# Coprinopsis cinerea Uses Laccase Lcc9 as a Defense Strategy To Eliminate Oxidative Stress during Fungal-Fungal Interactions

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**ABSTRACT** Frequently, laccases are triggered during fungal cocultivation for overexpression. The function of these activated laccases during coculture has not been clarified. Previously, we reported that *Gongronella* sp. w5 (w5) (Mucoromycota, Mucoromycetes) specifically triggered the laccase Lcc9 overexpression in *Coprinopsis cinerea* (Basidiomycota, Agaricomycetes). To systematically analyze the function of the overexpressed laccase during fungal interaction, *C. cinerea* mycelia before and after the initial Lcc9 overexpression were chosen for transcriptome analysis. Results showed that accompanied by specific utilization of fructose as carbohydrate substrate, oxidative stress derived from antagonistic compounds secreted by w5 appears to be a signal critical for laccase production in *C. cinerea*. A decrease in reactive oxygen species (ROS) in the *C. cinerea* wild-type strain followed the increase in laccase production, and then *lcc9* transcription and laccase activity stopped. By comparison, increased $H_2O_2$ content and mycelial ROS levels were observed during the entire cocultivation in *lcc9* silenced *C. cinerea* strains. Moreover, *lcc9* silencing slowed down the *C. cinerea* mycelial growth, affected hyphal morphology, and decreased the asexual sporulation in coculture. Our results showed that intracellular ROS acted as signal molecules to stimulate defense responses by *C. cinerea* with the expression of oxidative stress response regulator Skn7 and various detoxification proteins. Lcc9 takes part in a defense strategy to eliminate oxidative stress during the interspecific interaction with w5.

**IMPORTANCE** The overproduction of laccase during interspecific fungal interactions is well known. However, the exact role of the upregulated laccases remains underexplored. Based on comparative transcriptomic analysis of *C. cinerea* and gene silencing of laccase Lcc9, here we show that oxidative stress derived from antagonistic compounds secreted by *Gongronella* sp. w5 was a signal critical for laccase Lcc9 production in *Coprinopsis cinerea*. Intracellular ROS acted as signal molecules to stimulate defense responses by *C. cinerea* with the expression of oxidative stress response regulator Skn7 and various detoxification proteins. Ultimately, Lcc9 takes part in a defense strategy to eliminate oxidative stress and help cell growth and development during the interspecific interaction with *Gongronella* sp. w5. These findings deepened our understanding of fungal interactions in their natural population and communities.

**KEYWORDS** *Coprinopsis cinerea*, interspecific interaction, laccase, oxidative stress responses, reactive oxygen species

Interspecific interactions are critical factors involved in the ecology of fungi, as these live in complex organismal communities and often have to compete against each other for territory and to occupy the nutrient substrates [1, 2]. Fungal competition can be divided into primary resource capture for obtaining unoccupied resources, and secondary resource capture

for struggling for resources already colonized by other fungi [3]. When antagonistic relations occur, fungi usually employ several strategies, including developing attack or defense mechanisms and improvements in nutrient uptake, to gain an advantage over the competitor [3–5]. Aggressors successfully occupy the primary resources by various solutions, including good dispersal, rapid spore germination, rapid mycelial extension, and the ability to utilize organic compounds available in previously unoccupied resources [2, 3]. Also, they can increase the secretion of secondary metabolites or harming enzymes, or cause peroxide accumulation, and all this may be deleterious for the cellular integrity of a competitor [3, 6]. In contrast, a defender of resources may produce extracellular and intracellular enzymes that help detoxify of such noxious compounds [2]. For fungi harboring laccase genes, their laccase activities are often found enhanced after exposure to other fungi [7–11]. For example, the basidiomycetes *Pleurotus ostreatus* and *Agaricus bisporus* increase laccase production when cocultured with *Trichoderma* sp., indicating that laccases play roles during fungal confrontations [8].

Laccases (*p*-diphenol:oxygen oxidoreductases, EC1.10.3.2) are blue multicopper-containing phenol oxidases with a broad substrate specificity range that includes diverse aromatic compounds [12]. Fungal laccases have great potentials for biotechnological applications, but the biological functions of fungal laccase have not been fully confirmed, even though thousands of papers regarding laccase have been published [13]. Fungal laccases are suggested to be synergetic with other ligninolytic enzymes and involved in the biodegradation of natural polymers since many filamentous basidiomycetes laccases can cleave bonds in non-phenolic or phenolic subunits of lignin with or without mediators (reviewed in references 14 and 15). Because heterologous expression of a fungal laccase gene in *Pichia pastoris* can enhance the resistance of yeast to $H_2O_2$-mediated oxidative stress [16], and deletion of most isozyme genes of laccases results in a greater sensitivity to $H_2O_2$ than in the wild-type of *Podospora anserina* [17], fungal laccases are also supposed to react in response to oxidative stress in the environment [11]. Other studies suggest that laccases may play an essential role in several developmental and physiological functions such as sporulation, mycelial morphology, pigment production, fruiting body formation, and virulence in plant pathogenesis. For instance, the *abr2* gene product in *Aspergillus fumigatus* is involved in conidial pigmentation [18]. Silencing of gene *lcc1* of *Lentinula edodes* indicates involvement in mycelial morphology [19, 20]. Upon homologous overexpression of *lcc1*, *Hypsizygus marmoreus* displays faster mycelial growth and fruiting body initiation [21]. However, the role of the strongly induced laccases during fungal interspecific interactions is still unclear.

The litter-degrading ink-cap mushroom *Coprinopsis cinerea* has in total 17 non-allelic laccase genes, which present one of the largest groups of laccase genes ever described for a fungus [22]. Under different culture conditions, there was a differential expression of laccase isozymes in *C. cinerea*. Among them, Lcc1 and Lcc5 are the main laccases secreted in different culture media [23]. Previously, we developed a fungal coculture system between the basidiomycete *C. cinerea* and the mucoromycete *Gongronella* sp. w5. The latter triggered the transcription of the almost silent laccase gene *lcc9* from *C. cinerea* and the secretion of Lcc9 into the coculture broth, accompanied by a low expression of Lcc1 and Lcc5 [24, 25]. Based on the transcriptome analysis of *C. cinerea* and gene silencing analysis of *lcc9*, we demonstrate in this study that Lcc9 was used by *C. cinerea* as a defense strategy to eliminate the oxidative stress induced by *Gongronella* sp. w5, resulting in better growth of the *C. cinerea* mycelia and promoted generation of mitotic spores (oidia) during the interspecific interactions. Transcriptome analysis combined with qRT-PCR results also revealed that proteins involved in ROS elimination and the oxidative stress response regulator Skn7 were upregulated as further interspecies stress responses.

## RESULTS

**Overview of transcriptomes of *C. cinerea* during interspecific interaction with *Gongronella* sp. w5.** To reveal the function of the specifically activated Lcc9 [24, 25], *C. cinerea* mycelia, separately cocultured with *Gongronella* sp. w5 for 18 h and 28 h, were chosen for transcriptome analysis to get clues from the global gene-expression-pattern
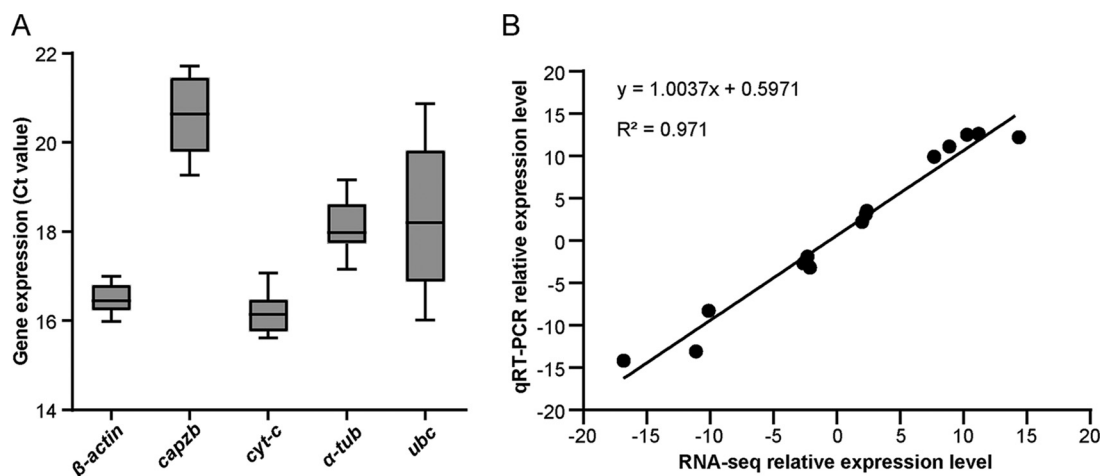
**FIG 1** qRT-PCR of five candidate reference genes (A) and 15 randomly chosen genes varied in RNA-seq (B). (A) The mean cycle threshold crossing-point ($C_T$) values of *β-actin*, *capzb* (F-actin capping protein beta subunit), *α-tub* (tubulin), *cyt-c* (cytochrome c), and *ubc* (ubiquitin) in *C. cinerea* across different time points of separated cocultures and single-species cultures. (B) qRT-PCR analysis to validate the RNA-seq data. The *x* axis that represents the RNA-seq data was calculated by the FPKM method. The *y* axis indicates the qRT-PCR results by using the $2^{-\Delta\Delta Ct}$ method. All experiments were performed independently in triplicate. The relative expression levels from RNA-seq and qRT-PCR obeyed a linear correlation ($y = 1.0037x + 0.5971$, $R^2 = 0.971$).

alterations before and after the initial *lcc9* overexpression. A total of 287 million raw sequencing reads were obtained from the transcriptomics analysis of in total 6 samples (3 per time point). After removal of the low-quality reads, a total of 268 million clean reads were obtained, with average reads of 44.7 million and average data of 6,716 million reads per sample. The clean reads were mapped to the genome of *C. cinerea* Okayama 7. The mapping rates of each sample were more than 93%. The sequence reads matched to 11,896 coding genes in the *C. cinerea* Okayama 7 genome, and 1,179 DEGs were calculated to be differentially expressed when comparing the transcriptome of 28 h to that of 18 h, with 695 genes upregulated and 484 genes downregulated.

Fifteen genes, including up- and downregulated genes, were randomly chosen and tested for expression profiling using qRT-PCR to validate gene expression data obtained through RNA-seq. As a prerequisite for getting solid results, the transcriptional levels of five candidate reference genes were detected at different time points of separated cocultures and single-species cultures of *C. cinerea* to find a stable reference gene. The mean cycle threshold crossing-point ($C_T$) values of the five genes ranged from 15 to 22 cycles among samples (Fig. 1A). Both the NormFinder algorithm and the BestKeeper algorithm identified *β-actin* (CC1G_13048, primers targeting 120 to 225 nt) as the most stable reference gene based on the NormFinder Stability value (0.037) and a higher correlation coefficient (standard deviation = 0.27, coefficient of variance = 1.66), respectively. Thus, the *β-actin* gene was chosen as the reference gene throughout the study. The expression values of the 15 randomly selected genes in *C. cinerea* mycelia in separated coculture with *Gongronella* sp. w5 for 28 h were then compared with 18 h. The results were consistent with the RNA-seq data (Fig. 1B), indicating the reliability and reproducibility of the sequencing results.

KEGG pathways were used for the functional classification of DEGs to investigate whether gene pathways in *C. cinerea* were changed during the cocultivation. DEGs were mapped to 70 KEGG pathways (Table S1-1). The 20 significantly most enriched pathways were interpreted to be involved in the following processes: amino acid metabolism, signal sensing and transduction, carbon metabolism, energy metabolism, lipid metabolism, and fungal secondary metabolites degradation (Table S1-1).

**Primary metabolic changes.** DEGs that participate in glycolysis/gluconeogenesis, citric acid cycle, and pentose phosphate metabolism pathways, including *gapdh* (glyceraldehyde 3-phosphate dehydrogenase, CC1G_09117), *cs* (citrate synthase, CC1G_12964), *mdh* (malate
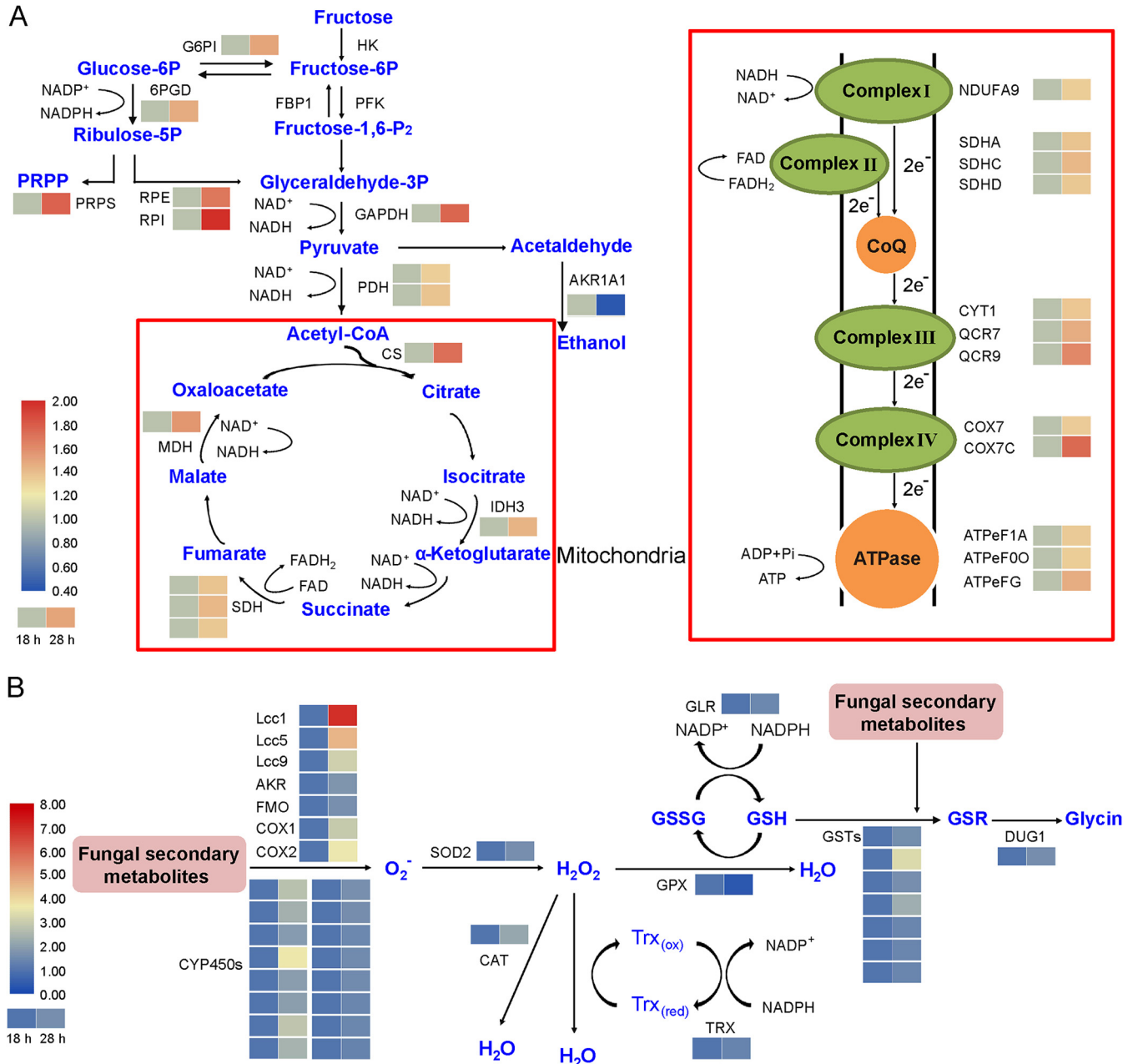
**FIG 2** Regulation of central carbon metabolism (A), oxidative phosphorylation (A), and oxidative stress responses (B) in *C. cinerea* at transcriptome level under interspecific interaction with *Gongronella* sp. w5 in separated coculture in SAHX medium with sucrose. Expression of genes at 28 h compared to 18 h coculture was analyzed. The heatmaps located beside each function indicate the logarithm to base 10 of fold changes in transcription. Abbreviations: *P*, phosphate; GSH, reduced form of glutathione; GSSG, disulfide form of glutathione; Trx (dx), thioredoxin; PRPP, 5-phosphoribosyl 1-pyrophosphate.

dehydrogenase, CC1G_07719), *rpe* (ribulose-phosphate 3-epimerase, CC1G_10281), and *rpi* (ribose-5-phosphate isomerase, CC1G_09808), were nearly all upregulated at 28 h compared to 18 h in separated cocultivation. Simultaneously, genes that are involved in the oxidative phosphorylation pathway were also upregulated, especially *qcr9* (ubiquinol-cytochrome c reductase, CC1G_00077) in complex III and *cox7* (cytochrome c oxidase subunit 7, CC1G_02587) in complex IV (Fig. 2A, Table S1-1 and S1-2).

Besides, key enzymes involved in β-oxidation breakdown of fatty acids and some amino acids into metabolites entering the citric acid cycle, such as ACAT (acetyl-CoA acetyltransferase, CC1G_14616), ECHS1 (enoyl-CoA hydratase, CC1G_02051), L-serine/threonine ammonia-lyase (CC1G_03788), and aspartate kinase (CC1G_11606), were upregulated
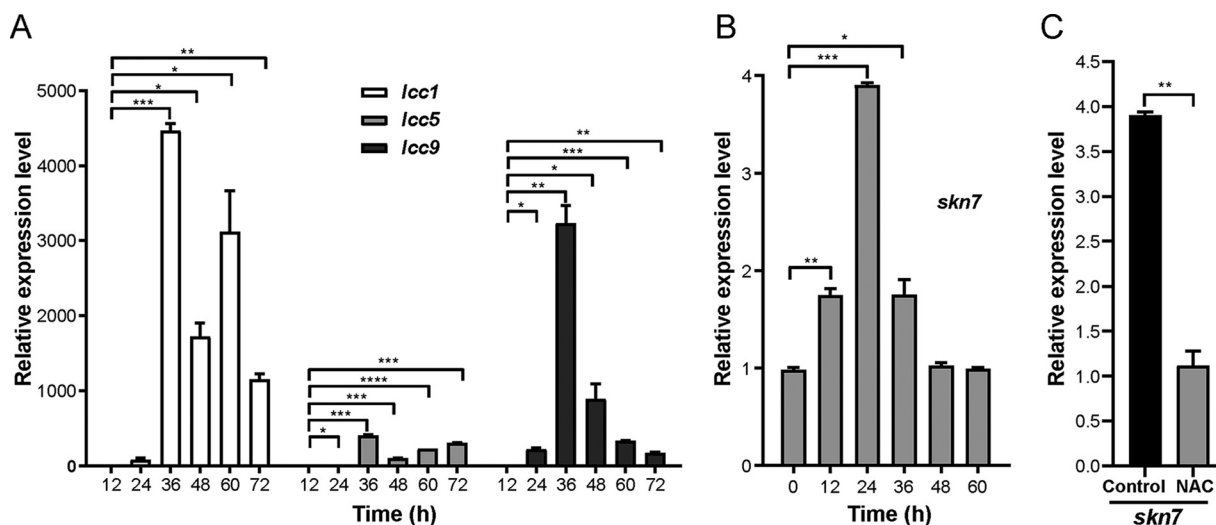
**FIG 3** Three laccases (A) and transcription factor Skn7 (B) were upregulated in expression in separated coculture of *C. cinerea* and *Gongronella* sp. w5 but ROS scavenger *N*-acetyl-L-cysteine (NAC) inhibited increase of *skn7* transcription (C). *C. cinerea* mycelia were collected every 12 h during separated coculturing with *Gongronella* sp. w5. Then they were ground, extracted for RNA, and analyzed for transcriptional levels by qRT-PCR. The transcription levels of genes of monocultures without NAC (A and B) or at 0 h of separated coculture in SAHX with sucrose (C) were set as baselines. The data in panel C were detected at 24 h of separated coculture and analyzed using Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Data in panels A to C show mean ± SD, $n = 3$.

(Table S1-1). Particularly, genes involved in tryptophan metabolism were mostly enriched in DEGs and increased in expression here. For example, *aadc* (aromatic-L-amino-acid decarboxylase, CC1G_02020), *tdo* (tryptophan 2,3-dioxygenase, CC1G_02499), *echs1*, *acat*, and *cat* (catalase, CC1G_09926) were all increased (Table S1-2).

**Degradation of fungal secondary metabolites.** Most of the significantly upregulated KEGG pathways at 28 h of separated cocultivation were related to degradation and metabolism of toxic metabolites, cell detoxification, and signaling and stress responses (Table S1-3), indicating that *C. cinerea* was exposed to various secondary metabolic compounds when confronted with *Gongronella* sp. w5. Our previous finding by NMR of HBA (*p*-hydroxybenzoic acid) obtained from *Gongronella* sp. w5 in culture broth [25] and the further GC-MS identification of a large number and types of benzene and naphthalene compounds among other metabolites from *Gongronella* sp. w5 culture broth further reinforced this hypothesis (Table S2).

The degradation processes of fungal secondary metabolites in fungi are largely unknown but most likely include a series of oxidation, reduction, and acylation reactions in extracellular and intracellular environments [26–29]. Among the DEGs, 11 CYP450 (cytochrome P450) genes were upregulated during the coculture process (Fig. 2B, Table S1-3). In fact, *cyp78A3* (CC1G_09431) and two *cyp98A3* (*cox1* and *cox2*, CC1G_03562 and CC1G_03564 [30]) encoding CYP subfamilies with oxidoreductases that catalyze hydroxylations of the phenolic ring of monolignols to participate in the degradation of phenolic compounds [31, 32] were 5.6, 6.7, and 11.2 times upregulated, respectively (Table S1-3). In addition, another monooxygenase of the UbiH hydroxylase family (CC1G_05962) that could modify a single position of the aromatic ring and is involved in hydroxylation of phenolic compounds [33] was 2.1 times upregulated.

Lignin-modifying enzymes are suggested to be also involved in the degradation of fungal secondary metabolites [26, 34, 35]. According to the transcriptomic data, laccases *lcc1* (CC1G_09614), *lcc5* (CC1G_00690), and *lcc9* (CC1G_05998) were 127.4, 1.5, and 7.7 times increased at the transcriptional level after 28 h compared to 18 h of separated cocultivation. Furthermore, as shown by qRT-PCR analysis in Fig. 3A, compared to the single-species cultures of *C. cinerea*, genes encoding for the three laccase isozymes Lcc1, Lcc5, and Lcc9 detected in separated coculture of *C. cinerea* and *Gongronella* sp. w5 [24, 25] were transcribed at 24 h and peaked at 36 h with hundreds

to thousands fold increase. Among which, *lcc1* and *lcc9* were strongly induced and responded quickly. However, in contrast to *lcc1* and *lcc5*, which were expressed up to 72 h, the expression of *lcc9* decreased significantly after 60–72 h.

**Antibiotic molecule and siderophore production.** Fungi defend their ecological niche mainly by producing antibiotic molecules, and the sesquiterpene lagopodin was reported to be an antibacterial compound of *C. cinerea* [36]. The CYP450 genes *cox1* and *cox2*, together with sesquiterpene synthase gene *cop6* (CC1G_03563), form a gene cluster for lagopodin biosynthesis and oxidative modifications [30, 36–38]. In our transcriptome data, the expression of the three genes of the cluster was in parallel upregulated, between about 6 to 12 times during 18 to 28 h of coculture. Moreover, most of the genes in terpenoid backbone biosynthesis, which could positively affect lagopodin production [38], were increased in expression (Table S1-4).

Gene *cpf2* (CC1G_04211) in the coprinoferrin siderophore gene cluster of *C. cinerea* for a putative L-ornithine-$N^5$-monooxygenase [39] for production of $N^5$-hydroxy-L-ornithine from ornithine as precursor of hydroxamate siderophores and competitive antimicrobial iron scavengers [40, 41], was 2.1 times upregulated. In contrast, the transcription in the cluster of genes for a non-ribosomal peptide synthetase (Cpf1, CC1G_04210) producing the coprinoferrin and for an uncharacterized cytidine deaminase-like protein (CC1G_04209 [39]) was not changed (Table S1-4).

**Oxidative stress response.** Extracellular fungal bioactive compounds cause oxidative stresses in cells. Following this fact, genes encoding for oxidative stress-related proteins, including aldo/keto reductases (AKRs), superoxide dismutases (SODs), glutathione S-transferases (GSTs), and catalases (CATs), were significantly upregulated in *C. cinerea* during the confrontation with *Gongronella* sp. w5 (Fig. 2B and Table S1-3). These proteins are suggested to play roles in oxidative stress signal transduction [11, 42–44], elimination of stress, and cellular redox balance maintenance [27, 45, 46]. The proteins participate in transforming the superoxide anion radical to $H_2O_2$ (SOD2, CC1G_03559, gene localizes together with the lagopodin biosynthesis cluster), reducing $H_2O_2$ to $H_2O$ to remove oxidative stress (CAT, CC1G_09926), and catalyzing 2′-deoxyribonucleoside diphosphate and thioredoxin disulfide to form ribonucleoside diphosphate and thioredoxin (Trx, CC1G_08103; Table S1-3). Although *gpx* (glutathione peroxidase, CC1G_07055) was downregulated, *glr* (glutathione reductase, CC1G_03850) and many *gst*s (glutathione transferases, CC1G_06890, CC1G_10591, and CC1G_14829), which are involved in catalyzing conjugation reactions of glutathione with toxic metabolites at its sulfhydryl site, were upregulated (Table S1-3). Furthermore, the *dug1* (Cys-Gly metallodipeptidase, CC1G_06968) gene, which plays a crucial role in degrading conjugated GSH [47], also increased in transcription (Fig. 2B and Table S1-3). These results indicated an increased glutathione flux in redox reactions and secondary metabolites in *C. cinerea* upon the confrontation with *Gongronella* sp. w5.

**Transcription factors.** Many potential transcription factors (TFs) were among the DEGs in our transcriptome analysis, but only three were significantly upregulated, and one was significantly downregulated in expression during the separated cocultivation of *C. cinerea* and *Gongronella* sp. w5 (Table S1-5). Of these upregulated TFs, the general transcription factor *tfIID* (CC1G_11509) was 2.2 times more transcribed. A candidate for GATA-type transcription factor gene *sreA* (CC1G_14734), which is involved in metabolisms of amino acids and especially tryptophan, and in iron homeostasis and sterol metabolism [48], was 2.1 times increased in expression. A candidate of *samB* (CC1G_08324), encoding for an MYND-type Zinc finger protein crucial for morphogenesis [49], was also significantly upregulated. Interestingly, transcription of *cbf11* (CC1G_13688) and *cbf12* (CC1G_03194) for two antagonistic CSL (CBF1/Su(H)/Lag-1) paralogs was increased and decreased about 2.2 times, respectively. They were reported to positively and negatively regulate development, cell wall synthesis, as well as stress response [50].

Of the $|\log_2 \text{FoldChange}| > 0.75$ TFs, one was *creb1* (cAMP response element-binding protein 1, CC1G_09350), which could enforce the GSH recovering capacity by directly upregulating genes for GSH synthesis and redox cycling [51]. For $|\log_2 \text{FoldChange}| < -0.75$ DEGs, there were candidates for *znf1* (a C2H2 zinc-finger transcription factor, CC1G_00774) for activation of core genes in glycolysis, pyruvate metabolism, and stress responses (1.8
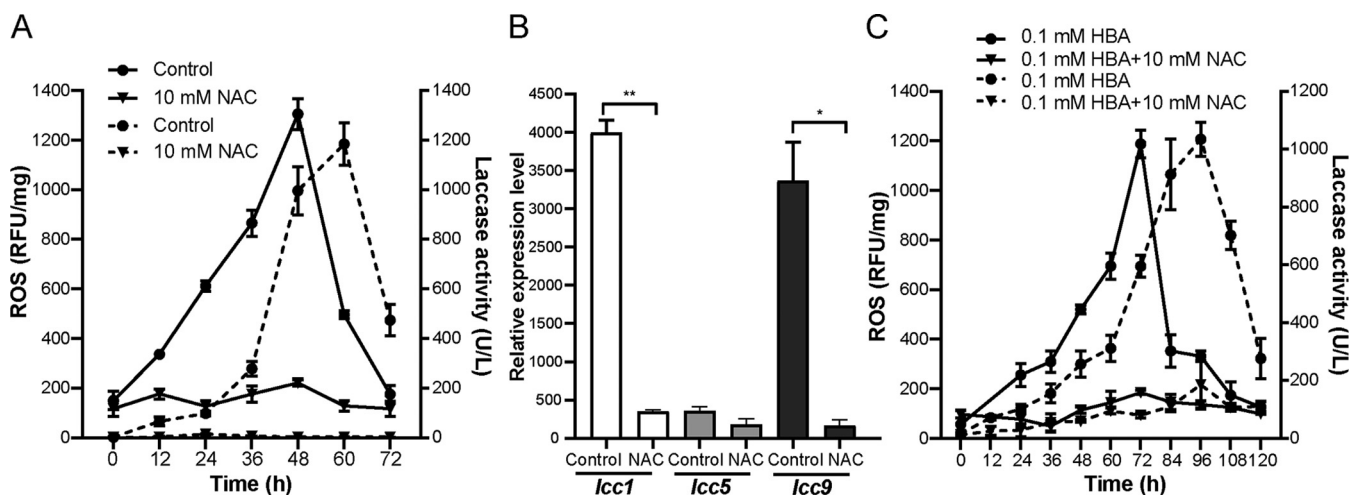
**FIG 4** ROS was involved in the synthesis of laccase during *C. cinerea*-*Gongronella* sp. w5 interspecific interaction in separated coculture. (A, B) ROS increased in *C. cinerea* separated cocultured with *Gongronella* sp. w5 in SAHX with sucrose (A) and added ROS scavenger NAC decreased laccase activity (A) and transcripts (B) of *C. cinerea*. Control represented separated cocultures without NAC. (C) NAC decreased laccase activity in monoculture of *C. cinerea* in SAHX medium using fructose as carbon source with 0.1 mM HBA added. Solid lines and dotted lines represent ROS values and laccase activities, respectively. The data were analyzed using Student's *t* test (*, $P < 0.05$; **, $P < 0.01$). Data show mean $\pm$ SD, $n = 3$. RFU, relative fluorescence units.

times downregulated) [52], for GATA-type transcription factor gene *gat1* (CC1G_05900) mediating nitrogen metabolite-repression (1.7 times downregulated) [53, 54], and for *hap1* (CC1G_08004) involved in ergosterol biosynthesis and efficient utilization of oxygen (1.8 times downregulated) [55] (Table S1-5).

Two TF genes were impressive because of their overall high read counts, although their expression changed only slightly between 18 and 28 h of separated coculture. Highest expressed was the general amino acid control transactivator/cross-pathway control protein A gene *cpcA* (*gcn4*, CC1G_05406) with a bZIP domain that acts as a repressor of protein synthesis while it induces TFs of stress-response genes under various stress conditions [56, 57]. The other highly expressed TF contains a leucine zipper domain and shares 68.7% sequence similarity with Skn7 of *S. cerevisiae*. Skn7 is an oxidative stress-responsive transcription factor shown in other fungi to contribute to the expression of detoxification genes (CC1G_08281 [58, 59]). As verified further by qRT-PCR (Fig. 3B), transcription of Skn7 was upregulated from 0 h to 36 h in separated cocultivation of *C. cinerea* with *Gongronella* sp. w5, and peaked at 24 h, indicating a function in oxidative stress response and regulation of expression of detoxification proteins. Moreover, Skn7 showed no increase at the transcription level after NAC (*N*-acetyl-L-cysteine) as ROS scavenger was added into the culture broth at 0 h of cocultivation, further supporting a role in stress response (Fig. 3C).

**ROS is involved in the synthesis of laccase during *C. cinerea*-*Gongronella* sp. w5 interspecific interaction.** Based on the transcription data, we hypothesized that the oxidative stress in *C. cinerea* derived from antagonistic compounds secreted by *Gongronella* sp. w5 is a signal for induction of laccase production in *C. cinerea*. Thus, the intracellular ROS concentration in *C. cinerea* and the laccase activity during the separated coculture were analyzed to verify this hypothesis. As shown in Fig. 4A, the ROS level in *C. cinerea* increased rapidly after the start of fungal cocultivation, with a peak at 48 h and a sharp decline afterwards. Notably, the laccase and ROS curves shared the same growth trends, with a lag-phase in between with which the growth curve of laccase activity followed that of the intracellular ROS concentration. The laccase activity curve thus peaked 12 h after the highest ROS level reached and fell sharply with a delay of also 12 h (Fig. 4A). ROS scavenger NAC added to the coculture system at 0 h further supported the above hypothesis. NAC prevented an increase in intracellular ROS concentration (Fig. 4A) and, as tested for both *lcc9*, *lcc5* and *lcc1* transcription (Fig. 4B) as well as total enzyme activity levels (Fig. 4A), the expression of laccase in *C. cinerea* under coculture conditions.

Previously, HBA as produced by *Gongronella* sp. w5 was evidenced as a principle to induce *C. cinerea* laccase activity in cocultures [25]. In order to rule out an indirect influence of NAC added in coculture due to a possible changed behavior of *Gongronella* sp. w5 and to link the transmission of the ROS signal in *C. cinerea* with induction of laccase production, HBA at 0.1 mM concentration was employed in *C. cinerea* SAHX (sucrose DL-asparagine XH medium) single species-cultures with fructose as the carbon source. The addition of HBA continuously increased the intracellular ROS level in the first 72 h of culture incubation. Consistent with the results of the cocultures and with a time lag compared to ROS, it also increased the laccase activity of *C. cinerea*, which peaked at 96 h of single-species cultivation. The decrease of laccase activity at 108 h followed the drastic decrease in the ROS level at 84 h. However, in treatments with 10 mM NAC, HBA had an inducing effect neither on ROS generation nor on the laccase production (Fig. 4C), suggesting that HBA was not a direct inducer of laccase activity.

**Laccase expression in *C. cinerea lcc9* silencing strains.** Laccase *lcc9* silencing strains were constructed based on two antisense constructs, pYSK-*lcc9*-antisense-1 and pYSK-*lcc9*-antisense-2 [60], to further evaluate the role of *lcc9* during interspecific fungal interaction. Three strains of pYSK-*lcc9*-antisense-1 (R1-7, R1-15, and R1-18 as positive strains, plus R1-C transformed with p*Cc*Ade8 as a control strain) and five strains of pYSK-*lcc9*-antisense-2 (R2-6, R2-9, R2-11, R2-19, and R2-23 as positive strains, plus R2-C as a p*Cc*Ade8 control strain) were randomly chosen from transformations for laccase activity determination in liquid cocultivation with *Gongronella* sp. w5. As shown in Fig. 5A and B, the laccase activities of the two control strains R1-C and R2-C were similarly increasing to that of the wild-type strain, but the eight *lcc9* silencing strains in comparison showed much lower laccase activities over the whole time of cocultivation ($< 500$ U/liter) (Fig. 5A and B). Separated cocultivation experiments provided similar tendencies of laccase activities for the clones (not shown) and further suggested that the fragment *lcc9* antisense 2 performed better than the antisense 1 on *lcc9* silencing. As a result, silencing strains R2-6, R2-11, R2-19, and R2-23 were chosen for further experiments.

Native-PAGE of culture samples taken from 48 h and 60 h coculturing showed the Lcc9 activity of the four silencing strains was at both time points significantly suppressed, accompanied by a partial reduction of Lcc1 and Lcc5 expression in several clones at the same time (Fig. 5C and D, $P < 0.05$, $P < 0.01$ or $P < 0.001$). qRT-PCR analysis, in which the *lcc9* transcription level of the wild-type strain at 36 h was set as the baseline, showed that *lcc9* transcripts were substantially downregulated in the four silencing strains compared to the wild-type at 36 h in separated coculture with *Gongronella* sp. w5 (Fig. 5E, $P < 0.01$ or $P < 0.001$). Among them, R2-19 had the highest downregulation observed (75%).

**Laccase is involved in cellular oxidative stress resistance of *C. cinerea* during coculture with *Gongronella* sp. w5.** To analyze whether the Lcc9 could protect *C. cinerea* against ROS, the intracellular ROS and $H_2O_2$ concentrations of the wild-type strain and four *lcc9* silencing clones were tested and compared during separated cocultivation with *Gongronella* sp. w5. The four silencing strains always showed 1.5–2 times higher concentrations of intracellular ROS and $H_2O_2$ than the wild-type strain during the entire cocultivation from 36 h to 72 h (Fig. 6A and B). Furthermore, the *lcc9* silencing strains also showed higher sensitivity than the wild-type strain to oxidative stresses caused by $H_2O_2$, guaiacol, or 1,3-dihydroxynaphthalene added at 36 h of cultivation to the cocultures. Within 36 h of further incubation with $H_2O_2$ and 1,3-dihydroxynaphthalene, ROS levels in the *lcc9* silencing strains increased above those of untreated control cultures (Fig. 6C). Their biomass was much less than that for the wild-type strain (Fig. 6D), although they all showed no apparent difference in single-species cultures of *C. cinerea* with the addition of oxidative stressors (not further shown).

**Lcc9 is involved in cell growth and cell morphology of *C. cinerea* during coculture with *Gongronella* sp. w5.** The enhanced *lcc9* production may be necessary for *C. cinerea* to confront stress and survive during coculture with *Gongronella* sp. w5. Thus, we compared the growth rate of the *C. cinerea* wild-type strain and three *lcc9* silencing strains R2-11, R2-19, and R2-23. All strains in single-species cultures showed
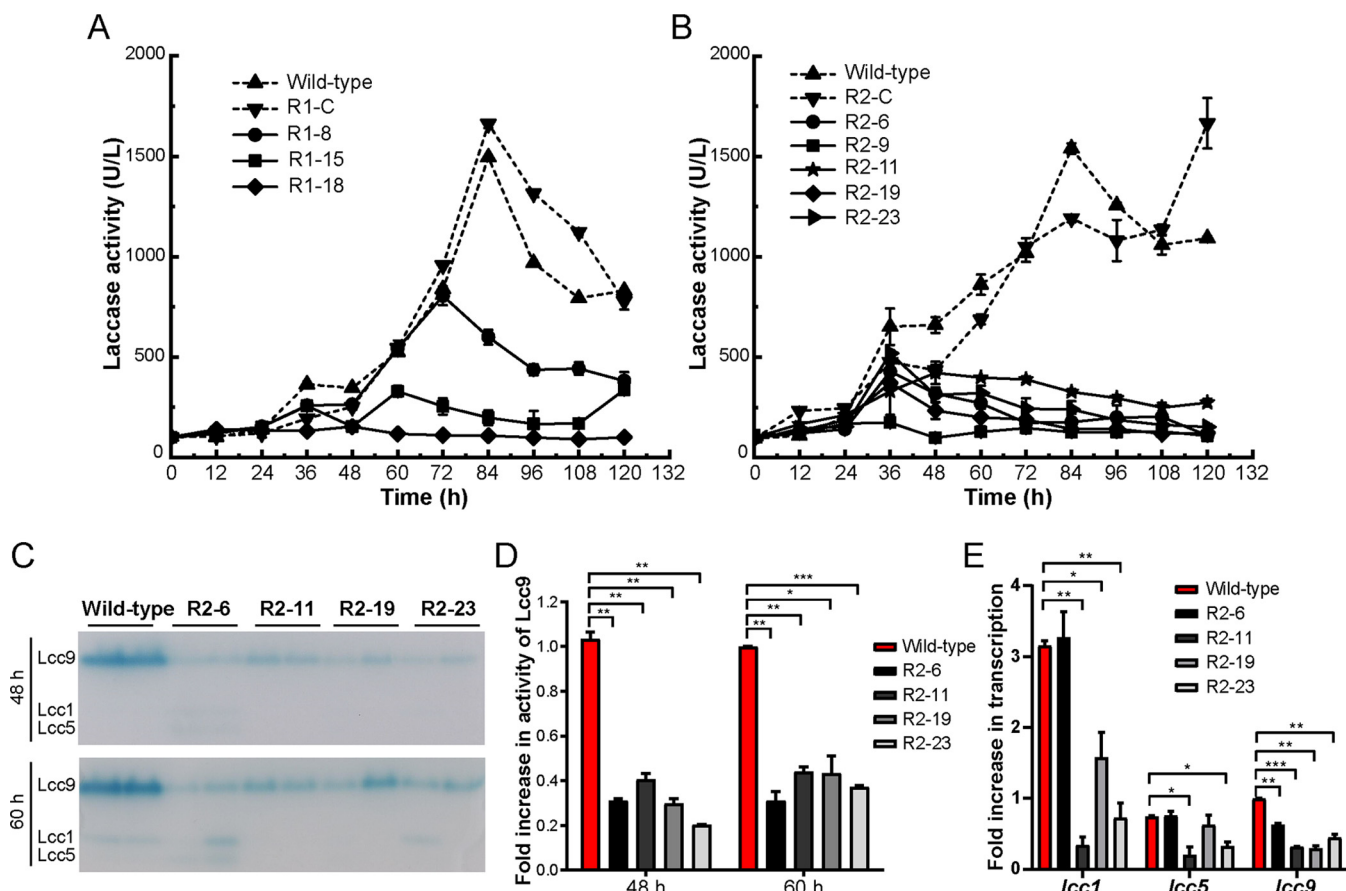
FIG 5 Lcc9 was downregulated in *lcc9* silencing strains. (A, B) The laccase activity of four transformants of pYSK-*lcc9* antisense 1 (A) and six transformants of pYSK-*lcc9* antisense 2 (B) tested in separated coculture with *Gongronella* sp. w5 was found to be decreased, using ABTS as the substrate. R1-7, R1-15, R1-18, R2-6, R2-9, R2-11, R2-19, and R2-23 were positive *lcc9* silencing strains represented by solid lines. pCcAde8 transformants R1-C and R2-C used as control strains are represented by dotted lines, as well as wild-type strains. (C) Native-PAGE of laccase activities of *C. cinerea* wild-type and of four *C. cinerea lcc9* silencing strains from 48 h and 60 h separated coculture with *Gongronella* sp. w5 indicates downregulation in the *lcc9* silencing strains. Equal amounts of culture supernatants (10 μl) were loaded onto the gels and activity-stained after gel-electrophoresis. (D) Quantification of *lcc9* activity from the separated cocultures deduced from gels as shown in panel C. (E) *lcc1*, *lcc5*, and *lcc9* laccase transcripts were shown by qRT-PCR analysis to be downregulated in the four *lcc9* silencing strains. The *lcc9* transcription level of wild-type strain at 36 h was set as the baseline. The data were analyzed using Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Data show mean ± SD, $n = 3$.

no difference in speed of mycelial growth on SAHX-fructose agar plates. However, when cocultured with *Gongronella* sp. w5, the expansion rate of the three *lcc9* silencing strains was much reduced (Fig. 7A and Fig. S1).

The mycelial morphologies of the *C. cinerea* wild-type strain and the *lcc9* silencing strains as cocultured with *Gongronella* sp. w5 on slides with the SAHX-sucrose agar medium are shown in Fig. 7B. *C. cinerea* mycelial extension fronts in the interspecific interaction zone were observed in regions still separated from *Gongronella* sp. w5 when the two fungi with hyphae had just reached each other at some places. Light microscopy results suggested that although *lcc9* silencing had no effect on the formation of an aerial mycelial mat by *C. cinerea*, it significantly reduced the mycelial density and the formation of hyphal side branches compared to the hyperbranching wild-type (Fig. 7B and C). Indeed, the hyphal branching patterns of the three *lcc9* silencing clones appeared more normal, comparable to the hyphal growth of the wild-type when growing unstressed on fungal media (Fig. S2). Moreover, after incubation for 7 days on SAHX-sucrose agar plates, there were many more mitotic oidia distributed in liquid droplets in the aerial mycelium of the *C. cinerea* wild-type strain than in the aerial mycelium of the three *lcc9* silencing clones (Fig. S2 and Table S3).

TEM observation results of *C. cinerea* hyphae collected from growth zones at the confrontation side with *Gongronella* sp. w5 showed that the hyphal cell walls of the
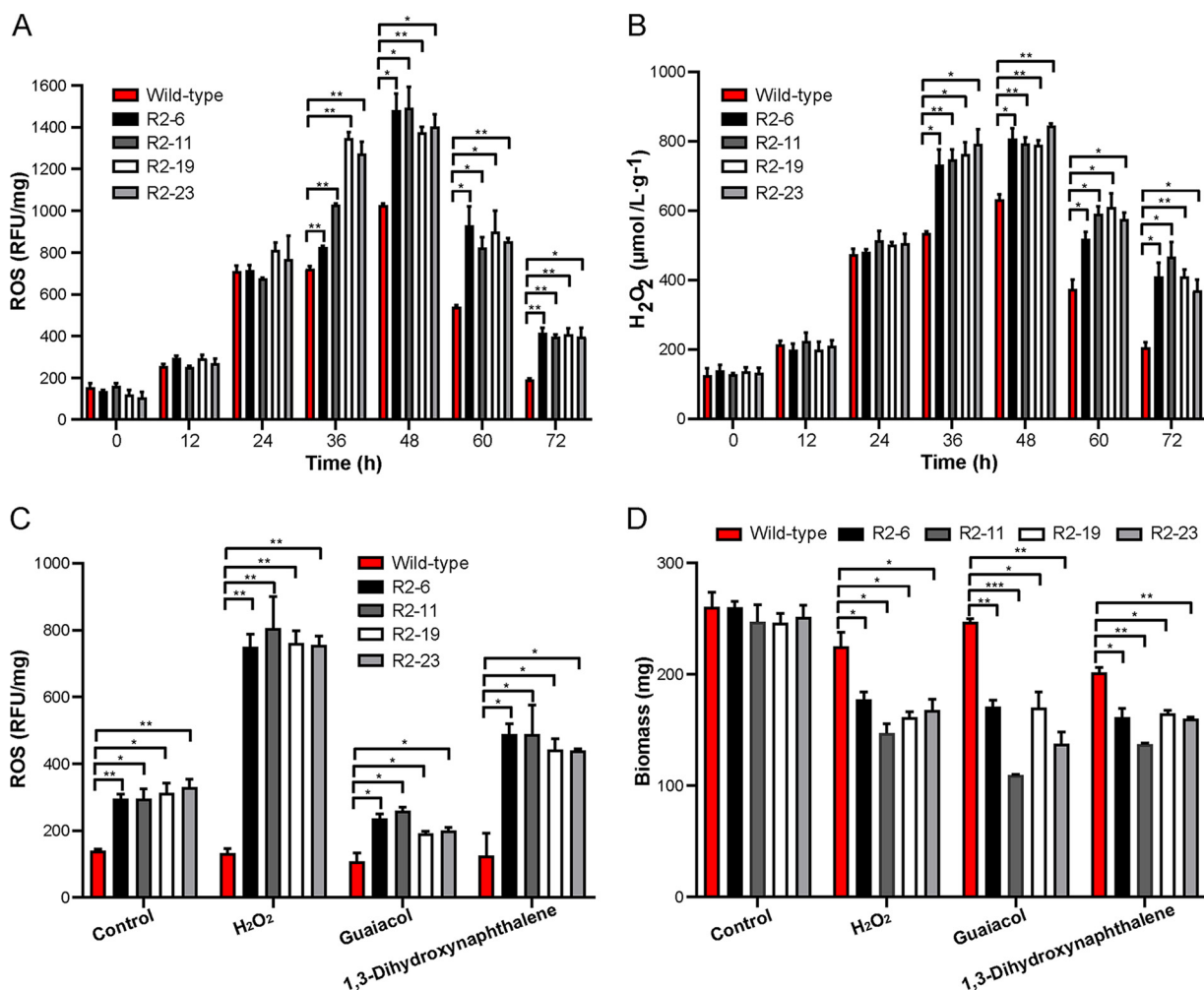
**FIG 6** Laccase was involved in cellular oxidative stress resistance of *C. cinerea* during interspecific interaction with *Gongronella* sp. w5. *C. cinerea* exhibited higher ROS level (A) and $H_2O_2$ content (B) in *lcc9* silencing strains compared to the wild-type strain after separated coculturing for 48 h in SAHX with sucrose with *Gongronella* sp. w5 sealed in sterile dialysis tubes. (C, D) The *lcc9* silencing strains showed higher ROS level (C) and less biomass (D) than the wild-type strain in separated cocultures when exposed to 100 mM $H_2O_2$, 2 mM guaiacol, and 0.5 mM 3, 5-dihydroxynaphthalene. Chemicals were added at 36 h of separated cocultivation and cocultures were incubated for further 36 h. The data were analyzed using Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Data show mean ± SD, $n = 3$.

wild-type strain comprised an inner layer (IL) and an outer layer (OL). However, the hyphal cell walls of the three *lcc9* silencing strains were thinner and did not have two clear layers, as randomly presented in Fig. 8 for R2-11. Thus, the development of cell wall was also affected during interactions with *Gongronella* sp. w5 after *lcc9* silencing. Furthermore, our results demonstrated that the vacuoles in the wild-type strain were large and few in most cells, whereas the silencing strains R2-11, R2-19, and R2-23 had a number of small vacuoles (Fig. 8 and data not shown).

## DISCUSSION

Most fungi will come across competitive or antagonistic organisms in their natural population and communities and will be subject to attack, defenses, and possibly defeat [3, 61]. Activating silent gene clusters for the production of antimicrobial secondary metabolites is among the promising strategies adopted in this warfare [62–64] and also induction of expression of various types of combative and protective enzymes, including laccase [3, 7, 10, 11, 24, 61, 65]. In some cases, however, the increase in laccase production is simply due to the co-production between the two species; only some laccase isoenzyme genes could be activated in specific combinatorial
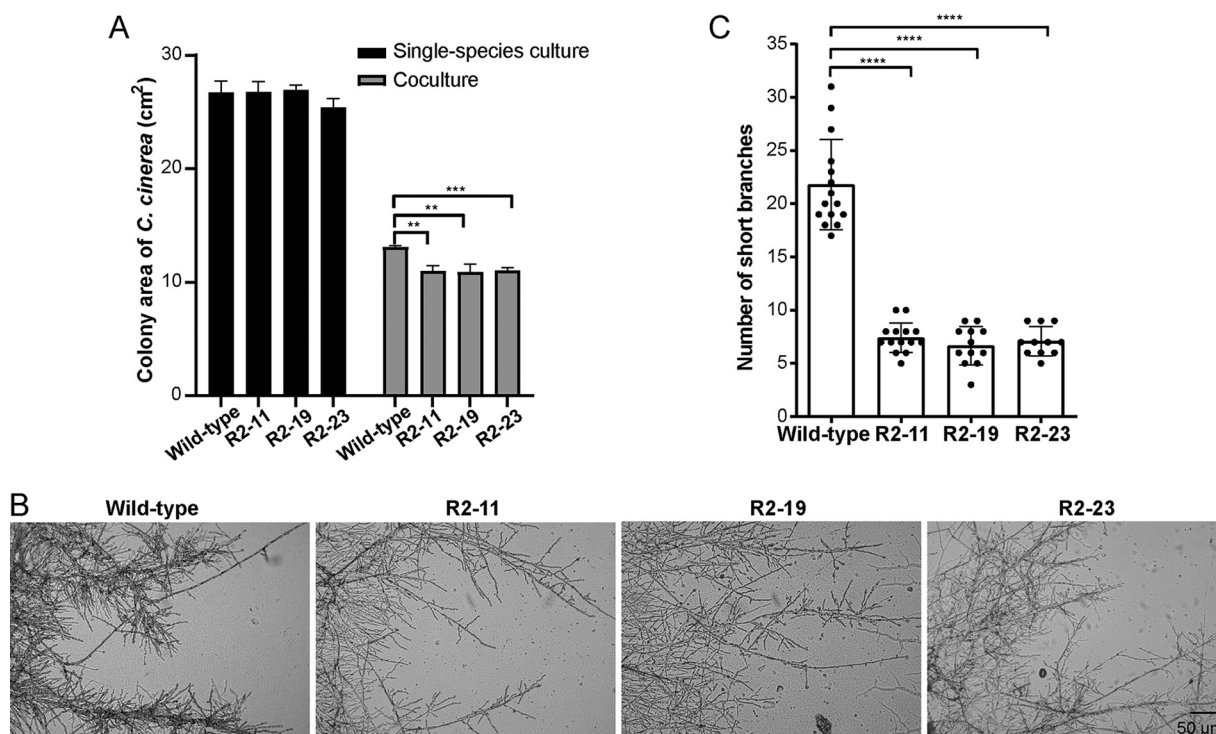
**FIG 7** Lcc9 was involved in cell growth and cell morphology of *C. cinerea* in coculture with *Gongronella* sp. w5 on solid medium. (A) Quantification of the colony area of the *C. cinerea* wild-type strain and three *lcc9* silencing strains under single-species culture or coculture with *Gongronella* sp. w5. (B) Mycelial growth fronts with short hyphal branches in the interspecific region with *Gongronella* sp. w5 of the *C. cinerea* wild-type strain and three *lcc9* silencing strains were cultured on microscope slides with SAHX medium with sucrose and observed by light microscopy. (C) Quantification of the numbers of short branches from 15 different randomly chosen views of the mycelia as shown in panel B. The data were analyzed using Student's *t*-test (**, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). Data show mean $\pm$ SD, $n = 3$. Scale bar, 50 $\mu$m.

cultivations [66, 67]. It is still unknown what role the upregulated laccases and those triggered newly in expression play during the fungal coculture. In this study, comparative transcriptomic analysis of *C. cinerea* and gene silencing of laccase *lcc9* were performed to reveal the function of enhanced-expressed laccase during the fungal *C. cinerea*-*Gongronella* sp. w5 interaction.

**Specific utilization of fructose as carbohydrate substrate and energy supply.** Our previous data showed that *Gongronella* sp. w5 in cocultivation with *C. cinerea* hydrolyzed almost all of the sucrose in the medium into glucose and fructose within 24 h and provided *C. cinerea* with mainly the fructose and traces of glucose [25]. Compared to the no-growth of *C. cinerea* in monocultures with sucrose as the sole carbon source [25, 68], *C. cinerea* grew fast in coculture in the first 48 h by using released fructose spurned by *Gongronella* sp. w5 [25]. Consistent with these results, the transcriptome results in this study showed that functions in the catabolic primary metabolisms, including glycolysis/gluconeogenesis, pentose phosphate pathway, citric acid cycle, and oxidative phosphorylation pathway for energy supply, were mostly upregulated (Fig. 2 and Table S1-2). The increased pentose phosphate pathway could also supply sufficient NADPH for the fast growth of *C. cinerea* in coculture. Meanwhile, catabolic pathways for energy generation involved in fatty acid and several amino acid metabolisms were also upregulated, together with adaptations in expression of potential TFs for nitrogen and amino acid regulation (Table S1-2).

In paired cultures of *P. ostreatus* with either *Dichomitus squalens* or *Trametes versicolor* and of *D. squalens* with *T. versicolor*, with glucose used as sole carbon and energy source, expression of the oxidative phosphorylation pathway is upregulated in *P. ostreatus* and *D. squalens*, and in addition, the expression of the TCA cycle in *P. ostreatus* [46, 69]. Expression of functions in glycolysis/gluconeogenesis is in addition
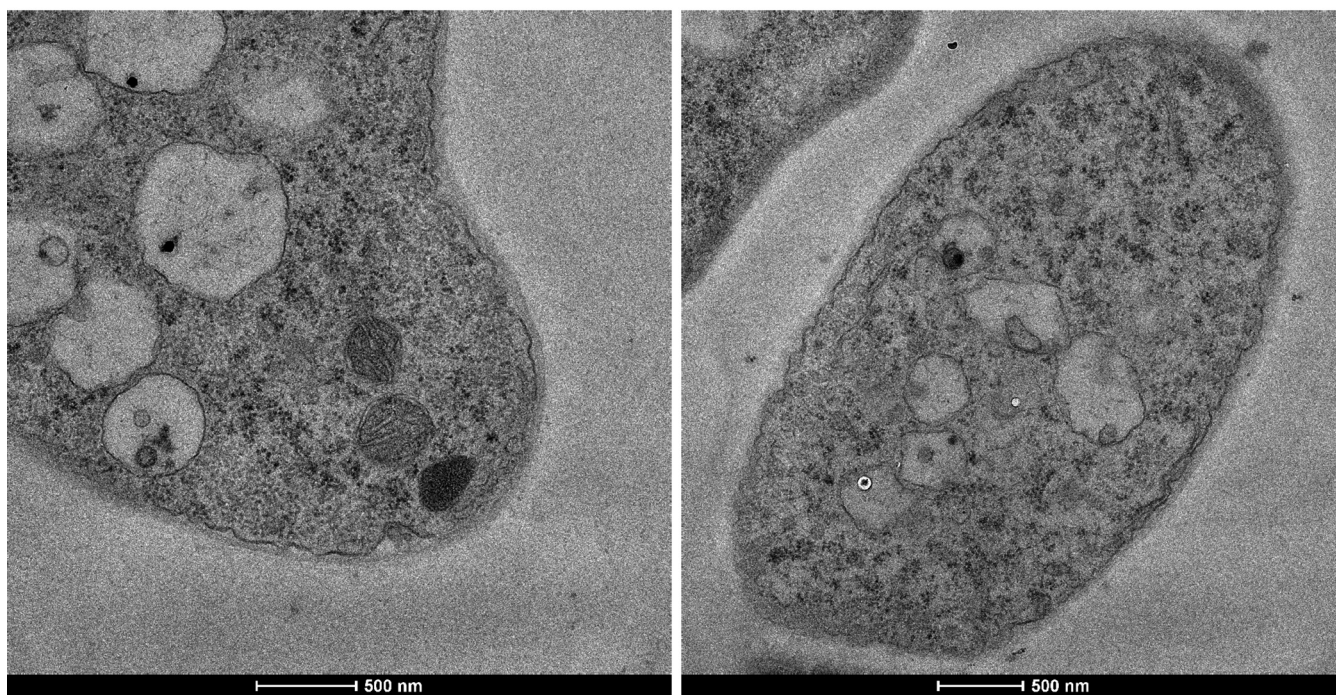
**Wild-type**
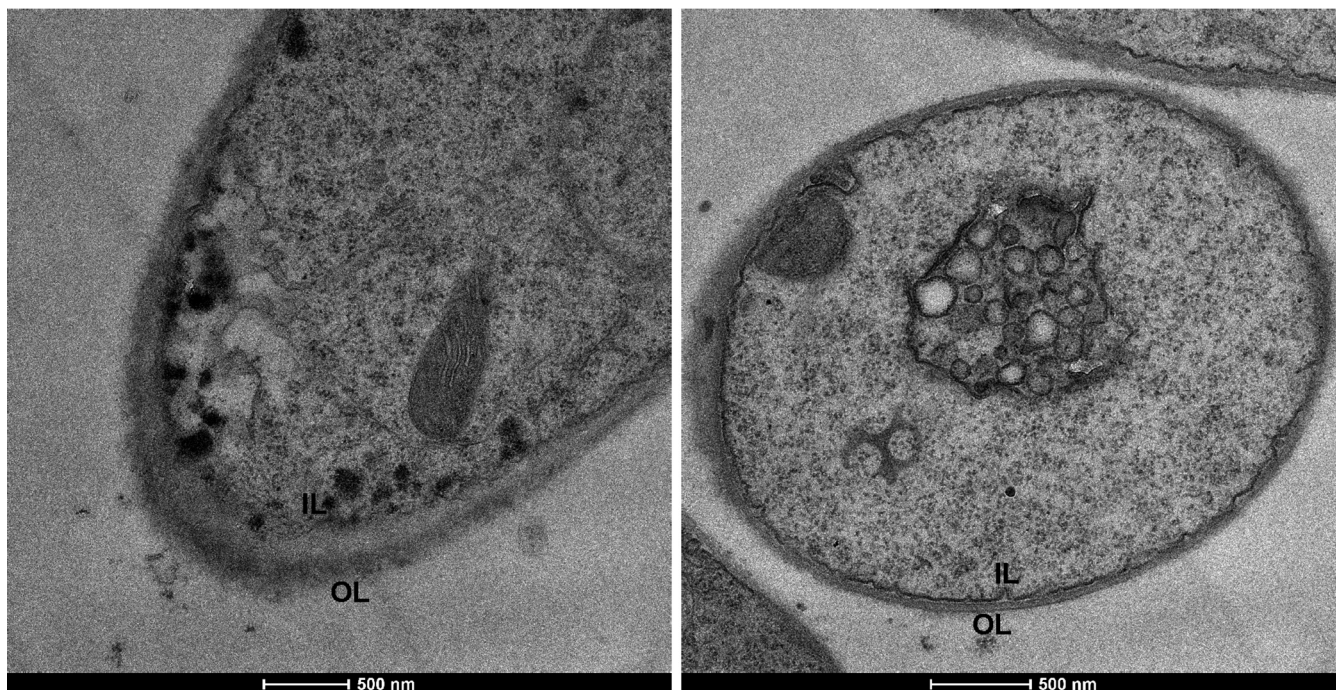


**R2-11**



**FIG 8** Lcc9 silencing affected cell walls of *C. cinerea* during coculture with *Gongronella* sp. w5. The hyphal cell walls with inner (IL) and outer layer (OL) and fungal vacuoles of the wild-type strain and *lcc9* silencing strain R2-11 are shown by transmission electron microscopy. Scale bar, 500 nm.

enhanced in *Pleurotus eryngii* var. *ferulae* in dual cultures with *Rhodotorula mucilaginosa* in a medium with a more complex mixture of carbon sources (glucose, corn powder, wheat bran) [70], similar to what was observed here in *C. cinerea* in separated coculture with *Gongronella* sp. w5 (Fig. 2 and Table S1-2). Direct competition between two fungi for the same nutrients is responsible for the increases in the TCA cycle and

the oxidative phosphorylation pathway [46, 69, 70]. Different preferences for supplied carbon sources between *P. eryngii* var. *ferulae* and *R. mucilaginosa* and specific utilization of fructose provided by *Gongronella* sp. w5 for *C. cinerea* might have also activated the upstream glycolysis pathway. Noteworthy is that all these examples of fungal cocultures had in common that specific laccases are selectively overexpressed in the basidiomycetes analyzed by transcriptomics [25, 46, 69, 70]. Moreover, pathways for toxic metabolite biodegradation are also induced in all species studied [46, 69, 70] (Table S1-3). ROS generation, oxidative stress responses, and protective responses are observed during the interspecies confrontations in *P. ostreatus*, *D. squalens*, and *C. cinerea* [46, 69] (Table S1-3) but not in *P. eryngii* var. *ferulae* in the confrontation with *R. mucilaginosa* that provided the antioxidant β-carotene [70].

**ROS and laccase induction in interspecies interactions.** Phenolic compounds are often effective inducers for laccase production. β-Carotene released by red basidiomycetous yeasts into the medium is confirmed to be the inducer of laccase activity in fungal coculture systems including *P. eryngii* var. *ferulae* [65, 70]. This phenomenon is also exemplified by inducing laccase production with phenolic compounds, such as *o*-toluidine, in single-species cultures of various basidiomycetes [71–74]. Based on our previous data [25] and that presented in this study (Table S2), we summarize that HBA and likely also other secondary organic compounds produced by *Gongronella* sp. w5 were the main inducers for the *lcc9* expression in *C. cinerea* (Fig. 2 and Fig. S3).

The detailed strategy of how antagonistic compounds induce the expression of laccase during fungal cocultivations is still under investigation. However, it is well known that extracellular agents and toxic compounds can destroy the intracellular redox balance, resulting in increased intracellular ROS concentrations [61, 75]. ROS is reported to play a central role in the molecular cross talk that occurs during interactions between fungi and organisms in their environment, including plants and other microbes, leading to interspecific recognition and eventually the activation of fungal defense reactions [43]. Several studies have reported that oxidative stress increases during fungal cocultivation [6, 11, 46]. Our data supported that compounds from *Gongronella* sp. w5 raised the ROS concentration in *C. cinerea* cells and suggested that *C. cinerea* encountered oxidative stress during cocultivation (Fig. 4, Fig. S3).

**Transcriptional regulation.** Unfortunately, little knowledge has yet been gained on relationships between increased oxidative stress and laccase production during fungal cocultivations. Interestingly in this study, ROS was speculated to be a signal to induce *lcc9* expression at the early stage of cocultivation (Fig. 4), and the signal might be transmitted into the nucleus by the oxidative stress response regulator Skn7 (Fig. 3, Fig. S3, and Table S1-5). *S. cerevisiae* Skn7 is activated following several phosphorelays, responsible for sensing upstream signals and controlling the expression of downstream antioxidative proteins [58, 59]. Deleting *skn7* in the yeast increases sensitivity to a wide variety of oxidizing agents [76] and decreases expression of detoxification genes such as *trx2*, *gpx2*, *ctt1*, *sod1*, or *sod2* [59]. Another study showed that Skn7 can interact with heat shock factor Hsf1 *in vivo*, may bind to the same sites as Hsf1, and is required to induce heat shock genes by oxidative stress [58]. In the human pathogen *Candida glabrata* or *Candida albicans*, a significant role of *skn7* in regulating antioxidant genes such as *trx2*, *trr1*, *tsa1*, and *cta1* was demonstrated [77], and many other ROS-detoxifying enzymes are regulated by *skn7* directly or indirectly [78]. Similarly, the expression levels of two genes encoding for Zn-superoxide dismutase and glutathione reductase for antioxidant defense are lower in *skn7* deleted mutants in *Aspergillus flavus* [79], catalase B expression in *A. nidulans* requires the oxidative stress Skn7 protein SrrA [80], and deletion of FoSkn7 in *Fusarium oxysporum* f. sp. *cubense* negatively affects expression of many genes encoding for ROS-detoxifying enzymes and heat shock proteins [81].

Lcc1 and Lcc5 are the main laccases secreted by *C. cinerea* monokaryons in liquid single-species cultures [23], while Lcc9 is the main laccase expressed during coculture with *Gongronella* sp. w5 (data from reference 24 and this study). In addition, with the qRT- PCR data indicating that *lcc1* was also induced strongly with *lcc9* here (Fig. 3A),

increased laccase activity in coculture might be produced as a sum effect of both upregulated genes. In phylogenetic analysis, the 17 laccase isozymes of *C. cinerea* branch into four subgroups, with laccases Lcc5 and Lcc9 grouped in a same branch in the same subgroup as Lcc1 [22]. This reinforced the fact that although the amino acid sequences of these three laccase isozymes are very similar, *lcc9* might be transcribed differently from *lcc5* and might specifically be expressed to deal with ROS. Similarly, upregulated *lcc1* might also contribute to ROS detoxification. In contrast to the promoter of *lcc1* and *lcc5*, one motif TCTAGA was found in the promoter region of *lcc9* (position −180 to −175), which is confirmed in *C. albicans* by CHIP-Seq as a binding motif of Skn7 [78], suggesting possible regulation of *lcc9* in *C. cinerea* by Skn7 when expressed under ROS.

Previously, Kombrink et al. (2019) reported transcriptome studies of *C. cinerea* homokaryon AmutBmut challenged by the bacteria *Bacillus subtilis* and *Escherichia coli*, with three laccase genes being overexpressed, which are *lcc9*, *lcc10*, and *lcc14* (JGI AmutBmut IDs 488706, 502564, and 208308) [82]. The activating principle is secreted by the bacteria into the medium and is, as here, the presence of *Gongronella* sp. w5, also inducing expression of the antibacterial lagopodin gene cluster with genes *cox1*, *cop6*, and *cox2* [36, 82]. Of these genes in *C. cinerea*, other than *lcc9*, the promoters of *lcc10* (position −284 to −279) and *cop6* (position −389 to −384) contained a TCTAGA sequence. The *lacc2* gene of *P. eryngii* var. *ferulae* (JGI ID 1521536) induced by the antioxidant β-carotene in cultivation with *R. mucilaginosa* in the absence of ROS stimulation, in contrast, has no such motif in its promoter, but it possesses a stress STRE element CCCCT (position −249 to −245) [70] to which in *S. cerevisiae* the zinc finger transcription factors Msn2 and Msn4 bind [83]. *P. ostreatus* has the same STRE element in its *lacc2* promoter (JGI ID 1067328) but none in the *lacc10* promoter (JGI ID 1089723), and both have no TCTAGA sequence, while both were overexpressed in the confrontation with *D. squalens* [69]. Despite oxidative stress, also neither of the laccase genes induced by fungal confrontations in *D. squalens* (*Dslcc11*, JGI ID 110823; *Dslcc5*, JGI ID 169869) contain a TCTAGA sequence but *Dslcc5* has a STRE element in its promoter (position −39 to −35). Finally, STRE elements are also found in the promoter of gene *cox1* (position −59 to −63) in *C. cinerea* as well as in the promoters of genes *clp1* (positions −143 to −138 and −98 to −102) and *CC1G_04209* (positions −211 to −207 and −205 to −201) in the coprinoferrin siderophore gene cluster, whereas the promoter of the upregulated *cpl2* gene contains a TCTAGA sequence (−138 to −133). In *S. cerevisiae*, Skn7 with some overlap in gene regulatory function may bind as Hsf1 to a heat shock element (HSE) AGAAnnTTC [58], but none of the basidiomycete genes discussed here had such sequence in their promoters. Summing up, a clear picture on stress regulation in expression of laccase genes in confrontations of different fungal species and usage of potential transcription factor binding sites is not evolving.

By the increased expression of *skn7* in interspecies interaction and a target site for Skn7 in the promoter region of *lcc9*, the oxidative stress factor Skn7 in *C. cinerea* is likely to directly participate in specific *lcc9* activation while this might not be the same case for the overexpressed laccase genes in fungal confrontations of other species. A recent literature overview on fungal *skn7* deletion mutants of various species indicates that the Skn7 transcription factor variably effects tolerance to oxidative and sometimes also osmotic stresses, cell wall integrity, hyphal growth, and asexual sporulation of different fungal species [84]. Many of these phenotypes reoccurred here in *C. cinerea* in interspecific interactions with *Gongronella* sp. w5 and in the *lcc9* silencing clones. Suggested from the results of the combined HBA-NAC treatment of *C. cinerea* single species cultures (Fig. 4C), compound HBA as an inducer of gene *lcc9* expression might act indirectly on the promoter in *C. cinerea* in a complex regulation network involving oxidative stress. Two potential benzoate-response elements were reported for *Aspergillus niger*, i.e., TAGGTAGCC and TAGTCA [85, 86]. These were neither found in the promoter of gene *lcc9* nor in the promoter of gene *skn7*.

**Lcc9 and ROS in fungal growth and morphology.** Lcc9 was expressed and secreted to the extracellular environment to relieve the oxidative stress caused by

secondary compounds from *Gongronella* sp. w5 and to then also lower the intracellular ROS level here (Fig. 5 and 6 and Fig. S3). Actually, maintenance of ROS detoxification is paramount for fungi. Several intracellular and extracellular defense mechanisms have been evolved to scavenge intracellular ROS [27]. Among them, laccases are seen as a resistant approach of some ascomycetes and basidiomycetes to organic compounds. In fact, gene deletion in the ascomycete *P. anserina* indicates constitutive laccases to play a protective role against aromatic compounds and $H_2O_2$ [17]. Expression of a heterologous laccase in *Pichia pastoris* increases the resistance of the yeast to $H_2O_2$-mediated oxidative stress by stimulating the activity of the glutathione-based antioxidative system (including glutathione, glutathione peroxidase, $\gamma$-glutamylcysteine synthetase, and glutathione reductase) [16]. In addition, fungal vacuoles are acidic compartments containing abundant hydrolases and lipases, and are involved in storage, osmoregulation, homeostasis, and detoxification [87]. According to the fact that endogenous ROS is associated with vacuole fragmentation in *S. cerevisiae* [88, 89], *lcc9* silencing leading to vacuole fragmentation might be attributed to the increased ROS level in *C. cinerea* (Fig. 8).

Several studies have suggested a clear link between laccases and fungal morphology and growth. For example, deletion of laccase-like fungal pigment multicopper oxidase genes in the human pathogenic *A. fumigatus*, the insect pathogenic *Metarhizium anisopliae*, and the dung fungus *P. anserina* all affect the pigment synthesis [17, 90, 91]. Laccases in *Cryptococcus neoformans* and *L. edodes* are associated with tension, thickness, and strength of cell walls [20, 92]. Moreover, it has been reported that laccase overexpression affects mycelial growth in *T. versicolor* [93], *P. ostreatus* [94–96], *H. marmoreus* [21], and *Trichoderma viride* [97]. Our research on *C. cinerea* suggested that Lcc9 facilitated reasonable fungal growth during cocultivation with *Gongronella* sp. w5 and under extracellular ROS stress (Fig. 6 and 7, and Fig. S1), provoked hyperbranching with bunches of multiple short side branches on parental hyphae in the wild-type when stressed by *Gongronella* sp. w5, helped in cellular morphology by maintaining vacuole and cell wall structures, and supported constitutive asexual sporulation (Fig. 7 and 8, Fig. S2 and S3).

Because laccase *lcc9* by its pattern of activation only functions in coculture, the opposing phenotypic effects seen in wild-type and *lcc9* silencing strains might be related to ROS regulation. In fact, the ability of ROS to influence cell morphology, disturb cell wall integrity, and affect cell growth is demonstrated in *C. albicans* [98]. For growth in *P. anserina*, the assimilation of complex plant biomass needs the production of external $H_2O_2$, and it has been shown that catalases are required in the protection of the fungal cells during this process [99]. Our work revealed that ROS was produced excessively in *C. cinerea* in the interspecific interaction with *Gongronella* sp. w5. After silencing *lcc9*, the levels of ROS and $H_2O_2$ were gradually increased (Fig. 6), then the cellular accumulation of ROS will have caused oxidative damage with harm to the growth and development of *C. cinerea* mycelium.

## MATERIALS AND METHODS

**Fungi and culture media.** *C. cinerea* Okayama 7 (#130; *A43*, *B43*, *ade8*) (ATCC No. MYA-4618) and *Gongronella* sp. w5 (China Center for Type Culture Collection No. AF2012004) were maintained on YMG agar (yeast malt glucose; per liter, 4 g yeast extract, 10 g malt extract, 4 g glucose, and 15 g agar) or PDA (potato dextrose agar; per liter, filtrate of 200 g boiled potato, 20 g glucose, and 15 g agar) plates at 4°C according to Pan et al. [24]. All culturing of fungi was done at 37°C.

**Classical mixed coculture, separated coculture, and chemical applications to cultures.** Classical mixed coculture and separated coculture were performed according to Hu et al. [25] in SAHX (sucrose DL-asparagine XH; per liter, 15.0 g sucrose, 1.5 g DL-asparagine, 1.0 g $KH_2PO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $Na_2HPO_4 \cdot 5H_2O$, 10.0 g $CaCl_2$, 1.0 mg $FeSO_4 \cdot 7H_2O$, 28.0 mg adenine, 2.0 mg $CuSO_4 \cdot 5H_2O$, and 50.0 $\mu$g vitamin $B_1$) medium using sucrose as the carbon source. In all classical mixed coculture experiments in this study, *C. cinerea* was always first inoculated and cultivated for 36 h before addition of homogenized *Gongronella* sp. w5 mycelium (2.5%, vol/vol), and the time zero h of cocultivation refers to the start of coculture or the 36 h of initial cultivation. For separated coculture, *Gongronella* sp. w5 were sealed in dialysis tubes (molecular mass cutoff of 50 kDa), which were immersed in flasks to avoid direct mycelial contact of the two species. When *p*-hydroxybenzoic acid (HBA, Sigma, St. Louis, MO, USA) was employed to induce laccase expression in single-species culture of *C. cinerea*, it was added at the final

concentration of 0.1 mM (solved in ethanol) at 0 h to liquid shaken SAHX cultures of *C. cinerea* (5% inoculum, vol/vol) that had been precultured for 36 h with 7.5 g/L fructose instead of sucrose as the carbon source. When *N*-acetyl-L-cysteine (NAC, Sigma, St. Louis, MO, USA) was used to remove the reactive oxygen species (ROS) in *C. cinerea* in separated coculture with *Gongronella* sp. w5 or when cultured alone with HBA induction, 10 mM final concentration was added at 0 h of cocultivation or initial cultivation, respectively. Cultures were incubated for another 4–5 d and laccase activity and the intracellular ROS levels were regularly assayed every 12 h. For stress tests with chemicals, 100 mM $H_2O_2$ (Sangon Biotech, Shanghai, China), 2 mM guaiacol (Sigma, St. Louis, MO, USA), or 0.5 mM 1,3-dihydroxynaphthalene (Sangon Biotech, Shanghai, China), which were secreted by *Gongronella* sp. w5 (Table S2) and effective in stress induction in *C. cinerea* in preliminary experiments, were added to separated cocultures of *C. cinerea* and *Gongronella* sp. w5 at 36 h of cocultivation, and the intracellular ROS levels and biomasses of *C. cinerea* were detected within 36 h of further cocultivation, respectively. The experiments were performed three times, always with triplicate cultures per test case.

**Measurement of *C. cinerea* biomass from cocultures.** After removing *Gongronella* sp. w5 from the dialysis bags, the total liquid cultures of *C. cinerea* in the flasks were filtered through Whatman No. 1 filter paper (GE Healthcare, Hangzhou, China). The *C. cinerea* mycelia were washed two times with distilled water, transferred into pre-weighed weighing bottles, and for measurements dried in an oven at 105°C to a constant weight.

**C. cinerea transcriptome sequencing.** For comparative transcriptome analysis, wild-type *C. cinerea* mycelia were harvested from the culture liquid after 18 h and 28 h in separated cocultivation with *Gongronella* sp. w5, respectively. The mycelia of *C. cinerea* were homogenized using glass beads in a freezing grinding instrument JXFSTPRP-CL (Jingxin Co., Ltd., Shanghai, China), and the total RNA of *C. cinerea* was extracted using the TRIzol reagent (Thermo Fisher, Catalogue No. 15596026) according to the manufacturer's protocol. The integrity and quantity of the isolated RNA were assessed on a NanoDrop spectrophotometer (Thermo Fisher, Waltham, MA, USA). RNA library preparation was carried out by poly(A)-selection of mRNA, cDNA synthesis, adenylation of 3′ ends, adapter ligation, DNA fragment enrichment, and purification. Library quality check was performed using the Agilent high sensitivity DNA assay on a Bioanalyzer 2100 system (Agilent, CA, USA). The library was then sequenced on NovaSeq 6000 platform (Illumina) by Shanghai Personal Biotechnology Co. Ltd. Transcriptome sequencing was performed for three independent samples per time point.

**Differentially expressed gene (DEG) calculation and analysis.** Raw sequencing data were filtered by deleting the sequences containing connectors and low quality reads using Cutadapt (v1.15) [100]. The clean reads were then mapped to the reference genes of *C. cinerea* Okayama 7 (GenBank, accession No. AACS00000000) [101] using HISAT2 (v2.0.5) [102]. HTSeq (v0.9.1) statistics was used to compare the Read Count values on each gene as the original expression of this gene, and then FPKM (Fragments Per Kilobase per Million) was used to standardize the expression [103]. Then, gene expression differences were analyzed by DESeq (v1.30.0) with $|log_2FoldChange| > 1$ and false discovery rate (FDR) $< 0.05$ [104]. In a few cases, DEGs with a threshold of $|log_2FoldChange| > 0.75$ or $< -0.75$ were considered. At the same time, R language Pheatmap (1.0.8) software package was used to perform bi-directional clustering analysis of all different genes of samples. Heatmap was gated according to the expression level of the same gene in different samples and the expression patterns of different genes in the same sample with Euclidean method to calculate the distance and Complete Linkage method to cluster. Go enrichment analysis was performed with topGo (v3.2), and the *P* value was calculated by hypergeometric distribution method. GO terms with a *P* value $\leq 0.05$ were considered significantly enriched. ClusterProfiler (v3.4.4) software was used to carry out the enrichment analysis of the KEGG pathway of differential genes, focusing on the significant enrichment pathway with *P* value $<0.05$.

**Quantitative reverse transcription-PCR (qRT-PCR) analysis.** To confirm results from the transcriptome analysis, 15 genes were randomly selected for qRT-PCR verification using a SYBR green kit (TaKaRa, Dalian, China) on a Roche LightCycler 96 real-time PCR system (Roche, Basel, Switzerland) according to the manufacturer's instructions (primers for qRT-PCR analysis are shown in Table 1). Three biological replicates and three technical replicates per sample were included for each cDNA template. Then, the relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [105]. In order to normalize the qRT-PCR data, five genes, including *β-actin*, *capzb* (F-actin capping protein beta subunit), *α-tub* (tubulin), *cyt-c* (cytochrome c), and *ubc* (ubiquitin) were chosen for their stable expression levels across different time points. They were classified and screened according to their suitability as internal standards using the NormFinder (https://www.moma.dk/normfinder-software/) and BestKeeper (https://www.gene-quantification.de/bestkeeper.html) algorithms [106, 107]. The most stable transcribed gene was chosen as endogenous reference gene.

Further, qRT-PCR analysis for *lcc1*, *lcc5*, *lcc9*, and *skn7* transcription was also done from separated cocultures at the indicated times using the same protocol as described above with the primers listed in Table 1.

**Intracellular ROS levels and $H_2O_2$ assay.** Intracellular ROS levels were detected using the fluorogenic probe DCFH-DA (2, 7-dichlorodihydrofluorescein diacetate) [108] according to the manufactural instructions (Beyotime Biotech, Shanghai, China). *C. cinerea* cells in liquid cultures were harvested at certain time points, washed twice with PBS (phosphate-buffered saline), resuspended in 10 $\mu$M DCFH-DA in PBS, and incubated at 37°C for 20 min in the dark. After PBS washing, *C. cinerea* hyphae were ground to a fine powder under liquid nitrogen and mixed with 1 ml cold PBS buffer. A total of 200 $\mu$l supernatant was measured with excitation and emission wavelengths of 488 nm and 525 nm, respectively, on a SpectraMax M5 instrument (Molecular Devices, San Jose, CA, USA). All measurements were performed in triplicate.

Intracellular $H_2O_2$ concentration measurements were analyzed in 96-well plates (Thermo Fisher, Waltham, MA) using an $H_2O_2$ detection kit (Beyotime Biotech, Shanghai, China) in which $Fe^{2+}$ could be oxidized, formed purple product with xylenol orange and be detectable on a SpectraMax M5 instrument

**TABLE 1** Primers used in this study[a]

| Name | Sequence (5′–3′) | Purpose |
|---|---|---|
| lcc9-antisense1-fwd | CTCCCATCTACACACAACAAGCTTATCGCCCGGGATTCTCATAGTTGTAAGTGCTGC | Cloning of lcc9 antisense fragment 1 |
| lcc9- antisense 1-rev | CACTGGCCCTCTGGTCAACTATAATATTATAGATGGGCCTTGGACCTGCCG | Cloning of lcc9 antisense fragment 1 |
| lcc9- antisense 2-fwd | CTCCCATCTACACACAACAAGCTTATCGCCCGGACCCACTTCCTCCTGGGGCA | Cloning of lcc9 antisense fragment 2 |
| lcc9- antisense 2-rev | CACTGGCCCTCTGGTCAACTATAATATTATCTCTCATGGTCGACGAAATCCAGATC | Cloning of lcc9 antisense fragment 2 |
| PF | ACATCCACCATCTCCGTTTTCTCCCAT | PCR of co-transformants |
| PR | TGACTATAGCAGCCTCCTACCACTG | PCR of co-transformants |
| qRT-13048-F | CTCTGGAGTTATGGTAGGAATGGGC | qRT-PCR of $\beta$-actin |
| qRT-13048-R | GATGCCATGTTCGATGGGGTACTTG | qRT-PCR of $\beta$-actin |
| qRT-00146-F | CCGCCAACAACTATGCCCGTGGTCA | qRT-PCR of $\alpha$-tubulin |
| qRT-00146-R | TCCTCCACCGAAGGAGTGGAAGACG | qRT-PCR of $\alpha$-tubulin |
| qRT-10735-F | TTCGCTCCTGGTGACGCTTCTA | qRT-PCR of cyt-c |
| qRT-10735-R | GCCGAAGATGCCGAAGAGGTTG | qRT-PCR of cyt-c |
| qRT-11833-F | AAGGCGAAGATTCAGGACAAGGAAGGTA | qRT-PCR of ubc |
| qRT-11833-R | CGAAGGCGGAGAACCAAGTGAAGAGTA | qRT-PCR of ubc |
| qRT-07869-F | GAAGAAGACTCTCACTCCGTCAACG | qRT-PCR of capzb |
| qRT-07869-R | CTGTGTGGTGAGCTGGAGCATGA | qRT-PCR of capzb |
| qRT-lcc9-F | ATGTCCAGGAAACTTTTCTCTCTCG | qRT-PCR of lcc9 |
| qRT-lcc9-R | ATGTTCGAGACCGTCATGGTACT | qRT-PCR of lcc9 |
| qRT-07201-F | AGCGCAAGCTGACCCCTTGGT | qRT-PCR of sga1 |
| qRT-07201-R | ACAAGTCCAGCCGCTGCCCC | qRT-PCR of sga1 |
| qRT-05652-F | CAAGGATCGACGGAGGGCGCA | qRT-PCR of tkl |
| qRT-05652-R | TCCACTTCCGAGCCGCCACC | qRT-PCR of tkl |
| qRT-02494-F | AGCAAGGTGGATGTCCACGAGTCC | qRT-PCR of vwfa |
| qRT-02494-R | GGCCACTGCCAGCGACTTGGT | qRT-PCR of vwfa |
| qRT-06452-F | TACGCTGGAATGGACGCCGGA | qRT-PCR of exg |
| qRT-06452-R | TGCCGCGGGCCCAAAAGTTCTTC | qRT-PCR of exg |
| qRT-03564-F | CACCTTCGCTTGTTCTACGGATCCA | qRT-PCR of cyp98A3 |
| qRT-03564-R | TCAACCCAGAAGCGTCCGGGAATAC | qRT-PCR of cyp98A3 |
| qRT-05291-F | CTAAGCAGCATAATGTGAAAGCCCT | qRT-PCR of Chi |
| qRT-05291-R | GGAAATTCCCAGTCAAAGTCGATACCA | qRT-PCR of Chi |
| qRT-11387-F | ATCGAGATGATCGAGCATGTCGGC | qRT-PCR of cfa |
| qRT-11387-R | TGATGACAGCGGCGGCCTTTTG | qRT-PCR of cfa |
| qRT-08324-F | TGCAGAGGAAGAGCGACCTCGGA | qRT-PCR of samB |
| qRT-08324-R | CCAGATGCTGAGAGGGAAACGGAGG | qRT-PCR of samB |
| qRT-09241-F | CCGGATTTGAAGACGATCAAGAGCTT | qRT-PCR of psd |
| qRT-09241-R | GAGAAGGTACATGGGAACGCCGATAG | qRT-PCR of psd |
| qRT-09422-F | TTATCGCATACTCGCCCCCTCGGA | qRT-PCR of akr |
| qRT-09422-R | AAGGCATCGACGATTGCCATGTTG | qRT-PCR of akr |
| qRT-14829-F | TGCTGGAGAGCAAGGTTGTGTCTCA | qRT-PCR of gst |
| qRT-14829-R | AAGGGAATGGTGTAGTGGGGCGAGC | qRT-PCR of gst |
| qRT-05005-F | GCCATCCGCTCCAGTGCCTTCAAT | qRT-PCR of cgl-II |
| qRT-05005-R | GCGGATGCTGATGTGGAGGAGGATG | qRT-PCR of cgl-II |
| qRT-02499-F | CAGTCAGAGAGTTGCCTATACTCTCA | qRT-PCR of tdo |
| qRT-02499-R | GGATGATGGTGGCAGAGAGTGAATG | qRT-PCR of tdo |
| qRT-07229-F | CATCTTAGCTGGCTCGGCTTCTTC | qRT-PCR of nrt |
| qRT-07229-R | ACGATGTTGGAGTTGGCGACTTCG | qRT-PCR of nrt |
| qRT-lcc1-F | ATGTTCAAGAACCTCCTCTCGT | qRT-PCR of lcc1 |
| qRT-lcc1-R | ACGTTCGCGTTGGTGAGGGTCAT | qRT-PCR of lcc1 |
| qRT-lcc5-F | ATGTCGTTTGCTTGGAAAGCAT | qRT-PCR of lcc5 |
| qRT-lcc5-R | TGGTCATAGTATCTTGGTTGCCAAT | qRT-PCR of lcc5 |
| qRT-08281-F | TGTACAAGATGCTCGAAGACCCA | qRT-PCR of skn7 |
| qRT-08281-R | TTCATGTCCTTTACGACGAAACAGTC | qRT-PCR of skn7 |

[a]Five candidate reference genes chosen were $\beta$-actin, capzb (F-actin capping protein beta subunit), $\alpha$-tub (tubulin), cyt-c (cytochrome c), and ubc (ubiquitin). Fifteen randomly chosen genes for qRT-PCR to validate gene expression data in the RNA-seq experiment were lcc9 (laccase 9), sga1 (glucoamylase), tkl (TKL/TKL-ccin protein kinase), vwfa (VWFA domain protein), exg (glucan 1, 3-beta-glucosidase), cyp98A3 (cytochrome P450 98A3), Chi (endochitinase), cfa (cyclopropane-fatty-acyl-phospholipid synthase), samB (MYND-type Zinc finger protein), psd (phosphatidylserine decarboxylase), akr (aldo/keto reductase), gst (glutathione S-transferase), cgl-II (galectin-2), tdo (tryptophan 2,3-dioxygenase), and nrt (nitrate transporter).

at wavelengths of 560 nm. *C. cinerea* cells were harvested, washed twice with PBS, ground to a fine powder in liquid nitrogen, and mixed with 1 ml cold PBS buffer. After that, 50 $\mu$l supernatant was mixed with 100 $\mu$l $H_2O_2$ detection reagent and incubated for 30 min at room temperature before testing. The concentrations of $H_2O_2$ in the samples were calculated according to a standard curve obtained by testing the absorbance of different dilutions of $H_2O_2$.

**Laccase assay.** Aliquots of culture broth from classical mixed coculture, separated coculture, and single-species culture systems were withdrawn every 12 h. Laccase activity was determined with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) (0.5 mM) as the substrate according to Bourbonnais and Paice [109]. One activity unit (U) was defined as the amount of laccase required for oxidizing 1 $\mu$mol of ABTS per minute. Reactions with heat-treated enzyme samples (5 min, 100°C) were used as control.

Activity staining of laccases was performed in 100 mM citrate-phosphate buffer (pH 4.0) after poly-acrylamide gel electrophoresis (PAGE) using ABTS as the substrate, according to Pan et al. [24].

**Data availability.** Transcriptomic data are publicly available in National Center for Biotechnology Information (NCBI) under BioProject number PRJNA755756. The data sets generated/analyzed during the present study are available and included either within this article or in the supplemental material. The DNA sequence of the 18S rRNA gene for molecular identification of *Gongronella* sp. w5 was previously deposited in the GenBank database (accession no. EU253966) [110]. In addition, the GenBank accession numbers of the genome sequence of *C. cinerea* Okayama 7 [101] and *Gongronella* sp. w5 [111] were AACS00000000 and GCA_001650995.1, respectively.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.
**SUPPLEMENTAL FILE 1**, PDF file, 0.7 MB.
**SUPPLEMENTAL FILE 2**, XLSX file, 0.1 MB.

## ACKNOWLEDGMENTS

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