Laccase Gene *Sh-lac* Is Involved in the Growth and Melanin Biosynthesis of *Scleromitrella shiraiana*

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ABSTRACT

Scleromitrella shiraiana causes the popcorn disease in mulberry trees resulting in severe economic losses. Previous studies have shown that melanin may play a vital role in establishing the pathogenicity of fungi. In the present study, we identified the melanin produced in *S. shiraiana* belongs to DHN melanin by gas chromatography-mass spectrometry, and cloned the laccase *Sh-lac*, a potential DHN melanin biosynthesis gene from *S. shiraiana*. We obtained two stable *Sh-lac* silenced transformants using RNAi, ilac-4 and 8 to elucidate the DHN melanin biosynthetic

pathway in *S. shiraiana*. The melanin production of ilac-4 and ilac-8 was significantly reduced, and their vegetative growth was also suppressed. Results such as these led to a proposal that *Sh-lac* played a key role in DHN melanin formation in *S. shiraiana* and may function differentially with other melanin biosynthetic genes. The inhibition of melanin was accompanied by the decrease of oxalic acid and the adhesion of hyphae was impaired. Our results indicated that laccase was an important enzyme in the synthesis of melanin and might play a critical role in the pathogenicity of *S. shiraiana*.

Mulberry trees (*Morus* sp.) are cultivated widely in the temperate, subtropical, or tropical regions of the world, and can grow in a wide range of climatic, topographical, and soil conditions (Ercisli and Orhan 2007; Özgen et al. 2009). Mulberry trees have been grown to rear silkworms for a long time, but recently there has been a surge in the demand for mulberry fruits owing to their taste and nutritive value. Mulberry fruits also contain phytonutrient compounds like polyphenol pigment antioxidants, minerals, and vitamins that have proven health benefits. Mulberry alkaloids can activate macrophages. Macrophages then stimulate the human immune system to fight against health threats (Kim et al. 2013). However, mulberry fruits are susceptible to diseases from pathogens such as *Ciboria carunculoides* (Sieglar and Jenkins) Whetzel, *Ciboria shiraiana* (Henn.) Whetzel, and *Scleromitrella shiraiana* (Henn.) Imai (Kishi 1998; Kohn and Nagasawa 1984; Whetzel and Wolf 1945). The fruit bodies of these pathogens release ascospores to infect blossoms and then cause the mummification of fruits (Fig. 1). This kind of disease is also called popcorn disease (Blain 1931; Sieglar and Jenkins 1923). Traditional methods are not effective in the prevention and treatment of the disease.

The pathogen *S. shiraiana*, SX-001 strain, was isolated from diseased mulberry fruits. *S. shiraiana* produces large amounts of black polymer-like material on potato dextrose agar (PDA) medium and the sclerotia formed in the diseased fruit is coated with a thick black substance (Li and Rollins 2009). These black substances have the chemical properties of melanin, such as black color, insolubility in cold or boiling water and organic solvents, resistance to degradation by hot or cold concentrated acids, bleaching by oxidizing agents, and solubilization and degradation by hot alkali solutions (Butler and Day 1998; Wu et al. 2008). Butler et al. (2009) confirmed that the melanin produced by *Sclerotinia sclerotiorum* is a 1,8-dihydroxynaphthalene (1,8-DHN). Melanins are biological macromolecules formed by oxidative polymerization of phenolic

or indolic compounds that form complexes with proteins and carbohydrates (Langfelder et al. 2003; Watt et al. 2009). Melanin plays a protective role against various adverse external environmental conditions such as UV irradiation, osmotic pressure, and biotic stresses (Butler and Day 1998; Henson et al. 1999). Melanin is also closely related with pathogenicity of some plant and animal pathogens (Butler et al. 2001; Ebbole 2007; Jacobson 2000; Langfelder et al. 2003; Thomma 2003; Thywissen et al. 2011).

Fungi synthesize DHN (1,8-DHN) melanin and L-3,4-dihydroxyphenylalanine (L-dopa) melanin. DHN melanin is synthesized by a polyketide pathway and is produced by a series of redox reactions (Gomez and Nosanchuk 2003). The scytalone dehydratase (SCD), 1,3,6,8-tetrahydroxynaphthalene reductase (4THNR), and/or 1,3,8-trihydroxynaphthalene reductase (3THNR) are important enzymes involved in redox reactions in DHN melanin biosynthesis pathway (Andersson et al. 1996; Lee et al. 2003). The final step of DHN polymerization to DHN melanin is putatively catalyzed by laccases (Tetsch et al. 2005; Upadhyay et al. 2013) (Fig. 2). In contrast, the L-dopa melanin is synthesized by laccases, and its production requires exogenously supplied phenolic precursors such as L-dopa (Williamson et al. 1998). However, the study of laccase catalyzed DHN polymerization reaction is rare. A mutant of putative laccase, *Pgr1*, failed to form melanin, which suggested *Pgr1* involved in the oxidation of DHN in *Cochliobolus heterostrophus* (Tanaka et al. 1991, 1992). One recent study demonstrated that a laccase (LAC2) was associated with melanin production and pathogenicity in *Colletotrichum orbiculare* (Lin et al. 2012). Laccase is a copper-containing enzyme that catalyzes the oxidation of a phenolic substrate by coupling it to the reduction of oxygen to water (Liu and Nizet 2009). Laccases are ubiquitously found in many organisms and are involved in many other biological activities (besides melanin biosynthesis) such as in morphogenesis, pathogenesis, and lignin degradation (Baldrian 2006). For example, a laccase-like enzyme, relative to the fungal pathogenicity in *Botrytis cinerea*, can degrade several phytoalexins of grapevines (Sbaghi et al. 1996). In the present study, we isolated *S. shiraiana*, one of the pathogens causing the mulberry popcorn diseases to study its morphology and biochemistry. We hypothesize that the copious amounts of melanin produced by the fungus are related to the pathogenicity of the fungus. We investigated the roles of a laccase gene *Sh-lac* in the

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melanin biosynthetic pathway of *S. shiraiana*. Our results will help understand the etiology of the popcorn disease at the molecular level. Development of an agent that can specifically inhibit the production of melanin or degradation of melanin will be an effective means for prevention and control of the mulberry popcorn disease.

MATERIALS AND METHODS

Isolation of fungal strains and culture conditions. The diseased mulberry fruits were collected from the mulberry orchard in Southwest University, Chongqing, China. The drupelets of diseased mulberry fruit were rinsed with water, air-dried, and disinfected with 70% alcohol for 30 s followed by 0.1% mercuric chloride surface-sterilized for 5 min (Zhang et al. 2009). The drupelets were then rinsed five times with sterile distilled water to ensure complete cleansing of alcohol and mercuric chloride. A sterile scalpel was used to slice the drupelets, which were then placed on the PDA medium and incubated at 25°C in darkness.

Fig. 1. The symptom of mulberry popcorn disease. The white mummified mulberry fruit.

The target pathogen was isolated and named SX-001 strain. The strain was routinely subcultured on PDA at 25°C to maintain vigor and purity. A sterile cellophane membrane was placed on the medium before incubation to collect the hyphae from cultures grown on solid medium.

Nucleic acid isolation, cDNA production, and real-time RT-PCR analysis. The hyphae were collected, frozen in liquid nitrogen, and stored at –80°C until further use. Genomic DNA was isolated according to a previously described method of Yelton et al. (1984). Total RNA was extracted with the RNAiso Plus (Takara, Dalian, China) according to the manufacturer's instructions and treated with RNase-free DNase I to remove contaminated DNA. First-strand cDNA was synthesized using a PrimeScript RTase based PrimeScript RT-PCR Kit (Takara, Dalian, China). For RT-PCR, 1 µg of total RNA was reverse transcribed as the template. Real-time RT-PCR was performed using SYBR Green (Takara) on StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA). The expression level of *Sh-lac* was analyzed via qPCR with the primer pairs RT-LAC02F/RT-LAC02R. The cDNA abundance in the sample was normalized using the *actin* housekeeping gene as a control. *S. sclerotiorum actin* gene (SS1G_08733) primers pair ActinF/ActinR were used to amplify the *actin* gene from *S. shiraiana* cDNA (Yu et al. 2012). PCR amplification and sequencing showed that this pair primer is suitable for amplified *actin* gene in *S. shiraiana*.

Melanin preparation. *S. shiraiana* inocula were maintained on PDA and then inoculated into a 250-ml flask containing 100 ml of malt extract (ME) medium and incubated at 25°C for 17 days with continuous shaking at 200 rpm. Melanin was prepared and purified as described by Wu et al. (2008), with slight modification as chloroform and isoamyl alcohol (1:5) were replaced with chloroform and ethyl alcohol (1:1).

Sample preparation and melanin identification. Inquiry sample and standard were dissolved in acetonitrile and then centrifuged at 10,000 rpm for 10 min. Supernatant (100 µl) was rotary evaporated to dryness. Derivatization was performed with 50 µl of methyl-trimethyl-silyl-trifluoroacetamide at 37°C for 1 h. Samples were analyzed by gas chromatography-mass spectrometry (GC-MS) (Agilent Technologies Inc., CA). The separation was done with a DB-5MS column (30 m × 0.25 mm I.D. with a 0.25 µm). The injection volume was 1.0 µl and carrier gas was helium at 1.0 ml/min. The oven program started at an initial temperature of 50°C for 3 min. Temperature was then increased at a rate of 5°C/min up to 300°C, maintained at 300°C hold for 3 min with a 10:1 split ratio. Ionization was performed by electronic impact (EI), setting the electron multiplier to 70 eV.

Construction of gene silencing vectors and *S. shiraiana* transformation. The pCIT vectors were kindly provided by Yang Yu (College of Plant Protection, Southwest University, Chongqing,

Fig. 2. 1,8-Dihydroxynaphthalene melanin biosynthetic pathway of *Sclerotinia shiraiana*.

China). The primer pair *idz* LAC-F and *idz* LAC-R were used to amplify a 417-bp *Sh-lac* coding sequence to obtain the sense fragments to construct the *Sh-lac* silencing vector. We introduced two restriction sites *Hind*III and *Bam*HI at the 5' terminus of the sense primer, and another two restriction sites *Eco*RI and *Pst*I at the 5' terminus of the antisense primer. The primer sequences are listed in Supplementary Table S1. The PCR product was sequenced and digested with *Pst*I and *Bam*HI, and then ligated into vector pCIT (Yu et al. 2012) between the intron and *TtrpC*, to produce the intermediate vector pCIT *idz*LAC-I. The same PCR products were digested with *Hind*III and *Eco*RI and then ligated into previous constructed intermediate vector pCIT *idz*LAC-I between the intron and *PtrpC* to yield vector pCIT *idz*LAC-II. The pCIT *idz*LAC-II was digested with *Xho*I and *Sac*I to obtain a fragment in which the two 417-bp fragments were inserted in opposite orientations between *PtrpC* and *TtrpC*. This fragment was then ligated between *Xho*I and *Sac*I sites of pCAMBIA3300, which carries hygromycin B phosphotransferase gene, to create the *Sh-lac* silencing vector pCHidzLAC. The vector pCHidzLAC was transformed into the wild-type strain SX-001. The candidate transformants were screened on PDA containing hygromycin B at 30 µg/ml. The vector pCHidzSCD was constructed for silencing *Sh-SCD* using the same method.

Melanin production assay. *S. shiraiana* mycelia were preserved on PDA. Each flask, containing 50 ml of ME medium, was inoculated with five equal-sized mycelium (to reduce systematic errors caused by the operation) and incubated at 25°C, 200 rpm for 17 days. Three replicates were performed per treatment. ME medium was filtered through two layers of filter paper (Sangon Biotech, Shanghai, China). The filtrate was scanned using a DU800 spectrophotometer (Beckman Coulter). Melanin sample extracted from the mycelium showed that absorbance at wavelengths from 200 to 400 nm was interfered by the liquid medium. Also absorbance was low at wavelengths greater than 500 nm, and this could affect the accuracy of the detection results. Thus, 420 nm was selected to measure the yield of melanin.

Chromatography. Chromatographic separation of oxalic acid was performed using Waters Acquity UPLC I-CLASS system (Waters Corp., MS); ACQUITY UPLC HSS T3 column (1.8 µm, 2.1 × 50 mm) was used. The mobile phase used was: KH₂PO₄ (90% A; 0.5% wt/vol, pH 2.0) and TBA (10% B; tetrabutylammonium, 20 mM, do not adjust pH) at a flow rate of 0.5 ml/min. The chromatogram was monitored at a wavelength of 210 nm. The

column temperature was maintained at 30°C and the injection volume of each sample and standard solution was 0.8 µl. Data were processed with Waters Empower 2 software for analysis.

RESULTS

Isolation and identification of *S. shiraiana*. We identified *S. shiraiana* from the diseased mulberry fruit through internal transcribed spacer (ITS) alignment using the primer pair ITS1/ITS4 (White et al. 1990). The ITS sequences cloned from the genome of *S. shiraiana*'s apothecia confirmed its identity (Supplementary Fig. S1). We named it SX-001 strain. The *S. shiraiana* colony darken when cultured for about 4 days on PDA medium. The colony almost stopped growing on the tenth day and showed remarkable blackening (Fig. 3A and B). At 20 to 25 days, we saw the appearance of milky liquids on the colony surface (Fig. 3C and D). The milky liquids contain many microspores, which germinated to form colonies. Sclerotia typically have two structures: the hard black outer layer is called the pseudoparenchyma and the loose inner layer is called the prosenchyma (Fig. 2F to I). Sclerotia are the dormant pathogen structures and only overwintered sclerotia have the vitality to form fruit bodies.

***S. shiraiana* hyphae disorganize mulberry drupelets and form sclerotia in diseased fruits.** *S. shiraiana* ascospores are the only source of primary infection and infect mulberry inflorescences. The disease manifested itself with the growing mulberry fruits, and we observed the formation of sclerotia in drupelet. The mulberry fruits finally turned to mummified white fruits. We observed fibrous, interwoven, and interspersed hyphae in the paraffin sections of diseased mulberry fruits, and the hyphae disorganize drupelet tissue into small granules (Fig. 4A). The small granules in the drupelet tissue provide adequate nutrition for *S. shiraiana*.

Paraffin sections produced at different stages of the disease showed a large number of small black particles gathered around a black spheroid structure (Fig. 4B). The small black particles are ingredients of the outer wall of sclerotia. These black particles probably shed from the mycelium (Fig. 4C) and are parts of the fractured hyphae.

Identification of melanin. Extract ion chromatography (EIC) studies identified the black substance from *S. shiraiana* hyphae as 1,8-DHN, which is a monomer of DHN melanin. The results

Fig. 3. Morphology of *Scleromitrium shiraiana*. **A and B**, The front and reverse of *S. shiraiana* grown on potato dextrose agar (PDA) medium for 10 days and colony was blackened. Bar: 1 cm. **C**, *S. shiraiana* grown on PDA and much liquid secreted containing microspores after 25 days. Bar: 1 cm. **D**, A partial enlarged view of the upper end of the colonies in C. **E**, Microspore micrographs are in the optical microscope. Bar: 20 µm. **F**, The sclerotia of pathogens of mulberry popcorn disease. Bar: 1 cm. **G**, Overall microstructure sclerotia. Bar: 200 µm. **H**, Sclerotia pseudoparenchyma and black particles. Bar: 50 µm. **I**, Sclerotia prosenchyma. Bar: 40 µm.

showed that DHN appeared at 24.4 min as shown on the EIC chart (Fig. 5A). DHN can be detected at the same site from inquiry melanin sample. Mass spectrometric analysis indicated *S. shiraiana* melanin was indeed DHN melanin (Fig. 5). In addition, *S. shiraiana* was treated with tricyclazole (5-methyl-1,2,4-triazol[3,4]benzothiazole). Tricyclazole is an agrochemical that acts specifically on DHN melanin pathway by inhibition reductase, 1,3,6,8-tetrahydroxynaphthalene reductase (4THNR) and/or 1,3,8-trihydroxynaphthalene reductase (3THNR). The results showed that tricyclazole at 60 µg/ml significantly inhibited the growth of fungi and the melanin production of *S. shiraiana* (Supplementary Fig. S2).

Cloning of the *Sh-lac* from *S. shiraiana*. We cloned the laccase gene to identify the laccase responsible for melanin biosynthesis and pathogenicity of *S. shiraiana*. We obtained an approximately 1,240-bp amplified fragment from *S. shiraiana* cDNA using a pair of degenerate primers targeted to conserved regions of the fungal laccase gene. Sequence BLAST analysis of the fragment exhibited 97% identities to the laccase (SS1G_11927) of *S. sclerotiorum* 1980,

indicating that it encoded a homolog of laccase. We named this homolog *Sh-lac*. The full-length *Sh-lac* gene contains an open reading frame (ORF) of 2,016 bp, and encodes 672 amino acids. The *Sh-lac* ORF comparison with the laccase gene sequence amplified from genomic and cDNA revealed one intron (86 bp) with the consensus GT-AG rules (Breathnach et al. 1978; Mount 1982). A putative signal peptide consisting of 18 amino acid residues was identified at the N terminus (Fig. 6A). It predicted that *Sh-lac* protein is an extracellular protein. We found three typical cupredoxin domains in laccase: Cu-oxidase_3 (PF07732), Cu-oxidase (PF00394), and Cu-oxidase_2 (PF07731) (Fig. 6A). The degree of overall sequence identity of putative *Sh-lac* and putative laccase protein BC1G_10329 of *Botrytis cinerea* B05.10, hypothetical protein SS1G_11927 of *S. sclerotiorum* 1980 was approximately 72 and 77%, respectively. But, *Sh-lac* protein showed high sequence conservation in sequence alignment with *Magnaporthe oryzae* (MGG_13464) and *S. sclerotiorum* laccases (Supplementary Fig. S3). Our study provides sufficient evidence that *Sh-lac* is a laccase of *S. shiraiana*. The neighbor-joining phylogenetic analysis of *Sh-lac* sequence with other plant-pathogenic fungi laccase sequences showed that all laccases were clustered in two groups. Ascomycetes are in group I, while basidiomycetes are in group II (Fig. 6B).

High expression level of *Sh-lac* is related to melanin production in *S. shiraiana*. RT-PCR studies showed that the expression level of *Sh-lac* increased from the fourth day forward until the twelfth day (Supplementary Fig. S4). This increase coincided with the beginning of fungal colony blackening or production of melanin. On the second day, when the mycelia were completely white, the *Sh-lac* expression level was low. The expression of *Sh-lac* started to increase from the fourth day, when the colony began to darken and continued to increase until the twelfth day, when the fungal colony stopped growing, and the melanin production was reduced.

***Sh-lac* silenced transformants show decrease in melanin production.** We transformed the *S. shiraiana* wild-type strain SX-001 using the vector pCHidZLAC (Fig. 7A), wherein the *Sh-lac* gene was transformed. Segmental amplification of hygromycin B phosphotransferase gene (Fig. 7B) confirmed eight transformants. We named the transformants ilac-1 to ilac-8. *Sh-lac* silencing caused the formation of abnormal colonies on PDA (Fig. 7C and D). Transformant colonies changed color from dark olive brown (WT) to maroon or light brown. We hypothesized that a lighter color of colonies was associated with a lower production of melanin. In addition, transformant hyphae were fluffy. We examined the relative expression levels of *Sh-lac* in the transformants and WT strain SX-001 after multiple subculture (Fig. 7E).

We chose stable transformants ilac-4 and ilac-8 for further studies (Fig. 8B). The melanin productions in these two transformants were significantly lower than WT and Pint (empty vector transformant) by measuring the filtrate absorbance at 420 nm (Fig. 8A and C, Supplementary Fig. S5). Also notable was that the colonies adhesion of ilac-4 and ilac-8 was reduced on the PDA. Although it is difficult to measure the adhesion of colonies on PDA, it is widespread and the colonies rolled up or separated from the PDA medium in ilac-4 (9 of 10 colonies observed) and ilac-8 (10 of 10 colonies observed) (Supplementary Fig. S6). However, all of the WT mycelia were firmly rooted in the culture medium and were difficult to peel. The results indicated ilac-4 and ilac-8 attenuate adhesion compared with WT.

Silenced *Sh-lac* inhibited the vegetative growth of *S. shiraiana*. Vegetative growth was inhibited in the *Sh-lac* silenced transformants (Fig. 7C and D). In order to verify whether the vegetative growth and melanin are relevant in *S. shiraiana*, we obtained scytalone dehydratase (SCD) silencing transformant using the same method. *Sh-SCD* is another key gene in the DHN melanin biosynthesis pathway in *S. shiraiana*. The expression of *Sh-SCD* in resultant transformant iSCD-2 was 35.3% decreased compared with the WT. Meanwhile, the melanin production of iSCD-2 was also reduced. However, transformant iSCD-2 was a reddish brown colony on PDA

Fig. 4. Mulberry drupelet paraffin sections. **A**, Infected drupelet that was full of hyphae. **B**, Sclerotia is forming in diseased fruit and sclerotia pseudoparenchyma is composed of small black particles. **C**, Small black particles are formed by the fragmentation from fracture hyphae. Bar: 100 µm. H: hyphae, BP: black particle.

Fig. 5. Identification of inquiry melanin sample. **A**, Standard DHN extract ion chromatography (EIC) chart. **B**, Standard DHN mass spectrometry chart. **C**, Inquiry sample EIC chart. **D**, Inquiry sample mass spectrometry chart. **E**, DHN standard or inquiry melanin sample monomer derivatization.

Fig. 6. Analysis of *Sh-lac*. **A**, Schematic representation of domains in *Sh-lac* protein. SP: signal peptide at the N-terminal. *Sh-lac* protein contains three multicopper oxidase domains: Cu-oxidase_3, Cu-oxidase, and Cu-oxidase_2. **B**, Neighbor-joining phylogenetic tree of *Sh-lac* and laccases from other fungi. The bootstrap values (%) obtained with 1,000 replicates are indicated on branches. Solid triangle indicates *Sh-lac* from *Scleromitrla shiraiana*. Fungi belong to ascomycetes in the group I, and belong to basidiomycetes in the group II.

with normal vegetative growth (Supplementary Fig. S7). This indicated that the inhibition of vegetative growth was not related to the reduction of melanin in *S. shiraiana*.

Low melanin levels affect the virulence of *S. shiraiana*. It's a technical difficulty to test pathogenicity on mulberry fruit. Therefore, we indirectly tested transformant pathogenicity by detecting the oxalic acid that is a virulence factor in many sclerotia forming phytopathogens. We detected oxalic acid in culture filtrates of the different strains by ultra performance liquid chromatography (UPLC). WT strain contain 2.13 mM oxalic acid, whereas CK (sterile ME medium), ilac-4, and ilac-8 did not produce measurable amounts of oxalic acid (Fig. 9; Supplementary Fig. S8). Meanwhile, the oxalic acid concentration of WT treated with tricyclazole (0.49 mM) and iSCD-2 (0.98 mM) was also significantly lower than that in the WT strain. Since oxalic acid production is proportional to melanin production, either directly or indirectly, our results suggest that melanin may very possibly be implicated in *S. shiraiana* pathogenicity.

DISCUSSION

Characteristics of *S. shiraiana*. In the present study, we isolated strains of *S. shiraiana* that cause the popcorn disease in mulberry fruits. The strain was named SX-001 strain and elucidated

the mechanism of the formation of sclerotia in *S. shiraiana*-infected mulberry fruits. Paraffin sections of diseased fruits showed that the fungal hyphae invade the infected mulberry drupelet and destroy the host structure. After that, the hyphae disintegrate into small black particles, which make up the outer wall of the sclerotia. The sclerotia of pathogenic fungi are dormant structures with a hard, dense black wall that can withstand adverse environmental impacts (Amselem et al. 2011). Melanin has been implicated in sclerotia formation and pathogenicity in *S. sclerotiorum* (Butler et al. 2009), which is closely related to *S. shiraiana*.

***S. shiraiana* produces DHN melanin.** Our GC-MS studies confirmed that *S. shiraiana* produces DHN melanin. The biosynthesis of DHN melanin starts with the synthesis of pentaketide and proceeds to 1,3,6,8-tetrahydroxynaphthalene (4THN) (Wheeler and Stipanovic 1979). Subsequently, tetrahydroxynaphthalene reductase (THNR) catalyzes the reduction of 4THN to generate scytalone. The scytalone dehydratase (SCD) then catalyzes the dehydration of scytalone to produce 1,3,8-trihydroxynaphthalene (3THN), the reduction of 3THN to vermelone, and the dehydration of vermelone to 1,8-DHN (Kihara et al. 2004a, b; Thompson et al. 2000; Vidal-Cros et al. 1994). Tricyclazole specifically inhibits the reductases involved in DHN melanin biosynthesis in black fungi (Andersson et al. 1996; Liao et al. 2001). The conidial pigmentation

Fig. 7. *Sh-lac* silencing transformants. **A**, Construction of *Sh-lac* gene silencing vectors. **B**, Eight *Sh-lac* silencing transformants genomic PCR validation by amplification part sequence of the hygromycin B phosphotransferase gene. P indicates transforming plasmid, WT indicates wild-type *Sclerotinia shiraiana* strain SX-001, H indicates a negative control with H₂O as template, and Pint indicates empty vector-transferred strain (the same below). **C**, *Sh-lac* silencing transformants (1 to 8), empty vector-transferred strain (Pint), and WT colony morphology on potato dextrose agar (PDA) medium after 9 days. Bar: 5 mm. **D**, Hyphal growth rates of *Sh-lac* silencing transformants. Growth rates were examined on PDA medium at 25°C after 10 days. **E**, Expression of *Sh-lac* gene in silencing transformants on PDA after 10 days. Bars indicate standard error. Three biological replicates, each containing three technical replicates for each sample, were performed.

of *Colletotrichum lagenarium* and *M. oryzae* are synthesized via DHN melanin pathway, and the pigmentations were restrained by tricyclazole treatment (Howard and Valent 1996; Perpetua et al. 1996). In the present study, tricyclazole at 60 $\mu\text{g/ml}$ can significantly inhibit the growth and melanin production of *S. shiraiana*.

***Sh-lac* is not only involved in the synthesis of melanin, but also related to the vegetative growth.** The 1,8-DHN is oxidized and

polymerized to form melanin (Bell and Wheeler 1986). We identified a laccase gene, *Sh-lac*—implicated in melanin synthesis—in *S. shiraiana*. Laccase is involved in the DHN polymerization reaction to form melanin in the DHN-melanin biosynthetic pathway (Lin et al. 2012; Upadhyay et al. 2013). *Sh-lac* has a predicted signal peptide and is an extracellular protein. Generally, melanin is located in either external or within the cell walls in fungi

Fig. 8. Reduction of melanin in *Sh-lac* silencing transformants ilac-4 and ilac-8. **A**, WT, Pint (empty vector transformant), ilac-4, and ilac-8 were incubated on potato dextrose agar for 17 days. The reduced melanin of colonies in silencing transformants is shown. Bar: 5 mm. **B**, The relative expression levels of *Sh-lac* gene in transformants ilac-4 and ilac-8. **C**, The relative melanin yield in transformants ilac-4 and ilac-8. Letters a and b indicate statistical differences, $P < 0.05$. The experiments were repeated at least three times.

Fig. 9. The chromatogram of detected oxalic acid in wild type (WT) and transformants by ultra performance liquid chromatography. Oxalic acid peak appears at 0.943 min under the detection condition. Arrows indicate the corresponding oxalic acid sites. TCZ indicates wild-type strain SX-001 treated with tricyclazole. CK is a negative control (sterile malt extract medium). The experiments were repeated twice.

(Ellis and Griffiths 1974). In order to verify the function of *Sh-lac* in the melanin synthesis, we silenced *Sh-lac* gene by RNA interference (RNAi). Most of the *Sh-lac* silenced transformants had reduced melanin and slow growth rate. We selected two stable silenced transformants ilac-4 and ilac-8 for further study. The melanin yield of ilac-4 and ilac-8 and growth rate were significantly lower than WT. In order to clarify whether inhibition of the vegetative growth of *S. shiraiana* was associated with reduced melanin production by silencing *Sh-lac* gene, another key gene *Sh-SCD* in DHN melanin biosynthesis pathway was silenced. The colony of iSCD-2 transformant was reddish brown with normal vegetative growth. The distinctive growth pattern of the transformant suggested the possibility that *Sh-lac* gene is involved in the vegetative growth of *S. shiraiana*. Laccase is a multifunctional factor in organisms. In addition to its participation in DHN melanin synthesis, laccase is also involved in morphogenesis, pathogenesis, and lignin degradation (Baldrian 2006). Transformants ilac-4 and ilac-8 are probably deficient in the morphogenesis, and lead to slow growth.

The fungus *Gaeumannomyces graminis* var. *tritici* is a phytopathogenic ascomycete that was known to produce DHN melanin and complete its infection with melanized hyphae (Edens et al. 1999). It has been reported that a purified laccase of *G. graminis* catalyzed the polymerization of 1,8-DHN into melanin in vitro (Edens et al. 1999). In the present study, different strain filtrates and standard 1,8-DHN were used in assay. Compared with WT and Pint, the catalytic abilities of ilac-4 and ilac-8 filtrate were decreased (Supplementary Fig. S9). These results also suggested that *Sh-lac* was involved in the final step in the DHN melanin biosynthesis.

Reduced melanin inhibited the production of oxalic acid.

Oxalic acid secretion by sclerotinia is deemed an essential determinant of its pathogenicity (Godoy et al. 1990; Noyes and Hancock 1981; Zhou and Boland 1999). Oxalic acid is a key pathogenicity factor in *S. sclerotiorum* and helps in the formation of sclerotia (Bolton et al. 2006; Cessna et al. 2000). Oxalic acid accumulates early during pathogenesis and increases in concentration as host colonization advances. Concomitant with the increase in oxalic acid is a decrease in extracellular host pH from 5 to 4, which enhances the activity of the fungus in the host tissues (Magro et al. 1984). *S. shiraiana* is also a sclerotia forming phytopathogen and the medium pH value reduced from 5.50 to 3.823 after 17 days inoculation in ME medium (Supplementary Table S2); therefore, we explored the possibility of oxalic acid production in medium. Our results showed WT produced 2.13 mM of oxalic acid. However, oxalic acid was not detected in ilac-4, ilac-8, and CK. In addition, the oxalic acid content was significantly reduced in *Sh-SCD* silenced transformant and WT treated with tricyclazole. Since, oxalic acid secretion is consistent with melanin production in different transformants and WT, we infer that melanin is associated with oxalic acid production in *S. shiraiana*.

Conclusions. *S. shiraiana* is one of the main pathogens of mulberry popcorn disease. The sclerotia is formed by fractured hyphae of pathogen in diseased mulberry fruit. *Sh-lac* gene plays a key role in DHN catalysis to DHN melanin. Silenced *Sh-lac* gene resulted in inhibition of the production of melanin, vegetative growth, and oxalic acid. Our study indicated that the gene *Sh-lac* can be used as a target for mulberry popcorn disease control.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

SignalP 4.1 Server: <http://www.cbs.dtu.dk/services/SignalP/>
 MultiLoc: <http://abi.inf.uni-tuebingen.de/Services/MultiLoc/>
 Search for Conserved Domains:
<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>
 Broad Institute's Fungal Genome Initiative (FGI):
<http://www.broadinstitute.org/annotation/fgi/>
 GenBank: <http://www.ncbi.nlm.nih.gov/genbank/>