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REVIEW

Mechanisms of Broad Host Range Necrotrophic Pathogenesis in *Sclerotinia sclerotiorum*

Xiaofei Liang and Jeffrey A. Rollins†

First author: State Key Laboratory of Crop Stress Biology for Arid Areas and College of Plant Protection, Northwest A&F University; and second author: Department of Plant Pathology, University of Florida, P.O. Box 110680, Gainesville 32611-0680.
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ABSTRACT

Among necrotrophic fungi, *Sclerotinia sclerotiorum* is remarkable for its extremely broad host range and for its aggressive host tissue colonization. With full genome sequencing, transcriptomic analyses and the increasing pace of functional gene characterization, the factors underlying the basis of this broad host range necrotrophic pathogenesis are now being elucidated at a greater pace. Among these, genes have been characterized that are required for infection via compound appressoria in addition to genes associated with colonization that regulate oxalic acid (OA) production and OA catabolism. Moreover, virulence-related secretory proteins have been identified, among which are candidates for manipulating host activities apoplastically and cytoplasmically. Coupled with these mechanistic studies, cytological observations of the colonization process have blurred the heretofore clear-cut biotroph versus necrotroph boundary. In this review, we reexamine the cytology of *S. sclerotiorum* infection and put more recent molecular and genomic data into the context of this cytology. We propose a two-phase infection model in which the pathogen first evades, counteracts and subverts host basal defense reactions prior to killing and degrading host cells. Spatially, the pathogen may achieve this via the production of compatibility factors/effectors in compound appressoria, bulbous subcuticular hyphae, and primary invasive hyphae. By examining the nuances of this interaction, we hope to illuminate new classes of factors as targets to improve our understanding of broad host range necrotrophic pathogens and provide the basis for understanding corresponding host resistance.

Additional keywords: biochemistry and cell biology, genetics and resistance, mycology.

Conceptual models of the evolutionary and mechanistic interplay between plants and pathogens have developed primarily from the study of biotrophic and hemibiotrophic interactions. In these models (Jones and Dangl 2006; Oliveira-Garcia and Valent 2015; Zipfel 2008), pathogens rely on effector proteins to suppress or avoid host basal defense or pathogen associated molecular pattern (PAMP)-triggered immunity (PTI). Correspondingly, plant hosts evolve to 'recognize' these effector proteins and elicit a rapid, often hypersensitive defense response, termed effector-triggered immunity (ETI).

Within these models, host-specialized necrotrophic pathogens are also represented where necrotrophic effectors and host-selective toxins form the basis of an interaction generally referred to as an inverse gene-for-gene interaction (Friesen et al. 2008; Wolpert et al. 2002). In this version of the model, necrotrophic effectors and host-selective toxins target susceptibility genes to trigger programed cell death conducive to necrotrophic colonization (Lorang et al. 2012; Oliver et al. 2012). This inversion of the standard gene-for-gene model goes beyond genetic analogy. Functionally, necrotrophs can utilize host resistance genes found in gene-for-gene resistance but as a consequence of triggering all or specific components of the HR resistance pathway, susceptibility is achieved (Friesen and Faris 2012).

The broad host range necrotrophs are yet another important category of phytopathogens. These pathogens cause huge economic damage; yet, a working model of host–pathogen compatibility is woefully incomplete, and it is unclear whether effectors function in suppressing or triggering PTI and ETI reactions and how these activities contribute toward virulence and host susceptibility.

Sclerotinia sclerotiorum (Lib.) de Bary exemplifies the model of a broad host range necrotroph. It infects over 600 plant species and incites rapid host tissue maceration in a nondiscriminant manner. Although natural variation in host resistance and pathogen virulence exists, there is little evidence of host specialization or a host genotype-to-pathogen isolate interaction. Superficially, disease develops in an unsophisticated and direct manner, involving the killing of host cells and subsequent nutrient uptake. Mechanistically, the release of toxins

†Corresponding author: J. A. Rollins; E-mail: rollinsj@ufl.edu

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(e.g., oxalic acid [OA]) and cell wall degrading enzymes (CWDEs) appear to constitute the primary arsenal (Bolton et al. 2006). However, over the past several years, the infection process of *S. sclerotiorum* has been viewed as much more sophisticated than previously thought. For instance, characterization of genes involved in OA biosynthesis and degradation have demonstrated that full virulence requires a dynamic control of OA accumulation through multiple mechanisms; moreover, the ability of OA-minus mutants to infect and colonize hosts to varying degrees in the absence of OA accumulation suggests that the role of OA appears better described as a colonization factor rather than an essential compatibility factor (Liang et al. 2015a; Xu et al. 2015). Furthermore, small secretory proteins, functioning in both host defense suppression and in inducing host cell death, have been identified. Accordingly, evidence supporting the host internalization and intercellular translocation of pathogen-secreted proteins, processes not typically associated with a "brute force" necrotrophic pathogen, is coming to light (Kabbage et al. 2015; Lyu et al. 2016b). These new findings point to a nuanced interaction of this aggressive, broad-host range necrotrophic pathogen with its many hosts. The challenges ahead are to identify and assign the temporal, spatial, and tissue-specific functions to the unknown virulence factors guiding this specific form of plant disease system and leverage this for quantitative disease resistance breeding and phenotypic screens for identifying stage-specific defenses.

The objective of this review is to recall the cytology of *S. sclerotiorum* infection and put more recent molecular and genomic data into this context. We summarize past histology and cytology studies of the infection and colonization process, and categorize known genetic factors defining the broad host range necrotrophic trait, specifically factors regulating infection-related morphogenesis, OA accumulation dynamics, factors effecting aggressiveness, and the production of toxins and hydrolytic enzymes. From these, we hypothesize that *S. sclerotiorum* infects in a two-phase manner. In phase one the pathogen uses well-orchestrated strategies to overcome host basal defense reactions for establishing basic compatibility. Spatially this is achieved through compatibility factor/effector production in infection structures including compound appressoria, bulbous subcuticular hyphae, and primary invasive hyphae. In phase two, the pathogen relies on toxins (OA in particular) and hydrolytic enzymes to disrupt and degrade host tissues, causing rapid tissue maceration. Here we present and discuss key findings that have shaped this model and our current knowledge of *S. sclerotiorum* pathogenesis. We hope this will serve to improve the understanding of the *S. sclerotiorum* infection process and point the way toward a deeper appreciation of the complexities underlying host–pathogen interactions involving aggressive necrotrophic pathogens. For readers interested in learning more comprehensively about the pathogenic biology and genomic features of *S. sclerotiorum*, several additional papers and reviews are recommended (Amselem et al. 2011; Bolton et al. 2006; Derbyshire et al. 2017; Hegedus and Rimmer 2005; Kabbage et al. 2015; Lumsden 1979; Mbengue et al. 2016; Purdy 1979; Rollins et al. 2014; Wei and Clough 2016).

INFECTION CYTOLOGY SUGGESTS A TWO-PHASE COMPATIBILITY MODEL

Modified hyphae in the form of compound appressoria are the primary means by which *S. sclerotiorum* initiates infection (Abawi 1975; Huang and Kokko 1992; Jamaux et al. 1995; Purdy 1958; Sutton and Deverall 1983). These hyphae are derived indirectly from ascospores in the presence of exogenous nutrients which the pathogen mainly absorbs from flowers or other senescent tissues during field infection (Abawi 1975). Multicellular compound appressoria are also formed by other Sclerotiniaceae species (e.g., *Monilinia fructicola*, *Botrytis cinerea*) and necrotrophic non-Sclerotiniaceae species (e.g., *Fusarium* spp., *Rhizoctonia* spp.) (Boenisch and Schafer 2011; Hofman and Jongebloed 1988; Rittenour and Harris 2010).

In *S. sclerotiorum* and the closely related *B. cinerea*, a compound appressorium (also known as an infection cushion) develops from a hyphal tip through a series of recurrent events including growth retardation, swelling, and subsequent bifurcation (Abawi 1975; Sedun and Brown 1987). Contact with inert impenetrable surfaces which are often but not always hydrophobic (e.g., dialysis tubing, cellophane, parafilm, plastics, and glass) efficiently triggers differentiation (Abawi 1975; Li et al. 2015a, 2018a; Purdy 1958; Tariq and Jeffries 1984) and the multicellular complexity is positively correlated with penetration resistance (Abawi 1975; Tariq and Jeffries 1984). Within a compound appressorium, hyphal tips are compactly aligned and independent hyphal tips initiate independent penetrations (Huang et al. 2008; Lumsden and Wergin 1980). Compared with a single-celled appressorium, the localized organization of numerous hyphal tips within a continuous plane presumably increase the local accumulation of hydrolytic enzymes, toxins and defense-suppressive factors. In turn, concerted and localized penetrations increase the survival chance of penetration pegs and the speed of initial infection establishment.

Compound appressoria are covered by a layer of mucilaginous material with a putative adhesive function (Lumsden and Dow 1972; Tariq and Jeffries 1986). Both mechanical force and localized cutinolysis have been suggested to be important for cuticle penetration. Lumsden and Dow (1972) observed an inward cuticle depression at the penetration site, supporting the importance of mechanical force. Tariq and Jeffries (1986), however, observed that the appressorium tip contains a large number of 50 to 100 nm intracellular vesicles, and secretes extraplasmalemmal vesicular material toward the host cuticle. They suggested that the penetration peg contains no or a very thin layer of cell wall, which would be unable to hold the huge turgor pressure required for mechanical force generation.

Compound appressorium-derived penetration pegs only breach the cuticle layer without penetrating into the underlying host epidermal cell (Huang et al. 2008; Liang et al. 2015a; Lumsden and Dow 1972; McKeen 1973; Purdy 1958; Tariq and Jeffries 1986). From these penetration pegs, bulbous and multilobed subcuticular vesicles form, which then branch to produce subcuticular infection hyphae that spread horizontally beneath the cuticle to comprise the leading colonization front. This initial growth is followed by subapical branching of smaller ramifying hyphae that grow inter- and intracellularly into the epidermal and mesophyll cells (Jamaux et al. 1995; Lumsden and Dow 1972; Lumsden and Wergin 1980). Compared with ramifying hyphae, the subcuticular hyphae are thicker, granular, and stain lightly with safranin similar to the subcuticular infection vesicle (Lumsden 1979). It is evident that the *S. sclerotiorum* subcuticular hyphae can spread several cell layers ahead of killed epidermal cells (Kabbage et al. 2013, 2015; Liang et al. 2015a), leading to the proposition that the infection of *S. sclerotiorum* involves a transient biotrophic interaction stage (Kabbage et al. 2015). Ramifying hyphae are typically formed through branching of subcuticular infection hyphae. These thin hyphae typically sense and penetrate at the anticlinal cell wall junctions, causing a loosening and swelling of the epidermal cell wall (Huang et al. 2008; Liang et al. 2015a). The strikingly different morphology and colonization patterns between subcuticular infection hyphae and ramifying hyphae indicate the potential for distinctive functional specialization. Likely, subcuticular hyphae are more important in defense suppression and infection establishment whereas the ramifying hyphae are more important in initiating killing and cell wall degradation. Under this scenario, a basic compatibility establishment, involving the suppression or subversion of physical (callose and papilla deposition) and chemical (ROS, glucosinolates) defense barriers, would be critical for the "necrotrophic" infection success of *S. sclerotiorum*. Aspects of this infection cytology as observed on onion bulb epidermal peels is presented in Figure 1.

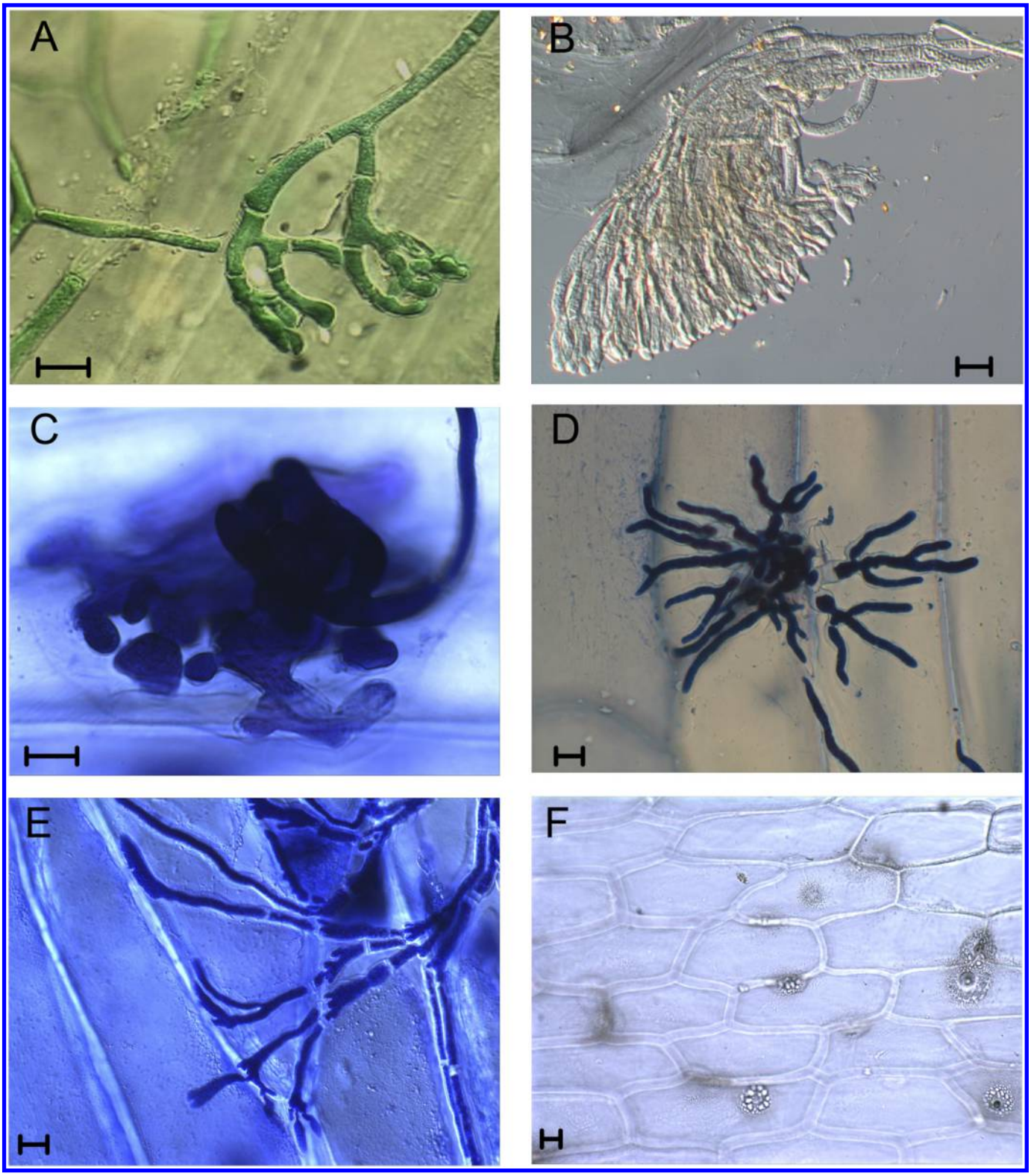

FIGURE 1

The infection and colonization process of *Sclerotinia sclerotiorum* from observations on onion epidermal peels. **A,** Early stage development of wild-type compound appressorium 12 h postinoculation, stained with bromophenol blue. **B,** Mature wild-type compound appressorium 16 h post-inoculation, unstained and physically dislodged from the epidermal cell to view the structure in profile. **C,** Bulbous wild-type subcuticular hyphae formed beneath a compound appressorium 16 h postinoculation, stained with trypan blue. **D,** Wild-type subcuticular hyphae developed from bulbous hyphae at the site of penetration 20 h postinoculation, stained with trypan blue. **E,** Trypan blue stained wild-type intra- and intercellular infection hyphae, 24 h postinoculation. **F,** Host cell papillae formation beneath the site of failed penetration 24 h postinoculation with a penetration deficient mutant. Scale bar, 20 μm.

Based on the cytological observations described above and molecular mechanisms discussed below, we propose that infection by *S. sclerotiorum* fits a two-phase model in which the pathogen first evades, counteracts and subverts host basal defense reactions, perhaps in a large extent independent of OA (discussed below). Following this stage, the pathogen switches gears and initiates killing and host cell wall degradation through OA, OA-independent toxins, as well as cell wall degrading enzymes. In the following sections, we discuss the infection mechanisms of *S. sclerotiorum* in the context of this two-phase model diagrammed in Figure 2.

COMPOUND APPRESSORIA, THE START OF HOST MANIPULATION

To date, seven genes regulating compound appressorium development have been identified, these are *SMK3* (Bashi et al. 2016), *Ss-caf1* (Xiao et al. 2013), *Ss-ggt1* (Li et al. 2012), *Ss-odc2* (Liang et al. 2015b), *Ss-rhs1* (Yu et al. 2016b), *Ss-pth2* (Liberti et al. 2013), *Ss-nsd1* (Li et al. 2018a), and *Ss-sac1* (Jurick and Rollins 2007) (Table 1). Among them, *SMK3*, *Ss-caf1*, *Ss-ggt1*, *Ss-nsd1*, and *Ss-odc2* gene mutants exhibit specific developmental or penetration defects in pathogenicity that could be fully bypassed by wound inoculation. *SMK3*, *Ss-sac1*, Ss-*ggt1*, and *Ss-caf1* are key components of cellular signaling processes. The observation that genetic mutants defective in compound appressorium formation exhibit virulence defects that can be rescued by wounding the host strengthens the important role of compound appressorium in the infection process (Bashi et al. 2016; Li et al. 2012; Liang et al. 2015b; Xiao et al. 2013).

Fungal appressorium penetration conservatively requires a protein complex made up of NADPH oxidases (Nox) and the tetraspanin PLS1 (Gourgues et al. 2004; Segmüller et al. 2008; Siegmund et al. 2013). This Nox-tetraspanin complex is critically required for appressorium tip growth reorientation and penetration peg formation in *Magnaporthe oryzae* (Dagdas et al. 2012; Ryder et al. 2013). The Nox-tetraspanin complex functions by regulating septin organization, and in turn controls actin dynamics. In *S. sclerotiorum*, two Nox-encoding genes, *Ss-nox1* and *Ss-nox2*, have been characterized (Kim et al. 2011). *Ss-nox1* but not *Ss-nox2* contributes toward fungal virulence, but whether *Ss-nox1* regulates compound appressorium formation and cuticle penetration has not been reported. Among the compound appressorium defect mutants, *Ss-odc2* encodes an oxalate decarboxylase which catabolizes OA to produce formate and carbon dioxide (Liang et al. 2015b). *Ss-odc2* transcript accumulation is confined to compound appressoria and is essential for proper compound appressorium formation and host penetration (Liang et al. 2015b). These results suggest a possibility that OA negatively regulates appressorium development. In accordance, deletion of *Ss-pth2*, which encodes a carnitine acetyl transferase involved in fatty acid mobilization and OA biosynthesis, causes a significant increase in compound appressorium formation frequency (Liberti et al. 2013). Consistent with the idea of OA as a negative regulator of compound appressoria, OA-minus mutants created via CRISPR-CAS9 mediated gene disruption and by gene deletion overproduce appressoria and are not defective in penetration (Li et al. 2018b).

For germ tube-derived simple appressoria in *Magnaporthe* and *Colletotrichum* spp., a melanized cell wall layer is critical for turgor pressure-mediated physical force generation, and thus penetration success. (Howard and Ferrari 1989; Kubo et al. 1996; Lin et al. 2012). The *S. sclerotiorum* compound appressorium is melanized with dihydroxynaphthalene (DHN) derived compounds (Butler et al. 2009). One *S. sclerotiorum* polyketide synthase (SsPKS13) regulates melanin accumulation in compound appressoria but does not affect melanin accumulation in sclerotia. Loss of function *Sspks13* mutants are still fully pathogenic (Li et al. 2018b). Similarly, deletion of the melanin biosynthesis genes *SCD1* and *THR1* affects sclerotial development and vegetative growth, but not pathogenicity (Liang et al. 2018). In *B. cinerea*, gene deletion mutants of melanin-biosynthetic pathway genes retain full virulence (Schumacher 2016; Zhang et al. 2015). It thus appears that a role for melanin in appressorium function akin to that found in fungi producing simple appressoria to support turgor generation is lacking in Sclerotiniaceae fungi. Enzymatic activities are presumably more important for compound appressorium-mediated penetration. Chief among these are likely to be cutinolytic enzymes. The *S. sclerotiorum* genome encodes four putative cutinases, one of which *SsCutA* has been characterized for transcripts accumulation patterns (Bashi et al. 2012). *SsCutA* expression exhibits a sharp induction upon solid surface contact and increases steadily at the early phases of infection (from 1 h postinoculation to 24 h postinoculation). This expression pattern supports a functional role, although deletion of its orthologous gene in *B. cinerea* does not affect penetration or virulence (van Kan et al. 1997). The specific factors and activities responsible for penetration success that are independent of the normal compound appressorium development process are worthy of more focused investigation.

HOST DEFENSE SUPPRESSION ACTIVITIES AT THE EARLY INFECTION PHASE

Known defense-suppressive factors. Necrotrophic pathogens colonize hosts differently from biotrophs and hemibiotrophs and may encounter different defense responses (Glazebrook 2005; Mengiste 2012). Yet, PAMP signatures are usually highly conserved between necrotrophs, biotrophs, and hemibiotrophs; thus, innate immunity suppression is anticipated as a common theme for all types of pathogens. On onion epidermal strips, *S. sclerotiorum* penetration mutants trigger intense papilla and ROS depositions surrounding the penetration sites (Fig. 1F) (X. Liang and J. A. Rollins, *unpublished data*), demonstrating that early-phase defense suppression may be critical for its infection success.

Small secretory proteins play critical roles in host PTI suppression for biotrophic and hemibiotrophic pathogens. Emerging evidence suggests that they also contribute toward *S. sclerotiorum* infection. The secreted chorismate mutase enzyme Ss-Cmu1 represents a striking example. In *Ustilago maydis*, this enzyme presumably translocates inside host cells and dampens salicylic acid (SA) synthesis by shunting chorismate into the phenylpropanoid pathway to promote infection (Djamei et al. 2011). *Ss-Cmu1* is highly up-regulated during *Brassica napus* infection (Nováková et al. 2014). Surprisingly, Ss-Cmu1 is predicted to be secreted, a feature not predicted from homologs of other necrotrophic fungal genomes (Kabbage et al. 2015). This and other evidences have led to the speculation of a biotrophic phase associated with *S. sclerotiorum* infection (Kabbage et al. 2015). Other putative effectors include the *Ss-ITL* gene which encodes a secreted integrin-like protein and is highly up-regulated during early infection (Zhu et al. 2013). Gene silenced mutants for *Ss-ITL* exhibit reduced virulence and cause elevated expression of the *Arabidopsis thaliana* defense genes *PDF1.2* and *PR-1*. An *A. thaliana* transgenic line overexpressing *Ss-ITL* was more susceptible to infections by *S. sclerotiorum* and *B. cinerea*. These data together suggest the defense-suppressive functions of Ss-ITL. Recently, the *S. sclerotiorum* predicted secretome was categorized bioinformatically (Guyon et al. 2014; Heard et al. 2015), providing a list of candidate effectors for further functional studies. Conserved domains related to chitin-recognition or chitin-binding, the CFEM cysteine-rich motif, a subtilisin protease inhibitor, the cerato-platanin domain, nuclear localization signals and transcription factors were identified in the secretome. It is interesting to note here there are six *S. sclerotiorum* proteins with LysM effector domains relative to two LysM-domain proteins observed in the sister

species *B. cinerea* (Derbyshire et al. 2017; van Kan et al. 2017). The secretome also contains sequences homologous to the *Colletotrichum hingginsianum* effector candidate CHEC80, CHEC91, and the *Magnaporthe oryzae* effector AvrPita (Guyon et al. 2014). It appears that *S. sclerotiorum* encodes a wide range of effector functions beyond cell death elicitation.

The full virulence of *S. sclerotiorum* also requires the detoxification of ROS and host-derived secondary metabolites, important components of PTI defense reactions (Stotz et al. 2011). Disruption of a Cu/Zn superoxide dismutase *Ss-Sod1* significantly impairs virulence (Rolke et al. 2004; Veluchamy et al. 2012; Xu and Chen 2013). Interestingly, disturbance of *S. sclerotiorum* redox status also negatively affects the OA accumulation level (Kim et al. 2011), indicating a tight linkage between ROS signaling and OA accumulation. Genes that are functional in responses to osmotic, high salt, and cell wall stresses also contribute to virulence (Fan et al. 2016; Lyu et al. 2016a; Veluchamy et al. 2012; Xu and Chen 2013; Yu et al. 2015). Detoxification of host compounds may be mediated by Ss-BGT1, a putative brassinin glucosyltransferase which glucosylates the phytoalexin brassinin (Pedras and Hossain 2006; Sexton et al. 2009). *Ss-BGT1* gene expression is significantly induced during infection of *Brassica napus* leaves and in response to phytoalexin exposure. Additionally, two putative nitrilases/cyanide hydratases are encoded in the *S. sclerotiorum* genome and may play an important role in detoxifying glucosinolate breakdown products in Brassicaceae hosts.

Do oxalic acid or oxalic acid independent factors play a larger role in host defense suppression? The importance of OA as a virulence factor has been demonstrated by studies with both *S. sclerotiorum* and its hosts. In *S. sclerotiorum*, severe reduction in virulence has been observed in both UV-induced and genetically defined 'oxalate-minus mutants' (Godoy et al. 1990; Liang et al. 2015a; Xu et al. 2015). On the host side, overexpression of oxalate degrading enzyme (e.g., oxalate oxidase, oxalate decarboxylase) enhances resistance against *S. sclerotiorum* infection in a variety of plant species (Calla et al. 2014; Cunha et al. 2010; da Silva et al. 2011; Davidson et al. 2016; Dias et al. 2006; Ghosh et al. 2016; Kumar et al. 2016); moreover, *Medicago truncatula* transgenic lines with a reduced capacity to degrade oxalate, engineered by knocking down expression of the oxalyl-CoA synthetase gene (*Mtaae3*) show increased susceptibility to *S. sclerotiorum* infection (Foster et al. 2016). During pathogenesis, OA plays a range of virulence functions including the creation of a low pH environment to facilitate hydrolytic enzyme activities (Cotton et al. 2003; Favaron et al. 2004; Marciano et al. 1983; Rollins 2003), chelation of calcium to weaken the host cell wall structure and reduce calcium exposure toxicity (Dutton and Evans 1996; Heller and Witt-Geiges 2013), and triggering apoptotic programmed cell death to allow necrotrophic colonization (Kim et al. 2008). Importantly, OA appears to manipulate host cell death fate from a resistance-related autophagy to a susceptibility-related apoptosis (Kabbage et al. 2013). Evidence for these and other activities have been presented in several publications to which the reader is referred (Bolton et al. 2006; Criscitiello et al. 2013; Kabbage et al. 2013, 2015). These findings suggest that oxalic acid is the primary necrotrophic effector in *S. sclerotiorum* playing multifaceted roles and more broadly, high levels of OA accumulation have been implicated in the evolution of broad host-range necrotrophy within the family Sclerotiniaceae (Andrew et al. 2012).

Genetically defined biosynthesis pathway mutants accumulate no detectable OA under a range of in vitro and in planta conditions. Remarkable among the findings during phenotypic characterization of these biosynthetic mutants are the conclusions that (i) these OA-minus mutants retain the ability to infect all tested plants among its broad host range (Li et al. 2018b; Liang et al. 2015a; Xu et al. 2015); and (ii) on a limited number of hosts, lesions spread beyond primary lesions and may fully colonize assayed host tissues. This OA-independent colonization capacity has been attributed to the relative pH buffering capacity of host tissue (Xu et al. 2015). Restricted lesion hosts were determined to have higher buffering capacity than hosts fully colonized by OA-minus mutants (Xu et al. 2015). Regardless of the specific physiological roles, these finding place pH dynamics again at the forefront of pathogenic success and raise important questions regarding the specific pH-independent functions of OA. Loss of function *oah1* mutants created recently using

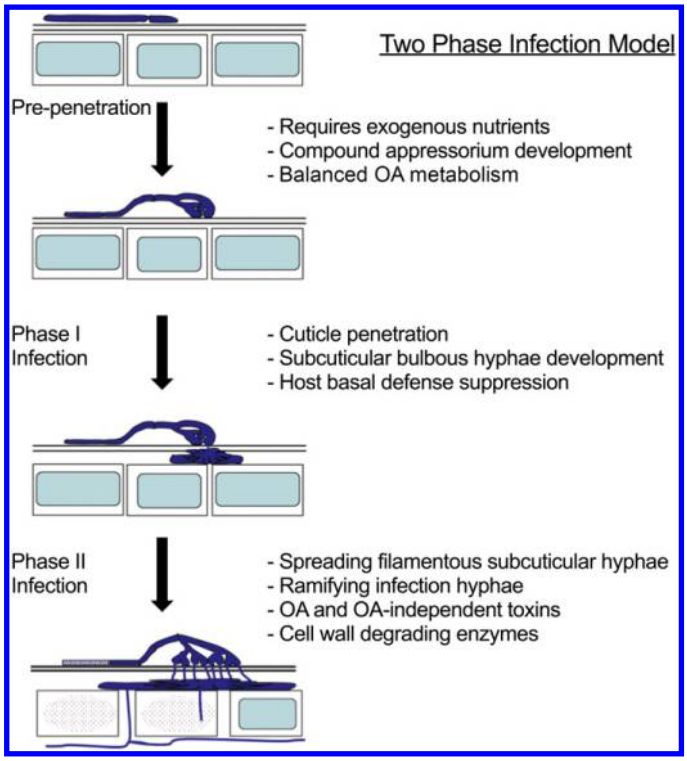

FIGURE 2

Schematic representation of a two-phase *Sclerotinia sclerotiorum* infection model with key cellular events indicated for each infection phase.

CRISPR technology in three independent wild-type backgrounds and comparison with the *oah1* knockout mutant previously created by Xu et al. (2015) reconcile previously reported phenotypic incongruences among *oah1* gene deletion mutants (Liang et al. 2015a; Xu et al. 2015). Multiple examined CRISPR-mediated mutants in all three wild-type backgrounds produced essentially identical phenotypes when compared with the Xu et al. (2015) mutant (Li et al. 2018b). All mutants fail to produce oxalic acid, over-produce compound appressoria on artificial surfaces, and produce functional sclerotia in culture. In host tissues in which lesions can expand, symptom development is obviously different from wild type with less water soaking maceration, a decreased breakdown of chlorophyll, and in some interactions, a reduced rate of colonization (Li et al. 2018b; Xu et al. 2015). Thus, many hosts produce limited lesions when infected by OA-minus mutants and although some host tissues are colonized in the absence of OA, the full range of disease symptoms is not observed. Thus, OA is an important virulence factor that plays a primary role in host colonization rather than in establishing basal host–pathogen compatibility.

The proposition that OA mainly functions at the colonization stage is further supported by additional studies with the wild-type pathogen. Heller and Witt-Geiges (2013) traced the infection-related calcium oxalate depositions based on a potassium pyroantimonate histological staining procedure. At the early infection stage, calcium oxalate is not detected surrounding surface hyphae, compound appressorium, and subcuticular infectious hyphae, but rather in the vesicles of plant surface hyphae. The lack of potassium pyroantimonate staining surrounding early fungal infection structures persist even after calcium chloride treatment, suggesting that OA concentration is maintained at a low level. In contrast, abundant calcium oxalate depositions are observed at the colonization center at the late infection phase, where the host tissues become fully macerated. In another study, Davidson et al. (2016) generated transgenic soybean plants overexpressing oxalate oxidase, these OA-degrading transgenic lines block lesion expansion but not primary lesion formation following *S. sclerotiorum* inoculation. On detached leaflets, primary lesions form similarly between the wild type and the OA-degrading line 18 to 24 h postinoculation although the wild type lesions accumulate significantly more OA. Histological observation shows that during this early period, *S. sclerotiorum* aggressively penetrates and infects both lines, producing subcuticular, intercellular and vascular hyphae with similar densities. At three days postinoculation, plant tissue damage is similar in appearance between the wild-type host and the transgenic oxalate oxidase over-expression host but infectious hyphae formed on the transgenic lines are highly vacuolized and degenerated. The authors suggested a two-phase model for lesion establishment and lesion expansion to explain the lack of lesion expansion by wild-type *S. sclerotiorum* when inoculated on the oxalate oxidase over-expressing line (Davidson et al. 2016). The results of this study (Davidson et al. 2016) are congruent with those in which OA accumulation is eliminated by mutation of the pathogen (Li et al. 2018b; Liang et al. 2015a; Xu et al. 2015) in that both produce only limited lesions on soybean when OA is reduced or eliminated. These independent studies support a two-phase model of pathogenesis.

While the importance of OA in virulence appears to be colonization phase-specific, experimental evidence suggest its virulence functions encompass necrosis inducement as well as defense suppression (Kabbage et al. 2013; Kim et al. 2008; Williams et al. 2011). Based on observations with a redox-regulated GFP reporter, Williams et al. (2011) showed that OA induces an immediate lowered redox environment which suppresses host basal defense reactions. Moreover, Arabidopsis plants infected with UV mutagenesis-generated OA mutants show restricted colonization and undergo cytological changes consistent with host autophagy. Arabidopsis lines with known mutations in the general autophagic pathway are unable to mount an oxidative burst and exhibit increased colonization by these OA mutants (Kabbage et al. 2013). At the same time, OA also induces ROS-dependent apoptosis in promoting necrotic lesion development (Kim et al. 2008). Worthy of note, many studies concerning the virulence functions of OA have been most based on these UV-induced "OA-minus" A mutants. These mutants differ significantly from OA-minus mutants generated by gene-specific mutagenesis in terms of OA accumulation and morphological phenotypes. The UV-induced mutant strains still accumulate a low-level OA and the genetic basis for their pathogenicity defects has not been fully characterized. The availability of genetically-defined OA-minus mutants created through gene deletion (Xu et al. 2015) or gene disruption (Li et al. 2018b) should be utilized for further study of the role and phase-specificity of OA during *S. sclerotium* colonization.

BIOCHEMCIAL PATHWAYS AND MECHANISMS REGULATING OXALIC ACID BIOSYNTHESIS

The importance of OA in *Sclerotinia* pathogenesis warrants a closer look at its metabolism. In filamentous fungi, OA is produced from either the hydrolysis of oxaloacetate, or the oxidation of glyoxylate or glycoaldehyde (Dutton and Evans 1996). In *S. sclerotiorum*, deletion of the *Ss-oah1* gene (encoding oxaloacetate acetylhydrolase [OAH]) completely abolishes OA accumulation (Liang et al. 2015a; Xu et al. 2015). Similarly, loss of function mutants for OAH-encoding genes abolishes OA accumulation in other Ascomycota fungi (Chen et al. 2010; Gombert et al. 2011; Han et al. 2007; Pedersen et al. 2000). Thus, Ascomycota OA biogenesis relies predominately on OAH-mediated oxaloacetate hydrolysis. Biochemically, several metabolic pathways can generate oxaloacetate, namely pyruvate carboxylation mediated by pyruvate carboxylase, malate oxidation mediated by malate dehydrogenase, citrate lysis catalyzed by citrate lyase, and aspartate deamination mediated by aspartate aminotransferase. Aspartate aminotransferase utilizes amino acid substrates whereas pyruvate carboxylase, malate dehydrogenase, and citrate lyase mainly utilize simple carbon metabolites derived from glycolysis, TCA and glyoxylate cycles. Spatially, OAH is a cytoplasmic enzyme and is only accessible to oxaloacetate in the cytosol pool. On the other hand, oxaloacetate generated in the mitochondrial TCA cycle or the peroxisomal glyoxylate cycle, derived from malate dehydrogenase activity localized in these organelles respectively, cannot diffuse across the membrane barrier (Munir et al. 2001). Rather, malate or citrate are exported and transformed into oxaloacetate by cytoplasmic malate dehydrogenase and citrate lyase activities respectively.

Müller and Frosch first proposed that the TCA cycle could transform citrate into OA in *Aspergillus niger* (Müller and Frosch 1975). This hypothesis was based on the presence of TCA cycle enzymes in cell-free extracts with the transformation activity. Later, Kubicek et al. (1988) reported a cytoplasmic OA biosynthesis pathway where oxaloacetate is synthesized from pyruvate and CO_2 by pyruvate carboxylase. In *S. sclerotiorum*, gene deletion mutants of a carnitine acetyl transferase, *Ss-pth2*, fail to grow on media with glycerol, acetate, or oleic acid as the sole carbon source; interestingly, the mutant could efficiently utilize glycerol but not acetate, or oleic acid for OA production (Liberti et al. 2013). Carnitine acetyl transferase catalyzes the reversible transformation between acetyl-CoA and acetyl-carnitine, a critical step for acetyl transfer across membrane barrier and cellular organelles (only acetyl-carnitine but not acetyl-CoA can transfer across the plasma membrane barrier). Ss-Pth2 belongs to a group of Ascomycota proteins namely PTH2/CRAT1, which conservatively encode two versions of proteins, localizing in mitochondria and in peroxisomes, respectively; this localization pattern coordinates the acetyl shuttling among the cytoplasm, peroxisomes, and mitochondria (Hynes et al. 2011). The inability of Δs*s-pth2* to utilize glycerol,

acetate, or oleic acid as the sole carbon source for growth is likely due to a deficiency in energy generation and intermediate metabolite production. The efficient utilization of glycerol for OA production by Δ*ss-pth2*, on the other hand, indirectly supports pyruvate-derived OA generation through pyruvate carboxylation (Fig. 3, left). Interestingly, the Δ*ss-oah1* mutant, defective in OA production, hyper-accumulates fumaric acid, a malate-derived catabolite (Xu et al. 2015). Malate is the metabolic intermediate of both TCA and glyoxylate cycle, supporting the involvement of the TCA and the glyoxylate cycles in OA production (Fig. 3, left). The characterization of a loss of function pyruvate carboxylase mutant would be helpful in discerning the relative importance of pyruvate or malate as oxaloacetate precursor.

In the Basidiomycota fungi, both glyoxylate/glycoaldehyde oxidation and oxaloacetate hydrolysis play significant roles in synthesizing OA (Munir et al. 2001). In the Basidiomycota fungus *Fomitopsis palustris*, the TCA and glyoxylate cycles are coupled for OA biosynthesis (Munir et al. 2001; Sakai et al. 2006). During glucose fermentation, the activity of isocitrate lyase (the branch point enzyme for the glyoxylate cycle) is much higher than the

TABLE 1
List of characterized genes regulating *Sclerotinia sclerotiorum* pathogenesis

Gene name	Mutant phenotypes					Gene functions	Citation
	Mutant generation approach	Compound appressorium formation	Virulence defect on prewounded tissue	Oxalate production phenotype	Mutant phenotypes in addition to virulence defect		
SMK3	Knock out	Abolished	No	Not assessed	Unable to produce sclerotia; increased aerial hyphae formation; increased sensitivity to cell wall stresses	Slt2 MAPK, signaling transduction	(Bashi et al. 2016)
Ss-caf1	T-DNA, Silencing	Abolished	No	Hyperaccumulation	Increased sensitivity to salt and osmotic stresses; defective sclerotial development (reduced melanization, smaller size, looser interior texture, failed carpogenic germination)	Putative secretory protein with a Ca2+-binding EF-hand motif	(Xiao et al. 2013)
Ss-rhs1	Silencing	Reduced	Yes	Slightly reduced	Fewer but larger sclerotia, normal carpogenic germination; slightly reduced growth rate	A secretory protein containing Rhs/YD repeats	(Yu et al. 2016b)
Ss-ggt1	Knock out	Abolished	No	Not assessed	Sclerotia development defect (increased sclerotia initials, delayed sclerotia maturation, thickened and disorganized rind layer, failed carpogenic germination)	γ-Glutamyl transpeptidase, involved in oxidative stress responses	(Li et al. 2012)
Ss-nsd1	Knock out	Abolished	No	Not assessed	Excessive spermatia, loss of sclerotium structural integrity, disrupted ascogonia formation, abolished apothecium development	A GATA-type IVb zinc-finger transcription factor	(Li et al. 2018a)
Ss-odc2	Knock out	Reduced	No	Reduced in vitro, but not in planta	Elevated inoculum nutrient level increases appressorium formation, but not cuticle penetration on soybean leaflets	Oxalate decarboxylase	(Liang et al. 2015b)
CNA1	Silencing	Normal	Yes	Normal	Fungal cell wall more susceptible to hydrolytic enzymes, and β-1,3-glucan synthase inhibitor	Calcineurin, a calcium and calmodulin-dependent Ser/Thr protein phosphatase	(Harel et al. 2006)
Ss-pth2	Knock out	Increased	Yes	Carbon source dependent; slightly reduced in planta	Increased transcripts accumulation of the glyoxylate cycle genes *Ss-mls1* and *Ss-icl1* and the oxalate biosynthetic gene *Ss-oah1* on MM medium with glucose as the carbon source	Carnitine acetyl transferase, involved in acetyl-CoA transfer and metabolism	(Liberti et al. 2013)
Ss-sac1	Knock out	Abolished	Yes	Normal	Reduced radial growth; aberrant sclerotia unable to germinate carpogenically; abundant microconidia	Adenylate cyclase, cAMP signaling	(Jurick and Rollins 2007)
Ss-oah1	Knock out, T-DNA, gene disruption	Increased	Yes	Abolished in vitro and in planta	Reduced radial growth at higher pH; increased fumaric acid accumulation in culture	Oxaloacetate acetylhydrolase, oxalic acid biosynthesis	(Li et al. 2018b; Liang et al. 2015a; Xu et al. 2015)
Ss-pac1	Knock out	Not assessed	Not assessed	Significant reduction	Reduced radial growth at higher pH; aberrant sclerotia development (lacking the melanized rind layer)	Zinc finger transcription factor activated by alkaline pH	(Rollins 2003)

(*Continued on next page*)

[a] *SsPemG1* gene-silenced mutants exhibit an increase in virulence.

activity of isocitrate dehydrogenase (the branch point enzyme for the TCA cycle); moreover, glucose strongly induces the glyoxylate cycle enzyme activities (e.g., malate synthase). Such metabolic coupling would force more glucose-derived carbon to feed into OA (through the glyoxylate cycle) than into carbon dioxide (through the TCA cycle). The *S. sclerotiorum* malate synthase gene *Ss-mls1* has been functionally characterized. The Δ*ss-mls1* loss of function mutant exhibits a severe defect in oleic acid utilization in accordance with its functional importance in the glyoxylate cycle. Yet, Δ*ss-mls1* was not affected in virulence when inoculated with glucose-containing medium (Liberti et al. 2007). Moreover, *Ss-mls1* gene expression is glucose-repressed rather than glucose-induced (Liberti et al. 2013). Further assessment of the OA production potential of the Δ*ss-mls1* mutant on defined carbon source media would help clarifying the importance of glyoxylate cycle in OA production.

Taken together, current data show that *S. sclerotiorum* utilizes multiple metabolic sources to generate oxaloacetate but relies exclusively on OAH activity encoded by the *oah1* gene to catalyze OA production. Oxaloacetate acetylhydrolase is a cytosolic enzyme

TABLE 1
(*Continued from previous page*)

Gene name	Mutant phenotypes					Gene functions	Citation
	Mutant generation approach	Compound appressorium formation	Virulence defect on prewounded tissue	Oxalate production phenotype	Mutant phenotypes in addition to virulence defect		
Ss-nox1	Silencing	Not assessed	Not assessed	Reduced	Unable to produce sclerotia	MADPH oxidase, ROS generation	(Kim et al. 2011)
Ss-sop1	Silencing	Not assessed	Not assessed	Not assessed	Reduced radial growth; aberrant sclerotia formation; increased sensitivity to salt, osmotic, and cell wall stresses; reduced sensitivity to oxidative stress	A microbial opsin homolog, also similar to proton extruding pumps	(Lyu et al. 2016a)
Ss-Bi1	Silencing	Not assessed	Not assessed	Normal	More excessive hyphal tip branching; increased sensitivity to tunicamycin and H_2O_2	BAX inhibitor-1-like protein	(Yu et al. 2015)
Ss-MADS	Silencing	Not assessed	Not assessed	Not assessed	Reduced aerial growth	Transcription factor, Mcm1 ortholog	(Qu et al. 2014)
Ss-SOD1	T-DNA, Knock out	Not assessed	Not assessed	Reduced in one but not another characterized strain	Increased sensitivity to ROS stress; abolished sclerotia development in one but not another strain	Cu/Zn superoxide dismutase, oxidative stress response	(Xu and Chen 2013; Veluchamy et al. 2012)
Scat1	Knock out	Not assessed	Not assessed	Not assessed	Reduced radial growth; smaller and less-melanized sclerotia; increased sensitivity to cell wall and osmotic stresses	Type A catalase	(Yarden et al. 2014)
Ss-ITL	Silencing	Not assessed	Not assessed	Not assessed	Reduced radial growth; denser hyphae, more excessive branching; smaller sclerotia	Integrin-like protein, suppresses host defense reactions when expressed in *Arabidopsis*	(Zhu et al. 2013)
Ss-SSVP1	Silencing	Not assessed	Not assessed	Not assessed	Slightly reduced radial growth	Small cysteine-rich, secretory protein, demonstrated to be internalized within host cells and translocates among cells, interacts with plant QCR8 and induces cell death	(Lyu et al. 2016b)
Ss-CVNH	Silencing	Not assessed	Not assessed	Not assessed	Reduced growth rate; reduced sclerotia formation	Cysteine-rich, small secreted protein, with a carbohydrate-binding module	(Lyu et al. 2015)
Ss-CP1	Knock out	Not assessed	Not assessed	Not assessed	Normal colony morphology, growth rate, and sclerotial development	Small secreted protein with a cerato-platanin domain, induces cell death and plant defense reactions, interacts with plant PR1 protein	(Yang et al. 2018)
Ss-PemG1[a]	Silencing	Increased efficiency	Not assessed	Not assessed	Higher growth rate, higher tolerance toward salt and SDS stresses; higher cellulase and pectinase activities	High sequence similarity to a characterized elicitor in *M. grisea*	(Pan et al. 2015)
Ss-FKH1	Silencing	Not assessed	Not assessed	Not assessed	Reduced growth rate; abolished sclerotia formation; increased sensitivity to osmotic and oxidative stresses	Forkhead transcription factor	(Fan et al. 2016)
Ss-axp	Knock out	Not assessed	Not assessed	Not assessed	Not assessed	Arabinofuranosidase/ β-xylosidase precursor	(Yajima et al. 2009)
Ss-Xyl1	Knock out	Not assessed	Not assessed	Not assessed	Aberrant colony morphology, reduced growth rate, fewer sclerotia which cannot produce apothecia	A putative endo-b-1, 4-xylanase	(Yu et al. 2016a)

(Kubicek et al. 1988). Thus, synthesized OA must be properly processed and transported to avoid cellular toxicity. In *F. palustris*, a six transmembrane domain-encoding gene named *FpOAR* confers OA tolerance when expressed in *Saccharomyces cerevisiae*, indicating a membrane localization and a function in OA secretion (Watanabe et al. 2010). No apparent FpOAR homolog exists in the *S. sclerotiorum* genome. It remains to be determined how OA is exported in *S. sclerotiorum* and other fungi. Such an understanding may provide targets for fungicide development or host engineering to resist cytoplasmic accumulation of OA.

S. sclerotiorum OA accumulation can be affected by a range of factors such as carbon source, nitrogen source, and medium C:N ratio. Among these factors, ambient pH might be the most prominent. Both OA biosynthesis and *Ss-oah1* gene expression are regulated by ambient pH feedback, being strongly induced by neutral ambient pH (Maxwell and Lumsden 1970; Rollins 2003; Rollins and Dickman 2001). This neutral pH induction is controlled by the Pal/PacC pH signaling pathway (Peñalva et al. 2008), as OA accumulation and *Ss-oah1* transcript accumulation can be abolished by deleting the *Ss-Pac1* gene (Kim et al. 2007; Rollins 2003), a zinc finger transcription factor and the central regulator of Pal/PacC pathway (Fig. 3, right). This negative pH feedback control may enable *S. sclerotiorum* to steadily maintain an acidic pH environment, to which it is best adapted. During vegetative growth, OA secreted by *S. sclerotiorum* can acidify the ambient pH to a level below 3.0, a level which many fungi cannot tolerate. Moreover, Δ*ss-oah1* mutants, which cannot accumulate OA or significantly acidify buffered growth media show a severe growth defect at neutral pH (Liang et al. 2015a). Oxalate decarboxylase activity represents another form of OA accumulation regulation. The *S. sclerotiorum* genome encodes two oxalate decarboxylase (ODC) enzymes which catabolize OA into formate and CO_2 (Liang et al. 2015b; Magro et al. 1988). Deletion of one of the encoding genes (*Ssodc2*) but not the other (*Ssodc1*) reduces oxalate accumulation and disrupts normal compound appressorium development and function (Liang et al. 2015b).

FACTORS INVOLVED IN KILLING AND MACERATION OF HOST CELLS

Symptomatically, *S. sclerotiorum* causes tissue maceration following infection indicating rapid cell death and host cell wall

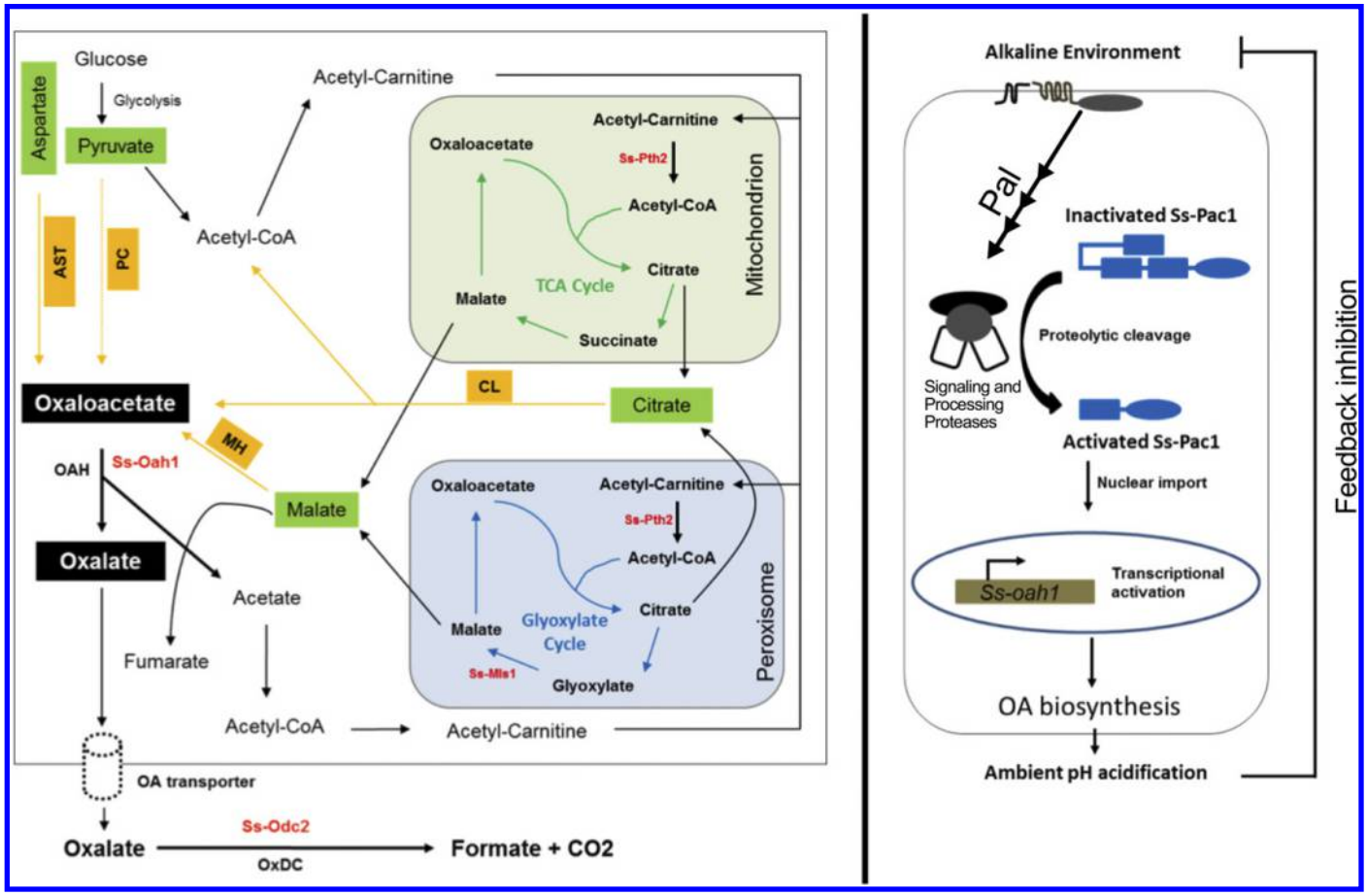

FIGURE 3

Sclerotinia sclerotiorum oxalic acid (OA) metabolism and regulation. Left, proposed OA metabolic pathway. OA production depends on oxaloacetate acetylhydrolase (OAH, Ss-Oah1)-mediated hydrolysis of oxaloacetate. Biochemically, the oxaloacetate precursor may be generated from four metabolite precursors (green boxes: aspartate, pyruvate, malate, and citrate) catalyzed by four cytosolic enzymes: aspartate aminotransferase (AST), pyruvate carboxylase (PC), malate dehydrogenase (MH), and citrate lyase (CL), respectively. Malate and citrate could be derived from the TCA cycle or the glyoxylate cycle. Oxalic acid minus mutants *ss-oah1* hyperaccumulates fumarate (Xu et al. 2015), indicating elevated availability of cytoplasmic malate. Following production and export, OA can be catabolized to produce formate and CO_2 catalyzed by oxalate decarboxylase (OxDC, Ss-Odc2). Red text indicates characterized *S. sclerotiorum* genes. Right, pH regulation of OA biosynthesis via PacC (Ss-Pac1)-dependent activation of *Ss-oah1* gene transcription. Alkaline ambient pH is sensed and transduced through the Pal/PacC pathway (Peñalva et al. 2008) resulting in a final proteolytic processing step to activate the transcription factor Pac1 (a PacC ortholog). The OAH encoded by *Ss-oah1* is positively regulated by Pac1, resulting in the accumulation of OA and subsequent acidification of the ambient environment leading to a dampening of the Pal signaling pathway.

degradation during colonization. Toxins and host cell wall degrading enzymes (CWDEs) are thought to play critical roles in promoting these processes. OA, the primary necrotrophic effector in *S. sclerotiorum*, is known to contribute toward both killing and host cell wall degradation in different ways including pH acidification, calcium chelation, wilt, induction of programed cell death and disruption of chloroplast function (Dutton and Evans 1996; Guimaraes and Stotz 2004; Kim et al. 2008; Noyes and Hancock 1981; Tu 1989). The toxic effect of OA is wide ranging, and OA accumulation level variation has been related to host range evolution within the Sclerotiniaceae family (Andrew et al. 2012). Despite these demonstrated roles for OA, mutants which do not produce OA have the capacity to colonize some hosts under laboratory conditions (Liang et al. 2015a; Xu et al. 2015), suggesting that fumaric acid (Xu et al. 2015) or other factors may at least partially compensate for the lack of OA during colonization.

In addition to OA, the *S. sclerotiorum* genome encodes proteinaceous factors able to induce necrosis, such as necrosis and ethylene-inducing peptides (Dallal Bashi et al. 2010), endopolygalacturonases (Bashi et al. 2013; Zuppini et al. 2005), and a cutinase (Zhang et al. 2014). Recently, two small secretory necrosis-inducing protein, Ss-SSVP1 and Ss-CP1, have been demonstrated to contribute toward full virulence and have been characterized in detail (Lyu et al. 2016b; Yang et al. 2018). Ss-SSVP1 induces host cell death upon transient expression in *Nicotiana benthamiana*, either with or without a signal peptide, suggesting that the protein functions inside the host cell. Importantly, based on fluorescent protein tagging, SsSSVP1 is demonstrated to be internalized inside and translocated among host cells. SsSSVP1 interacts with the subunit 8 of cytochrome b-c1 complex (QCR8), a highly conserved mitochondrial protein in plants; silencing of QCR8 causes abnormal plant development and cell death. Likely, SsSVP1 promotes infection by inducing QCR8 mislocalization and thus necrosis. QCR8 is highly conserved in plant species, indicating the broad spectrum of Ss-SSVP1 virulence function. Ss-CP1 is a small secreted protein with 138 amino acids. It belongs to the cerato-platanin protein family and induces necrosis-like cell death when transiently expressed in *N. benthamiana*. *Arabidopsis thaliana* plants stably expressing *Ss-CP1* exhibit hallmarks of an activated salicylic acid defense pathway and show enhanced disease resistance. Ss-CP1 localizes in the apoplastic space and interacts with plant PR1; however, the protein region required for this interaction is dispensable for plant immunity activation (Yang et al. 2018).

The rapid tissue maceration associated with *S. sclerotiorum* infection is a result of highly active plant cell wall degradation. This activity may be mediated by pectolytic activity, and endo-polygalacturonases in particular. The *S. sclerotiorum* genome encodes five endo-polygalacturonases, and their expression during infection and in response to pH and nutrient conditions have been well-characterized (Bashi et al. 2012; Cotton et al. 2003; Kasza et al. 2004; Li et al. 2004). However, none of the endo-polygalacturonase has hitherto been functionally analyzed via gene mutation. The *S. sclerotiorum* and *B. cinerea* genomes encode a similar number of carbohydrate-active enzymes (CAZyme) as their hemibiotrophic and saprophytic relatives, suggesting that gene content variations are not key characteristics distinguishing different trophic life styles, on the other hand gene expressional regulations may play an more important role (Amselem et al. 2011). Despite functional redundancy commonly observed with cell wall degrading enzymes, gene deletion of an arabinofuranosidase/β-xylosidase precursor gene and an endo-b-1, 4-xylanase encoding gene caused significant virulence reduction in *S. sclerotiorum* (Yajima et al. 2009; Yu et al. 2016a).

SUMMARY AND FUTURE PERSPECTIVES

S. sclerotiorum is an economically devastating pathogen that has provided significant insights into the mechanisms of necrotrophic colonization and broad host range specificity. Over the past several years, a number of virulence genes have been identified and characterized from *S. sclerotiorum* (Table 1). Defining the functions of these genes has demonstrated that this devastating necrotroph interacts with plant hosts in a much more nuanced manner than previously thought. In this review, we have proposed a two-phase infection model in which the pathogen suppresses defense reactions in the early phase and induces host necrosis and host cell wall degradation in the late phase. Moreover, different infection structures may have different functional specializations (Fig. 2). In the future, while it is important to identify novel virulence factors, it is equally important to pinpoint the temporal and spatial specificities of the identified virulence functions, so as to place gene functions in an infection phase-related context.

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