A single laccase acts as a key component of environmental sensing in a broad host range fungal pathogen
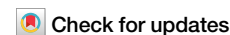
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Secreted laccases are important enzymes on a broad ecological scale for their role in mediating plant-microbe interactions, but within ascomycete fungi these enzymes have been primarily associated with melanin biosynthesis. In this study, a putatively secreted laccase, *Sslac2*, was characterized from the broad-host-range plant pathogen *Sclerotinia sclerotiorum*, which is largely unpigmented and is not dependent on melanogenesis for plant infection. Gene knockouts of *Sslac2* demonstrate wide ranging developmental phenotypes and are functionally non-pathogenic. These mutants also displayed indiscriminate growth behaviors and enhanced biomass formation, seemingly as a result of their inability to respond to canonical environmental growth cues, a phenomenon further confirmed through chemical stress, physiological, and transcriptomic analyses. Transmission and scanning electron microscopy demonstrate apparent differences in extracellular matrix structure between WT and mutant strains that likely explain the inability of the mutants to respond to their environment. Targeting *Sslac2* using host-induced gene silencing significantly improved resistance to *S. sclerotiorum*, suggesting that fungal laccases could be a valuable target of disease control. Collectively, we identified a laccase critical to the development and virulence of the broad-host-range pathogen *S. sclerotiorum* and propose a potentially novel role for fungal laccases in modulating environmental sensing.

Laccases are a broadly conserved class of multicopper oxidase (MCO), which are known for their capacity to oxidize otherwise recalcitrant phenolic compounds, both directly and indirectly through the activity of mediators[1]. While these enzymes have garnered industrial interest in recent years as drivers of xenobiotic bioremediation, they play a pivotal role in development across a range of species and can be found in the genomes of plants, animals, fungi, oomycetes, and bacteria[1–3]. One of their most important roles in natural ecosystems is to mediate the interplay between lignin production and degradation in interactions between plants and fungi. Higher plants utilize laccases in the biosynthesis and polymerization of lignin, which acts as a structural compound facilitating plant development and helps to defend plant tissue in response to pathogenesis[3]. Intriguingly, many wood rot fungi similarly use secreted laccases in the degradation of lignin, in which terminal phenolic lignin is directly oxidized by laccases and non-phenolic lignin is degraded through the activity of mediators, which are oxidized by the enzyme[4]. This interplay between production and degradation is critical to carbon cycling in the environment and the maintenance of healthy soils.

Unlike wood rot fungi, which are almost exclusively basidiomycetes, laccase activity within ascomycete fungi is typically associated with melanin/pigment deposition on fungal tissue, with most laccase knockout mutants within the phylum demonstrating reduced pigmentation[5–11]. The best studied examples of such laccases are *Abr2* and *ya*, from *Aspergillus fumigatus* and *Aspergillus nidulans*, respectively, which function in melanization through the polymerization of phenolic monomers into mature melanin in fungal cell walls[5]. Intriguingly, the effect on fungal development and growth within these various knockouts is highly variable, likely due to the expanded repertoire of laccases and possible functional redundancy[2]. This redundancy

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has been suggested by the broad substrate overlap observed within individual species' laccase repertoires and demonstrated in mutants of the *Cochliobolus heterostrophus* laccase *ChMCO1*, in which mutant pigmentation defects could be complemented through the chemical induction of other laccases in vitro[11,12]. In the plant pathogen *Fusarium oxysporum* f. sp. *lycopersici*, multiple distinct laccases were knocked out, and while some differences in sensitivity to oxidative/chemical stress could be measured, no clear changes to development or virulence were observed[13].

In some cases, however, roles for specific laccase genes have been noted, such as in *Colletotrichum gloeosporioides*, *Colletotrichum orbiculare*, and *Setosphaeria turcica* where individual laccase gene knockouts result in reduced virulence on host plants[6-8]. These laccases and the laccase *Mlac1* from the entomopathogenic fungus *Metarhizium anisopliae* are also deficient in appressorium formation[6-8,10]. Given that these laccases are almost always associated with pigmentation in ascomycetes, it has broadly been assumed that a lack of melanin/pigment deposition is driving the wide variety of phenotypes observed in knockouts, although a causal relationship has never been confirmed[7-9]. It is known, however, that laccase-mediated melanization is not a general requirement for pathogenicity given the unpigmented nature of the entomopathogenic fungus *Beauveria bassiana*. Laccases studied within this species appear highly involved in virulence, through either scavenging insect immune response-generated reactive oxygen species or the biosynthesis of the secondary metabolite oosporein[14-16].

Sclerotinia sclerotiorum is a broad-host-range pathogen of dicotyledonous plants and is distinct from many of the previously mentioned fungi in that its melanin biosynthesis pathway has been relatively well studied. Three genes, encoding a scytalone dehydratase (*SCD1*), a trihydroxynaphthalene reductase (*THR1*), and a polyketide synthase (*PKS13*), with putative upstream roles in melanin biosynthesis have been knocked out and characterized[17,18]. Although mutants were deficient in melanization of either sclerotia (*SCD1* and *THR1*) or compound appressoria (*PKS13*), no changes in lesion size were observed, suggesting that melanin deposition does not significantly alter *S. sclerotiorum* virulence[17,18]. This has a parallel in the closely related species *Botrytis cinerea* in which melanogenic genes are dispensable for proper growth and virulence[19]. Of note, the *B. cinerea* laccase Bclcc2 is broadly believed to be involved in the oxidation of environmental antimicrobials[20,21]. In this study we sought to characterize a *S. sclerotiorum* laccase gene, *Sslac2*, which is the putative ortholog of the *B. cinerea* laccase *Bclcc2*, and elucidate the role of this gene in fungal development and host-microbe interaction. Unlike Bclcc2, which lacks a clear role in development or virulence, our data shows that Sslac2 is crucial for the pathogen's environmental sensing, as Δ*Sslac2* strains exhibit significant alterations in development, infection, and response to environmental cues[21]. This is unlike other fungal systems, such as *C. orbiculare*, where orthologous laccases between related species have been shown to be functionally interchangeable[8]. We also discuss the developmental and virulence phenotypes seen in Δ*Sslac2* mutants, demonstrate the potential for targeting fungal laccases to achieve increased resistance in host plants, and consider the broader context of laccases within fungal and pathogen biology.

Results

Sslac2 is the primary laccase expressed during pathogenesis
Some fungal laccases play a role in plant pathogenicity, but fungi typically maintain multiple laccases within their genomes, so an evaluation of the *S. sclerotiorum* laccase repertoire was performed[2]. Genomic analysis identified 7 putative laccases within the *S. sclerotiorum* genome, all of which contained predicted secretion signal domains, but which varied in length and cupredoxin domain architecture (Fig. 1A). While enzyme secretion is typically mediated by the presence of a signal peptide on the N-terminus of a protein, the C-termini of fungal laccases is additionally processed during secretion and is considered critical to laccase activity[22]. The canonical motif mediating this activity is a C-terminal DSGL, and Sslac2-6 contain a conserved DSGx motif in their C-termini, although this is apparently missing in Sslac1 and Sslac7 (Supplementary Fig. 1)[22]. To determine the laccases most important
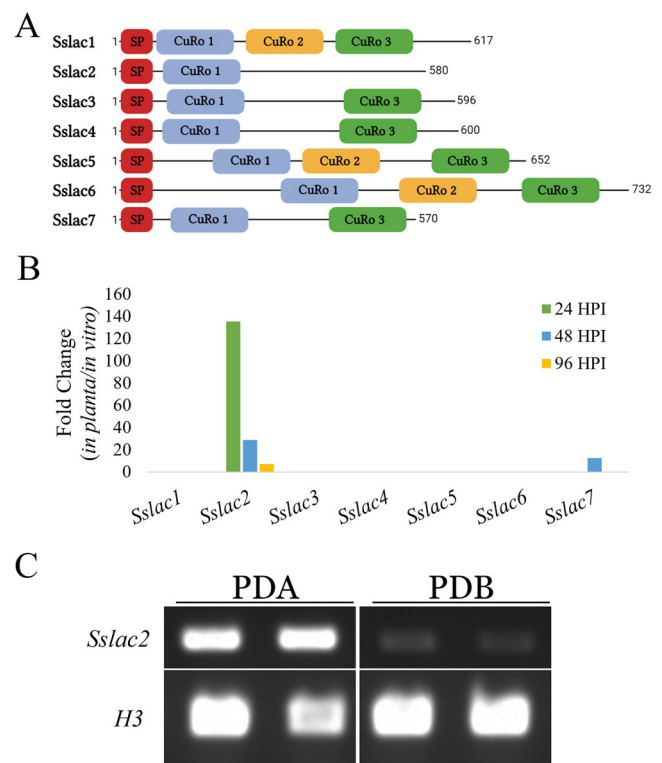

Fig. 1 | Schematic representation and expression of laccase genes in the genome of *S. sclerotiorum*. A Length and domain architecture of the seven putative laccases in the *S. sclerotiorum* genome. SP refers to a predicted secretion signal peptide. CuRo 1, 2, and 3 correspond to the three cupredoxin domains characterized in *Melanocarpus albomyces* (cd13854, cd13901, and cd13880, respectively). **B** Relative transcript fold change of the seven laccases identified in the *S. sclerotiorum* genome during infection of soybean when compared to in vitro culture growth. Fold change values are from the transcriptomic analysis performed in Westrick et al.[23]. **C** RT-PCR of *Sslac2* and Histone 3 (*H3*) expression when grown on Potato Dextrose Agar (PDA) or in Potato Dextrose Broth (PDB).

during *S. sclerotiorum* pathogenesis, a transcriptomic dataset generated from *S. sclerotiorum* infection of soybean at 24, 48, and 96 h post inoculation (HPI) was analyzed to identify which homologs were upregulated *in-planta* compared to an in vitro culture control[23]. Surprisingly, only *Sslac2* appeared to be highly upregulated during infection, particularly in the early stages of disease development (Fig. 1B). Because the onset of infection requires the pathogen to interact with plant surfaces, it was considered whether any *S. sclerotiorum* laccases were distinctly upregulated on solid surfaces. To examine this, a separate transcriptomic dataset was analyzed from *S. sclerotiorum* grown on potato dextrose agar (PDA) or in potato dextrose broth (PDB), as these two media are nearly identical outside of surface rigidity produced by the agar[24]. Similar to pathogenesis, *Sslac2* appeared to be the primary laccase upregulated (Supplementary Fig. 2). This upregulation was additionally validated through RT-PCR (Fig. 1C). *In toto*, these data indicate that *Sslac2* is likely upregulated upon deposition of the pathogen on plant material or other inducive surfaces.

Laccase and sclerotial production are abolished in Δ*Sslac2* knockout mutants
To evaluate the role of Sslac2 in disease and development, a CRISPR-Cas9 assisted method was used to generate two independent *Sslac2* gene knockouts. Surprisingly, Δ*Sslac2* strains completely lost the ability to produce sclerotia, and this was accompanied by an increase in the formation of aerial hyphae (Fig. 2A). Laccase production of these mutants was also evaluated by growing WT (1980) and mutant strains on media containing 0.2 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) or tannic acid and evaluating pigment production, as oxidation by laccases is
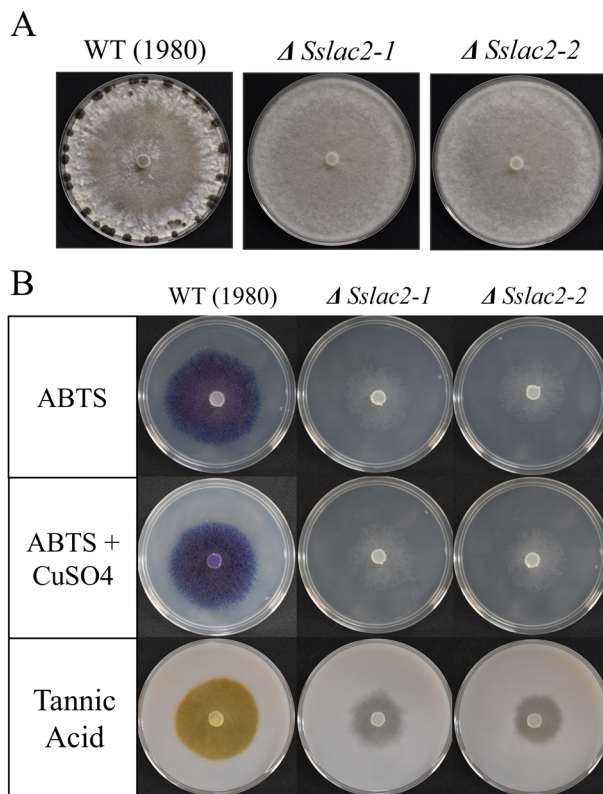
Fig. 2 | General phenotype and laccase production of WT (1980) and laccase mutants. A WT and mutant strains 2 weeks after inoculation of PDA plates. **B** WT and mutant strains grown for 24 h on PDA supplemented with 0.2 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 0.2 mM ABTS + 0.6 mM CuSO$_4$, or 2.5 mg/ml tannic acid.

known to generate a violet/blue pigment from ABTS and a brown pigment from tannic acid[6,21]. These pigments were clearly observed in plates growing the WT (1980) strain but were completely absent in the mutants (Fig. 2B). All strains were additionally grown on ABTS and copper sulfate (CuSO$_4$) in an attempt to complement this laccase deficiency through the activation of other laccase homologs, as CuSO$_4$ has been shown to induce laccase production in related species[11,25]. The addition of CuSO$_4$ did not appear to increase laccase production in either of the knockout mutants, suggesting that Sslac2 is the primary secreted laccase utilized by *S. sclerotiorum* during growth and development on solid surfaces (Fig. 2B).

Sslac2 expression affects fungal tropism

Initial observations of *ΔSslac2* strains indicated a growth defect, as reduced radial growth could be observed on PDA (Fig. 3A). In contrast, when grown in liquid culture on PDB *ΔSslac2* mutants grew to a significantly higher mass than WT (1980), suggesting that the initial growth defect was specific to radial growth (Fig. 3B). A clear increase in aerial hyphae was noted in older cultures of *ΔSslac2* (Fig. 2A), so *ΔSslac2* and WT (1980) strains were grown for 2 weeks to assess whether the mutants were growing downward into the agar as well. At 2 weeks post inoculation (WPI), WT hyphae rested in a thin layer atop the agar, whereas *ΔSslac2* strains visibly penetrated the agar surface (Fig. 3C). Remarkably, when grown on split plates, *ΔSslac2* strains grew over the high barrier separating the plate sections and continued to grow into the neighboring chamber (Supplementary Fig. 3). When confronted with such barriers, the WT strain typically enters dormancy and produces survival structures (Supplementary Fig. 3). Thus, *ΔSslac2* may be unable to sense the environmental triggers leading to dormancy and the production of sclerotia.

To assess if the agar penetration and slow radial growth phenotypes were caused by defects in directional growth or a failure to respond to dormancy triggers, all strains were grown on PDA with and without a top layer of sterile cellophane to halt agar penetration. While a significant defect in radial growth was measured on PDA, this defect disappeared when grown on cellophane (Fig. 4). These data and the noted sclerotial phenotype in the mutants suggest that the mutant grows in a random, indiscriminate manner,

Fig. 3 | Phenotypes of WT and mutant strains grown on liquid and solid media. A WT and *ΔSslac2-1* one day after inoculation on 60 mm PDA plates. **B** Hyphal dry weight of WT and mutant strains grown in PDB for 48 h. **C** Cross section of PDA colonized with WT and mutant strains 2 weeks post inoculation (WPI). Statistical analysis utilized a Student's *t*-test on three biological replicates of each strain (***<0.001). Scale bars represent 1 mm. The source data underlying this figure can be found in Supplementary Data 1.
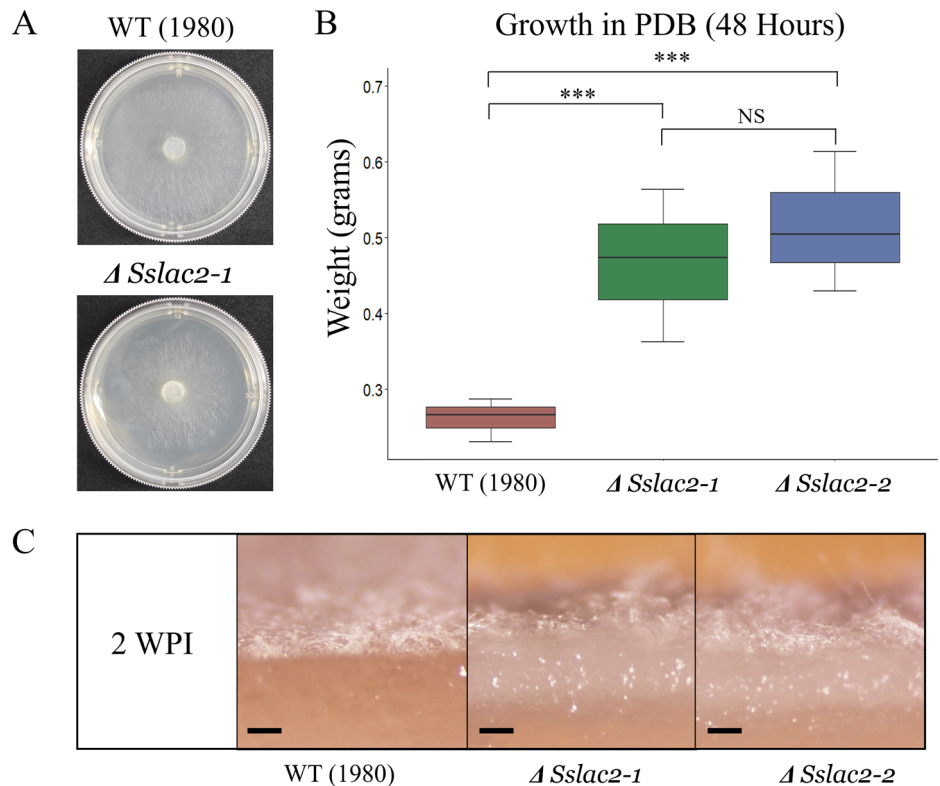
whereas the WT prioritizes radial growth and is able to respond to environmental cues. This suggests that Sslac2 plays a role in hyphal thigmotropism and the recognition of environmental triggers leading to the differentiation of morphological features.

Sslac2 is required for penetration structure formation and oxalic acid (OA) production

Because *Sslac2* is highly upregulated during the early stages of infection (Fig. 1B), we assessed the ability of ΔSslac2 mutants to generate canonical compound appressoria (the penetration structures formed by *S. sclerotiorum* to infiltrate plant tissue). Strikingly, ΔSslac2 mutants generated severely malformed and far fewer compound appressoria when grown on inductive surfaces such as glass slides, suggesting that Sslac2 may be required for proper signaling and differentiation of penetration

structures on host surfaces (Fig. 5A, B). Exogenously applied cAMP has been shown to rescue appressorium defects in G-protein-coupled receptor mutants;[26] however, the addition of cAMP to the glass slides used for compound appressorium production did not rescue this defect in the ΔSslac2 mutants.

After penetrating into host tissue, *S. sclerotiorum* secretes copious amounts of OA, an organic acid that facilitates host tissue acidification as well as directly induces cell death and subverts defenses in the host[27–29]. As OA is the dominant acid secreted by *S. sclerotiorum*, its production can be assessed using bromophenol blue plates which shift from blue to yellow as the medium is acidified. A failure to acidify bromophenol blue plates has been broadly used as a hallmark of OA deficiency in *S. sclerotiorum*, and while ΔSslac2 mutants were capable of acidifying the media to a limited degree, they were clearly deficient relative to WT (1980) (Fig. 5C)[18,30,31].
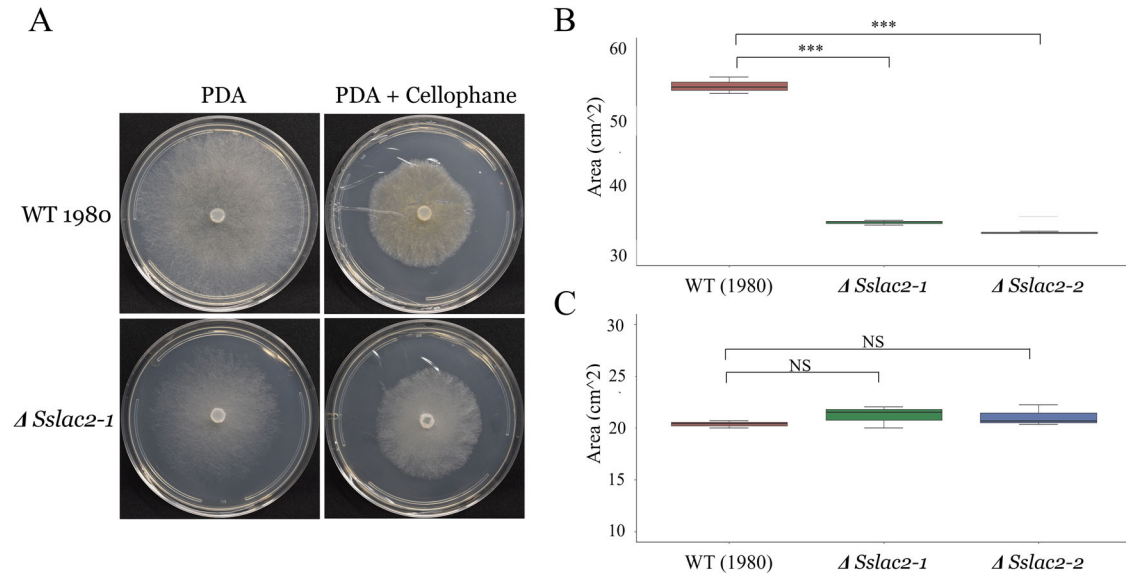

Fig. 4 | Growth of WT and mutants on PDA and cellophane covered PDA. A WT and ΔSslac2-1 strains 48 h after inoculation. **B** Quantification of colony area on PDA. **C** Quantification of colony area on PDA covered in cellophane. Statistical analysis utilized a Student's *t*-test on three biological replicates of each strain (***<0.001). The source data underlying this figure can be found in Supplementary Data 1.

Fig. 5 | Compounds appressorium formation and OA secretion. A Comparison of compound appressorium formation between WT (1980) and ΔSslac2-1 strains. **B** Quantification of canonical compound appressoria and attempted compound appressoria generated by the WT and mutant strains, respectively. **C** Growth of the WT and mutant strains on bromophenol blue plates to qualitatively assess OA production. Statistical analysis utilized a Student's *t*-test on three biological replicates of each strain (***<0.001). Scale bars represent 100 μm. The source data underlying this figure can be found in Supplementary Data 1.
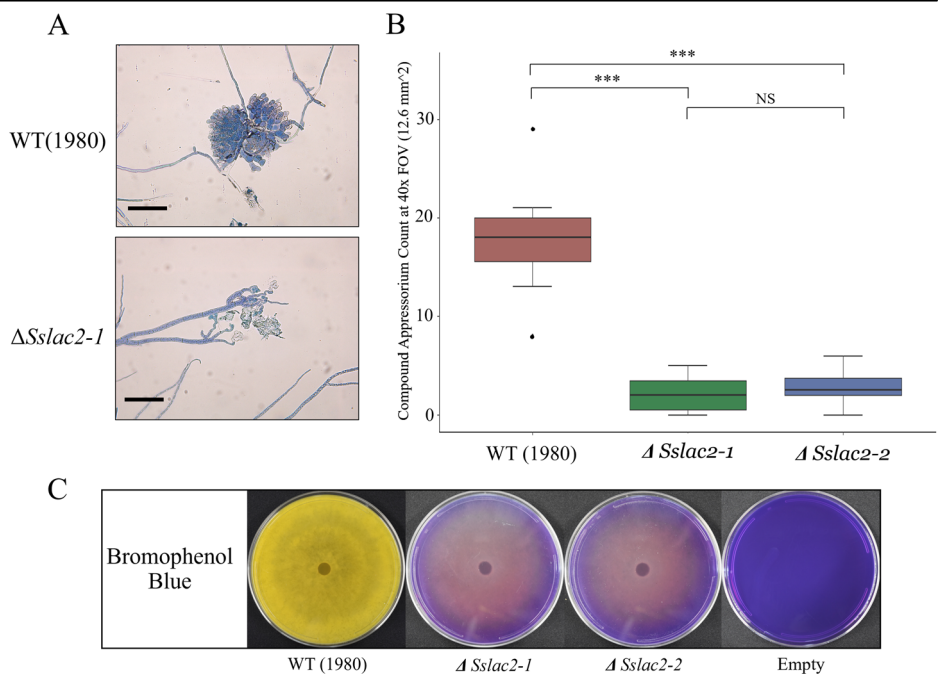
Given the importance of both penetration and OA production in *S. sclerotiorum* pathogenic development, it is unsurprising that these mutants were essentially non-pathogenic (Fig. 6A). To assess whether this defect in pathogenicity was due only to the loss of host penetration, WT and *ΔSslac2* strains were inoculated onto damaged soybean leaves, which provide direct access to the hosts tissues and have been shown in other systems to complement penetration structure defects (Fig. 6B). Additionally, in order to assess the role that OA may be playing in the *ΔSslac2* virulence, mutant strains were compared with a previously described *ΔSsoah1* strain deficient in OA production due to the deletion of oxaloacetate acetylhydrolase, which converts oxaloacetic acid to OA[31]. As expected, WT (1980) was capable of infecting both damaged and undamaged soybean leaves and *ΔSsoah1* colonization was limited to the area around the plant vasculature as previously reported[30]. In contrast, even with damaged tissue, *ΔSslac2* strains were incapable of inducing lesions on soybean leaves, indicating that the previously observed loss of pathogenicity is not solely the result of defects in penetration and acidification (Fig. 6B). Because soybean is a moderately resistant host of *S. sclerotiorum*, we additionally tested *ΔSslac2* infection of the more susceptible host *Nicotiana benthamiana*. No lesions were observed on undamaged leaves, but limited lesions could be seen on damaged leaves, albeit to a far lesser extent than either WT (1980) or *ΔSsOah1* (Fig. 6C). While the *ΔSslac2* strain is able to marginally colonize wounded *N. benthamiana* leaves, overall, it is clear that this mutant's virulence defect extends beyond penetration structures and OA production and likely involves the loss of other virulence factors or an inability to adequately respond to a hostile host environment.

Transcriptional activity of *ΔSslac2* is less responsive to environmental factors

Many of the defects observed in *ΔSslac2* mutants suggest a failure to respond to environmental factors, but it's unknown if this failure is specific to features such as pH and surface recognition or more generalized. To address this question, an RNA sequencing analysis was conducted to compare WT (1980) and *ΔSslac2-1* strains in both defined glucose minimal media (GMM) and GMM with the addition of soybean green stem extract (GrSt) to simulate the presence of plant material. A principal component analysis was conducted to examine the variation in gene expression profiles, and unsurprisingly, a large transcriptional shift was observed in the WT strain with the addition of GrSt, reflecting the pathogen's response to additional proteins, carbohydrates, and plant chemical signals (Fig. 7A). Remarkably, the *ΔSslac2-1* is largely non-responsive to the addition of GrSt (Fig. 7A). This is further visualized by the quantification of differentially expressed genes in *ΔSslac2-1* and WT strains, in which a drastically increased number of genes demonstrate both up- and down-regulation in the WT when exposed to

GrSt (Fig. 7B, Supplementary File 1). To further validate the noted deficiency in OA production by *ΔSslac2* mutants, we analyzed the expression of the critical enzyme *Ssoah1* (Sscle_10g075560), revealing ~160x lower expression than the WT (Fig. 5C; Supplementary Data 2).

ΔSslac2 environmental sensing defect is likely due to alterations in fungal cell wall structure

Laccases within ascomycete fungi have primarily been associated with detoxification of exogenous compounds and cell wall modification, so the sensitivity of *Sslac2* to antifungal plant defense compounds and cell wall stressors was assessed[5,6,8–12,16,21]. WT and *ΔSslac2* mutants were grown on PDA supplemented with different upstream and downstream components of the plant phenylpropanoid pathway[21,32]. *ΔSslac2* mutants were significantly more susceptible to all tested compounds (Supplementary Fig. 4A). This result is somewhat surprising, as the *ΔBclcc2* strain is known to show greater resistance to the phytoalexin resveratrol than WT *B. cinerea*.

To assess the susceptibility of *ΔSslac2* mutants to cell wall stressors, all strains were additionally grown on plates amended with poacic acid, calcofluor white (CFW), or Congo red (Supplementary Fig. 4B)[33]. Both mutants demonstrated significantly greater susceptibility to poacic acid and CFW and moderately increased susceptibility to Congo red (Supplementary Fig. 4B). As this difference in susceptibility could be due to either alterations in hyphal cell wall architecture or a general difficulty in responding to chemical stresses, as is suggested by Supplementary Fig. 4A, we attempted to form protoplasts of both strains using a typical cocktail of cell wall degrading enzymes from *Trichoderma harzianum*. While protoplasts could be efficiently generated from WT (1980) hyphae, *ΔSslac2* hyphae remained largely intact, suggesting that alterations to the hyphal cell wall may be reducing the efficiency of the enzyme cocktail (Supplementary Fig. 4C).

Additional evidence for alterations to cell wall structure can be seen in the assessment of surface hydrophobicity through hyphal wetting, which show dramatically decreased hyphal hydrophobicity in the *ΔSslac2* mutants (Supplementary Fig. 5). This alteration may help to additionally explain the accelerated growth of the mutants in liquid culture, as nutrient exchange through liquid media may be more efficient through a more hydrophilic hyphal cell wall, as was noted in hydrophobin mutants of *Trichoderma* spp[34]. An assessment of the hyphal surface was made with scanning electron microscopy, and while clear differences in the cell surface were difficult to discern, obvious changes to hyphae and hyphal growth patterns were observed (Fig. 8A). WT growth typically consists of thin hyphae branching to evenly disperse across an environment. In contrast, *ΔSslac2* mutant hyphae were noticeably thicker in diameter and often found to grow in bundles (Fig. 8A). This may be due to increasingly hydrophilic hypha

Fig. 6 | Virulence of WT and mutant strains.
A Comparison of WT (1980), *ΔSslac2-1*, and *ΔSslac2-2* during infection of soybean leaves.
B Infection of damaged and undamaged soybean tissue by WT (1980), *ΔSslac2-1*, *ΔSslac2-2*, and *ΔSs-oah1*. **C** Infection of damaged *N. benthamiana* at 48 and 96 h post infection (HPI) by WT (1980), *ΔSslac2-1*, *ΔSslac2-2*, and *ΔSs-oah1*.
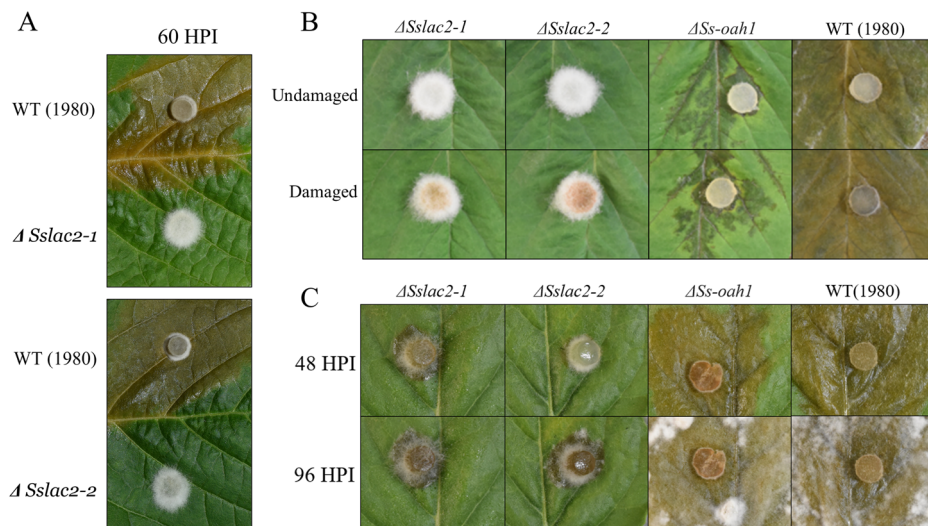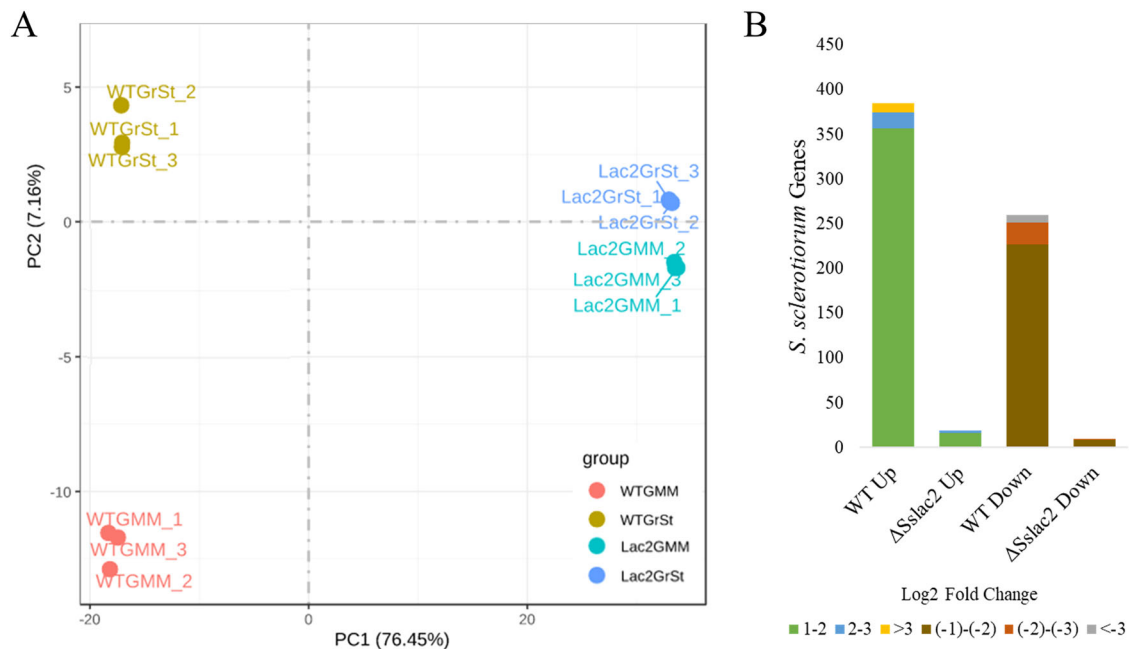
Fig. 7 | Gene expression profiles of WT (1980) and *ΔSslac2-1*. A PCA plot comparing expression profiles of WT (1980) and *ΔSslac2-1* grown in GMM or GMM + GrSt. **B** Number of genes found to be differentially regulated between GMM and GMM + GrSt for each strain. Colors within the bars represent genes categorized by their log$_2$ fold-change (FC) (FDR < 0.05; log$_2$ FC > 1 or <−1). Up = upregulated in GMM + GrSt vs GMM. Down = downregulated in GMM + GrSt vs GMM. The source data underlying Fig. 7 can be found in Supplementary Data 2.
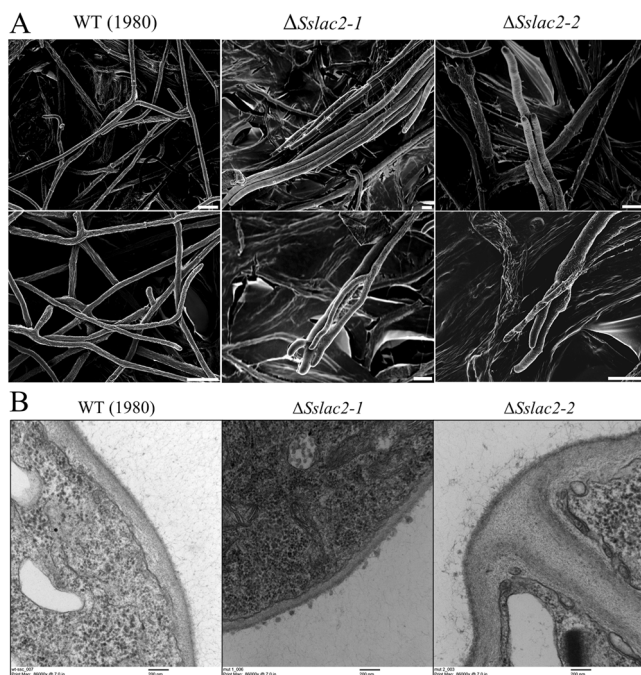

Fig. 8 | Electron micrographs of *S. sclerotiorum* mutants and WT. A Scanning electron micrographs of WT and mutant strains of *S. sclerotiorum*. White scale bars correspond to 10 microns. **B** Transmission electron microscopy (TEM) photos of the cell walls and extracellular matrices of WT (1980), *ΔSslac2-1*, and *ΔSslac2-2*. R Resin, FC fungal cell, CW cell wall, arrows denote the fungal extracellular matrix. Black scale bars correspond to 200 nm.

adhering to one another and may be either a cause or an effect of the previously noted defect in growth directionality.

A higher resolution analysis of cell well structure was conducted through transmission electron microscopy (TEM) to assess potential changes in cell wall architecture in the mutant strains. While no structural modifications could be confirmed in the *ΔSslac2* strains given the inherent variability in *S. sclerotiorum* cell walls relative to hyphal age, a clear textural difference in the extracellular matrix coating the cells was seen (Fig. 8B; Supplementary Fig. 6). This apparent failure by the laccase mutants to properly assemble their exterior cell wall ultrastructure may contribute to the previously noted changes in their physiochemical properties and altered responses to external environmental signals (Fig. 7; Supplementary Fig. 5).

Host-induced gene silencing (HIGS) targeting *Sslac2* induces resistance to *S. sclerotiorum* in soybean

Given the clear importance that Sslac2 plays in both the virulence and development of *S. sclerotiorum*, we considered the value that this gene may have as a target of HIGS-mediated disease control. Targeted gene silencing using stable transgenic lines has been shown to provide robust disease control against *S. sclerotinia* in some pathosystems, so *Sslac2* gene silencing was first assessed using a viral vector. To achieve this, a segment of *Sslac2* was cloned into a modified Bean pod mottle virus (BPMV) vector which was then used to biolistically inoculate soybean seedlings[35]. Infected material was used to inoculate new seedlings with either empty vector (EV) or *Sslac2*-targeting BPMV, which were subsequently infected with WT *S. sclerotiorum*. Stem lesions were measured over a week of infection, and significantly smaller lesions were observed on plants in which *Sslac2* was being silenced (Fig. 9). The reddening seen on BPMV-*Sslac2* stems in response to infection indicates a successfully induced resistance response by soybeans against *S. sclerotiorum* invasion. This finding indicates that the gene silencing of *Sslac2* likely serves to limit the virulence of the pathogen, while allowing the host time to mount a more successful defense (Fig. 9)[32].

Discussion

In this study a novel laccase, Sslac2, was identified within the broad-host-range fungal pathogen *S. sclerotiorum* and found to be critical for the proper regulation of an array of developmental and virulence traits. The phenotypes observed from *ΔSslac2* mutants appear surprisingly expansive, in contrast, some of these phenotypes have only been noted in individual laccase mutants of other ascomycetes (Fig. 10). The mutants most closely

Fig. 9 | Assessment of *S. sclerotiorum* virulence in plants utilizing virus-induced gene silencing (VIGS) to silence expression if *Sslac2* during infection. A Visual appearance of lesions from *S. sclerotiorum* infection soybeans containing an empty vector (EV) strain of Bean pod mottle virus (BPMV) and soybeans containing BPMV targeting *Sslac2*. **B** Quantification of lesion lengths from infection of BPMV-EV and BPMV-*Sslac2* soybeans. Statistical analysis utilized a Student's *t*-test on six biological replicates of each construct. Experiment was repeated twice (*<0.05, **<0.01, ***<0.001). The source data underlying this figure can be found in Supplementary Data 1.
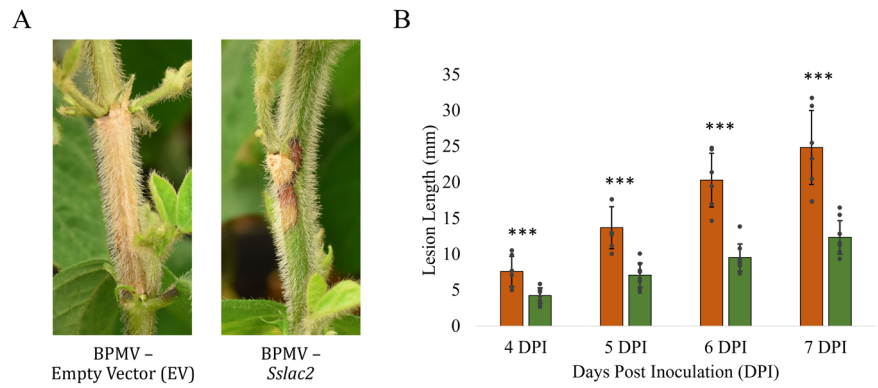
resembling *ΔSslac2* are that of *ΔStLAC2*, from the northern corn leaf blight pathogen *S. turcica*, and *Lac1*, from the anthracnose pathogen *C. gloeosporioides* (Fig. 10). In both cases, laccase knockout mutants were similar to *ΔSslac2* in that they had severe defects in appressorial production, leading to a loss of pathogenicity on intact plant tissue and drastically reduced virulence on wounded tissue[6,7]. *ΔStLAC2* additionally displays a similar increased susceptibility to cell wall stressors and altered hydrophobicity, with an identical hyphal wetting phenotype[6]. A primary difficulty in comparing the spectrum of biological roles mediated by individual laccases is that many of these functions have not been assessed across all species. In the mulberry pathogen *Scleromitrula shiraiana*, knockdown mutants of the laccase *Sh-lac* generated significantly less OA than WT strains, similar to *ΔSslac2*[9]. Unfortunately, environmental acidification has not been assessed outside of these two systems, so it is unknown whether this is a common feature of laccase mutants (Fig. 10).

Many of the assessed laccase mutants have some defect in melanin/pigment formation, and it is typically suggested that subsequent phenotypic alterations are due to melanogenic defects, but we argue that this explanation is unlikely in *S. sclerotiorum* (Fig. 10)[6,8,9]. *S. sclerotiorum* is known to generate melanin through the DHN melanin pathway and multiple components of this pathway have been deleted and characterized[17,18]. Although a loss of melanization was seen in sclerotia and compound appressoria, there was no effect on fungal virulence. This agrees with expansive studies on the melanogenic genes from the closely related fungus *B. cinerea*, none of which was found to play a clear role in infection[19].

S. sclerotiorum has seven putative laccases in its genome, all of which have predicted secretion signal peptides on their N-termini, but only five of which (Sslac2-6) contain the canonical C-terminal motif DSGx (Fig. 1A; Supplementary Fig. 2)[22]. While *Sslac2* is the only of these laccases with clear induction *in-planta* from our transcriptomic analysis, existing *S. sclerotiorum* expressed sequence tag libraries suggest that other laccases are expressed at distinct developmental stages (sclerotial development, carpogenic germination apothecial formation), possibly playing a more classic role in melanin deposition (Fig. 1B)[36]. Given the overlapping substrate ranges of many laccases, some amount of redundancy is expected and has been demonstrated in other systems[11,12]. Additionally, it was demonstrated in *C. orbiculare* that *ΔLac2* mutants could be functionally complemented with orthologous laccases from related species. The unexpected severe phenotype in *ΔSslac2* mutants is surprising given that the closest characterized ortholog, *B. cinerea* laccase *Bclcc2*, does not significantly contribute to development or virulence. Knockout mutants of *Bclcc2* displayed no alterations in virulence and appear to be primarily involved in oxidation of environmental phenolics, although both *ΔSslac2* and *ΔBclcc2* strains show abolished tannic acid oxidation activity[20,21]. Laccases are a class of multi-copper oxidase requiring a core of copper to catalyze oxidation, and while no phenotypes similar to *ΔSslac2* have been observed in *B. cinerea* laccase mutants, a nearly identical phenotype has been observed in mutants of the copper transporter *BcCcc2*[37]. Knockouts of *BcCcc2* show reduced melanization, malformed and reduced compound appressoria, no pathogenicity

on unwounded tissue, and reduced virulence on wounded tissue, all of which we observed in *ΔSslac2* strains. These deficiencies were attributed to the failure of the *ΔBcCcc2* mutant to provide copper to copper-containing enzymes, of which laccases were major likely recipients, and suggests that *B. cinerea* may utilize laccases other than *Bclcc2* to perform similar functions to *Sslac2*[37].

Our data show that *ΔSslac2* mutants are reduced in hydrophobicity relative to WT strains, as observed through enhanced hyphal wetting (Supplementary Fig. 5). Typically, surface hydrophobicity is largely mediated by the presence of surface hydrophobins, the presence of which are both ubiquitous among and unique to filamentous fungi[38]. It's surprising that surface hydrophobicity would be affected after the deletion of a single laccase because the *S. sclerotiorum* hydrophobins should still be intact; however, TEM analysis of the WT and *ΔSslac2* strains suggests that the extracellular matrix (ECM) of mutant strains may be more intrinsically disordered than the WT (Fig. 8B; Supplementary Fig. 6). Such a change in the ECM, the component of hyphae that interacts most directly with the environment, may help to explain why the mutant hyphae were observed to "stick" to one another and why hydrophobins may by incapable of maintaining hydrophobicity. This loss of hydrophobicity may also explain the enhanced growth of the mutants in liquid culture, as a similar phenotype has been observed in more hydrophilic strains of *Trichoderma*[34]. Additionally, modifications to the ECM could undermine fungal cell receptor activity, including G-protein-coupled receptors (GPCRs) or ion channels. This could help to explain the aberrant environmental sensing phenotypes observed in this study. The defect in environmental sensing might also explain many of the developmental phenotypes we observed, including the observed reduction in OA secretion and canonical compound appressoria formation (Fig. 5). This study is the first to provide evidence for roles of fungal laccases in ECM formation and environmental sensing.

Other Sslac2-driven modifications to cell wall composition are likely, as we show that *ΔSslac2* is very resistant to protoplasting by a cocktail of cell wall degrading enzymes (Supplementary Fig. 4C). Such a phenotype was additionally observed in knockout mutants of *Rho1*, a GTPase, from *F. oxysporum*, which displayed strikingly similar developmental defects to *ΔSslac2*, including a growth defect specific to solid surfaces, attenuated virulence on plants, increased susceptibility to cell wall stressors, and resistance to protoplasting[39]. Broadly, *Rho1* orthologues in yeast are known to play a role in polarized cell growth through regulation of the actin cytoskeleton and can directly interact with the β-1,3-glucan synthase in fungal cell walls[39]. This activity likely extends to filamentous fungi as well, given the growth morphology and cell wall alterations observed in *ΔRho1* from *F. oxysporum*) and *ΔRhoA* (*A. nidulans*), and supports a connection between cell wall composition and the thigmotropic phenotypes observed in *ΔSslac2* (Figs. 7 and 8; Supplementary Fig. 3-6)[39,40]. The precise interplay between cell wall biosynthesis and thigmotropism is currently unclear, but such alterations may additionally play a role in the attenuated virulence of *ΔSslac2* strains, as plants are known to activate defenses in response to fungal cell wall components (Fig. 6)[41]. If the cell wall of *ΔSslac2* mutants were

Avirulent on unwounded plants	Reduced virulence on wounded plants	Appressorial defects	Alteration to pigmented structures	Reduced oxalic acid production	Altered Stress tolerance
ΔSslac2	ΔSslac2	ΔSslac2	ΔSslac2	ΔSslac2	ΔSslac2
ΔLac2	ΔLac1	ΔLac2	Δya	KD-Sh-lac	ΔMLac1
ΔLac1	ΔStLAC2	ΔLac1	ΔAbr2		ΔBclcc2
ΔStLAC2		ΔMLac1	ΔLac2		ΔStLAC2
		ΔStLAC2	KD-Sh-lac		Δlcc3
			ΔStLAC2		

ΔSslac2 – This Study
ΔStLAC2 – S. turcica (Ma et al. 2017)
ΔLac1 – C. gloeosporioides (Wei et al. 2017)
ΔLac2 – C. orbiculare (Yu Lin et al. 2012)
Δya – A. nidulans (Tsai et al. 1999)

ΔBclcc2– B. cinerea (Schouten et al. 2008)
ΔMLac1 – M. anisopliae (Fang et al. 2010)
KD-Sh-lac – S. shiraiana (Lu et al. 2016)
ΔAbr2 – A. fumigatus (Upadhyay et al. 2016)
Δlcc3 – F. oxysporum (Cordoba Cañero and Roncero, 2008)

Fig. 10 | Comparison of laccase mutant phenotypes from filamentous ascomycetes. Photos represent experimental evidence of ΔSslac2 mutants presenting aberrant phenotypes. The names of laccase genes characterized in other Ascomycete fungi and their associated references are below.

modified in a way that increases the release of such components, as was predicted in ΔRho1, then a reduction in virulence would be expected. Moreover, compromised virulence in the mutants could be the result of heightened susceptibility and/or impaired transcriptional response to plant-produced antifungal compounds, as evidenced by in vitro plate assays and transcriptomic analysis (Supplementary Fig. 4A; Fig. 7).

Given the clear and pivotal role that Sslac2 plays in virulence, we chose to evaluate it as a target of host induced gene silencing (HIGS) to achieve disease resistance in soybeans. An initial screen using a viral vector confirmed that silencing Sslac2 significantly increases plant resistance to S. sclerotiorum (Fig. 9). A drawback of such an approach is that viral vectors are often only capable of partial gene silencing, given the relatively low viral titer in plants used for this assay[32]. We are currently generating stable transgenic soybean lines expressing hairpin dsRNA targeting Sslac2 and will be evaluated for resistance to S. sclerotiorum infection.

In summary, this study characterizes a single fungal laccase (Sslac2) critical for proper development, virulence, and environmental sensing in the broad-host-range fungal plant pathogen S. sclerotiorum. Future work will focus on elucidating the chemical substrates of Sslac2 and precise mechanisms by which this protein mediates fungal thigmotropism and responses to environmental stimuli. Efforts will also focus on evaluating Sslac2 and other fungal laccases as targets of gene silencing for disease control.

Materials and methods
Plant and fungal growth
All soybean and N. benthamiana plants were maintained in the greenhouse or growth chamber at 24 ± 2 °C with 16-h light/8-h dark photoperiod cycle. Plants were watered daily and supplemented with fertilizer (Miracle-Gro) every week.

All S. sclerotiorum cultures were maintained on potato dextrose agar (PDA) plates or PDA supplemented with 50 µg/ml hygromycin in the case of knockout strains. Liquid cultures were grown in potato dextrose broth. Cellophane assays were conducted by autoclaving pre-cut rings of cellophane prior to being placed on 100 × 15 mm petri dishes containing PDA.

Fungal transformation
The wild-type S. sclerotiorum strain used in the generation of all mutants was the type strain referred to as 1980 (ATCC Product Name: 18683), as this strain was used to generate the genome sequence used for genomic and RNA-Seq analysis (NCBI BioProject Acc. PRJNA15530). The ΔSsoah1

mutant was additionally generated in a WT (1980) background[31]. Gene knockouts were generated in S. sclerotiorum using a CRISPR-Cas9 method in combination with a modified form of the protocols described in Rollins et al.[42]. and Westrick et al.[43] Split markers targeting Sslac2 were generated using polymerase chain reaction (PCR) by amplifying 5–600 bp regions upstream (Sslac2-LF-F and Sslac2-LF-R) and downstream (Sslac2-RF-F and Sslac2-RF-R) regions of Sslac2. These amplicons were designed to contain 20 bp sequences with homology to the 5′ and 3′ regions, respectively, of the hygromycin resistance cassette (HygR; 1.8 kb) found in pCRISPR-Cas9-TrpC-Hyg[18]. The hygromycin resistance cassette was amplified using the two flanking regions and the HygR were connected through fusion PCR as described in Szewczyk et al.[44]. for a product of ~3kb[44]. Split markers were generated from this product by using primers internal to HygR (Hyg Split F and Hyg Split R) in conjunction with Sslac2-RF-F and Sslac2-RF-R, yielding two amplicons with an overlapping region of ~400 bp.

Two small guide RNAs (sgRNAs) targeting Sslac2 were designed using the E-CRISP Design Tool (http://www.e-crisp.org/E-CRISP/index.html), generated using the GenCrispr sgRNA Screening Kit (L00689; Genscript Biotech Corp.), and diluted to a concentration of 4 µM. Alt-R S.p. Cas9 nuclease 3NLS (1081058; IDT) was diluted to a concentration of 4 µM and combined with sgRNA at a 1.2-to-1 ratio (3.6 µl of sgRNA to 3 µl of Cas9 protein) and incubated at room temperature for 5 min to assemble the RNP complex. These complexes were combined with 1 µg of each split-marker and transfected into S. sclerotiorum protoplasts using the polyethylene glycol (PEG) transformation described in Rollins et al.[42].

Transformants capable of surviving on PDA containing 50 µg/ml hygromycin were subjected to 5 rounds of hyphal tipping before undergoing DNA extraction to confirm the replacement of Sslac2 with the HygR marker. Primers internal and external to Sslac2 and HygR were used to confirm deletion and primers targeting Histone 3 (H3 F and H3 R) were used as a control (Supplementary Fig. 7: Supplementary Table 1). DNA was extracted using the cetyl trimethyl ammonium bromide (CTAB) method described in Talbot et al.[45].

Virus-induced gene silencing (VIGS) assay and construct generation
A modified Bean pod mottle virus (BPMV) vector was used to assess Sslac2 as a potential target of VIGS for disease control[35]. In order to silence Sslac2 (Sscle_03g023030; SS1G_00974; XM_001598835), a 267 base pair sequence was selected within the mRNA of S. sclerotiorum strain 1980 (GenBank Accession XM_001590428). Total RNA was extracted from S. sclerotiorum

using the Maxwell® RSC Plant RNA Kit, and cDNA was generated using an AMV first strand cDNA synthesis kit (New England Biolabs, Catalog # E6550). The segment was amplified through PCR with PstI and BamHI restriction sites incorporated onto the double-stranded cDNA using specific primers (PstI-VIGS-F and BamHI-VIGS-R) (Supplementary Table 1). The amplicon underwent gel purification (QIAquick Gel Extraction Kit®, QIAGEN), then both the amplicon and the viral vector RNA2 plasmid (pBPMV-IA-V1) were subjected to restriction digestion with PstI/BamHI before being ligated to form BPMV-Sslac2[35]. The vector plasmids were then transformed into DH5α competent cells using 5 µl of the purified ligation product per 50 µl of competent cells, a 30 min ice incubation, 45 s heat shock in a 42 °C water bath, and incubation in 500 µl of Luria broth (LB) for 1 h at 37 °C. Using glycerol stocks, midi preparations were conducted (Fast Ion Plasmid Midi Kit®, IBI Scientific) for subsequent biolistic inoculations. Biolistic inoculations were performed as described in McCaghey et al. [46].

Plant disease assays

Soybeans: For detached leaf assays, leaves were taken from the first trifoliate of 5–6-week-old plants (cv. Williams 82) and placed in petri dishes containing two layers of filter paper/paper towel and 7 milliliters of sterile water. Leaves were inoculated near the center with agar plugs of actively growing wild-type (1980) or mutant *S. sclerotiorum*. Damaged leaves were scored four times in a crisscross pattern using a sterile scalpel directly under the agar plug. Petri dishes were wrapped in parafilm, placed at room temperature, and photographed every twenty-four hours. All disease assays were conducted in triplicate.

For VIGS assays, 10–14 day old plants (cv. Traff) were rub-inoculated with lyophilized leaves infected with BPMV-Sslac2 as described in McCaghey et al. [46]. Plants were then allowed to grow an additional 5 weeks prior to being inoculated with WT (1980) *S. sclerotiorum* through cut petioles. Briefly, deep-well plates (100 mm × 55 mm) containing 75 ml of PDA were inoculated with WT (1980) and allowed to grow for two days. The petiole of the first trifoliate was cut ~2 cm from the main stem with a razor and a plug from the leading edge of mycelia was punctured using an inverted one-ml pipette tip. The inverted pipette tip with agar plug, was then slid onto the excised petiole. Lesions were measured with digital calipers 4–7 Days post inoculation (DPI). Three plants were inoculated per 1 L pot and 5 pots were tested for both the empty vector and BPMV-Sslac2 infected plants. The experiment was replicated twice.

N. benthamiana: Leaves were taken from 6–7 week-old plants and placed in petri dishes containing two layers of filter paper/paper towel and 7 ml of sterile water. Leaves were inoculated near the center with agar plugs of actively growing wild type (1980) or mutant *S. sclerotiorum*. Damaged leaves were scored four times in a crisscross pattern using a sterile scalpel directly under the agar plug. Petri dishes were wrapped in parafilm, placed at room temperature, and photographed every twenty-four hours.

Stress Testing

All stress test assays were performed in 60 mm × 15 mm petri dishes containing PDA supplemented with benzoic acid (150 µg/ml), cinnamic acid (150 µg/ml), ferulic acid (500 µg/ml), resveratrol (200 µg/ml), congo red (50 µg/ml), calcofluor white (250 µg/ml), or poacic acid (50 µg/ml). Cultures were allowed to grow for 24–48 h prior to being photographed. Colony areas were quantified in ImageJ[47]. All comparisons were tested in triplicate and the statistical significance of colony area differences was assessed with a Student's t-test.

Protoplasting assay

Protoplasting was done using a modified protocol from Rollins et al. [42]. Briefly, three agar plugs of each strain were grown for two days in petri dishes containing PDB at room temperature. Agar plugs were excised with tweezers and a scalpel, and each sample was washed with water and then protoplast buffer (0.8 M MgSO₄·7H₂O, 0.2 M Sodium citrate·2H₂O, pH 5.5). Samples were roughly chopped with a sterile razor blade and placed in 17 ml of protoplast buffer. For each sample, 100 mg of lysing enzyme from

Trichoderma harzianum (Sigma Aldrich, L1412) was dissolved in 3 ml of Novozyme buffer (1 M Sorbitol, 50 mM Sodium citrate·2H₂O) and then filtered through a 0.45 µm filter directly into to protoplast buffer containing the sample. All samples were incubated in a 28 °C shaker at 120 RPM for 3 hours before being filtered through four layers of Miracloth to collect protoplasts. Samples were centrifuged at 3000 × *g* for 10 min to pellet protoplasts, which were subsequently reconstituted in 1 ml protoplast buffer before quantification with a hemocytometer. All samples were run in triplicate. The statistical significance of protoplast count differences was assessed with a Student's *t*-test (*<0.05, **<0.01, ***<0.001). The experiment was replicated twice.

Assay of laccase production

Laccase production was assessed using either 0.2 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 0.2 mM ABTS + 0.6 mM CuSO₄, or 2.5 mg/ml tannic acid amended to PDA plates (100 mm × 15 mm)[6,21]. Agar plugs of actively growing hyphae from each strain was used to inoculate the center of plates and were allowed to grow for 24 h prior to being photographed. Laccase production was associated with the accumulation of bluish/purple pigments in the case of ABTS and brown pigment in the case of tannic acid. CuSO₄ amendment was utilized as a known inducer or laccase production[25].

Hydrophobicity assay

Three-week-old cultures of all strains were topped with 100 µl of either H₂O or H₂O + 0.01% Triton X-100. Photos were taken 2 minutes after treatment to observe mycelial soaking.

Compound appressoria observation/quantification

Agar plugs of actively growing mycelium were collected and placed face down on glass slides and then incubated in the dark at room temperature in a sealed container overnight (~16 h). A scalpel was used to cut the agar plugs away from hyphae which had grown onto the glass slide and the plugs were removed. Mycelia were stained with 0.05% trypan blue for 1 h before being rinsed with water to remove the dye. As the mutant strains are substantially less hydrophobic than the wild-type and therefore attach poorly to the glass slide, the water rinse was done by carefully removing the dye and replacing it with water. This process was repeated 10× for each agar plug. Stained mycelia were then observed under a compound microscope to observe the production of compound appressoria. The statistical significance of compound appressorium count differences was assessed with a Student's *t*-test.

RT-PCR of *Sslac2* on PDA and PDB

WT (1980) *S. sclerotiorum* was grown for 48 h at room temperature either in a 125 ml Erlenmeyer flask containing PDB on a rotary shaker (120 RPM) or on a plate of PDA (100 mm × 15 mm). Samples in PDB were then removed from the broth and flash frozen in liquid nitrogen and ground into a powder with a mortar and pestle. Samples on PDA had liquid nitrogen poured directly onto plates to flash freeze mycelium and the underlying PDA, then a thin layer of mycelia was scraped off using a pre-chilled scalpel. Total RNA was extracted from frozen mycelia using the Maxwell® RSC Plant RNA Kit, and cDNA was amplified using an AMV first strand cDNA synthesis kit and was normalized to 50 ng/µl for each sample (New England Biolabs, Catalog # E6550). *Sslac2* was amplified using specific detection primers (Sslac2 Det F and R) for 30 cycles. The raw gel is included as Supplementary Fig. 8.

Scanning and transmission electron microscopy (SEM and TEM)

For SEM samples were grown on PDA plates embedded with 10 mm Whatman filters shortly after plates were poured, allowing for a thin layer of agar to cover the filters. Samples were grown for 2 days until they had completely covered the filter paper, before being submerged in a chemical fixative (78% - Ultrapure ddH₂O, 10% - 10× PBS, 10–37% formaldehyde, 2–50% glutaraldehyde) overnight. Samples were treated with 1% osmium tetroxide for 30 min at 22 °C. Samples were subsequently washed with a series of increasing ethanol concentrations (30–100% [vol/vol]), followed by

critical point drying and sputter coating with platinum. Scanning electron microscopy (SEM) of samples was performed using a LEO 1530 microscope. TEM samples were grown on PDA overlayed with cellophane for two days before 10 mm circles were peeled off and placed in the above fixative overnight. Sample preparation, sectioning, and imaging was conducted by the UW Madison Medical School Electron Microscope Facility on a Philips CM120 STEM.

Genomic and RNA-Seq analysis

For the genomic analysis, known laccase proteins from *Cucurbita maxima*, *Melanocarpus albomyces*, *Myrothecium verrucaria*, and *Saccharomyces cerevisiae* were used as queries in a BLASTp search of *S. sclerotiorum* proteins in the National Center for Biotechnology Information (NCBI) Genbank repository, as described in Feng et al.[2]. All identified genes were assessed for a secretion signal peptide using SignalP 6.0[48]. Protein features were described using the NCBI Conserved Domain database after querying through BLASTp.

For the RNA-Seq analysis, WT (1980) and *Sslac2* mutant strains were grown in 1% GMM liquid culture for 3 days before cultures were moved to either fresh media alone or fresh media containing 1% soybean SE and allowed to grow for 4 h prior to flash freezing in liquid nitrogen. RNA was extracted with a Maxwell RSC Plant RNA Kit (AS1500). Differential gene expression values were generated using the bioinformatic pipeline described in Westrick et al.[43].

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

RNA sequencing data was deposited in NCBI's Gene Expression Omnibus (GEO) under project GSE246683. Source data underlying the figures can be found in Supplementary Data Set 1. Strains generated in this manuscript are available upon request.

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Author contributions

N.M.W. and M.K. conceived the project. N.M.W., M.K., E.D., M.B., C.M.H. and D.L.S. contributed to experimental design. Experiments were conducted by N.M.W. and E.D. All authors contributed to the writing and editing of this manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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