Oxaloacetate acetylhydrolase gene mutants of *Sclerotinia sclerotiorum* do not accumulate oxalic acid, but do produce limited lesions on host plants

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SUMMARY

The oxaloacetate acetylhydrolase (OAH, EC 3.7.1.1)-encoding gene *Ss-oah1* was cloned and functionally characterized from *Sclerotinia sclerotiorum*. *Ss-oah1* transcript accumulation mirrored oxalic acid (OA) accumulation with neutral pH induction dependent on the pH-responsive transcriptional regulator *Ss-Pac1*. Unlike previously characterized ultraviolet (UV)-induced oxalate-deficient mutants ('A' mutants) which retain the capacity to accumulate OA, gene deletion $\Delta ss-oah1$ mutants did not accumulate OA in culture or during plant infection. This defect in OA accumulation was fully restored on reintroduction of the wild-type (WT) *Ss-oah1* gene. The $\Delta ss-oah1$ mutants were also deficient in compound appressorium and sclerotium development and exhibited a severe radial growth defect on medium buffered at neutral pH. On a variety of plant hosts, the $\Delta ss-oah1$ mutants established very restricted lesions in which the infectious hyphae gradually lost viability. Cytological comparisons of WT and $\Delta ss-oah1$ infections revealed low and no OA accumulation, respectively, in subcuticular hyphae. Both WT and mutant hyphae exhibited a transient association with viable host epidermal cells at the infection front. In summary, our experimental data establish a critical requirement for OAH activity in *S. sclerotiorum* OA biogenesis and pathogenesis, but also suggest that factors independent of OA contribute to the establishment of primary lesions.

Keywords: necrotroph, oxalate, oxalic acid, pathogenesis, phytopathogen, *Sclerotinia*, virulence.

INTRODUCTION

The Ascomycota fungus *Sclerotinia sclerotiorum* (Lib.) de Bary is one of agriculture's most devastating necrotrophic fungal plant

pathogens. It infects over 400 plant species and accounts for an estimated \$200 million in annual crop losses in the USA (Bolton *et al.*, 2006). During pathogenesis, *S. sclerotiorum* accumulates millimolar levels of oxalic acid (OA) which has been implicated as being critical to its broad host range necrotrophic pathogenicity (Andrew *et al.*, 2012). The most compelling evidence for the importance of OA in pathogenesis comes from ultraviolet (UV)-induced oxalate-deficient mutants ('A' mutants), reported to be non-pathogenic on *Phaseolus vulgaris* (Godoy *et al.*, 1990) and *Arabidopsis thaliana* (Dickman and Mitra, 1992). These mutants have since been shown to accumulate low levels of OA and to produce limited lesions on various hosts (Cessna *et al.*, 2000; Kim *et al.*, 2008; Williams *et al.*, 2011). In addition to pathogenicity, OA accumulation may also affect the growth and developmental processes of *S. sclerotiorum*. The 'A' mutants exhibit reduced growth and aberrant development, including fluffy hyphae and abolished sclerotium formation, whereas, in the wild-type (WT), low ambient pH promotes both sclerotium morphogenesis and vegetative growth (Rollins, 2003; Rollins and Dickman, 2001). These effects of OA may be derived from its effect on ambient pH, as well as its metabolic connections with primary metabolism.

OA is a strong dicarboxylic organic acid synthesized by a broad range of pathogenic and non-pathogenic organisms, including bacteria, fungi, plants and some mammals. In filamentous fungi, OA biogenesis has been demonstrated to occur through the hydrolysis of oxaloacetate, the oxidation of glyoxylate or the oxidation of glycolaldehyde (Dutton and Evans, 1996). Previous biochemical studies have indicated that the conversion of oxaloacetate to OA and carbon dioxide, catalysed by oxaloacetate acetylhydrolase (OAH, EC 3.7.1.1), is the primary activity associated with OA biogenesis in Ascomycota fungi, including *S. sclerotiorum* (Kubicek *et al.*, 1988; Maxwell, 1973; Ruijter *et al.*, 1999). This enzymatic function has been further supported by the abolition of OA accumulation through *oah* gene disruption in *Aspergillus niger* (Pedersen *et al.*, 2000a, b), *Botrytis cinerea* (Han *et al.*, 2007), *Cryphonectria parasitica* (Chen *et al.*, 2010) and *Penicillium chrysogenum* (Gombert *et al.*, 2011). In

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S. sclerotiorum, neutral pH strongly induces OA accumulation and OAH activity (Maxwell, 1973; Rollins and Dickman, 2001). This neutral pH induction is dependent on the zinc finger ambient pH-dependent transcriptional regulator *Ss-Pac1* (Rollins, 2003; Rollins and Dickman, 2001). Diverse carbon sources can support OA biogenesis in *S. sclerotiorum* with intermediates derived from the tricarboxylic acid (TCA) cycle or the glyoxylate cycle depending on the carbon growth substrate (Liberti *et al.*, 2013).

To determine the role of OAH in *S. sclerotiorum* OA metabolism and to evaluate its requirement for pathogenesis, we isolated and performed gene deletion analysis of a putative OAH-encoding gene *Ss-oah1*. We demonstrate that *Ss-oah1* is required for OA accumulation, and that it affects pH-responsive growth, morphogenesis and virulence of *S. sclerotiorum* on a variety of plant hosts. The observation that the *Ss-oah1* knockout mutants ($\Delta ss-oah1$ mutants) retain the ability to form primary lesions suggests that factors other than OA contribute significantly towards the establishment of initial host compatibility, i.e. primary lesions. Histological features of infection suggest that *S. sclerotiorum* can initially colonize its hosts to a limited degree without OA accumulation, and that the infection and colonization process involves a dynamic regulation of OA accumulation.

RESULTS

Ss-oah1 gene cloning and sequence analysis

The *Ss-oah1* coding sequence was initially identified among expressed sequence tags (ESTs) as the most abundant clone within a cDNA library derived from neutral pH-treated vegetative hyphae (Amselem *et al.*, 2011). The genomic sequence of the *Ss-oah1* locus was obtained by PCR-based genome walking. Following the *S. sclerotiorum* genome annotation (Amselem *et al.*, 2011), the *Ss-oah1* genomic sequence was confirmed to be identical with Broad Institute database locus ID SS1G_08218.3 (GenBank Accession XM_001590428). The encoded protein, *Ss-Oah1*, exhibits 90.7% and 62.7% sequence identity with the OAH protein from *B. cinerea* and *A. niger*, respectively, and contains a conserved active site serine residue (S^{278}) present in all other enzymatically characterized OAH enzymes (Joosten *et al.*, 2008).

Ss-oah1 transcript accumulation is alkaline induced and requires *Ss-Pac1*

Previously, we have reported that the zinc finger transcription factor *Ss-Pac1* is required for neutral pH-induced OA accumulation in *S. sclerotiorum* (Rollins, 2003). Here, we compared *Ss-oah1* transcript accumulation levels between the WT and *Ss-pac1* gene deletion mutant ($\Delta ss-pac1$) over a range of buffered (pH 3.0–7.0) growth media (Fig. 1A). *Ss-oah1* transcript accumulation was

Fig. 1 Northern hybridization analysis of *Ss-oah1* transcript accumulation in response to ambient pH, nutrients and development. Ethidium bromide-stained 28S rRNA is shown as a loading control. (A) Effect of ambient pH. Vegetative hyphae treated for 4 h at the indicated pH values. WT, wild type. (B) Effect of pH and carbon source. Vegetative hyphae treated for 4 h in MM-YEG medium containing glucose, polygalacturonic acid (PGA), PGA + glucose, glycerol or no carbon source. Each carbon source was evaluated at three different pH values (pH 3, 5 and 7). (C) Effect of developmental stages. M, mycelia collected from 4-day-old stationary potato dextrose broth (PDB) culture; S, sclerotia at developmental stage 3–4; A, apothecia at developmental stage 4–5; CA induction, compound appressoria induced by growing hyphae on cellophane overlaid on potato dextrose agar (PDA) medium; IT, infected tomato leaflets [2 days post-inoculation (dpi)]; T, mock-inoculated tomato leaflets (2 dpi).

detected in all pH treatments in both strains; however, as the pH increased from 3.0 to 7.0, the hybridization signal intensity increased dramatically in the WT, but not in the $\Delta ss-pac1$ mutant. Consistent with direct regulation by Ss-Pac1, four PacC binding sites (5'-GCCARG-3') were identified in the *Ss-oah1* promoter region at 773, 1094, 1108 and 1742 bp upstream of the predicted translation start site (Table S1, see Supporting Information).

Ss-oah1 transcript accumulation was further examined in liquid growth medium with different carbon sources [glucose, polygalacturonic acid (PGA), glucose + PGA, glycerol and no carbon] across three pH values (Fig. 1B). Strong neutral pH induction occurred in the WT, but not in the $\Delta ss-pac1$ mutant, for all carbon sources. In the WT, *Ss-oah1* transcript levels were slightly depressed in cultures lacking glucose, particularly under 'no carbon' conditions, but neutral pH induction remained constant. We also examined the effect of exogenous OA supplementation at pH 4.8 and observed no inductive or repressive effect with a range of OA concentrations from 0 to 40 mM (data not shown). A comparison of *Ss-oah1* transcript accumulations across different life stages revealed similar accumulation levels among vegetative hyphae, sclerotia and infected host, but no accumulation in developing apothecia and a small decrease during compound appressorium development (Fig. 1C). This decrease (two-fold between 36 and 48 h, and five-fold between 48 and 72 h) was validated by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (Table S2, see Supporting Information).

Ss-oah1 is required for OA biogenesis in *S. sclerotiorum*

Ss-oah1 gene knockout mutants were generated with the strategy illustrated in Fig. S1A (see Supporting Information). Two genetically pure gene knockout mutants, designated $\Delta ss-oah1$ KO1 and KO2, were obtained and further verified by Southern blot analysis (Fig. S1B). Hybridization probes designed to the *Ss-oah1* coding sequences and downstream of the coding sequence produced band patterns consistent with the expected homologous DNA integration event illustrated in Fig. S1A.

The first evidence that Ss-Oah1 is required for OA accumulation was an observed defect in medium acidification by the $\Delta ss-oah1$ mutants. Potato dextrose agar (PDA) medium supplemented with bromophenol blue (violet, > pH 4.6; yellow, < pH 3.0) changed colour rapidly from violet to yellow following WT growth, whereas plates inoculated with the $\Delta ss-oah1$ mutants remained violet, even after complete plate colonization (Fig. 2A). The medium colonized by the 'A' mutants (Godoy *et al.*, 1990) acidified at a slower rate and to a reduced extent compared with the WT (Fig. 2A).

We quantified OA accumulation for strains growing in 0.5 M 3-(*N*-morpholino)propanesulfonic acid (MOPS)-buffered YPSu (pH 7.0), a growth medium highly conducive for OA accumulation in the WT (Fig. 2B). Although the WT and complemented (Com) strain accumulated OA rapidly, reaching approximately 10 mg/mL by 48 h post-inoculation, no OA accumulation occurred in the

Fig. 2 The $\Delta ss-oah1$ mutants (KO1, KO2) are defective in oxalic acid accumulation. (A) Medium acidification is indicated by a colour change from violet to yellow. (B) Oxalic acid accumulation kinetics in 0.5 M 3-(*N*-morpholino)propanesulfonic acid (MOPS)-buffered YPSu shake culture (pH 7.0). Each data point represents the mean $\pm$ standard deviation based on measurements from three independent cultures. WT, wild type; Com, complementation strain; KO1, KO2, $\Delta ss-oah1$ gene deletion mutants; A1, A2, A3, UV-induced oxalic acid-deficient mutants.

$\Delta ss-oah1$ mutants over a time course of 192 h. Under the same condition, the 'A' mutants accumulated significant OA, but to levels lower than the WT, reaching approximately 6 mg/mL by 48 h post-inoculation (Fig. 2B). The pH values of the media in which the WT and Com strain were grown decreased rapidly and stabilized near pH 4.0 by 48 h post-inoculation, whereas the values for the $\Delta ss-oah1$ mutants and the 'A' mutants stayed above pH 6.0 (data not shown). We also examined OA accumulation in infected leaves of soybean, common bean and tomato. In all three hosts, OA was detected in the WT and Com strain, but not in the $\Delta ss-oah1$ or 'A' mutants (Table S3, see Supporting Information). The abolished *in vitro* and *in planta* OA accumulation of the $\Delta ss-oah1$ mutants demonstrated that Ss-Oah1 is required for OA biogenesis in *S. sclerotiorum*.

$\Delta ss-oah1$ mutants differ from the 'A' mutants in pH-responsive radial growth and in development

On PDA medium, the growth rates of the $\Delta ss-oah1$ and 'A' mutants were 65% and 50% of the WT, respectively. Compared with the WT, the $\Delta ss-oah1$ mutants showed no growth reduction on PDA medium buffered at pH 3.6, slight growth reduction on medium buffered at pH 5.4, but severe growth reduction on medium buffered at pH 6.9 [Fig. S2 (see Supporting Information), KO2 data not shown]. The 'A' mutant, in contrast, exhibited reduced radial growth on medium buffered at all tested pH values (Fig. S2, 'A1' and 'A3' data not shown). The *Ss-oah1* Com strain grew in the same manner as the WT under all pH conditions (Fig. S2). In PDA culture, the $\Delta ss-oah1$ mutants differentiated loose, unmelanized stroma-like aggregates (covering the entire colony) rather than discrete, compact and melanized sclerotia (Fig. 3A); the 'A' mutants did not form stromatic tissue, but developed fluffy aerial hyphae. The $\Delta ss-oah1$ mutants, but not the 'A' mutants, were defective in compound appressorium formation on artificial surfaces, including Petri dishes (data not shown), cover slides (data

not shown) and parafilm (Fig. 3B). The Com strain formed discrete sclerotia, but with delayed development and at a lower frequency than the WT. These Com strains did not form compound appressoria on artificial inductive surfaces. We screened more than 150 complementation transformants from two independent complementation constructs encompassing different lengths of the *Ss-oah1* gene locus, but still failed to obtain a strain fully complemented in sclerotium and appressorium development. In older PDA cultures, the 'A' mutants produced spermatia abundantly (c. 10^8 per plate), whereas the WT, $\Delta ss-oah1$ mutants and Com strain produced them sporadically and at levels less than 10^3 per plate (data not shown).

$\Delta ss-oah1$ mutants exhibit significantly attenuated virulence

The $\Delta ss-oah1$ mutants and Com strain could not infect efficiently as a result of a defect in appressorium development (data not shown). This made it difficult to assess the importance of OA accumulation for cuticle penetration. Instead, the role of OA in virulence, independent of penetration, was assessed by performing pathogenicity tests by wound inoculation. The tests were performed on a variety of hosts (soybean, common bean, tomato, canola, sunflower, *Arabidopsis*). In all cases, the $\Delta ss-oah1$ and 'A' mutants produced limited lesions compared with the WT and Com strain (Table S3, Fig. 4 and data not shown). Although the WT and Com strain produced light brown spreading lesions, the $\Delta ss-oah1$ and 'A' mutants produced restricted brown–green lesions defined by a thin, dark border (Fig. 4). Over time, lesions generated by the $\Delta ss-oah1$ and 'A' mutants appeared dark green, reminiscent of 'green islands' or 'green necronissia', whereas the surrounding uncolonized leaf tissue became yellow and senescent (Fig. S3, see Supporting Information). In detached soybean leaf inoculations, vascular colonization of the $\Delta ss-oah1$ and 'A' mutants was accompanied by red host pigment accumulation (presumably anthocya-

Fig. 3 Colony morphology and compound appressorium formation phenotypes. (A) Potato dextrose agar (PDA) colony morphology [10 days post-inoculation (dpi)]. (B) Compound appressoria formation on parafilm (3 dpi). (C) Microscopic assessment of compound appressorium development. Scale bars represent 50 μ m. WT, wild type; Com, complementation strain; KO1, KO2, $\Delta ss-oah1$ gene deletion mutants; A1, A2, A3, UV-induced oxalic acid-deficient mutants.

Fig. 4 Virulence assays on detached tomato leaves (A), detached soybean leaves (B), detached *Arabidopsis* leaves (C) and intact *Arabidopsis* plants (D). Leaves were wounded prior to inoculation. Photographs in (A), (B) and (C) were taken 2, 7 and 2 days post-inoculation, respectively. WT, wild type; Com, complementation strain; KO1, KO2, $\Delta ss-oah1$ gene deletion mutants; A1, A2, A3, UV-induced oxalic acid-deficient mutants.

nin) and restricted lesion development on emergence from the vascular tissue (Fig. S3). On *Arabidopsis* plants, the infections of the $\Delta ss-oah1$ and 'A' mutants were confined to the inoculated leaves (Fig. 4). OA treatment partially restored the virulence defects in the $\Delta ss-oah1$ and 'A' mutants [Fig. S4 (see Supporting Information) and data not shown]. On all tested hosts, the $\Delta ss-oah1$ mutants were more virulent than the 'A' mutants (Table S3 and data not shown), probably as a result of their different growth rates.

Green fluorescent protein (GFP)-labelled strains were used to investigate the fate of colonizing hyphae. These strains infected with the same characteristics as their parental strains (data not shown). Lesions generated by the GFP-labelled $\Delta ss-oah1$ and 'A' mutants expanded slowly and were delimited within 3 days. Under microscopic observation, the WT hyphae at the infection front exhibited strong GFP fluorescence at all surveyed time points [1, 3 and 5 days post-inoculation (dpi)], as shown in Fig. 5. The infectious hyphae of the $\Delta ss-oah1$ mutants were more closely aligned to one another relative to the WT, and exhibited strong GFP fluorescence

early in the colonization process (Fig. 5, 1 dpi). Over time, the GFP fluorescence faded from the $\Delta ss-oah1$ hyphae, coincident with an increase in host cell autofluorescence (Fig. 5, 3 and 5 dpi). The infectious hyphae of the 'A' mutant behaved in a very similar manner to those of the $\Delta ss-oah1$ mutants (data not shown).

The $\Delta ss-oah1$ and 'A' mutants elicit elevated host defence reactions

3,3'-Diaminobenzidine (DAB) precipitation, indicative of H_2O_2 accumulation, was observed within hyphae at the infection front, as well as within the surrounding mesophyll cells, with more intense staining observed in the $\Delta ss-oah1$ and 'A' mutant infections relative to the WT and Com strain infections (Fig. 6A). Under observation by fluorescence microscopy with UV excitation, particles stained by aniline blue (indicative of callose deposition) were observed to accumulate more extensively at the infection fronts of the $\Delta ss-oah1$ and 'A' mutants compared with the WT and Com strain (Fig. 6B).

Fig 5 Viability of infectious hyphae at the infection front. Detached soybean leaves were wounded prior to inoculation with green fluorescent protein (GFP)-labelled strains; sampled leaf tissues were observed directly under the microscope. Images of the same panel from left to right show views from bright field (I), GFP fluorescence with autofluorescence background (II) and GFP fluorescence alone (III). Scale bars represent 100 μ m. Note that the $\Delta ss-oah1$ KO2 mutant produces more GFP fluorescence and has a denser subcuticular growth habit relative to the wild-type (WT). dpi, days post-inoculation.

Fig. 6 Host defence reactions elicited on detached soybean leaflets. To visualize the deposition of H₂O₂ and callose, leaf samples were stained with 3,3'-diaminobenzidine (A) or aniline blue (B). The scale bars in (A) and (B) represent 1 mm and 100 μ m, respectively. WT, wild type; Com, complementation strain; KO2, $\Delta ss-oah1$ gene deletion mutant; A2, UV-induced oxalic acid-deficient mutant.

Comparative cytology of WT and $\Delta ss-oah1$ infection of onion epidermal peels

Under laboratory conditions, WT *S. sclerotiorum* penetrated the cuticle of onion epidermal strips and differentiated bulbous subcuticular hyphae between 12 and 18 h post-inoculation (Fig. S5A, see Supporting Information). The subcuticular bulbous hyphae differentiated filamentous hyphae and spread by intercellular and subcuticular growth (Fig. S5C,F). In contrast, the $\Delta ss-oah1$ mutants required wound inoculation to penetrate the cuticle barrier. Following penetration, these mutants differentiated subcuticular, filamentous hyphae, similar in appearance to those of the WT, except that the hyphae were more densely aligned to one another (data not shown). Using a plasmolysis assay to gauge host cell viability, the tips of subcuticular hyphae for both the WT and $\Delta ss-oah1$ mutants were frequently observed above viable

epidermal cells, often growing over two or three cells before a trailing host cell death was observed (Fig. S6, see Supporting Information). For the first 24 h, the subcuticular hyphae represented the forward-most position in host colonization; simultaneously, the leading hyphal colonization front of intercellular hyphae was always associated with nonviable host cells in both WT and $\Delta ss-oah1$ colonization.

To assess the spatial accumulation of OA during onion epidermal colonization by the WT, cross-polarized light microscopy was utilized to detect OA crystals. Crystals were observed with the characteristic bipyramidal shape of dihydrate calcium oxalate and the highly birefringent ovoid and biconcave shapes of monohydrate calcium oxalate. No crystal accumulation was observed following inoculation with the $\Delta ss-oah1$ mutants (data not shown). With the WT, OA crystals rarely accumulated in the proximity of epiphytic hyphae or compound appressoria prior to

cuticle penetration; in contrast, OA crystals frequently accumulated directly at the point of penetration (Fig. S7A,B, see Supporting Information). At advanced infection stages, OA crystals accumulated abundantly within fully disrupted epidermal cells and old compound appressoria at the lesion centre (Fig. S7C,D). In contrast, OA crystals rarely accumulated in the proximity of spreading subcuticular hyphae, except when they became stressed or lost viability (indicated by cell browning and the loss of GFP fluorescence). OA crystals were also frequently observed to accumulate below viable epidermal cells in the adhering adaxial cell wall matrix ahead of the colonization front, and became increasingly abundant as colonization proceeded (Fig. S7D,E).

DISCUSSION

OA is a dicarboxylic acid produced by a broad spectrum of fungal species (Dutton and Evans, 1996). In these fungi, OA biosynthesis is primarily catalysed by glyoxalate dehydrogenase or OAH activity. We demonstrate here that *Ss-oah1* gene deletion abolishes OA accumulation both *in vitro* and *in planta* in *S. sclerotiorum*. This inability to accumulate OA exists even when mutant hyphae are cultured in media strongly buffered at neutral pH with nutrients highly conducive for OA accumulation (Rollins, 2003). Thus, OA biogenesis in *S. sclerotiorum* appears to rely exclusively on OAH-mediated C–C cleavage of oxaloacetate. This result is consistent with previous detection of OAH enzyme activity, but not glyoxalate dehydrogenase enzyme activity, in *S. sclerotiorum* (Maxwell, 1973), and with the lack of a predicted glyoxalate dehydrogenase-encoding gene in the *S. sclerotiorum* genome (Amselem *et al.*, 2011). Our results and reports from other Ascomycota fungi, demonstrating that *oah* gene disruption abolishes OA biogenesis (Chen *et al.*, 2010; Gombert *et al.*, 2011; Han *et al.*, 2007; Pedersen *et al.*, 2000a, b), suggest that oxaloacetate is the predominant precursor for OA production in Ascomycota fungi. This finding contrasts with what is known from Basidiomycota fungi, where OAH and glyoxalate dehydrogenase activities can both contribute significantly to OA biogenesis (Munir *et al.*, 2001).

From our transcript profile results, ambient pH appears to be the primary exogenous regulator of *Ss-oah1* gene expression in vegetative hyphae. Other factors, including carbon source and exogenous OA, have a much smaller or no influence, respectively. Development has a stage-specific influence, with no detectable transcript accumulation during apothecium development and subtle changes in accumulation dynamics during appressorium development. Our previous report indicating that *Ss-Pac1* is required for high levels of OA production (Rollins, 2003), and the results reported here indicating that *Ss-Pac1* is required for neutral pH induction of *Ss-oah1* transcript accumulation, lead us to hypothesize that *Ss-Pac1* directly and positively regulates *Ss-oah1* transcription by promoter binding. This hypothesis is further supported by the presence of four *PacC* consensus binding sites

(Tilburn *et al.*, 1995) within the intergenic region upstream of the *Ss-oah1* translation start site. These data suggest a pH feedback regulation model in which *Ss-Oah1*-mediated accumulation of OA leads to ambient environmental acidification, resulting in a down-regulation of *Ss-Pac1* signalling, *Ss-oah1* transcription, OAH activity and OA accumulation. Such a feedback regulatory mechanism might be conserved among many Ascomycota fungi, as alkaline pH also strongly induces OA accumulation in *A. niger* and *B. cinerea* (Kubicek *et al.*, 1988; Manteau *et al.*, 2003; Ruijter *et al.*, 1999). In addition, *PacC* binding sites are enriched within the upstream intergenic regions of all known Ascomycota *oah* orthologues (Table S1). The prevalence of this pH modulation strategy and its biological significance should be further investigated with comparative studies of the expression and function of *oah* orthologues from different pathogenic and non-pathogenic Ascomycota species.

The reintroduction of the WT *oah1* gene into $\Delta ss-oah1$ mutants fully restored OA accumulation to the levels and kinetics of the WT. These same Com strains, however, were only partially restored for sclerotium development and not restored for compound appressorium development. This result remained unchanged when a longer intergenic region of the *Ss-oah1* locus was used for complementation. Thus, the involvement of OA metabolism in sclerotium and compound appressorium development is more complex than the accumulation of OA alone. This conclusion is supported by the fact that the sclerotium defect seen in the $\Delta ss-oah1$ mutants could not be restored by decreasing the growth medium pH or by adding exogenous OA (data not shown). Possibly, *S. sclerotiorum* requires precise and dynamic regulation of OA metabolism to support these developmental events, which is not fully achieved by ectopic complementation. Alternatively, epigenetic processes that attenuate these developmental stages in the absence of OA accumulation may not be reversed on genetic complementation. Under either scenario, we are unable to draw firm conclusions regarding the role of *Ss-oah1* or OA accumulation in the regulation of sclerotium and compound appressorium developmental processes. As discussed below, however, the roles for *Ss-oah1* in virulence are strongly supported by the mutant and Com strain data.

The $\Delta ss-oah1$ mutants exhibited a significant virulence defect on a range of hosts, resulting in the formation of limited lesions. This virulence phenotype was fully restored via genetic complementation and partially restored by the application of exogenous OA. Host defence reactions, including reactive oxygen species (ROS) accumulation and callose deposition, were associated with the limited lesions, as reported previously for the OA-deficient 'A' mutants (Williams *et al.*, 2011). We also observed the persistence of chlorophyll, evident as 'green islands', within the restricted colonized lesion, indicating that chloroplast disruption observed during WT colonization (Heller and Witt-Geiges, 2013; Tariq and Jeffries, 1985) is primarily mediated by OA accumulation. These

results demonstrate the critical requirement of Ss-Oah1-mediated OA biosynthesis during *S. sclerotiorum* pathogenesis, and are consistent with the multifaceted virulence functions associated with OA during pathogenesis (Bateman and Beer, 1965; Cessna *et al.*, 2000; Cotton *et al.*, 2003; Errakhi *et al.*, 2008; Guimaraes and Stotz, 2004; Heller and Witt-Geiges, 2013; Kabbage *et al.*, 2013; Kim *et al.*, 2008; Lampl *et al.*, 2013; Noyes and Hancock, 1981; Rollins and Dickman, 2001; Williams *et al.*, 2011).

Using a variety of histological and microscopic approaches, Heller and Witt-Geiges (2013) recently demonstrated that OA accumulates minimally in the proximity of subcuticular hyphae and that oxalate crystal formation is limited to advanced and late stages of the infection process (36–72 h). A similar spatial distribution pattern of oxalate crystals was observed in this study, except that we also observed the deposition of crystals at the sites of cuticular penetration. These results are consistent with an emerging model suggesting that only low levels of OA accumulate in the early infection process of *S. sclerotiorum* (Billon-Grand *et al.*, 2012; Heller and Witt-Geiges, 2013). The specific roles of the host and the pathogen in the regulation of this accumulation will be of great interest going forward. The transient living cell interaction between subcuticular hyphae and epidermal cells strongly indicates that *S. sclerotiorum* interacts with its hosts in a much more nuanced manner beyond the disruption and consumption of host cells. This scenario is consistent with the recently reported observations concerning the growth of *S. sclerotiorum* infectious hyphae in living plant tissue (Kabbage *et al.*, 2013). These results indicate the need for high-resolution spatial and temporal analysis of infection-related transcript, protein and metabolite accumulation to better understand the complexities of necrotrophic colonization.

Given the importance of the UV-induced OA-deficient mutants ('A' mutants) in establishing the requirement for OA in pathogenesis (Godoy *et al.*, 1990) and the continued use of these mutants as tools for dissecting the functions of OA in pathogenesis and host response (Kabbage *et al.*, 2013; Kim *et al.*, 2008; Williams *et al.*, 2011), we compared the phenotypes of the 'A' mutants with those of the $\Delta ss-oah1$ mutants created in this study. This comparison revealed that the two classes of mutant differed in several respects: (i) the $\Delta ss-oah1$ mutants failed to accumulate OA under any tested condition, whereas the 'A' mutants accumulated low levels of OA in culture, but not *in planta*; (ii) the 'A' mutants, but not the $\Delta ss-oah1$ mutants, exhibited fluffy mycelial growth and produced spermatia abundantly; (iii) the $\Delta ss-oah1$ mutants, but not the 'A' mutants, were deficient in compound appressorium differentiation; and (iv) acid pH conditions fully rescued the growth defect of the $\Delta ss-oah1$ mutants, but not the 'A' mutants. These differences in phenotypic characters and the observation of identical *Ss-oah1* nucleotide sequences in the WT and A2 mutant (data not shown) suggest that the 'A' mutants have a mutation(s) at a locus other than *Ss-oah1*. Despite the observed phenotypic

differences in growth and development and the independence of the underlying mutation(s), the $\Delta ss-oah1$ and 'A' mutants are strikingly similar in virulence and symptom development phenotypes, and the application of exogenous OA partially restores virulence to both. Given these similarities and the lack of detectable OA accumulation *in planta*, we suggest that either mutant class may be used for the further evaluation of the functions of OA during *S. sclerotiorum* pathogenesis. Although the 'A' mutants remain genetically undefined, they offer an experimental advantage in their ability to form appressoria and directly penetrate unwounded host tissue.

Although the $\Delta ss-oah1$ and 'A' mutants showed significant virulence reduction, both categories of mutant could successfully induce the formation of primary lesions with fungal hyphae remaining viable during lesion establishment. Thus, *S. sclerotiorum* can establish limited compatibility with its host in the absence of OA. Although a relationship between early, abundant OA accumulation and broad host range aggressiveness is observed in the Sclerotiniaceae, additional OA-independent 'toolbox genes' may play roles in the establishment of host–pathogen compatibility in host specialist pathogens within this family (Andrew *et al.*, 2012). The fact that the $\Delta ss-oah1$ and 'A' mutants could still establish limited lesions suggests that such additional factors may also function in host generalists, such as *S. sclerotiorum*. Consistent with this proposition, we have observed the hyperaccumulation of OA crystals during failed attempts to infect onion epidermal peels by non-adapted pathogens within the Sclerotiniaceae family (Rollins and Kim, unpublished data). In addition, hyperaccumulation of OA via genetic mutation (Kim *et al.*, 2007) or hypovirus infection (Li *et al.*, 1996) does not increase symptom development and, indeed, can decrease virulence. Thus, OA accumulation alone may not be sufficient to confer full virulence in host generalists, such as *S. sclerotiorum*.

Two major virulence functions that may support the establishment of necrotrophic host compatibility include defence suppression as well as toxicity towards host cells. Secreted proteins functioning in defence suppression, such as Ss-ITL and Ss-Cm1, have been reported recently (Djamei *et al.*, 2011; Kabbage *et al.*, 2013; Zhu *et al.*, 2013). In addition, recently, a large set of candidate effector proteins with unknown and proposed functions in host damage and host defence suppression/avoidance have been categorized bioinformatically from the *S. sclerotiorum* genome (Guyon *et al.*, 2014). The establishment of *S. sclerotiorum* primary infection may involve an active suppression and avoidance of host basal defence reactions and associated cell death independent of OA accumulation. With regard to the factors triggering toxicity towards host cells, in addition to OA, programmed cell death (PCD)-triggering endopolygalacturonases have been identified from *S. sclerotiorum* (Bashi *et al.*, 2013; Zuppini *et al.*, 2005). In the closely related necrotroph *B. cinerea*, a range of necrosis-inducing factors, including xylanase (Brito *et al.*, 2006; Noda *et al.*,

2010), polygalacturonases (Kars *et al.*, 2005), cerato-platanin family protein (Frias *et al.*, 2011), botcinic acid and botrydial (Dalmis *et al.*, 2011), have been demonstrated to contribute to virulence. Gene clusters homologous to the *B. cinerea* botcinic acid biosynthesis cluster have been identified from the *S. sclerotiorum* genome (Dalmis *et al.*, 2011), and this gene cluster is up-regulated during infectious development (Rollins, unpublished data). It is reasonable to assume that the *S. sclerotiorum* infection relies on an arsenal of toxic necrotrophic factors in addition to OA.

In summary, functional analysis of the *Ss-oah1* gene has demonstrated that the encoded OAH activity is essential for *S. sclerotiorum* OA biogenesis. The absence of OA accumulation during infection does not prevent the establishment of a primary lesion, but does prevent the production of a spreading lesion and associated host colonization. These data support the view that the interaction between *S. sclerotiorum* and its hosts involves a dynamic regulation of OA accumulation levels. In addition, factors other than OA may contribute significantly towards *S. sclerotiorum* pathogenesis, possibly via both defence suppression/avoidance and disruption of host function. Further research efforts to identify and characterize OA-independent virulence factors would provide a better understanding of *S. sclerotiorum* virulence, and thus new targets for resistance to this broad host range necrotrophic pathogen.

EXPERIMENTAL PROCEDURES

Fungal strains and maintenance

Sclerotinia sclerotiorum WT isolate 1980 and the derived UV-induced mutants (A1, A2, A3) (Godoy *et al.*, 1990) were maintained on PDA. The Δ *ss-oah1* strains (KO1, KO2) were maintained on PDA supplemented with 100 µg/mL hygromycin and the Com strains (compl1 and compl2) were maintained on PDA supplemented with 10 µg/mL Bialaphos (Phyto Technology Laboratories, Overland Park, KS, USA) or 200 µg/mL nourseothricin (Werner BioAgents, Jena, Germany), respectively.

Cloning, deletion and complementation of the *Ss-oah1* gene locus

The full-length *Ss-oah1* coding sequence was initially identified as a cDNA clone from hyphae transferred from acidic (pH 3) growth conditions and incubated at neutral (pH 7) conditions for 4 h. The corresponding genomic sequence and flanking sequences were isolated from total genomic DNA by PCR and Genome Walking™ (Clontech, Mountain View, CA, USA) using the provided arbitrary primer 1 (AP1), gene-specific upstream primer GWOAH-F1 (5'-GGCGGCCAGTCGAGAATGAAGTAGTGGTAAGAGTTG-3') and gene-specific downstream primer GWOAH-R1 (5'-GGCGGCCTATCATCTAGTAAGTGGTGGTCTAG-3') with *Ascl* restriction enzyme recognition sites (italic) added to the 5' ends of the gene-specific primers. Amplicons (c. 1.0 and 1.6 kb, respectively) were cloned into pCR2.1 TOPO vectors

(Invitrogen, Carlsbad, CA, USA). The AP1-GWOAH-F1 amplicon was isolated from its vector as an *Ascl*-*NotI* fragment and ligated into the pGWOAH-R1 *Ascl*-*NotI* site. Subsequently, the hygromycin resistance gene cassette from pGEMHPH (Hutchens, 2005) was isolated as an *Ascl* fragment and ligated into the pGWOAH-F1 *Ascl* site that now intervened between the *Ss-oah1* upstream and downstream sequences. The final construct represents 1.0 kb of the 5' *Ss-oah1* untranslated region (UTR) sequence beginning 7 bp upstream of the *Ss-oah1* start codon, and 1.6 kb of the 3' *Ss-oah1* UTR sequence beginning 32 bp downstream of the stop codon, with the intervening *Ss-oah1* coding sequence deleted and replaced with the hygromycin resistance gene cassette in the opposite transcriptional orientation relative to the original *Ss-oah1* locus. Ten micrograms of this gene replacement construct were linearized by *NotI* restriction enzyme digestion and transformed into WT *S. sclerotiorum* protoplast, as described previously (Rollins, 2003). Hygromycin-resistant transformants ($n = 52$) were screened by Southern blot hybridization with the DIG DNA Labeling and Detection Kit (Roche Diagnostics, Indianapolis, IN, USA).

Two independent *Ss-oah1* gene complementation vectors were constructed by cloning genomic PCR amplicons derived from F1 and R1 primer pairs (oah_Compl_F1 5'-AACCAGACTGGCTGCCAGCAAGATACA-3' with oah_Compl_R1 5'-TCACATGAGCTGACACGCTGCGGGTAA-3'), and from F2 and R2 primer pairs (oah1_Compl-F2 5'-CACAGCCTAGTTTGGTGATG-3' with oah1_Compl-R2 5'-GGTAGGTGCACAATGCAGA-3'). The amplicons were initially cloned into pGEMT-easy (Promega, Madison, WI, USA) for sequence verification. The F1-R1 amplicon was subcloned into the *NotI* site of plasmid pBARKS1 carrying the *bar* gene resistance cassette for Bialaphos selection (Pall and Brunelli, 1993). This complementation vector (pOAH1_compl1) contained the *Ss-oah1* genomic coding sequence with 2.9 kb of upstream DNA sequence and 712 bp of downstream genomic sequence. The second complementation vector (pOAH1_compl2) constructed in pD-NAT1 (Kück and Hoff, 2006) contained the *Ss-oah1* genomic coding sequence with 1.2 kb of upstream DNA sequence and 343 bp of downstream genomic sequence. Both plasmids were used to transform protoplasts of the *Ss-oah1* gene deletion mutants and selected on regeneration medium containing 10 µg/mL Bialaphos or 200 µg/mL nourseothricin as appropriate. Following the screening of more than 75 transformants for each construct, identical phenotypes were obtained with both plasmids and, as such, only strains from the pOAH1_compl2 transformants (Com) were presented.

Treatments, sample collection and gene expression analysis

pH treatments of vegetative hyphae were carried out as described previously (Rollins, 2003); citric acid–sodium phosphate buffers (pH range from 3.0 to 7.0) were used to generate pH-buffered YPSu media [containing, per litre, 4 g of yeast extract (Difco, Sparks, MI), 15 g of sucrose, 1 g of K₂HPO₄ and 0.5 g of MgSO₄]. For carbon source gene expression studies, starter cultures were established in *Aspergillus* minimal medium (Barratt *et al.*, 1965) with 10 g/L yeast extract (MM-YEG). The initial pH was adjusted to either pH 4.0 for the Δ *ss-pac1* strain, or pH 6.5 for the WT. Hyphae from starter cultures were washed with sterile water, collected by vacuum filtration and transferred to the pH/carbon series media. The treatment medium was MM-YEG or MM-YEG with the glucose replaced with 5 g PGA, 5 g PGA + 10 g glucose, 10 mL glycerol or no carbon source. Each

carbon source was evaluated at three different pH values buffered with citric acid–sodium phosphate (pH 3), citric acid (pH 5) or piperazine-*N,N'*-bis-2-ethanesulfonic acid (pH 7). Samples were collected following a 4-h treatment period. ³²P-labelled *Ss-oah1* coding sequence was used as the probe for Northern blot hybridization analysis. The WT RNA and $\Delta ss-pac1$ RNA samples were separated on the same gel, transferred onto the same membrane and hybridized within the same tube to allow for direct comparison of the hybridization signals.

OA treatments of vegetative hyphae were carried out in YPSu medium buffered at pH 4.8 with citric acid–sodium phosphate buffer. Fresh mycelia (approximately 2 g) collected from actively growing YPSu cultures were pretreated in buffered YPSu medium (pH 4.8) for 4 h before being divided for OA treatments. For treatments, OA was added to the medium to a final concentration ranging from 0 to 40 mM and the medium pH values were readjusted to pH 4.8. Following a 4-h incubation, mycelia were harvested and lyophilized for total RNA extraction and Northern blot analysis.

Analysis of *Ss-oah1* transcript accumulation at different developmental stages was conducted with hyphae collected from stationary potato dextrose broth (PDB) culture at 4 dpi, with compound appressoria induced by the inoculation of mycelial plugs on cellophane (catalogue no. 165–1779, Bio-Rad, Hercules, CA, USA) overlaid on PDA medium, with infected tomato tissue (2 dpi), with sclerotia at developmental stage 3–4 (Li and Rollins, 2009) and with apothecia at developmental stage 4–5 (Veluchamy and Rollins, 2008). qRT-PCR was used to quantify *Ss-oah1* gene expression during compound appressorium development. Total RNAs were isolated with the RNeasy Mini Kit (Qiagen Sciences, Valencia, CA, USA), treated with DNases (Qiagen Sciences) and reverse transcribed with the iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. Five-fold-diluted cDNA products were used as templates for subsequent PCRs. The primer pair *SsOah1F* (5'-GAAGTTGTCCCTGAAGAAG-3')/*SsOah1R* (5'-GTGCGTGCAATCAACACAATA-3') was used to amplify a 96-bp amplicon of *Ss-oah1*. The *S. sclerotiorum* histone H3 gene was used as the endogenous reference gene with the primer pair H3-F1 (5'-ATGGCTCGTACCAAGCAAAC-3')/H3-R1 (5'-AGAGCACCAATAGCGGAAGA-3') to amplify a 27-bp amplicon. iTaq™ Universal Probes Supermix (Bio-Rad) was used to set up the PCR mixes. The PCRs were carried out on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The thermocycle programme consisted of 30 s at 95 °C and 44 cycles of 10 s at 95 °C, 10 s at 55 °C and 30 s at 60 °C. The $\Delta\Delta C_t$ method was used to calculate the relative transcript accumulation levels of different samples (Livak and Schmittgen, 2001).

Radial growth and OA accumulation

Radial mycelial growth assays in PDA cultures were carried out by the methods described previously (Kim *et al.*, 2007; Rollins, 2003). Citric acid–sodium phosphate buffer was used to generate pH-buffered PDA medium. The relative growth rate was calculated from the slopes of the linear regions of the growth curve.

For medium acidification assays, PDA medium was adjusted to pH 7.0 with NaOH, autoclaved and supplemented with 50 mg/L bromophenol blue before being poured into 5-cm Petri dish plates. For OA quantification in YPSu-MOPS medium, 500 μ L of culture filtrates were sampled at each time point. OA quantification was performed with an enzymatic assay kit (Trinity Biotech., Wicklow, Ireland). OA standards with defined concentra-

tions (0–90 mg/L) were used to generate the standard curve. Serial dilutions were carried out to position samples in the range of the standard curve. During this study, we found that data extrapolation associated with our previous OA quantification protocol placed sample absorption values above the standard curve range, which resulted in an underestimation of the OA concentrations of the samples (Kim *et al.*, 2007; Rollins, 2003). The resulting underestimation of OA concentrations did not affect the reported accumulation kinetics or the conclusions drawn in previous studies, but did under-report the OA concentrations. The measurements reported here were derived from serial dilutions of media and reflect a more accurate report of OA accumulation in growth media.

To quantify *in planta* OA accumulations, soybean, common bean and tomato leaves were harvested at 3, 2 and 2 dpi, respectively. Lesion sizes were quantified with the Spot Advanced Software program (Diagnostic Instruments, Sterling Heights, MI, USA) before the lesions were excised and collected in 2-mL Eppendorf tubes. Tissues were lyophilized, broken into a fine powder with a metal spatula and suspended in 1 mL of distilled water. A 100- μ L volume of activated charcoal was added to each tube to absorb leaf pigments. The tissue suspensions were left on the bench for 30 min and centrifuged at 11 750 *g* for 10 min; the supernatants were then used for OA quantification.

Compound appressorium assay

Mycelial plugs with growing hyphal tips were placed on parafilm and incubated in a moisture chamber for 2 days to induce compound appressorium development. For microscopic observation, mycelial plugs were placed on the top of cover slides and incubated for 2 days.

Pathogenicity assays

Tomato (*Lycopersicon solanum* cv. Bonnie Best), common bean (*Phaseolus vulgaris* cv. Bush Blue Lake 47), soybean (*Glycine max* [L.] Merr. cv. Harosoy) and canola (*Brassica napus*) were grown in the glasshouse; *Arabidopsis thaliana* (ecotype Columbia) and sunflower (*Helianthus annuus*) plants were grown in a growth chamber at 20 °C with a 16-h day and 8-h night cycle using 15-W GE cool white fluorescent bulbs (General Electric Co., Fairfield, CT, USA). Mature, healthy and fully expanded leaves were excised from vigorously growing plants and immediately placed inside a moist chamber for pathogen inoculation. For wound inoculations, two 0.5-cm intersecting cuts were made through each leaf with a sharp scalpel blade and a mycelial plug was placed directly over the wound. For exogenous OA treatments, soybean petioles bearing trifoliate leaves were placed in small vials with double-distilled H₂O, 5 mM OA (adjusted to pH 5.8 with KOH) or 10 mM OA (adjusted to pH 5.8 with KOH), respectively; the trifoliate leaves were immediately wounded and inoculated with mycelial plugs. The petioles submerged in the vials were kept inside a moisture chamber to inhibit leaf wilting and to stimulate symptom development.

GFP-labelled strains

The *B. cinerea* codon-optimized *egfp* reporter gene (Leroch *et al.*, 2011) was used to generate the GFP-expressing p-Blunt-NAT-GFP via subcloning. The pD-NAT1 vector (Kück and Hoff, 2006) was double digested with

*Bam*HI and *Hind*III to release the nourseothricin acetyltransferase gene (*nat1*) expression cassette, which was ligated into the pCR®-Blunt (Invitrogen, Grand Island, NY, USA) vector to generate the pBlunt-NAT1 construct. The pOPTGFP vector, containing the *egfp* expression cassette and an incomplete hygromycin expression cassette, was *Sal*I digested and then self-ligated to obtain the pOPTGFP-*Sal*I construct. The *A. nidulans* *oliC* promoter-driven GFP expression cassette was released from the pOPTGFP-*Sal*I vector by *Spe*I and *Xho*I double digestion, which was then ligated into the pBlunt-NAT1 construct to generate the final construct p-Blunt-NAT-GFP. The p-Blunt-NAT-GFP construct was transformed into WT, $\Delta ss-oah1$ KOs and the A2 mutant to obtain constitutive GFP-expressing strains.

Onion epidermal infections

Yellow onion (*Allium cepa* var. *cepa*) bulbs were purchased from a local grocery store. Thick, fleshy bulb scale leaves were cut into 2 cm × 2 cm chunks and the inner epidermal strip was peeled off and placed on top of a glass slide with the cuticle layer up. Mycelial plugs with spreading hyphal tips were directly inoculated onto the epidermal strip surface. The epidermal strips were kept inside a moisture chamber for 12–24 h before microscopic examination. For inoculation with the $\Delta ss-oah1$ mutant, a 0.5-cm cross was cut on the epidermal strip with a scalpel before inoculation. The epidermal strips were placed in 0.75 M sucrose solution for 15 min to induce plasmolysis.

Staining and microscopic observation

Infected soybean plants were collected at 2 dpi to detect host defence reactions. DAB (Sigma-Aldrich, St. Louis, MO, USA) was used for H₂O₂ detection. DAB was dissolved in diluted HCl solution (pH 3.0) to a final concentration of 1 mg/mL; Na₂HPO₄ at a final concentration of 10 mM was added to stabilize the pH value at approximately pH 9.0. For staining, infected soybean leaves were immersed in DAB solution and shaken at 50 rpm for 6 h. The stained leaves were then fixed and cleared in the fixation solution (ethanol–acetic acid, 3:1). Aniline blue dissolved in 0.1 M Na₂HPO₄ to a final concentration of 0.05% was used to stain for callose. Cleared leaf tissues were stained overnight before examination by fluorescence microscopy.

Onion epidermal strips were either examined directly or stained with trypan blue solution for 5 h (dissolved in double-distilled H₂O to a final concentration of 0.5%) or safranin O solution for 5 min (dissolved in 70% ethanol to a final concentration of 0.1%) before examination. Cross-polarized light filters were utilized to detect birefringent crystals within infected tissues (Khan, 1995).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Generation and verification of *Ss-oah1* gene replacement mutants. (A) Schematic representation of the *Ss-oah1* genomic locus and expected gene replacement event. (B) Southern blot analysis of the wild-type (WT), knockout mutants (KO1, KO2) and complementation strain (Com). Left panel: *Hind*III (H)-digested total genomic DNA hybridized with probe 1. Right panel: *Hind*III (H)- and *Xba*I (X)-digested total genomic DNA hybridized with probe 2. UTR, untranslated region.

Fig. S2 Radial growth kinetics on potato dextrose agar (PDA) medium and PDA medium buffered with citric acid–sodium phosphate buffer at the indicated initial pH values. Data points represent the mean values from three independent colony replicates. The standard deviations for all data points did not exceed 0.3 and were not plotted. WT, wild type; A2, UV-induced oxalate-deficient mutant; KO1 and KO2, $\Delta ss-oah1$ gene deletion mutants; Com, complementation strain.

Fig. S3 'Green island/necronissia' lesions produced by the $\Delta ss-oah1$ mutant (KO2) on detached soybean (A) and detached canola (B) leaves. Leaves were wounded prior to inoculation. Leaves in (A) were photographed at 5 days post-inoculation. WT, wild-type.

Fig. S4 Exogenous oxalic acid (OA) treatment partially restored the virulence defect of the $\Delta ss-oah1$ mutants (KO1, KO2).

Following petiole immersion in OA (pH 5.8) or double-distilled H₂O, trifoliolate leaves were wounded and inoculated with mycelial plugs (3 days post-inoculation). CK, non-inoculated control.

Fig. S5 Cytological events related to the infection of *Sclerotinia sclerotiorum* on onion epidermal strips sampled between 12 and 24 h post-inoculation (hpi). (A) Compound appressorium-mediated cuticle penetration; arrowhead indicates the differentiation of nascent, bulbous subcuticular hyphae. (B) Papilla deposition and cell wall autofluorescence at the penetration sites. (C) Densely aligned subcuticular hyphae formed at the initial penetration site; arrowhead indicates the sheath layer surrounding subcuticular hyphae. (D) An established infection detailing where infectious hyphae had spread and killed epidermal cells over a large area. (E) A subcuticular hypha showing initial contact with the host plasma membrane (arrowhead); note the loss of plasmolysis activity of the penetrated cell, but not the two neighbouring cells. (F) Subcuticular hyphae penetrating into the anticlinal cell wall (arrowheads). P, penetration sites; PM, plasma membrane; SH, subcuticular hyphae. The scale bars represent 100 μ m.

Fig. S6 Frequent association between subcuticular hyphae and viable (plasmolysis-positive) onion epidermal cells observed with the wild-type (WT) and the $\Delta ss-oah1$ mutant (KO2). Plasmolysed onion epidermal cells in association with fungal subcuticular hyphae are marked with red dots. Scale bars represent 200 μ m. GFP, green fluorescent protein.

Fig. S7 Oxalic acid (OA) crystal accumulation dynamics during onion epidermal peel colonization by *Sclerotinia sclerotiorum* wild-type (WT). (A, B) Penetration stage OA crystals. Crystals were rarely observed surrounding epiphytic hyphae or compound appressoria on the cuticle surface, but were frequently observed at the penetration points. (C) OA crystals at the early infection stages. Crystals did not accumulate around subcuticular hyphae, but accumulated abundantly within old compound appressoria. (D, E) OA crystals at advanced infection stages. Crystals accumulated heavily within fully disrupted epidermal cells at the lesion centre, but occurred sporadically at the infection front; some crystals accumulated below (on the adaxial surface) viable epidermal cells in areas ahead of the colonization front, which became more abundant as colonization proceeded (dead epidermal cells in E are indicated by a red dot). CA, compound appressoria; P, penetration sites; SH, subcuticular hyphae; VH, vegetative hyphae. Scale bars represent 100 μ m.

Table S1 PacC binding sites (GCCARG) at the upstream intergenic regions of Ascomycota *oah* homologues. *oah* orthologues are defined as those showing gene order synteny conservation with known *oah* genes.

Table S2 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) quantification of *Ss-oah1* gene expression during compound appressorium development.

Table S3 Lesion development and oxalic acid (OA) accumulation.