A Forward Genetic Screen in *Sclerotinia sclerotiorum* Revealed the Transcriptional Regulation of Its Sclerotial Melanization Pathway

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Most plant fungal pathogens that cause worldwide crop losses are understudied, due to various technical challenges. With the increasing availability of sequenced whole genomes of these non-model fungi, effective genetic analysis methods are highly desirable. Here, we describe a newly developed pipeline, which combines forward genetic screening with high-throughput next-generation sequencing to enable quick gene discovery. We applied this pipeline in the notorious soilborne phytopathogen *Sclerotinia sclerotiorum* and identified 32 mutants with various developmental and growth deficiencies. Detailed molecular studies of three melanization-deficient mutants provide a proof of concept for the effectiveness of our method. A master transcription factor was found to regulate melanization of sclerotia through the DHN (1,8-dihydroxynaphthalene) melanin biosynthesis pathway. In addition, these mutants revealed that sclerotial melanization is important for sclerotia survival under abiotic stresses, sclerotial surface structure, and sexual reproduction. Foreseeably, this pipeline can be applied to facilitate efficient in-depth studies of other non-model fungal species in the future.

Keywords: DHN melanin, forward genetic analysis, next-generation sequencing, *Sclerotinia sclerotiorum*, sclerotial formation, soilborne phytopathogen, *SsSMRI*

Plant diseases are major threats to agriculture (Sturz et al. 1997), accounting for losses of at least 10% of the annual global harvest (Strange and Scott 2005). Among microbial pathogens, fungi represent the most diverse group of ecologically and economically relevant threats (Doehlemann et al. 1987), often causing catastrophes in the field and in postharvest products (Fisher et al. 2012; Fry 2008; Ullstrup 1972; Williamson et al. 2007). Despite the damage they inflict to crops, most plant fungal pathogens and, indeed, fungi in general are understudied. This is due to various difficulties, including culturability, lack of high-quality whole-genome sequences, multinuclear somatic inheritance patterns, and physical or chemical barriers inhibiting molecular biology experiments.

With the advent of modern high-throughput sequencing technologies, the number of fully sequenced fungal genomes is rapidly growing, and many belonging to species that have never been studied before. The availability of these sequenced genomes for numerous plant fungal pathogens is enabling reverse genetic analysis. Efficient targeted gene knockout methods are being developed for many of these understudied organisms. Some examples include homologous recombination protocols for gene replacement, RNA interference-mediated gene silencing, *Agrobacterium*-mediated insertional mutagenesis, and CRISPR-based gene editing (Bhadauria et al. 2009; Mullins and Kang 2001). Despite the many findings that reverse genetics studies have produced, it is inevitably limited by bias. Efficient forward genetic approaches are therefore needed for unbiased gene discovery.

Sclerotinia sclerotiorum (Lib.) de Bary is considered one of the most notorious soilborne fungal pathogens. This ascomycete has an extremely broad host range of over 600 plant species of mostly dicotyledonous and some monocotyledonous plants (Boland and Hall 1994; Liang and Rollins 2018). Major features of its development include the production of a vegetative resting structure, termed sclerotium, and a reproductive apparatus, named apothecium (Willetts and Wong 1980). Sclerotia can germinate myceliogenically to infect adjacent plants and

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carpogenically to spread ascospores in the air. Their highly-melanized outer surface has been hypothesized to be responsible for their long-term survival in soil, rendering this pathogen extremely hard to control in the field (Butler et al. 2009). Although many reverse genetics studies have been carried out on this fungus (Xia et al. 2020), only a couple of low efficiency mutant screens were attempted using UV irradiation or *Agrobacterium*-mediated T-DNA insertional mutagenesis (Godoy et al. 1990; Miller et al. 1989; Xu and Chen 2013). In addition, the lack of asexual conidia spores and the multinuclear nature of the vegetative hyphae (Willett and Wong 1980) of *S. sclerotiorum* also restrict the application of forward genetic methods. Thus, more efficient forward genetic methods are highly desirable.

Here, we describe an original high-throughput forward genetics pipeline for *S. sclerotiorum*. Using haploid, binucleate ascospores for mutagenesis, mutants defective in sclerotia formation were isolated. Next-generation sequencing (NGS) allowed us to identify the responsible candidate genes quickly. Using three sclerotial melanization-deficient mutants found from the screen, we demonstrate the feasibility of the pipeline for fast gene discovery. Considering its high efficiency, this pipeline has the potential to be broadly applied to other understudied fungi.

RESULTS

Development of a high-throughput forward genetics pipeline for *S. sclerotiorum*.

Since the first public release of the whole genome sequence of strain *S. sclerotiorum* 1980 in 2011, a growing number of reverse genetic methods have been developed to study this notorious pathogen. However, only a few forward genetic screens have been attempted, with low efficiency. Here, we designed a pipeline for efficient gene discovery in *S. sclerotiorum* (Fig. 1). First, ascospores were collected from apothecia of wild-type (WT) strain 1980 and were mutagenized by UV treatment. After testing different ascospore concentrations and UV exposure times (Supplementary Fig. S1), we settled on a UV exposure period of 15 s of irradiation, with 3,000 spores per plate inoculum. Under this UV dosage, 99% of WT ascospores are killed by UV irradiation. Through screening about 40,000 surviving spores, 32 mutants with visible morphological defects, i.e., discolored sclerotia or malformed or absent sclerotia, were identified (Supplementary Table S1). Upon transferring these morphologically distinguishable mutants from 96-well plates, we did not observe sectoring in their colonies on standard PDA (potato dextrose agar) plates, suggesting the homogeneity of the mutants. These mutants were further purified by at least three rounds of hyphal tip transfer to confirm that the mutant phenotypes were stable.

Identification of mutants with obvious morphological phenotypes.

Among these morphological mutants, three showed pigmentation defects during sclerotial formation. The WT strain formed black sclerotia (Fig. 2). However, the color of sclerotia is pinkish in *pnk1* (*pink 1*) and *pnk2* and brown in M98 (Fig. 2A). In addition, a large number of mutants did not form sclerotia (Fig. 2B and C). Although their ability to form sclerotia was abolished, every mutant exhibited slightly different mycelial morphology. M73, M77, L12, and R122 formed a hyphal ring at the edge of the plate where sclerotia normally form in the WT strain. WT mycelia grew flat on the surface of the media, while R10 produced extremely fluffy aerial mycelia covering up the entire plate. R7 and M30 produced small sclerotial primordia and white compact hyphal masses but could not develop further to form mature sclerotia (Fig. 2B). WT mycelia are hyaline. R1 and R213, however, had dark-colored mycelia (Fig. 2C). Viewed from both the front and back, mycelia of these two mutants

seemed highly melanized. Furthermore, the remaining eight mutants exhibited altered sclerotial size or number (Fig. 2D). WT usually forms 25 to 30 sclerotia on a standard 90-mm PDA plate. However, M35 and L10 formed more than 100 smaller-sized sclerotia on each plate. The other six mutants produced fewer sclerotia. Detailed phenotypic analysis of all 32 mutants is summarized in Supplementary Table S1. Taken together, this pipeline is highly effective in identifying mutants with desired observable phenotypes. Hereafter, we used the two pink-colored mutants *pnk1* and *pnk2* as a proof of concept for establishing an efficient gene discovery pipeline.

pnk1 and *pnk2* contain different mutations in the same gene.

To identify the molecular lesions in the *pnk* mutants, *pnk1* was first cultured for genomic DNA extraction and its full genome was sequenced by NGS. M98, which forms brownish sclerotia, was also subjected to NGS. The genomic sequence of M98 was used to identify background mutations that occur in both *pnk* and M98 to further reduce the number of candidate mutations. An NGS sequence analysis pipeline was modified for *S. sclerotiorum* from our previously used mapping-by-sequencing protocol for *Arabidopsis* (Huang et al. 2019). Using the pipeline guided by GATK best practices, a total 593 SNPs (single nucleotide polymorphisms) and 846 INDELs (insertions and deletions) were captured in *pnk1*, including mutations in exonic, intronic, upstream, downstream, and intergenic regions. After comparison with the M98 sequence, 103 SNPs and 166 INDELs were found to be unique to *pnk1*. Among them, only 21 SNPs and eight INDELs were in exons. We excluded synonymous, intronic, or intergenic mutations as candidates, since they are less likely to cause significant consequences to the encoded proteins. Since the mapped reads for 18 SNPs and all INDELs were not homogeneous, they were likely from mismatches of short reads to the reference genome. Therefore, these mutations were eliminated. Three remaining SNP mutations that led to nonsynonymous changes in the *pnk1* genome became the prime candidate mutations and were kept for further analysis (Fig. 3A). Among these three SNPs, one resulted in a premature stop codon while the other two caused single amino acid changes.

To determine which of the three mutations is responsible for yielding the pink sclerotia phenotype, the protein sequences of these genes were analyzed to examine whether these genes or their orthologs in other organisms have been previously studied with reported functions. From this literature search, one gene (*sscle_12g091490*) stood out, which encodes the ortholog of a transcriptional factor (TF), BcSMR1 (Sclerotial melanogenesis regulatory gene 1) from *Botrytis cinerea*. A mutation in *BcSMR1* led to orange-colored sclerotia in *B. cinerea*, which normally forms black sclerotia (Zhou et al. 2017). We therefore renamed this ortholog of BcSMR1 in *S. sclerotiorum* as SsSMR1, and it became our top candidate for *pnk1*. SsSMR1 and BcSMR1 share 93 and 87% amino acid similarity and identity, respectively.

Due to the similar color of sclerotia between *pnk1* and *pnk2*, Sanger sequencing was performed on SsSMR1 with DNA from *pnk2*. A different mutation also causing a premature stop codon in SsSMR1 was identified in *pnk2* (Fig. 3B). This suggests that mutations of SsSMR1 in both *pnk* mutants are likely responsible for causing the same pink sclerotia phenotype. SsSMR1 contains five exons and four introns, with the mutated nucleotides in *pnk1* and *pnk2* both occurring in exon 5 (Fig. 3C). As these truncation mutations in SsSMR1 likely cause loss-of-function of the protein, *pnk1* and *pnk2* were renamed *Sssmr1-1* and *Sssmr1-2*, respectively.

Protein BLAST and motifs analysis by MEME Suite (Bailey et al. 2009) using the full-length 937-aa SsSMR1 supported its role as a TF. SsSMR1 has predicted DNA-binding domains at its N-terminus, with two Cys₂His₂-zinc finger motifs and one

Zn₂Cys₆-binuclear motif. In addition, SsSMR1 contains a fungal-specific, 298 aa long TF domain and a nuclear localization sequence (NLS) at its C-terminus (Supplementary Fig. S2). The Y706* and Q749* transitions result in the formation of a truncated TF domain in *Sssmr1-2* and the loss of the NLS in both *Sssmr1-2* and *Sssmr1-1*. Sequence alignment of orthologs of SsSMR1 and BcSMR1 in other representative Ascomycota fungi *Colletotrichum lagenarium*, *Magnaporthe oryzae*, and *Bipolaris maydis* revealed that the two mutated amino acids are not conserved (Fig. 3D), however, these TF-related motifs are highly conserved (Supplementary Fig. S2). As *Botrytis* and *Sclerotinia*

are closely related, these results suggest that SsSMR1 is likely a transcription factor with similar functions.

Further phylogenetic analysis showed that orthologs of SsSMR1 are absent in prokaryotes, plants, and animals. It is only found in several classes of division Ascomycota and can be found nowhere else in the other lineages, including yeast (Supplementary Fig. S3). Meanwhile, SMR1 sequences from *S. sclerotiorum* and *B. cinerea* form a monophyletic Sclerotiniaceae group with a bootstrap of 100%. SsSMR1 is highly homologous to the orthologs from order Helotiales within the clade of Leotiomycetes fungi and is conserved in species of the classes Sordariomycetes and Dothideomycetes.

Fig. 1. A pipeline designed for forward genetic analysis of *Sclerotinia sclerotiorum*. Step 1: The haploid ascospores of the wild-type (WT) strain *S. sclerotiorum* 1980 are collected from apothecia that were germinated carpogenically from sclerotia. Step 2: The ascospores are subjected to UV mutagenesis. Step 3: After UV treatment, the germinating colonies from ascospores are transferred individually into 96-well potato dextrose agar (PDA) plates and are incubated for 8 to 10 days to observe for phenotypes. Step 4: The mutants showing target phenotypes (nonsclerotial formation or discolored sclerotia) are transferred onto a fresh PDA plate (90 mm diameter) for confirmation. Step 5: Confirmed mutants are subjected to DNA extraction. Step 6: Genomic DNA of the mutants are sequenced by next-generation sequencing (NGS). Step 7: After NGS data analysis, candidates can be identified for each mutant. Step 8: Target gene complementation or knockout approaches can be used to identify the exact gene responsible for the observed mutant phenotype. Step 9: The biochemical mechanism of the related proteins can be studied further by various means depending on the nature of the protein.

Targeted gene disruption and transgene complementation confirmed that *SsSMR1* is the responsible gene for *Sssmr1* phenotype.

To confirm that mutations in *SsSMR1* are responsible for the sclerotial pigmentation deficiency in *Sssmr1-1* and *Sssmr1-2*, targeted gene replacement by homologous recombination was performed in WT background (Fig. 4A). The *Sssmr1* transformants were selected on PDA with 50 µg of hygromycin per milliliter, and two independent positive transformants, *Sssmr1-3* and *Sssmr1-4*, were subjected to three rounds of hyphal tip transfer and protoplast purification to obtain pure knockout mutants. The pure disruption of *SsSMR1* in these knock-out transformants was verified by PCR (Fig. 4B). A 965-bp *SsSMR1* single band was present in WT but completely absent in the two transformants. The presence of selection marker gene hygromycin phosphotransferase (*hph*) in the transformants was further confirmed by the amplification of a 799-bp product. In addition, the upstream 1,567-bp and downstream 1,413-bp amplicons suggest that the gene replacement occurred as designed and did not disrupt other genes (Fig. 4B). After culturing on PDA medium for 14 days, pink sclerotia formed on the plate edges for both *Sssmr1-3* and *Sssmr1-4* (Fig. 4C), resembling the phenotypes of *Sssmr1-1* and *Sssmr1-2*. Therefore, knocking out *SsSMR1* leads to pink sclerotia.

Furthermore, when WT *SsSMR1* was cloned and transformed into *Sssmr1-1*, two complementing strains *SsSMR1*-C1 and *SsSMR1*-C2 (Fig. 4C) were recovered, which formed black sclerotia. Taken together, these data suggest that *SsSMR1* is the gene responsible for the pink sclerotia phenotype observed in the *Sssmr1* mutants isolated from our screen. It, indeed, has an important role in sclerotial melanization. Loss of *SsSMR1* blocks melanin biosynthesis, leading to pink sclerotia.

Sclerotia melanization does not contribute to virulence and compound appressoria formation, but it does contribute to sclerotial surface structure and apothecia formation.

We used a similar pipeline to analyze M98. Interestingly, a nonsynonymous change in the fourth exon of another melanin biosynthetic gene, *SsPKS12* (*sscle_12g091500*), which is adjacent to *SsSMR1* in the genome, was identified in M98 (Fig. 5A). This mutation results in an I584F change. Consistent with the phenotype of *Sspks12* observed by Wang and Rollins (2021), M98 formed brown sclerotia (Fig. 2A). These data indicate that the observed mutation in *SsPKS12* is most likely responsible for the M98 mutant phenotype.

Fig. 2. Colony morphology of mutants with sclerotial defects. **A**, Wild-type strain *Sclerotinia sclerotiorum* 1980 (WT) and mutants with discolored sclerotia. **B**, Mutants not forming sclerotia. **C**, Mutants with dark-colored mycelia. Photos were taken from both front and back of the plates. **D**, Mutants with altered sclerotial size or number. The WT strain and all mutants were cultured on potato dextrose agar plates for 2 weeks before the pictures were taken.

With the availability of these melanization-deficient mutants, we tested the biological roles of sclerotial melanization in *Sclerotinia* biology. *Sssmr1-1*, *Sssmr1-2*, *Sssmr1-3*, *Sssmr1-4*, and M98 mutants showed no significant differences from WT in growth rate (Fig. 5B), demonstrating that sclerotia melanization does not affect vegetative growth. Unlike in other fungi such as *M. oryzae*, in which melanin contributes greatly to pathogenicity (Howard and Ferrari 1989), sclerotial melanin does not seem to be required for establishing infection in *S. sclerotiorum*, as the *Sssmr1* mutants and M98 are not compromised in their pathogenicity and virulence, compared with the *oah1* mutant, which is an oxalate-deficient mutant exhibiting significantly attenuated virulence (Liang et al. 2015) (Fig. 5C). This is consistent with previous reports that disruption of *Sspks12* and *Sspks13*, two enzymes encoding genes required for melanin accumulation in sclerotia and apothecia and in compound appressoria (Li et al. 2018; Wang and Rollins 2021), respectively, did not affect virulence. Since *Sssmr1-1*, *Sssmr1-2*, *Sssmr1-3*, and *Sssmr1-4* behaved similarly, we chose *Sssmr1-1* as representative to perform assays hereafter.

When WT, *Sssmr1-1*, and M98 were cultured on glass slides for the observation of compound appressoria development and pigmentation, we did not observe any defects (Fig. 5D); they produced compound appressoria with wild-type pigmentation and

morphology. Thus, SsSMR1 and SsPKS12 do not seem to contribute to melanization of appressoria. Sclerotial melanization seems independent of melanization of appressoria, which is consistent with the previous report (Wang and Rollins 2021).

Next, the surface structure of these mutants was observed with a dissecting microscope. Compared with WT sclerotia, which are rough surfaced with many bumpy structures, sclerotia of M98 show reduced bumpiness, while the *Sssmr1-1* mutant is smooth-surfaced (Fig. 5E), indicating that SsSMR1 and SsPKS12 likely contribute to the surface structure of the sclerotia. When we produced apothecia, *Sssmr1-1* and M98 show significantly reduced ability to produce apothecia when compared with WT (Fig. 5F). And the apothecium produced from M98 was albino, which is consistent with the apothecial pigmentation defect in *Sspks12* mutants (Wang and Rollins 2021).

Sclerotial melanization is required for survival of sclerotia under physical and chemical stresses.

As highly melanized sclerotia were hypothesized to be responsible for resisting physical, chemical, and biological stresses (Bolton et al. 2006), we next tested whether sclerotia of the melanization deficiency mutants can withstand toxic bleach treatment or UV irradiation and germinate properly. After 60 min of bleach treatment or 20 min of UV irradiation, the

Fig. 3. Identification of the molecular lesions of the two *pnk* (*pnk*) mutants. **A**, Candidate mutations of *PNK1* after next-generation sequencing data analysis. The most likely gene, *SsSMR1* (*sscle_12g091490*), is highlighted in red. **B**, Sanger sequencing chromatograph of *SsSMR1* in *pnk2*. The nucleotide altered is marked in a red box. The nucleotide triplets coding amino acids are labeled in blue boxes. The asterisks represent nucleotides that remain unchanged. **C**, Gene structure of *SsSMR1*. The diagram was drawn using Illustrator for Biological Sequencing (Liu et al. 2015), including exons, introns, 5' untranslated region (UTR), and 3' UTR. The mutation sites in *Sssmr1-1* and *Sssmr1-2* are labeled with arrowheads. The scale bar is shown at the bottom. **D**, Protein sequence alignment of the region containing the two *Sssmr1* mutations with orthologs of *SsSMR1* in different fungal species. The amino acids changed in *Sssmr1-1* and *Sssmr1-2* are highlighted in red. The asterisks represent the conserved amino acids.

myceliogenic germination rate declined significantly by 67 and 51% for *Sssmr1-1* sclerotia and 37 and 21% for M98 sclerotia, respectively, as compared with WT (Fig. 5G and H). Taken together, melanization protects sclerotia from abiotic environmental stresses such as UV or toxic chemicals.

The melanin biosynthesis pathway is regulated by SsSMR1.

To analyze the role of the SsSMR1 TF in melanin biosynthesis in more detail, we carried out RNA sequencing (RNA-seq) analysis. As melanin biosynthesis occurs as the fungus reaches maturity, young (4-day-old) and old mycelia (9-day old) potato dextrose broth (PDB)-grown WT and *Sssmr1-1* were collected in triplicates for analysis (Fig. 6A). Distance matrix analysis of the samples confirmed that there is a high level of transcriptome similarity between biological replicates (Fig. 6B). Principal component analysis revealed that PC1, likely related to age, and PC2, likely related to genotype, explain 87 and 10% of the expression variance between samples (Fig. 6C). Agreeing with the morphological differentiation pattern, the transcriptomes of WT and *Sssmr1-1* are quite similar in young hyphae but differ substantially in older hyphae going through sclerotial melanization.

To determine which specific genes are regulated by SsSMR1, differentially expressed genes (DEGs) were identified using thresholds of $\log_2$ (fold-change) > 0.8 or < -0.8 and $p_{adj} < 0.05$ (Supplementary Table S2). Relatively few genes (282) were significantly affected in *Sssmr1-1* young hyphae compared with WT (Fig. 6D to E). Upon maturation, 1,057 and 1,179 genes are up- and down-regulated, respectively, in WT. This constitutes 20% of the 11,130 coding genes in *S. sclerotiorum*. The genes upregulated in old compared with

young WT hyphae are enriched in gene ontology (GO) terms related to secondary metabolite biosynthesis, copper ion transport, and melanin biosynthesis, consistent with the involvement of these processes in melanization during sclerotia development (Fig. 7A). Compared with WT, fewer genes were down-regulated and more genes were up-regulated in *Sssmr1-1* upon maturation. Specifically, 218 and 385 genes were found to be significantly down- and up-regulated, respectively in old *Sssmr1-1* mycelia compared with old WT mycelia. These 218 downregulated genes were enriched in GO terms related to melanin processes, further supporting the involvement of SsSMR1 in melanin biosynthesis (Fig. 7B).

Previous studies in *B. cinerea* and other fungi revealed that SMR1 regulates several genes directly involved in melanin biosynthesis (Schumacher 2016). From the literature, we deduced a hypothetical melanin biosynthesis pathway in *S. sclerotiorum* (Fig. 7C). Polyketide synthase (PKS) enzymes PKS12/13 along with YGH1 ("Yellowish-green" hydrolase 1) generate T4HN (1,3,6,8-tetrahydroxynaphthalene). This precursor is converted to T3HN (1,3,8-trihydroxynaphthalene) and then further to DHN (1,8-dihydroxynaphthalene) by sequential reactions catalyzed by BRN2 (T4HN reductase, also named THR1), BRN1 (T3HN reductase, also named THR2), and SCD1 (Scytalone dehydratase 1). Finally, a laccase, LAC1, converts DHN to DHN-melanin (Tsuji et al. 2001). Other proteins are also involved, such as the copper transporter CCC2 (Copper-transporting ATPase 2), which may import the copper necessary for laccases to function (Saitoh et al. 2010). In addition to these biosynthesis genes, various transcription factors, kinases, and the Velvet complex were also previously shown to play a role in melanin synthesis in other fungi (Schumacher et al. 2015; Yang et al. 2013).

Fig. 4. Generation of *Sssmr1* deletion mutants and complementation strains. **A**, The *SsSMR1* locus and the gene replacement design. The *SsSMR1* and hygromycin phosphotransferase genes (*hph*) are presented as orange and red rectangles, respectively. The primers labeled in the diagram were used to amplify the flanking sequences or for mutant screening. **B**, PCR verification of *SsSMR1* disruption. Genomic DNA isolated from wild type (WT) and deletion mutants *Sssmr1-3* and *Sssmr1-4* were used as PCR templates. Four pairs of primers for checking the insertion of *hph*, upstream and downstream of *hph* insertion and *SsSMR1* deletion, are listed and the sizes of amplified bands are shown in brackets. M = the lane for DNA marker. **C**, Colony morphology of WT, *Sssmr1-1*, *Sssmr1-2*, *Sssmr1-3*, *Sssmr1-4*, *SsSMR1-C1*, and *SsSMR1-C2*. All strains were cultured on potato dextrose agar plates for 2 weeks before the pictures were taken. Sclerotia collected from the plates are shown on the right. Scale bar = 5 mm.

We first investigated whether *S. sclerotiorum* homologs of these melanin biosynthesis-related genes are expressed in older hyphae and whether their expression depends on *SsSMR1* (Fig. 7D; Supplementary Table S3). Strikingly, key enzymes

PKS12/13, YGH1, BRN1/2, and SCD1 are all up-regulated in older WT mycelium compared with young mycelia in WT *S. sclerotiorum* (Fig. 7D), supporting their roles in melanization. Interestingly, the expression levels of all these enzymes are

Fig. 5. Testing *Sssmr1* and M98 mutants for defects in growth, virulence, and bleach or UV sensitivity. **A**, Gene structure of *SsPKS12*. The diagram was drawn using Illustrator for Biological Sequencing. Exons and introns are indicated in blue and gray boxes, respectively. The mutation site in M98 is labeled with an arrowhead. The scale bar is shown at the bottom. **B**, Mycelial growth of wild type (WT), *Sssmr1-1*, *Sssmr1-2*, *Sssmr1-3*, *Sssmr1-4*, and M98 over a period of 48 h with 12-h time intervals. **C**, Pathogenicity assay for WT, *Sssmr1-1*, *Sssmr1-2*, *Sssmr1-3*, *Sssmr1-4*, M98, and *oah1* on detached leaves of *Arabidopsis*. Representative photos were taken at 24 and 48 h postinoculation, respectively. **D**, Compound appressoria formation assay. Potato dextrose agar plugs colonized with WT, *Sssmr1-1*, and M98 were transferred onto glass slides and were then photographed at 2 days post-inoculation. The scale bar is 100 μ m. **E**, Two-week-old sclerotia of WT, *Sssmr1-1*, and M98 were photographed by a dissecting microscope. Scale bar = 1mm. **F**, Apothecia formation assay. Sclerotia of WT, *Sssmr1-1*, and M98 were cultured in Petri dishes (90 mm diameter). The red arrowhead indicates the sole apothecium produced by sclerotia of M98. **G** and **H**, The sensitivity to 15% bleach treatment (C) and UV irradiation (D) of the WT, *Sssmr1-1*, and M98 strains. Asterisks indicate significance (two asterisks [**] indicate $P < 0.01$) with one-way analysis of variance Tukey's post hoc test. No asterisk means no significance.

significantly reduced in the older mycelia of *Sssmr1-1*, suggesting that SsSMR1 regulates these key melanin biosynthetic enzymes. SsSMR1 itself is also strongly up-regulated in older mycelia, indicating it is regulated during development. The expression level of *SsSMR1* is not significantly altered in the *Sssmr1* mutant, suggesting that the point mutation did not

disrupt its transcription but, instead, affected its protein function. These data also suggest that SsSMR1 does not have a feedback role on its own transcription.

In contrast, the expression of putative upstream kinases, the Velvet complex, and other melanin-related TFs are not significantly affected in young or old mutant hyphae, suggesting that

Fig. 6. RNA sequencing (RNA-seq) analysis of wild type (WT) and *Sssmr1-1*. **A**, Young and old mycelia were harvested from WT and *Sssmr1-1* potato dextrose broth cultures at 4 and 9 days postinoculation, respectively. Photos were taken just before sample collection. **B**, Distance matrix between different RNA-seq samples. Heatmap showing Euclidean distance between different samples with values represented by a blue color scale. Hierarchical clustering of samples is shown by the top and left dendrograms. Different genotype and age groupings are denoted by colored bars on the top and left of the heatmap. **C**, Principal component analysis of gene expression in young and old WT and *Sssmr1-1* hyphae. First two principal components are shown. Age is represented by shape, and genotype is represented by color. **D**, Comparisons of significant differentially expressed genes (DEGs) between different ages and genotypes. Numbers represent DEG count for respective categories. Y = young mycelia, O = old mycelia. Thresholds used were $\log_2FC > 0.8$ or < -0.8 and $padj < 0.05$. **E**, Comparisons of significant differentially expressed genes between different ages and genotypes as compared with young WT mycelium. Upset plot showing DEGs shared between different groups. Vertical bars and corresponding value represent the distinct set between sets labeled with gray dots. Y = young mycelia, O = old mycelia.

Fig. 7. *SsSMR1* regulates the transcription of genes encoding melanin biosynthesis enzymes. **A**, Word cloud of gene ontology (GO) terms enriched in the gene set upregulated in old wild-type (WT) hyphae compared with young WT hyphae. Font size is proportional to fold enrichment (test ratio/reference ratio), and significance is represented by color. **B**, Word cloud of GO terms enriched in the gene set significantly downregulated in old *Sssmr1-1* hyphae compared with old WT hyphae. Font size is proportional to fold enrichment (test ratio/reference ratio), and significance is represented by color. **C**, Proposed *Sclerotinia* melanin biosynthesis pathway deduced from homologous genes in other fungi. Enzymes with red borders are regulated by *SsSMR1*. Gene codes can be found in Supplementary Table S3. **D**, Expression profile comparisons of *Sclerotinia sclerotiorum* homologs of known melanin biosynthesis-related genes. Heatmap showing mean-centered, variance-stabilized transformation of transcript counts with values represented by a diverging blue and red color scale. Names of *S. sclerotiorum* homolog genes are listed on the right, grouped by the type of regulator (denoted on left). Hierarchical clustering of samples was completed and is represented by the dendrogram above the heatmap. Different genotype and age groupings are denoted by colored bars on top of the heatmap. **E**, Gene diagram showing the *SsPKS12* and *SsSMR1* locus on chromosome 12. Genes found to be differentially regulated (threshold of $\log_2FC > 0.8$ or < -0.8 , $padj < 0.05$) in old *SsSMR1* hyphae compared with old WT hyphae are colored. **F**, Gene diagram showing the *SsPKS13* locus on chromosome 3. Genes found to be differentially regulated (threshold of $\log_2FC > 0.8$ or < -0.8 , $padj < 0.05$) in old *SsSMR1* hyphae compared with old WT hyphae are colored. **G**, Conserved binding motif identified in the promoter regions of melanin biosynthesis pathway genes. The x axis shows nucleotide positions, the y axis indicates sequence conservation at each position. The height of symbols represents the probability of each nucleotide occurring at this position. The diagram was drawn by WebLogo (Crooks et al. 2004). **H**, Potential binding motifs present in the promoter regions of all melanogenesis-related genes are marked with red lines. Blue triangles represent the start of coding regions of these genes. The diagram was drawn using Illustrator for Biological Sequencing. Scale bar is shown at the bottom.

SsSMR1 does not regulate these components. Thus, SsSMR1 appears to mainly regulate melanin biosynthesis genes. Interestingly, like in *B. cinerea*, some of these key melanin biosynthesis genes are encoded in tandem (Fig. 7E and F). *SsPKS12* and *SsSMR1* are encoded in a tail-to-tail orientation on chromosome 12, along with the loosely linked *SsLAC1* (*sscle_12g090390*). *SsSCD1*, *SsBRN2*, and *SsPKS13* are all encoded in the same cluster on chromosome 3.

DISCUSSION

To unbiasedly identify genes involved in a particular biological process, forward genetic approaches are often utilized in model organisms by screening populations after random mutagenesis. For most researchers, their first choices of mutagen are chemicals, such as ethyl methanesulfonate (EMS), as they can be easily applied with predictable nucleotide change consequences. Although we initially tested EMS on ascospores of *S. sclerotiorum*, the mutagenesis procedure failed as the protocol was too harsh for the fungal spores and difficult on the experimenter. Instead, UV mutagenesis, which is less laborious and more efficient compared with T-DNA insertional mutagenesis, proved to be highly effective for *S. sclerotiorum*. UV light has strong genotoxic effects that produces DNA damage, usually causing single nucleotide changes (Ikehata and Ono 2011). These mutations can be confidently captured by our NGS analysis pipeline.

Traditionally, following a mutant screen, positional cloning is done to map and identify mutated genes responsible for the mutant phenotypes. Mapping serves to reduce the genomic region for candidate identification. For example, in *Arabidopsis* EMS mutants, there are usually over 500 mutations throughout the genome in each mutagenized line. Mapping reduces the candidate mutations to less than five to ten in a narrow chromosomal region. However, genetic mapping is not feasible for mutants obtained from a homothallic fungus such as *S. sclerotiorum* that has not been shown to outcross in the lab. It is also not feasible for fungi with many nuclei in their sexual or asexual spores. Fortunately, based on our NGS analysis from the current mutants obtained from ascospore mutagenesis, the number of significant mutations in each mutant line is usually between 1 and 10. A large number of synonymous mutations or mutations in introns and untranslated regions can be initially excluded using bioinformatics analysis. The small number of more meaningful mutations is likely due to the haploid, binucleate nature of ascospores used for mutagenesis and the high relative dosage of UV we applied; ascospores are easily killed when an essential gene is mutated.

Due to the low mutation rate in each mutant, one disadvantage of our approach is the need to screen a large population. However, as most microorganisms can be easily manipulated in the lab with 96-well plates, large populations can usually be handled efficiently. From our population of 40,000, we identified two independent alleles of *Sssmr1*, suggesting that a similar sized population should already contain mutations in almost all genes of this organism (which possesses around 11,130 coding genes). As exemplified here, screening a large population is also helpful in narrowing down candidates, as independent alleles can harbor different mutations in the same gene, accelerating gene discovery.

Our *Sssmr1* mutants are useful tools for answering some of the *Sclerotinia* melanin-related questions. Many fungi produce melanin to protect themselves in adverse environments or against microbial degradation (Bell and Wheeler 1986; Butler and Day 1998). From our screen, we identified three melanization-deficient mutants. Discolored sclerotia isolates have been reported in *B. cinerea* (Zhou et al. 2018). Our study of *pnk* and M98 mutants facilitated the identification of the sclerotia

melanin biosynthesis pathway in this fungus. There are various types of melanin synthesized by species-specific pathways within the fungal kingdom (Toledo et al. 2017). However, the most-studied and prevalent fungal melanin is DHN melanin, produced via a polyketide pathway. Lazarovits et al. (2000) tested whether *S. sclerotiorum* produces DHN melanin by using tricyclazole, which specifically inhibits the reductases involved in the DHN melanin biosynthesis pathway. This is further supported by reverse genetic evidence provided by Liang et al. (2017), who found that the disruption of two DHN melanin pathway genes, *SCD1* and *THR1*, did not alter pathogenicity but resulted in slower growth, delayed sclerotial formation, reduced sclerotial numbers, and decreased resistance to UV irradiation in *S. sclerotiorum*. These two mutants displayed brown-colored sclerotia due to a later blockage of melanin synthesis. In agreement, our study showed that disruption of *SsSMR1* leads to global reduction of melanin biosynthesis pathway genes, resulting in pink sclerotia, increased sensitivity to UV light and toxic chemicals, smoother sclerotia surface, and reduced formation of apothecia. These results confirm that melanin is important for carpogenic growth and survival of *S. sclerotiorum* under bleach treatment or UV irradiation. However, melanin does not seem to be required for its vegetative growth and virulence.

BcSMR1 and SsSMR1 share 87% amino acid identity, and they may exert the same function in the two closely related fungi. A previous study showed that BcSMR1 only regulates the expression levels of its adjacent PKS-encoding gene, since genes in secondary metabolites biosynthesis pathways are often physically linked (Schumacher 2016). However, our RNA-seq data demonstrate that most of DHN melanogenic genes are down-regulated in *Sssmr1-1* background during sclerotial development, including the genes not clustered with *SsSMR1*, suggesting that SsSMR1 likely serves a more global role to regulate the expression of genes involved in melanogenesis. Strikingly, a specific “(T/G)CTCC(A/T/G)” element is detected in the promoter regions of all genes mentioned in the hypothetical melanin biosynthesis model (Fig. 7G and H), which is consistent with a previous study on the DNA-binding activity of the SsSMR1 ortholog of a yeast-like fungus, *Aureobasidium melanogenum* (Jiang et al. 2020). Besides, such a conserved element is not observed in the promoter region of *SsSMR1*, supporting our conclusion that SsSMR1 does not have autoregulation activity. From our current study, melanin biosynthesis is not affected in compound appressoria. As disruption of *SsPKS13* blocks melanin accumulation in compound appressoria (Li et al. 2018), it is possible that SsSMR1 predominately functions at specific growth stages including vegetative growth, sclerotium development, and apothecium formation but not during appressoria development. It is also possible that other regulators are functioning independently or in concert with SsSMR1 during appressoria maturation. Together, these analyses indicate that SsSMR1 serves as a master TF regulator of melanin biosynthesis during stages of the *Sclerotinia* life cycle, and it may directly bind to these elements existing in the promoter regions of key melanin biosynthetic enzymes to stimulate their expression.

In some plant-pathogenic fungi, such as *M. oryzae* (Howard and Ferrari 1989), *C. lagenarium* (Kubo et al. 1987), and *Verticillium dahliae* (Tokousbalides and Sisler 1979), melanin contributes to pathogenicity by influencing the development of penetration appressoria structures. However, in other fungi, such as *Alternaria alternata* (Thomma 2003), melanin production might not be essential for fungal pathogenicity. In our study, sclerotial melanization pathway is not needed for virulence of *S. sclerotiorum* or melanization of appressoria, as revealed through the analysis of the melanin-deficient mutants. This is consistent with previous report that different pathways are contributing to melanization of sclerotia and appressoria melanization does not

seem to be needed for virulence (Wang and Rollins 2021). This could be due to the different appressoria structure of *S. sclerotiorum*, which may not need DHN melanin to enforce host entry.

In conclusion, our study establishes a pipeline for effective screening of mutants with phenotypes of interest and subsequent cloning by NGS. From the initial screen, we successfully identified 32 mutants with mitotically stable morphological phenotypes. Notably, many of these phenotypes have not been reported before in *S. sclerotiorum*, demonstrating the potential of this pipeline for novel gene discovery. Furthermore, detailed analysis of three melanization-deficient mutants exemplifies the feasibility of the whole pipeline, which could be used theoretically in many non-model eukaryotic organisms to isolate genes involved in particular biological processes.

MATERIALS AND METHODS

Fungal strains and culture conditions.

The WT strain *S. sclerotiorum* 1980 was provided by J. Rollins at the University of Florida. Fungal cultures were maintained on PDA (Shanghai Bio-Way Technology) medium at room temperature and were stored on PDA slants at 4°C or as sclerotia. For transformant screening, hygromycin B (Sigma) was added to a final concentration of 50 µg/ml. Bacteria used in this study were grown in Luria-Bertani (Bio Basic) medium.

UV mutagenesis.

Ascospores were generated from sclerotia of WT strain *S. sclerotiorum* 1980 that were germinated carpogenically as described (Athukorala et al. 2010). Ascospores were stored on tin foil at 4°C in a desiccant in the dark. Ascospores were then resuspended and diluted with sterilized PDB (Shanghai Bio-Way Technology) to 10⁴ spores per milliliter. Spore suspension (300 µl) was evenly spread on PDA plates (90 mm diameter) and dried. Then these plates were irradiated under UV light (TL-2000 Ultraviolet Translinker) for 15 s, with an energy strength of 9,000 mJ/cm². The plates were incubated at room temperature for 2 days, until growing fungal colonies were visible, after which the colonies were individually picked and transferred to a 96-well plate by sterile toothpicks. Each well of the 96-well plate contained 150 µl of PDA. Mutants with desired phenotypes were visually identified in around 7 days and were transferred onto fresh PDA plates. The plates were incubated for 2 weeks to confirm the morphological defects.

Genomic DNA extraction of *S. sclerotiorum* mutants for NGS.

Actively growing fungal mycelia cultured in PDB for 5 to 7 days were harvested and ground to a fine powder in liquid nitrogen. The cetyltrimethylammonium bromide (CTAB) method was used to extract genomic DNA from the mutants (Cubero et al. 1999). The crudely extracted DNA was then purified with the DNeasy Plant Maxi kit (Li et al. 2002) before samples were sent for NGS with a commercial service (Novogene, Bioinformatics Technology Co. Ltd.). DNA degradation and contamination were monitored on 1% agarose gels. Paired-end library preparation, DNA-seq, and read quality control of samples were completed by Novogene, using Illumina sequencer NovaSeq 6000, resulting in 14 to 17 million clean reads per sample. Clean reads were aligned to the *S. sclerotiorum* genome (ASM185786v1).

NGS data analysis.

The sequence reads from NGS were mapped to the reference genome of WT strain *S. sclerotiorum* 1980 by BWA-MEM (Li 2013). Mutation sites were identified by SAMtools with default parameters (Li et al. 2009). Annotation of the mutations was performed with germline short variant discovery (SNPs + Indels) based on GATK best practices (Auwerwa et al. 2013). False

mutations in repetitive sequences were manually removed as previously described (Huang et al. 2019).

Sanger sequencing of *SsSMR1* in *pnk2*.

Full-length *SsSMR1* was amplified with *SsSMR1*-F and *SsSMR1*-R primers and genomic DNA of *pnk2* isolated by CTAB method was used as template. A 3,376-bp PCR product was then sequenced using five primers (*SsSMR1*-Seq-1F-5R) by Novogene in China. All primers are listed in Supplementary Table S4.

Target gene knockout and complementation.

The split marker method was used to generate *SsSMR1* gene replacement constructs (Catlett et al. 2003). In brief, 1,394-bp upstream and 1,223-bp downstream flanking fragments of *SsSMR1* were amplified with the primer pairs *SsSMR1*/1F-2R and *SsSMR1*/3F-4R, respectively, and were fused with *hph* fragments amplified with the primers HYG/F-HYG/R from *pCH-EF-1* plasmid (shared by D. Jiang from Huazhong Agricultural University in China) (Yu et al. 2004). All primers used for PCR are listed in Supplementary Table S4. The amplified PCR products were cotransformed into protoplasts of WT as described (Yu et al. 2004). Transformants were selected on fresh PDA plates supplemented with hygromycin B. The individual germinating transformants were transferred onto new PDA plates with hygromycin B. Fresh mycelia were used to perform colony PCR with two pairs of primers, 7F, H855-R and H855-F, 8R (Supplementary Table S4), to confirm the insertion of hygromycin gene. Positive transformants were inoculated onto new PDA plates with hygromycin B by hyphal tip transfer. Two more rounds of the same colony PCR and selection were conducted to accumulate more nuclei with hygromycin insertions. Then the transformants were cultured in PDB for 2 days. The fresh mycelia mat was used to produce protoplasts as mentioned above. The resulting protoplasts were diluted with PDB supplemented with hygromycin B to 10³ protoplasts per milliliter. A 1-ml suspension was evenly spread on PDA plates supplemented with hygromycin B and was dried. The germinating colonies were transferred onto individual PDA plates with hygromycin B. Then the CTAB method was used to extract DNA from transformants, which were later used as template to conduct PCR with four pairs of primers as shown in Figure 4B. The transformants with the target gene being homogeneously knocked out were chosen for further experiments.

For transgene complementation, genomic *SsSMR1* was PCR-amplified and was cloned into *pCH-EF-1*. The full-length genomic sequence of *SsSMR1* containing a 1,323-bp upstream region from the start codon was amplified, using specific primers (Supplementary Table S4). This fragment was digested, using restriction enzyme *Xma*I, and was ligated into *pCH-EF-1* vector to generate the *pCH-SsSMR1* construct. The above construct was introduced into *Sssmr1-1*, using a previously described protocol (Yu et al. 2012). Transformants were selected and were purified on fresh PDA plates supplemented with hygromycin B.

Colony morphology and growth rate determination.

WT strain, knockout mutants, and complementing transformants were grown on PDA plate for three to four days. Then, mycelial agar disks were taken from the colony margin with a sterilized hole punch (5 mm diameter), were transferred to the center of fresh PDA plates (90 mm diameter), and were incubated at room temperature. The colony diameter was measured every day until mycelia reached the edge of the PDA plate. The images of colony morphology were taken 14 days postinoculation (dpi).

Plant infection assay.

Pathogenicity of the strains was tested on detached leaves of *Arabidopsis thaliana* (ecotype Col-0) that were 4 weeks old. Mycelial

agar plugs of 2 mm in diameter were inoculated on detached leaves placed on moistened paper towels in Petri dishes (90 mm diameter), with one agar plug per leaf and five leaves per genotype. *oah1* is an oxalate-deficient mutant that exhibits significantly attenuated virulence (Liang et al. 2015) and served as the negative control. Photos were taken at 24 and 48 h postinoculation. The pathogenicity test was repeated three times with similar results.

Apothecia and compound appressoria formation.

Equal numbers of sclerotia were collected from WT, *Sssmr1-1*, and M98 and were used to generate apothecia as described (Athukorala et al. 2010). For compound appressoria formation, fresh mycelial plugs (5 mm diameter) with growing hyphal tips were placed on glass slides and were incubated in a humidity chamber for 2 to 3 days. Then compound appressoria were observed and photographed under a microscope.

Bleach treatment and UV irradiation assay on sclerotia.

For bleach treatment, sclerotia, formed from two-week-old PDA plates of WT, *Sssmr1-1*, and *Sssmr1-3*, were treated with 15% bleach (Cloromax) for 30, 60, and 90 min. For UV irradiation, sclerotia were irradiated under UV light (TL-2000 Ultraviolet Translinker, energy strength of 9,000 mJ/cm²) for 10, 20, and 30 min. For both assays, each treatment included 45 sclerotia from WT, *Sssmr1-1*, or *Sssmr1-3*. The treated sclerotia were then grown on PDA for 3 days and the germination rate was calculated by the number of germinated sclerotia divided by the total number of sclerotia treated. The assays were performed three times with similar results.

RNA-seq analysis.

WT and *Sssmr1-1* were cultured on PDA plates (90 mm diameter) and were incubated in the dark at room temperature for 3 days. Actively growing mycelia were transferred into flasks with 200 ml of fresh PDB media for an additional 4 to 9 days of growth in the dark at room temperature. Young mycelia were harvested from WT and *Sssmr1-1* PDB cultures at 4 dpi, while old mycelia were collected at 9 dpi. Young and old mycelial samples were collected in three biological replicates. Total RNA was extracted using TRIzol reagent according to manufacturer protocol (Invitrogen). Then, RNA degradation and contamination were monitored on 1% agarose gels. RNA integrity was assessed using the RNA Nano 6000 assay kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies).

Paired-end library preparation, RNA-seq, and read-quality control of samples were completed by Novogene using Illumina sequencers resulting in 20 to 23 million clean reads per sample. Clean reads were aligned to the *S. sclerotiorum* genome (ASM185786v1), using HISAT2 v2.2.0 on default parameters (Kim et al. 2019). SAMtools v1.10 was used to sort and index bam files (Li et al. 2009). Gene transcript counts were generated using featureCounts from the Subread v2.0.1 package on default parameters (Liao et al. 2014). GO term enrichment analysis using Fisher's exact test was done using annotations generated from Blast2GO (Götz et al. 2008). The R package DESeq2 v1.28.1 was used to identify DEGs between samples (Love et al. 2014). Gene rows were reorganized using Plots generated using the ComplexHeatmap, UpSetR, and ggplot2 packages in R (Conway et al. 2017; Gu et al. 2016; Villanueva and Chen 2019).

Statistical analysis.

One-way analysis of variance followed by Tukey's post hoc test were performed. The Scheffé multiple comparison was applied for testing correction. Normality test for all data were done in SPSS. Statistical significance was indicated with asterisks. *P* values are included in figure captions.

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