions of necrotrophs with their hosts are considerably more complex than previously considered. However, the association between the number of effectors in necrotrophs and broad host range remains unclear. To date, many interactions between host-specific necrotrophic fungal pathogen effectors and their hosts have been reported, including *Tsn1* in wheat as well as *SnTox1-Snn1* in the *Stagonospora nodorum*-wheat pathosystem, which presented an inverse gene-for-gene activity (Faris et al. 2010; Liu et al. 2004; Lyu et al. 2016b). The number of predicted secreted effector proteins in *C. shiraiana* was observed to be much lower than that in *B. cinerea* (521 proteins) and *Sclerotinia sclerotiorum* (363 proteins) (Amselem et al. 2011). Comparative secretome analysis of *S. sclerotiorum* has revealed that a diverse effector repertoire confers the ability to

infect a broad range of host species (Guyon et al. 2014). In the present study, a small number of predicted secreted effector proteins indicated a narrow host range of *C. shiraiana*, despite comparable numbers of CAZymes among the three necrotrophic fungi.

In fungi, P450 enzymes are known to possess diverse functions ranging from the synthesis of cell wall components to biodegradation of environmental chemicals and drug metabolism (Bhattacharya and Yadav 2018). Plants have the highest number of P450 genes in different taxa, followed by fungi (Doddapaneni et al. 2005). In the present study, the number of P450 genes was found to be conserved among the three fungal species of Sclerotiniaceae, which present a necrotrophic lifestyle, but was higher than that in the rest of the fungi examined. It is known that plant-pathogenic fungi utilize a variety of strategies to acquire carbon and nitrogen sources from their host plant and counteract the plant defense response. Therefore, we speculated that a high number of P450s could contribute to the biodegradation of environmental nutrition to adapt to the necrotrophic lifestyle. Moreover, we found that CYP503A1, a gene family involved in the biosynthesis of gibberellins in the rice pathogen *Fusarium fujikuroi* (Črešnar and Petrič 2011; Rojas et al. 2001; Troncoso et al. 2008), has expanded (data not shown). Although the production of gibberellins is not essential for the growth and development of fungi, they may help the fungi to survive in harsh environments. In addition, some studies have shown that gibberellins are related to pathogenicity (Salazar-Cerezo et al. 2018). Thus, the expanded gene family CYP503A1 may be involved in the biosynthesis of gibberellins or any other SMs in *C. shiraiana*.

Fig. 7. Expression pattern of predicted genes encoding pectin-degrading enzymes in *Ciboria shiraiana* at six stages (0, 1, 3, 6, 12, and 24 h after inoculation) of the infection process.

Conclusion.

The *C. shiraiana* genome sequencing and comparative analysis with a collection of Ascomycetes genome sequences shed light on the pathogenic mechanisms of this fungus, including secreted effector proteins, CAZymes, secondary metabolism, and P450. In addition, analysis of the key growth-related genes in the *C. shiraiana* life cycle provided molecular targets for the development of antifungal drugs. Following analysis of the *C. shiraiana* genome, efforts are underway to validate the function of all of the potential pathogenicity determinants, especially virulence- and growth-associated genes. The genome sequencing of *C. shiraiana* is the first step in understanding the pathogenic mechanisms of hypertrophy sorosis scleroteniosis at a genomic level. These efforts and ongoing functional identification will expand our knowledge of the interaction between mulberry and *C. shiraiana*, which may help to develop better disease control measures.

MATERIALS AND METHODS

DNA preparation.

The sequenced strain *C. shiraiana* CCTCC AF 2014019 WCCQ01, conserved in our laboratory, was originally isolated and purified from diseased mulberry fruit and deposited in the China Center for Type Culture Collection (Lü et al. 2013). The isolate was grown on potato dextrose agar at 25 °C for 3 to 5 days and three mycelia plugs (5 mm in diameter) were then transferred to potato dextrose broth and incubated at 25 °C and 220 rpm for 3 days. The fungal hyphae were collected from the liquid medium and genomic DNA was extracted by the sodium dodecyl sulfate (SDS) method. Briefly, 0.2 g of fresh hyphae was quickly homogenized in liquid nitrogen and mixed with 600 µl of solution A (200 mM Tris-HCl [pH 8.0], 250 mM NaCl, 25 mM EDTA, 0.5% SDS, 1% β-mercaptoethanol, and 8 µl of RNase) using a vortex mixer for 1 min and allowed to stand for 10 min. After that, 260 µl of 5 M potassium acetate was added to the extracted mixture and centrifuged at 12,000 rpm for 5 min. The supernatant was transferred into a new centrifuge tube and the same volume (500 µl) of isopropyl alcohol was added and centrifuged at 12,000 rpm for 5 min. Finally, the deposit was collected and washed thrice with 400 µl of 75% ethanol and the genomic DNA was resuspended in 60 µl of DNase-free water containing RNase A.

Genome sequencing and assembly.

The sequence of *C. shiraiana* genome was examined and assembled by Genedenovo Biotechnology Co., Ltd., Guangzhou, China. We first used high-throughput Illumina sequencing technology to perform paired-end sequencing of the *C. shiraiana* genome and then sequenced the genome with PacBio RSII. The genomic data from the two kinds of sequencing technology were assembled together using HGAP software (Chin et al. 2013). Polymerase reads were filtered for quality (eliminate length < 100, quality < 0.80), adapter, and other contamination, and the resulting 634,414 subreads were assembled using the HGAP assembler.

Karyotype analysis of *C. shiraiana*.

Three mycelia plugs (5 mm in diameter) were transferred to 25 ml of potato dextrose broth and incubated at 25°C and 220 rpm for 3 days. Then, 0.2 g of fresh fungal mycelia collected from potato dextrose broth was centrifuged at 4 °C and 4,000 rpm for 3 min, then fixed in 1 ml of 3:1 methanol and glacial acetic acid for 24 h at 4°C. The fixed mycelia were incubated with 500 µl of 1× phosphate-buffered saline (PBS) solution for 30 min and digested with 0.01% (wt/vol) lyticase and 0.01% (wt/vol) snailase for 2 h at 30°C. The digested mycelia were immersed in 500 µl of double-distilled H₂O for

30 min and smashed using a homogenizer after the addition of 200 µl of 1× PBS solution and centrifugation at 4°C and 4,000 rpm for 3 min. Then, two drops of the cell suspension were placed on a glass slide for staining with 10% Giemsa in phosphate buffer (pH 6.8) at room temperature for 6 h, and analyzed under a compound microscope using bright field at ×1,000 magnification (Leica DM 2500, Germany).

Synten analysis with *S. sclerotiorum*.

Synten analysis was performed at nucleotide and amino acid levels using MUMmer software and BLAST-2.2.26 (1e⁻⁵, soft masked) (Marçais et al. 2018), respectively. The minimum alignment similarity was set at 85%, 1:1 matches were selected in this alignment, and only best matches with local mapping of the reference to query were selected using Δ-filter. The best protein match was found between the two species by BLAST-based homology alignment, and the synteny region between the two genomes was determined based on their position on the genome. The picture was generated using mummerplot based on the best_delta results.

Genome annotation.

The ab initio gene predictor tool AUGUSTUS (Stanke et al. 2004) was used to predict gene models in the genome, trained with *S. sclerotiorum*. The AUGUSTUS gene prediction program was employed with options set for (i) *S. sclerotiorum*, (ii) coding sequence, and (iii) GFF (Stanke and Waack 2003). Prediction of rRNA was performed using rRNAmmer software (Lagesen et al. 2007), and prediction of the tRNA region and secondary structure of tRNA was accomplished by tRNAscan software (Lowe and Eddy 1997). TEs were identified by RepeatMasker, a software for genomic repeat identification that can be used for almost all species.

Analysis of gene family.

The analysis of gene family was based on the Treefam model, which defines a gene family as a group of genes that had descended from a single gene in the last common ancestor of the considered species (Li et al. 2006). Protein sequences from *S. sclerotiorum*, *S. borealis*, *Blumeria graminis*, *Pyronema omphalodes*, and *Saccharomyces cerevisiae* were downloaded from NCBI. The longest encoding sequence was selected to represent each gene, and sequences of less than 30 amino acids were removed. The genes that belong to one family were identified based on one of the following criteria: (i) they are phylogenetically separated from other genes or (ii) they lack homologs outside the fungal kingdom. Phylogenetic trees were constructed for each specific gene family and parameters such as Ka and Ks nucleotide substitution rates were also analyzed. The Ka/Ks values were used to analysis diversifying selection. A high pairwise Ka/Ks value under positive selection indicates that the protein had evolved rapidly in the species. The sequences from each family were aligned using MAFFT and RevTrans based on the protein sequences and codon alignments after translation. KaKs_Calculator 2.0 was used to obtain pairwise Ka/Ks values with significance level (Wang et al. 2010).

Analysis of secreted effector proteins.

Secreted effector proteins were analyzed by using online tools. SignalP was used to screen the signal peptide site, TMHMM version 2.0 was employed to predict the transmembrane helix structure, TargetP 1.1 was utilized to predict the signal peptide related to organelles that were removed, and PredGPI was used to predict glycosyl phosphatidyl inositol-anchor signals, which were also filtered out, and the remaining 120 sequences were considered as the potential secreted effector proteins (Amselem et al. 2011; Pedersen et al. 2012).

CAZymes and auxiliary proteins annotation.

Two different online tools were used to analyze CAZymes and auxiliary proteins; namely, CAT and dbCAN (E-value $<1.0 \times 10^{-5}$), respectively; and all of the results were combined and finally collected. The integration results were mainly based on dbCAN, supplemented with CAT (pfam rules method). One gene could retain only one optimal CAZy family result. The filtering criteria were identity ≥ 40 and query $\geq 70\%$, and were classified into reaction catalyzed such as GHs, CBMs, CEs, GTs, and PLs, as described in CAZY database.

CYP450 annotation.

The CYP450 analysis was based on an online database. The screening criteria (identity ≥ 40 , query $\geq 70\%$) were subjected to multiple filtering, and each gene retained only the best match.

TFs.

The analysis of TFs was based on the annotation results of the nr database, and the comment lines related to TFs were filtered from the annotation results and subjected to multiple filtering (E-value $< 1e^{-5}$, identity > 70 , query coverage $> 40\%$). The final 291 genes were noted to be putative TFs.

Secondary metabolism.

The antiSMASH database was used to search the SM gene clusters. Briefly, the potential biosynthetic gene clusters were identified by profile hidden Markov models (pHMMs), and 24 secondary metabolite classes of pHMMs describing the key biosynthetic enzymes were detected by HMMer3 software. Based on the detected secondary metabolite gene cluster, further detailed analyses were performed using a pHMM-based approach. All of the above-mentioned programs and software used have been integrated in antiSMASH. Finally, the putative product biosynthesis gene clusters between the genomes were obtained.

qRT-PCR expression analysis.

Primers (Supplementary Table S1) used for qRT-PCR were designed based on the genomic sequences of *C. shiraiana*. To analyze gene expression, the total RNA was extracted using the RNA Extraction Kit TransZol Plant (TransGenBiotech, Beijing, China) according to the manufacturer's protocol. First-strand cDNA was synthesized using the PrimeScript RT Reagent Kit (TaKaRa, Japan). The β -tubulin gene (*Ciboria_shiraiana05660*) was used as an internal reference, and the $2^{-\Delta\Delta Ct}$ method was employed to calculate the relative expression levels (Livak and Schmittgen 2001).

Plant growth conditions and *C. shiraiana* infection.

Tomato plants (*Solanum lycopersicum*) were cultured in incubator at 25 and 18°C (day and night, respectively) with a daily cycle of 16 h of light and 8 h of darkness. The leaves of 3-month-old tomato seedlings were used for infection experiments. Before an infection test, all of the leaves were surface sterilized with 75% ethanol for 1 min, then put in a petri dish covered with a damp filter paper. Fresh mycelium pieces cultured for 3 days in potato dextrose agar were used for infection. The leaves and agar mycelium were collected at 0, 1, 3, 6, 12, 24, 36, 48, and 72 h postincubation for RNA extraction. Each treatment contained three independent biological replicates and each replicate comprised three leaves. The primers used in the experiments are listed in Supplementary Tables S8 and S9.

AUTHOR-RECOMMENDED INTERNET RESOURCES

antiSMASH database: <https://fungismash.secondarymetabolites.org/#!/start>
CAT: <https://github.com/ComparativeGenomicsToolkit/Comparative-Annotation-Toolkit>

CAZY database:

<http://www.cazy.org/Welcome-to-the-Carbohydrate-Active.html>
CYP450 online database: <https://dmelson.uthsc.edu/cytochromeP450.html>
dbCAN: http://csbl.bmb.uga.edu/new/?page_id=13073
NCBI: <https://www.ncbi.nlm.nih.gov>
PredGPI: <http://gpcr.biocomp.unibo.it/predgpi>
RepeatMasker: <http://www.repeatmasker.org>
SignalP: <http://www.cbs.dtu.dk/services/SignalP/>
TargetP: <http://www.cbs.dtu.dk/services/TargetP>
TMHMM: <http://www.cbs.dtu.dk/services/TMHMM>

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