Fungal oxalate decarboxylase activity contributes to *Sclerotinia sclerotiorum* early infection by affecting both compound appressoria development and function

XIAOFEI LIANG¹, ELLEN W. MOOMAW² AND JEFFREY A. ROLLINS^{1,*}

¹Department of Plant Pathology, University of Florida, PO Box 110680, Gainesville, FL 32611-0680, USA

²Department of Chemistry and Biochemistry, Kennesaw State University, 1000 Chastain Road, MD# 1203, Kennesaw, GA 30144, USA

SUMMARY

Sclerotinia sclerotiorum pathogenesis requires the accumulation of high levels of oxalic acid (OA). To better understand the factors affecting OA accumulation, two putative oxalate decarboxylase (OxDC) genes (*Ss-odc1* and *Ss-odc2*) were characterized. *Ss-odc1* transcripts exhibited significant accumulation in vegetative hyphae, apothecia, early stages of compound appressorium development and during plant colonization. *Ss-odc2* transcripts, in contrast, accumulated significantly only during mid to late stages of compound appressorium development. Neither gene was induced by low pH or exogenous OA in vegetative hyphae. A loss-of-function mutant for *Ss-odc1* ($\Delta ss-odc1$) showed wild-type growth, morphogenesis and virulence, and was not characterized further. $\Delta ss-odc2$ mutants hyperaccumulated OA *in vitro*, were less efficient at compound appressorium differentiation and exhibited a virulence defect which could be fully bypassed by wounding the host plant prior to inoculation. All $\Delta ss-odc2$ phenotypes were restored to the wild-type by ectopic complementation. An *S. sclerotiorum* strain overexpressing *Ss-odc2* exhibited strong ODC, but no oxalate oxidase activity. Increasing inoculum nutrient levels increased compound appressorium development, but not penetration efficiency, of $\Delta ss-odc2$ mutants. Together, these results demonstrate differing roles for *S. sclerotiorum* ODCs, with Odc2 playing a significant role in host infection related to compound appressorium formation and function.

Keywords: appressorium, infection, oxalate, oxalic acid, pathogenesis, penetration, *Sclerotinia*.

INTRODUCTION

Sclerotinia sclerotiorum is a necrotrophic fungal phytopathogen causing multi-million-dollar losses each year in many important crops and vegetables (Bolton *et al.*, 2006). The pathogenic

success of *S. sclerotiorum* depends on complex changes in fungal development and metabolism comparable with the changes observed with biotrophic and hemibiotrophic pathogens. The general course of infection begins with airborne ascospores which must first saprotrophically colonize senescent or wounded tissues before entering healthy host tissues (Abawi *et al.*, 1975). The penetration of healthy tissues is achieved by modified hyphae that form compound appressoria (Abawi *et al.*, 1975; Tariq and Jeffries, 1984) ranging in developmental complexity from simple swellings to complex, bifurcating, multi-hyphal structures (Fig. S1, see Supporting Information). Each tip of a compound appressorium may penetrate the host cuticle and form vesicles of bulbous, subcuticular hyphae (Fig. S1), which give rise to filamentous subcuticular, cortical and ramifying hyphae (Lumsden and Dow, 1973). Coincident with this development is the production and accumulation of oxalic acid (OA), although the temporal and spatial regulation of this accumulation on a fine scale is just beginning to be documented (Heller and Witt-Geiges, 2013; Liang *et al.*, 2014).

The simple dicarboxylic organic acid OA contributes to *S. sclerotiorum* pathogenesis in myriad ways. During pathogenesis, OA elicits apoptotic-like programmed cell death (PCD) and wilting as a non-host-selective toxin (Errakhi *et al.*, 2008; Guimaraes and Stotz, 2004; Kim *et al.*, 2008; Lampl *et al.*, 2013; Noyes and Hancock, 1981), sequesters calcium (Heller and Witt-Geiges, 2013) and facilitates cell wall degradation by acting synergistically with polygalacturonases (PGs) (Bateman and Beer, 1965; Cotton *et al.*, 2003; Rollins and Dickman, 2001). In addition, OA suppresses host defence reactions and regulates the nature of host cell death (Cessna *et al.*, 2000; Dutton and Evans, 1996; Heller and Witt-Geiges, 2013; Kabbage *et al.*, 2013; Williams *et al.*, 2011). Mutants deficient in OA accumulation are severely affected in virulence (Godoy *et al.*, 1990; Liang *et al.*, 2014) and plants engineered to express OA-degrading enzymes, including oxalate oxidase (OxOx) and oxalate decarboxylase (OxDC), provide enhanced resistance against *S. sclerotiorum* infection (Donaldson *et al.*, 2001; Hu *et al.*, 2003; Livingstone *et al.*, 2005).

Several genetic factors have been identified that regulate OA accumulation in *S. sclerotiorum*. OA biogenesis occurs through oxaloacetate acetylhydrolase (*Ss-Oah1*)-mediated C–C cleavage

*Correspondence: Email: rollinsj@ufl.edu

of oxaloacetate (Liang *et al.*, 2014). Depending on the growth substratum, the tricarboxylic acid (TCA) cycle or the glyoxylate cycle may generate the oxaloacetate precursor (Liberti *et al.*, 2013). Neutral pH strongly induces OA biosynthesis and this induction requires the activation of *Ss-oah1* gene transcription by the zinc finger transcription factor *Ss-Pac1* (Liang *et al.*, 2014; Rollins, 2003).

In addition to OA biogenesis, OA accumulation can also be regulated by OA-degrading enzymes. Across phylogenetic lineages, three types of OA-degrading enzyme are known, specifically OxDC (EC 4.1.1.2), OxOx (EC 1.2.3.4) and oxalyl-CoA decarboxylase (OXC, EC 4.1.1.8). OxDC catalyses the production of formate and CO₂ from OA, and its activity occurs widely among fungi and bacteria (Mäkelä *et al.*, 2010). OxOx catalyses the oxidation of OA to produce CO₂ and H₂O₂, and its activity has been primarily reported from monocot plants. OxOx identified from the Basidiomycota fungus *Ceriporiopsis subvermisporea* is hitherto the only example identified outside of the plant kingdom (Escutia *et al.*, 2005). OXC converts activated oxalyl-CoA to formyl-CoA and CO₂ with thiamine pyrophosphate as the cofactor. Currently, this enzyme activity has been reported only from bacterial species (Baetz and Allison, 1989).

OxDC activity, but not OxOx activity, has been reported in *S. sclerotiorum* (Magro *et al.*, 1988). The *S. sclerotiorum* genome encodes two predicted OxDCs [Broad Institute locus ID: SS1G_08814 (*Ss-odc1*) and SS1G_10796 (*Ss-odc2*)], but no predicted OxOx. To characterize the role of OxDC activity on *S. sclerotiorum* OA accumulation and virulence, we carried out gene expression and deletion analysis of *Ss-odc1* and *Ss-odc2*. We found that the *Ss-odc2* gene down-regulates OA accumulation, its expression is strongly induced during compound appressorium development and gene deletion mutants are defective in appressorium development and infection at the pre-invasive stage. The requirement for OxDC-mediated OA catabolism for early infection indicates the existence of a fine-tuned regulation of OA accumulation during *S. sclerotiorum* pathogenesis.

RESULTS

Transcript accumulations of *Ss-odc1* and *Ss-odc2* genes

The *Ss-odc1* and *Ss-odc2* genes are predicted to encode proteins of 455 and 504 amino acids which share 59.2% sequence identity. Both *Ss-Odc* proteins are predicted, using SignalP, to contain an N-terminal secretion signal. Consistent with OxDC enzymes identified from fungi and bacteria, both *Ss-Odc* proteins contain a bicupin domain structure predicted by Pfam, conserved amino acids coordinating Mn²⁺ binding and acidic amino acids at the putative proton donor sites (Glu¹⁶² and Glu³³³ in the *Bacillus subtilis* OxDC) (Fig. S2, see Supporting Information). OxOxs from plants and fungi share similar features with OxDC, but OxOx

Fig. 1 Northern hybridization analysis of *Ss-odc1* and *Ss-odc2* transcript accumulation. Ethidium bromide-stained rRNA (28S) is shown as a loading control. (A) pH and oxalic acid (OA) treatments were conducted in citrate phosphate-buffered YPSu shaking culture for 4 h. (B) 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 induction on cellophane overlaid on potato dextrose agar (PDA) medium; abundant appressoria start to develop from 36 h post-inoculation (hpi); IT, infected tomato leaflets collected at 2 days post-inoculation (dpi); T, mock-inoculated tomato leaflets (2 dpi).

enzymes lack the conserved acidic residue at the putative proton donor sites (Fig. S2).

Northern blot hybridization was used to determine the effects of ambient pH, exogenous OA and tissue development stage on *Ss-odc1* and *Ss-odc2* transcript accumulation. Over a wide range of pH and OA treatment conditions, a hybridization band of similar signal intensity was observed for *Ss-odc1* in vegetative hyphae, whereas transcript accumulation of *Ss-odc2* was never detected (Fig. 1A). *Ss-odc1* transcript accumulation was detected at varying levels across all developmental life stages and during plant colonization (Fig. 1B). Compared with vegetative hyphae, the accumulation level was lower in sclerotia, apothecia and during mid to late stages of compound appressorium development (48 and 72 h post-cellophane induction). The *Ss-odc2* transcript, in contrast, accumulated significantly only during the mid to late stages of compound appressorium development (36, 48 and 72 h post-cellophane induction, Fig. 1B). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to quantitatively assess *Ss-odc2* transcript accumulation (Fig. S3, see Supporting Information). This analysis revealed a six-fold increase in infected

Fig. 2 Tissue specificity of *Ss-odc2* gene expression determined by promoter-driven green fluorescent protein (GFP) expression. Compound appressoria were induced on cellophane overlaid on potato dextrose agar (PDA) medium. GFP expression driven by the constitutive *oliC* promoter was used as a control. Arrowheads indicate compound appressoria. Scale bars: 200 μ m.

tomato leaflets, a 30-fold increase in sclerotia and apothecia, and a greater than 980-fold increase during compound appressoria development relative to vegetative hyphae. The exposure of vegetative hyphae to 20 mM exogenous OA resulted in a small (approximately two-fold) increase in *Ss-odc2* transcript accumulation relative to the untreated control (Fig. S3).

The tissue specificity of *Ss-odc2* gene expression was further characterized via *Ss-odc2* promoter-driven green fluorescent protein (GFP) expression. Fluorescence was not observed in vegetative hyphae growing on solid or liquid medium, nor was fluorescence induced by 4-h treatments with low pH, exogenous OA or starvation (data not shown). On cellophane-overlaid potato dextrose agar (PDA) medium, strong GFP fluorescence was observed in compound appressoria and in the hyphae immediately supporting their formation, but not in other vegetative hyphae (Fig. 2), which was in contrast with the universal GFP fluorescence driven by the constitutive *oliC* promoter (Fig. 2). On

PDA medium, fluorescence was strong in aerial hyphae growing at the colony centre and was weakly observed in developing sclerotia (Fig. S4, see Supporting Information). These patterns of fluorescence are consistent with the Northern hybridization and qPCR data.

$\Delta ss-odc2$ mutants differentiate compound appressoria less efficiently than the wild-type (WT)

Independent, genetically pure gene replacement mutants were obtained for both *Ss-odc1* and *Ss-odc2* (Fig. S5A, B, see Supporting Information). For *Ss-odc1* gene knockout (KO) analysis, WT and transformant genomic DNAs were digested with *EcoRV* for Southern hybridization. Hybridization with probe 1 [the *Ss-odc1* partial open reading frame (ORF)] yielded a 1.2-kb band for the WT, but no band for the KO mutant, whereas hybridization with probe 2 [the *Ss-odc1* 5' untranslated region (UTR)] yielded a 2.6-kb band for the WT and a 5.4-kb band for the KO mutant. These hybridization patterns are consistent with *Ss-odc1* gene replacement by homologous integration as illustrated in Fig. S5A. For *Ss-odc2* gene KO analysis, genomic DNAs were digested with *EcoRI* and hybridized with probe 1 (the *Ss-odc2* partial ORF), which yielded a 2.7-kb band for the WT and ectopic (Ect) strain, but no band for the KO mutants. Genomic DNAs were also digested with *PstI* and *XhoI* and hybridized with probe 2 (the *Ss-odc2* 3' UTR), which yielded a 5.4-kb band for the WT and a 3.2-kb band for the KO mutant. These patterns are consistent with *Ss-odc2* gene replacement by homologous integration as illustrated in Fig. S5B. The *Ss-odc2* Ect strain produced a band in addition to the 5.4-kb WT band (Fig. S5B), indicating a single ectopic integration event.

The $\Delta ss-odc1$ and $\Delta ss-odc2$ mutants grew in the same manner as the WT on PDA medium, and exhibited WT-like PDA colony morphology, development of sclerotia and apothecia (Fig. S6, see Supporting Information). The $\Delta ss-odc2$ mutants, however, were affected in appressorium development, forming much fewer cushion-shaped appressoria on parafilm compared with the control strains (Figs 3A, B, S7A, see Supporting Information). On cellophane-overlaid growth medium, ascospores of the $\Delta ss-odc2$ mutant initialized appressorium development similarly to the WT and Ect strains (Fig. 3C, top panels), but, over time, the mutant rarely differentiated more complex cushion-shaped appressoria (Fig. 3C, bottom panels).

$\Delta ss-odc2$ mutants are inefficient in primary lesion establishment

On a variety of plant hosts (tomato, common bean, soybean and celery), the virulence of the $\Delta ss-odc1$ mutant was indistinguishable from that of the WT; the $\Delta ss-odc2$ mutants, in contrast, exhibited varying degrees of virulence reduction depending on the

Fig. 3 $\Delta ss-odc2$ mutants are less efficient at compound appressorium development. (A) Compound appressoria produced on parafilm surrounding mycelia-colonized agar plugs [3 days post-inoculation (dpi)]. (B) Trypan blue-stained appressoria remaining on parafilm after mycelial plug removal. Scale bars: 2 mm. (C) Compound appressoria formed from germinated ascospores on cellophane laid on top of quarter-strength potato dextrose agar (PDA). Note the lack of highly complex cushion-shaped appressoria in the $\Delta ss-odc2$ mutants at 5 dpi. The scale bars on the top and bottom panels represent 200 μ m and 1 mm, respectively. WT, wild-type; Ect, ectopic strain; Com, complementation strain.

plant host and tissue. Relative to control strains, the $\Delta ss-odc2$ mutants showed no virulence reduction on tomato leaves (Fig. S8A, see Supporting Information), a small virulence reduction on bean leaves (Fig. S8A) and a dramatic virulence reduction on bean petioles (Fig. S8A), soybean leaves (Fig. 4A, B) and celery stalks (Fig. S8B). Wounding prior to inoculation fully restored the virulence defect in all host tissues (Fig. 4B and data not shown).

The infection of the $\Delta ss-odc2$ mutants on soybean leaves was characterized in more detail. Two days post-inoculation (dpi), the

Fig. 4 $\Delta ss-odc2$ mutants are inefficient at primary lesion establishment on unwounded host tissues. (A) Typical primary lesions formed by the wild-type (WT) and the $\Delta ss-odc2$ mutant (*odc2-KO1*) on soybean leaf at 2 days post-inoculation (dpi). (B) Wounding prior to inoculation fully restored the virulence defect of the $\Delta ss-odc2$ mutant. Soybean leaf lesions were quantified at 2 dpi and the bars represent means + standard deviations for 15 unwounded and seven wounded inoculation replicates, respectively. (C) Trypan blue staining of fungal tissue and dead host cells at 14 h post-inoculation. Arrowheads indicate compound appressoria formation. Ect, ectopic strain; Com, complementation strain.

mutants showed an infection efficiency of 53% ($n = 60$), whereas the WT, Ect and complementation (Com) strains all showed infection efficiencies of 100% ($n = 15$ each). Primary lesions formed by the $\Delta ss-odc2$ mutants were sporadic and much smaller compared with all other strains at 2 dpi (Fig. 4A, B). Microscopic observation indicated that the mutants differentiated appressoria as early as 14 h post-inoculation (hpi), but at a much lower frequency compared with control strains (Figs 4C and S9, see Supporting Information). Expansion from a primary lesion to a secondary spreading lesion was delayed with the $\Delta ss-odc2$ mutants, but ultimately resulted in complete leaf colonization (data not shown).

Appressoria formed by the $\Delta ss-odc2$ mutants are functionally defective

Hyphae nutrient status is an important factor affecting compound appressorium development in *S. sclerotiorum*. On parafilm, the WT differentiated appressoria far less efficiently when inoculated from plugs of $1/2 \times$ PDA compared with $1 \times$ PDA medium, and rarely differentiated appressoria when the nutrient level dropped to $1/8 \times$ PDA (data not shown). The effect of relative nutrient strength on appressorium formation in the $\Delta ss-odc2$ mutants was further tested. The $\Delta ss-odc2$ mutants rarely formed appressoria with PDA or $1/2 \times$ PDA, but formed appressoria efficiently with $2 \times$ PDA (Fig. 5A). Thus, an elevated inoculum nutrient level partially rescued the appressorium formation defect in the $\Delta ss-odc2$ mutants.

Fig. 5 Inoculum with elevated nutrient levels increased $\Delta ss-odc2$ compound appressorium formation, but not penetration efficiency. (A) Mycelial plugs from $2 \times$ potato dextrose agar (PDA), $1 \times$ PDA and $1/2 \times$ PDA were placed on parafilm for appressorium induction; photographs were taken at 2 days post-inoculation. (B) Trypan blue staining of fungal tissue and dead host cells [24 h post-inoculation (hpi)]. a, wild-type (WT), PDA mycelial plug. b, $\Delta ss-odc2$ mutant, PDA mycelial plug. c, d, $\Delta ss-odc2$, $2 \times$ PDA mycelial plug. Arrowheads indicate compound appressoria formation. Scale bars: 1 mm.

We next tested whether this elevated inoculum nutrient treatment could restore the primary lesion formation deficiency of the $\Delta ss-odc2$ mutants. Twenty-four hours post-inoculation, the WT had infection efficiencies of 92% ($n = 37$) and 97% ($n = 37$) when mycelial plugs from $2 \times$ PDA and $1 \times$ PDA were used for inoculation, respectively. The $\Delta ss-odc2$ mutant, in contrast, had infection efficiencies of 25% ($n = 40$) and 27% ($n = 41$) under the corresponding medium conditions. Further microscopic examination indicated that $\Delta ss-odc2$ inoculation from $2 \times$ PDA mycelial plugs dramatically increased compound appressorium formation frequency and complexity, but not cuticle penetration efficiency (Figs 5B and S10, see Supporting Information).

Ss-odc2 encodes OxDC activity

Unexpectedly, the $\Delta ss-odc2$ mutants, but not the $\Delta ss-odc1$ mutant, consistently hyperaccumulated OA under a variety of liquid and agar-based culture conditions (Fig. S11, see Supporting Information). Preliminary efforts failed to detect OxDC activity in hyphae or culture supernatants of the WT. To determine whether *Ss-odc2* encodes OxDC activity, *Ss-odc2* was overexpressed in *S. sclerotiorum* under the control of the constitutive *oliC* promoter (Fig. S12A, see Supporting Information). An overexpression strain, designated as *oliC-odc2*, was identified on the basis of RT-PCR (Fig. S12A) and an OA accumulation assay (Fig. S12B). Assays for OxDC and OxOx activities were carried out with protein extracts from hyphae and culture supernatant. The *oliC-odc2* strain showed strong OxDC activity relative to the WT (Fig. 6A) and, similar to the WT, no detectable OxOx activity (data not shown). The optimal pH for OxDC activity was approximately pH 3.5 (Fig. 6B). OxDC activity was detected (using a qualitative OA degradation assay) in protein extracts from mycelia (Fig. S12C), but not in concentrated protein extracts from culture filtrates (data not shown). In protein extracts from mycelia, most activity was in the insoluble rather than the soluble fraction (Fig. S12C).

The *oliC-odc2* strain showed normal radial growth, plate morphology and compound appressorium development (data not shown). On detached soybean leaves, it exhibited WT virulence and OA accumulation (Fig. S12D, E). The *oliC-odc2* strain showed WT sensitivity to stress conditions, including OA (20 mM), cell wall and membrane stress [200 μ g/mL calcofluor white, 200 μ g/mL Congo red, 0.01% sodium dodecylsulfate (SDS)], osmotic stress (1 M sorbitol, 1 M NaCl) and H_2O_2 (5 mM) (data not shown), suggesting a minor role of *Ss-Odc2* in conferring stress tolerance. In addition, both the WT and *oliC-odc2* strain grew poorly on minimum medium supplemented with OA (50 mM, pH 4.0) as the sole carbon source, indicating that *Ss-odc2* gene overexpression alone is insufficient to allow for the use of OA as a sole carbon source (data not shown).

Fig. 6 *Ss-odc2* encodes oxalate decarboxylase activity. Resuspended fungal hyphae of the wild-type (WT) and *Ss-odc2* overexpression strain (oliC-odc2) were used to assay oxalate decarboxylase (OxDC) activity. (A) Formate production over time (expressed as micromoles per milligram of lyophilized tissue). (B) pH profile of OxDC activity extracted from the *Ss-odc2* overexpression strain.

DISCUSSION

OxDCs belong to the bicupin subclass of the cupin protein superfamily (Dunwell *et al.*, 2000; Khuri *et al.*, 2001). Since their first description (Shimazono, 1955), these enzymes have been isolated and characterized from over 20 fungal and bacterial species, and their activities have been used for a wide variety of biotechnological purposes (Mäkelä *et al.*, 2010). The *in vivo* biological functions of OxDCs, however, are still ambiguous. To date, no other OxDC-encoding genes have been functionally characterized in a filamentous fungus via gene disruption, and no discernible phenotype has been observed for the OxDC gene disruption mutants generated in the bacteria *Agrobacterium tumefaciens* and *B. subtilis* (Costa *et al.*, 2004; Shen *et al.*, 2008). In this study, we characterized the expression patterns of two *odc* genes, *Ss-odc1* and *Ss-odc2*, and reported the effects of their deletions on *S. sclerotiorum* growth, development and pathogenesis. The Δ *ss-odc1* mutant was indistinguishable from the WT in all phenotypes assayed. The lack of an obvious phenotype associated with

deletion of the *Ss-odc1* gene was surprising, given the conservation of the bicupin domain structure, including residues known to be critical for OxDC activity. In addition, *Ss-odc1* transcripts accumulated to a significant level across a broad range of tissues, with the exception of developing and maturing compound appressoria, where *Ss-odc2* transcripts exclusively accumulated. These characteristics indicate a conservation of function between *Ss-Odc1* and *Ss-Odc2* with tissue specialization. The lack of evidence for this hypothesis based on the Δ *ss-odc2* phenotype may indicate dramatically different catalytic efficiencies between the two enzymes, or a different catalytic activity altogether for *Ss-Odc1*. Assays to measure specific activity and cellular localization are planned to better understand the biochemical and biological functions of *Ss-Odc1*. In contrast with Δ *ss-odc1*, the four independent Δ *ss-odc2* mutants hyperaccumulated OA and were uniformly deficient in compound appressorium development, leading to a penetration-based virulence defect which could be fully bypassed by wounding prior to inoculation. Although elevated nutrient levels enabled the Δ *ss-odc2* mutants to differentiate abundant compound appressoria on the plant surface, these appressoria did not penetrate the cuticle as efficiently as those formed by the WT. Thus, *Ss-Odc2*-mediated OA degradation contributes to *S. sclerotiorum* early infection by affecting both compound appressoria development and function.

Compound appressoria are hyphal tip-derived, multi-cellular, infection structures reported from a variety of pathogenic fungi, including *Sclerotinia* and *Botrytis* spp. (Purdy, 1958; Sharman and Heale, 1977), *Rhizoctonia* spp. (Hofman and Jongebloed, 1988) and *Fusarium* spp. (Boenisch and Schafer, 2011; Rittenour and Harris, 2010). Compared with single-cell appressoria, their complex, yet compact, structural organization may enable a more rapid and elevated accumulation of hydrolytic enzymes, toxins and defence-suppressive factors surrounding penetration sites. Moreover, the concerted penetration efforts initiated from independent appressoria tips could enable the fungus to combat host defence reactions more efficiently. It is probable that phytopathogens of different lineages have evolved compound appressoria convergently to achieve more efficient penetration during transitions from saprotrophic to pathogenic phases. In this study, the Δ *ss-odc2* mutants, which formed compound appressoria less efficiently, showed no or minor virulence defects on tomato and common bean leaves, but significant virulence defects on soybean leaves, celery stalks and common bean leaf petioles. The previously reported Δ *ss-ggt1* mutant, which fails to differentiate compound appressoria (Li *et al.*, 2012), could successfully penetrate detached tomato and bean leaves, but not soybean leaves, celery stalks or common bean leaf petioles (X. Liang and J. A. Rollins, unpublished data). These results indicate a plant- and tissue-dependent variation in penetration resistance against *S. sclerotiorum* infection and, on the pathogen side, the critical requirement of compound appressoria for the penetration of

plants and tissues with unique physical or chemical properties. Consistent with this observation, the appressorium complexity of *S. sclerotiorum* has been suggested to be proportional to penetration resistance (Tariq and Jeffries, 1984).

OxDC has been proposed to contribute to catabolic energy generation by functioning in concert with intracellular formate dehydrogenase (FDH, EC 1.2.1.2.) (Watanabe *et al.*, 2005). In the brown rot fungus *Postia placenta*, such a nutritional function is supported by the cooperative up-regulation of a putative OxDC-encoding gene, a putative formate transporter and three putative FDHs in cellulose relative to glucose growth medium (Martinez *et al.*, 2009). In this study, we observed that a decrease in the inoculum nutrient level significantly reduces appressorium formation efficiency of the WT; in addition, the appressorium development defect of the $\Delta ss-odc2$ mutants could be partially restored by increasing nutrients within the inoculum medium. Potentially, *S. sclerotiorum* compound appressorium development requires correct nutritional regulation and, during this process, Ss-Odc2 contributes to energy production through the mechanism proposed above. The *S. sclerotiorum* genome encodes one putative formate transporter (GenBank Accession number: XM_001595515) and one putative FDH (GenBank Accession number: XM_001590223). Expressed sequence tag (EST) data support the expression of both genes during pathogenesis (Amselem *et al.*, 2011). The existence and biological significance of such an OxDC–FDH-coupled OA catabolic pathway await experimental validation.

In addition to energy generation, OxDC activity has also been suggested to function in OA detoxification and pH homeostasis regulation (Micales, 1997). This hypothesis is supported by the observation that acidic ambient pH favours OxDC accumulation or enzymatic activity (Azam *et al.*, 2002; Dutton *et al.*, 1994; MacLellan *et al.*, 2009; Mäkelä *et al.*, 2014; Micales, 1995; Shimazono, 1955), as well as the cell wall localization of OxDCs (Antelmann *et al.*, 2007; Azam *et al.*, 2001; Micales, 1997). During compound appressorium development, the dense alignment of hyphal tips within a compound appressorium could dramatically elevate local OA levels. Although such an elevation could facilitate epidermal cell disruption and penetration, it may also expose the hyphae to toxic levels of OA. Moreover, compound appressorium development and function might require an appropriate ambient pH environment, as is the case for pathogenesis and sclerotia development (Rollins, 2003; Rollins and Dickman, 2001). The role of OxDC activity in OA stress tolerance, however, was not observed in this study, as the $\Delta ss-odc1$ mutant, the $\Delta ss-odc2$ mutants and the *Ss-odc2* gene overexpression strain all showed WT-like OA sensitivity (data not shown). In addition, we observed no effect of ambient pH or OA treatment on the accumulation of *Ss-odc1* or *Ss-odc2* transcripts. The possibility that Ss-Odc2 functions by affecting ambient pH signal transduction is open to future experimental tests.

When the *Ss-odc2* gene is overexpressed in *S. sclerotiorum*, no or negligible OxDC activity is secreted into the medium and the bulk of the activity is associated with the insoluble fraction of the mycelial protein extract. This finding supports the cell wall localization of Ss-Odc2. Although the OxDC activity of Ss-Odc2 was unequivocally demonstrated, the extent to which it affects ambient OA accumulation levels is not straightforward. Constitutive overexpression of the *Ss-odc2* gene significantly down-regulates OA accumulation in potato dextrose broth (PDB) shaking culture, but not *in planta*. Consistent with these findings, the $\Delta ss-odc2$ mutants showed elevated OA accumulation under various hyphal culture conditions, but not during plant infection. Such variation between *in vitro* and *in planta* accumulation levels might be related to the cell wall localization of Ss-Odc2. It is probable that, during plant infection, most secreted OA diffuses away from the fungal cell wall, making it inaccessible to the cell wall-bound OxDC. In liquid shaking culture, OA secreted into the medium would come into repeat contact with the cell wall-bound enzyme and be degraded.

In sum, we demonstrate that Ss-Odc2-mediated OxDC activity functions in early infection establishment of *S. sclerotiorum*. Although the specific physiological roles of OxDC activity have yet to be determined, the findings reported here demonstrate that OA accumulation is developmentally regulated and this activity is required for penetration-dependent infection on many hosts. Methods to quantitatively measure local OA accumulation and local ambient pH with high spatial resolution would be of great value to refine our understanding of the dynamics of these factors during host infection and colonization.

EXPERIMENTAL PROCEDURES

Fungal strains and plant materials

The *S. sclerotiorum* WT '1980' isolate was maintained and propagated on PDA (Difco, Sparks, MD, USA) medium; mutants ($\Delta ss-odc1$, $\Delta ss-odc2$, *Ss-odc2*-Ectopic) and the overexpression strain (*oliC-odc2*) derived from '1980' were maintained and propagated on PDA supplemented with 100 µg/mL hygromycin. The $\Delta ss-odc2$ complementation strain (*Ss-odc2*-Com) and the *Ss-odc2* promoter GFP fusion strain (*ProOdc2*-GFP) were maintained on PDA supplemented with 200 µg/mL nourseothricin. Dry sclerotia and filter papers colonized by vegetative hyphae were kept in paper envelopes at –20 °C for long-term storage.

Common bean (*Phaseolus vulgaris* cv. Bush Blue Lake 47), soybean (*Glycine max* [L.] Merr. cv. Harosoy) and tomato (*Lycopersicon esculentum* cv. Bonnie Best) plants were grown in the glasshouse under natural sunlight, with a temperature in the range 16–25 °C. Leaves and petioles excised from approximately 1-month-old plants were used for inoculation. Celery (*Apium graveolens*) was purchased from a local grocery store and the stalks were washed with running water and cut into 3–5-cm pieces for inoculation.

Sequence analysis, gene expression and treatments

Protein domains and putative signal peptides were identified using Pfam (<http://pfam.sanger.ac.uk/search>) and the SignalP 4.1 platform (<http://www.cbs.dtu.dk/services/SignalP/>), respectively. Sequence alignment of OxDC homologues was carried out using the CLUSTALW method built into MEGA 5.0 software (Tamura *et al.*, 2011).

For Northern blot hybridization, total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA electrophoresis, blotting, hybridization and ensuing detection were carried out as described by Solanas *et al.* (2001). Partial ORF sequences of the *Ss-odc1* and *Ss-odc2* genes were used as hybridization probes by labelling with digoxigenin (DIG) using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics, Indianapolis, IN, USA). qRT-PCR and RT-PCR assays were carried out as described by Li and Rollins (2009) and Liang *et al.* (2014). The *S. sclerotiorum* histone H3 gene was used as the endogenous reference gene and the primer pair H3-F1/H3-R1 was used to amplify a 278-bp amplicon (Table S1, see Supporting Information). The primer pair SsOdc2DF/SsOdc2RT-R was used to amplify a 192-bp amplicon of *Ss-odc2* in the qRT-PCR assay, and the primer pair SsOdc2DF/SsOdc2DR was used to amplify a 448-bp amplicon of *Ss-odc2* in the RT-PCR assay (Table S1).

pH and OA treatments were carried out in YPSu medium [containing, per litre, 4 g yeast extract (Difco), 15 g sucrose, 1 g K₂HPO₄ and 0.5 g MgSO₄]. Fresh, actively growing vegetative hyphae from a 4-day-old shaking culture (100 rpm, room temperature) were washed with double-distilled H₂O, and harvested onto filter paper by vacuum filtration. Harvested hyphae were then cultured for 4 h in YPSu medium buffered at pH 7.0 (with citric acid–sodium phosphate buffer) before being harvested again and divided among pH/OA treatment media (YPSu with the pH buffered between pH 3.1 and pH 6.8, with or without 25 mM exogenous OA). The vegetative hyphae were treated for 4 h and collected for total RNA extraction. We also determined the effect of OA at a fixed pH (pH 4.8) with treatments carried out as described by Liang *et al.* (2014). For OA treatment of mycelia in the qRT-PCR assay, vegetative hyphae collected from 4-day-old stationary PDB + 20 mM OA were used. For the validation of the *Ss-odc2* overexpression transformant with RT-PCR, vegetative hyphae collected from PDB shaking culture were used. Tissues representing different developmental stages were collected as described by Liang *et al.* (2014).

Gene replacement and complementation

Overlap PCR was used to generate the *Ss-odc1* and *Ss-odc2* gene replacement constructs (de Hoog *et al.*, 2000). The approximately 1.5-kb 5' and 3' flanking sequences of the *Ss-odc1* gene were amplified with primer pairs Odc1LF/Odc1LR and Odc1RF/Odc1RR, respectively (Table S1), whereas the 5' and 3' flanking sequences of the *Ss-odc2* gene were amplified with primer pairs Odc2LF/Odc2LR and Odc2RF/Odc2RR, respectively (Table S1). Fragments of the 3' and 5' truncated hygromycin resistance cassettes were amplified with the primer pairs Hyg5'F/Hyg3'R and Yg5'F/Hyg3'R, respectively (Table S1). The LR and RF primers were designed such that an overlapping 27-bp sequence exists between the 5'/3' flanking sequence fragment and the 3'/5' truncated hygromycin fragment. Primer pairs of Hy3'RNest/Odc1LFNest and Hy3'RNest/Odc2LFNest (Table S1) were used to generate fused 5' PCR fragments,

whereas primer pairs of Yg5'FNest/Odc1RRNest and Yg5'FNest/Odc2RRNest (Table S1) were used to generate fused 3' PCR fragments. The purified 5' and 3' PCR fragments (approximately 1 µg/µL) for each gene were mixed in an equal molar ratio and directly transformed into the '1980' protoplasts and selected with 100 µg/mL hygromycin. Putative gene KO mutants were identified by PCR and further verified by Southern hybridization. To complement the $\Delta ss-odc2$ mutant, a 3.9-kb DNA sequence encompassing the coding and flanking genomic locus was PCR amplified with the primer pair Odc2ComSpelF/Odc2ComXhoIR (Table S1) and ligated into the pD-NAT1 vector (Kück and Hoff, 2006). The construct was directly transformed into the $\Delta ss-odc2$ (KO1) protoplast and selected with 200 µg/mL nourseothricin.

Vectors for *Ss-odc2* promoter-driven GFP expression and *Ss-odc2* gene overexpression

To generate the *Ss-odc2* promoter-driven GFP expression vector pBlunt-NAT-Odc2-GFP, a 1660-bp 5' UTR of the *Ss-odc2* gene was PCR amplified from a genomic DNA template with the primer pair Odc2BamHIGFPS/Odc2SpelGFPAs (Table S1). *BamHI*/*SpeI* double digestion was then used to replace the *Aspergillus nidulans* *oliC* promoter sequence within the pBlunt-NAT-*oliC*-GFP vector (Liang *et al.*, 2014) with the *Ss-odc2* 5' UTR.

To generate the *oliC* promoter-driven *Ss-odc2* overexpression vector pBlunt-Hyg-*oliC*-*odc2*, the primer pair Odc2ORFNcoIS/Odc2ORFEcoRIAs was used to PCR amplify the *Ss-odc2* ORF from a cDNA template (Table S1). *NcoI*/*EcoRI* double digestion was then used to replace the *Bcgfp* coding sequence of the *poliC*-GFP-Sall vector (Liang *et al.*, 2014) with the *Ss-odc2* ORF, resulting in *poliC*-*odc2*-Sall. The hygromycin phosphotransferase expression cassette was cut from the pSO1 vector (Warwar *et al.*, 2000) and ligated into the pCR®-Blunt vector (Zero Blunt® PCR Cloning Kit, Invitrogen) via *BamHI* digestion, resulting in the vector pBlunt-Hyg. *SpeI*/*XhoI* double digestion was then used to ligate the *oliC*-*odc2* expression cassette from *poliC*-eGFP-Sall into pBlunt-Hyg, resulting in the final vector pBlunt-Hyg-*oliC*-*odc2*.

Phenotype analysis

Radial growth, apothecia induction, ascospore collection and OA quantification were carried out as described by Li and Rollins (2010) and Rollins (2003). The assays for compound appressorium development were carried out by inoculating a PDA–mycelia agar plug onto parafilm or onto cellophane overlaid on PDA medium, or by spreading an ascospore suspension (10⁵/mL) onto cellophane overlaid onto quarter-strength PDA. To examine the effect of nutrients on appressoria formation, mycelial plugs from 2 × PDA, 1 × PDA and 1/2 × PDA were used for parafilm inoculation. To stain appressoria, trypan blue (0.5% in double-distilled H₂O) was added to the parafilm surface after mycelial plug removal. Unwounded and wounded inoculations, and lesion size quantification, were carried out as described by Li *et al.* (2012). To visualize fungal infection structures and host cell death, infected plant tissues were cleared with 3 : 1 ethanol–acetic acid solution overnight and then stained with trypan blue (0.5% in double-distilled H₂O) for 1 day.

Fluids from the PDB stationary culture and PDB shaking culture were used directly for OA quantification. For PDB stationary culture, four mycelial plugs with spreading hyphal tips were inoculated into 25 mL of

PDB medium within a 9-cm-diameter Petri dish, and the dishes were kept at room temperature for fungal growth. For PDB shaking culture, eight mycelial plugs with spreading hyphal tips were inoculated into a 200-mL flask containing 50 mL medium and shaken at 100 rpm.

To measure OA within PDA cultures, mycelial agar plugs were collected at different time points. For each time point, six 5-mm-diameter mycelial plugs located at even intervals between the inoculation site and the colony front were pooled into a 2-mL Eppendorf centrifugation tube; the collected plugs were weighed, lyophilized and ground into a fine powder with a metal spatula; 1 mL of double-distilled H₂O was added to suspend the powder. After centrifugation (10 000 g, 5 min), the supernatant was used for OA measurement as described previously (Liang *et al.*, 2014). Final OA concentrations were expressed as mg/g wet plug unit. Similar methods were used to measure OA accumulation in PDA mycelial plugs collected during compound appressorium induction on parafilm. The quantification of *in planta* OA accumulation was carried out as described by Liang *et al.* (2014).

OA degradation and OxDC activity assay

Eight mycelial plugs were inoculated into a 200-mL flask containing 50 mL of PDB medium and shaken for 4 days at 100 rpm. Mycelia were collected by vacuum filtration and washed three times with double-distilled H₂O before being collected again and re-inoculated (approximately 0.5 g wet weight/50 mL medium) into buffered PDB medium (pH 3.0, buffered with citrate phosphate buffer). After two additional days of shaking culture, the culture fluid and vegetative hyphae were collected for protein extraction and OA-degrading activity assay. For protein extraction, the culture fluids were concentrated 500-fold with an Amicon UltraCentrifugal Filter Unit (molecular weight cut-off, 10 kDa; Millipore, Billerica, MA, USA), with the final protein concentration being approximately 1 mg/mL. To extract protein from the vegetative hyphae, around 200 µL of lyophilized and ground hyphal tissue were resuspended in 200 µL of ice-cold citrate phosphate protein extraction buffer (0.1 M, pH 3.0) and ice incubated for 30 min.

To test for OA degradation activity, exogenous OA (adjusted to pH 4.0 with NaOH) was added to a 100-µL protein extraction suspension to a final concentration of 10 mM. The reactions were then incubated at 37 °C for 30 min before being quenched with 1 µL of 10 M NaOH. OA concentrations within the reaction mixture were then compared based on qualitative colour development with the OA detection kit (Trinity Biotech, Bray, Wicklow, Ireland). As a negative control, the reaction mix was quenched directly before incubation. To determine whether OA degradation was cell wall associated, the slurry mycelial protein extract suspension (total volume, 200 µL) was centrifuged at 13 500 g for 5 min, the supernatant was collected and the pellet was resuspended in 200 µL of citrate phosphate buffer. OA degradation activity was compared between the supernatant and the resuspended fractions.

The level of OxDC activity of resuspended tissue samples was determined using an FDH-coupled assay. For the time course, assay mixtures consisted of 50 mM citrate phosphate buffer, pH 3.0, 0.1% Triton X-100, 50 mM potassium oxalate, pH 3.0, and resuspended, homogenized tissue (0.01 mg) (total volume, 100 µL). Reactions were initiated by the addition of substrate, incubated at ambient temperature (21–23 °C) and quenched by the addition of 2 M NaOH (10 µL). The amount of formate produced

was determined by an end-point assay (Schütte *et al.*, 1976) consisting of 50 mM potassium phosphate, pH 7.8, 0.09 mM NAD⁺ and 0.6 U/mg of FDH (final volume, 1 mL). The absorbance at 340 nm was measured after overnight incubation at 37 °C, and formate was quantified by comparison with a standard curve generated by spiking pre-quenched assay mixtures with known amounts of sodium formate. Assay mixtures for the construction of the activity pH profile contained a final concentration of 50 mM citrate phosphate buffer, 0.1% Triton X-100 and 50 mM potassium oxalate, with both the buffer and substrate solution pH adjusted to the interrogated pH.

The level of OxOx activity was determined using a continuous assay in which H₂O₂ production was coupled to the horseradish peroxidase (HRP)-catalysed oxidation of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Requena and Bornemann, 1999). Reaction mixtures contained 25 U HRP, 5 mM ABTS, 50 mM potassium oxalate and 0.01 mg resuspended homogenized tissue dissolved in 50 mM citrate phosphate buffer, pH 3.0, 0.1% Triton X-100 and 50 mM potassium oxalate, pH 3.0 (total volume, 1.0 mL). Assays were monitored at 650 nm and an extinction coefficient of 10 000 M⁻¹ cm⁻¹ for the ABTS radical product was assumed in these experiments. Control samples omitted HRP in order to distinguish between H₂O₂ production and any oxalate-dependent dye oxidation activity by the resuspended homogenized tissue.

ACKNOWLEDGEMENTS

XL was supported by a Graduate Alumni Fellowship provided by the University of Florida and funding from the National Sclerotinia Initiative. We gratefully acknowledge Ms Ulla Benny for laboratory support. The authors declare no conflict of interest.

REFERENCES

- Abawi, G.S., Polach, F.J. and Molin, W.T. (1975) Infection of bean by ascospores of *Whetzelinia sclerotiorum*. *Phytopathology*, **65**, 673–678.
- Amselem, J., Cuomo, C.A., van Kan, J.A., Viaud, M., Benito, E.P., Couloux, A., Coutinho, P.M., de Vries, R.P., Dyer, P.S., Fillinger, S., Fournier, E., Gout, L., Hahn, M., Kohn, L., Lapalu, N., Plummer, K.M., Pradier, J.M., Quevillon, E., Sharon, A., Simon, A., ten Have, A., Tudzynski, B., Tudzynski, P., Wincker, P., Andrew, M., Anthouard, V., Beever, R.E., Beffa, R., Benoit, I., Bouzid, O., Brault, B., Chen, Z., Choquer, M., Collemare, J., Cotton, P., Danchin, E.G., Da Silva, C., Gautier, A., Giraud, C., Giraud, T., Gonzalez, C., Grossetete, S., Guldener, U., Henrissat, B., Howlett, B.J., Kodira, C., Kretschmer, M., Lappartient, A., Leroch, M., Levis, C., Mauceli, E., Neuveglise, C., Oeser, B., Pearson, M., Poulain, J., Poussereau, N., Quesneville, H., Rosale, C., Schumacher, J., Segurens, B., Sexton, A., Silva, E., Sirven, C., Soanes, D.M., Talbot, N.J., Templeton, M., Yandava, C., Yarden, O., Zeng, Q., Rollins, J.A., Lebrun, M.H. and Dickman, M. (2011) Genomic analysis of the necrotrophic fungal pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea*. *Plos Genet.* **7**, e1002230.
- Antelmann, H., Töwe, S., Albrecht, D. and Hecker, M. (2007) The phosphorus source phytate changes the composition of the cell wall proteome in *Bacillus subtilis*. *J. Proteome Res.* **6**, 897–903.
- Azam, M., Kesarwani, M., Natarajan, K. and Datta, A. (2001) A secretion signal is present in the *Collybia velutipes* oxalate decarboxylase gene. *Biochem. Biophys. Res. Commun.* **289**, 807–812.
- Azam, M., Kesarwani, M., Chakraborty, S., Natarajan, K. and Datta, A. (2002) Cloning and characterization of the 5'-flanking region of the oxalate decarboxylase gene from *Flammulina velutipes*. *Biochem. J.* **367**, 67–75.
- Baetz, A.L. and Allison, M.J. (1989) Purification and characterization of oxalyl-coenzyme A decarboxylase from *Oxalobacter formigenes*. *J. Bacteriol.* **171**, 2605–2608.
- Bateman, D.F. and Beer, S.V. (1965) Simultaneous production and synergistic action of oxalic acid and polygalacturonase during pathogenesis by *Sclerotium rolfsii*. *Phytopathology*, **55**, 204–211.

- Boenisch, M. and Schafer, W. (2011) *Fusarium graminearum* forms mycotoxin producing infection structures on wheat. *BMC Plant Biol.* **11**, 110.
- Bolton, M.D., Thomma, B.P. and Nelson, B.D. (2006) *Sclerotinia sclerotiorum* (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen. *Mol. Plant Pathol.* **7**, 1–16.
- Cessna, S.G., Sears, V.E., Dickman, M.B. and Low, P.S. (2000) Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. *Plant Cell*, **12**, 2191–2200.
- Costa, T., Steil, L., Martins, L.O., Völker, U. and Henriques, A.O. (2004) Assembly of an oxalate decarboxylase produced under α K control into the *Bacillus subtilis* spore coat. *J. Bacteriol.* **186**, 1462–1474.
- Cotton, P., Kasza, Z., Bruel, C., Rascle, C. and Fevre, M. (2003) Ambient pH controls the expression of endopolygalacturonase genes in the necrotrophic fungus *Sclerotinia sclerotiorum*. *FEMS Microbiol. Lett.* **227**, 163–169.
- Donaldson, P.A., Anderson, T., Lane, B.G., Davidson, A.L. and Simmonds, D.H. (2001) Soybean plants expressing an active oligomeric oxalate oxidase from the wheat *gf-2.8* (germin) gene are resistant to the oxalate-secreting pathogen *Sclerotinia sclerotiorum*. *Physiol. Mol. Plant Pathol.* **59**, 297–307.
- Dunwell, J.M., Khuri, S. and Gane, P.J. (2000) Microbial relatives of the seed storage proteins of higher plants: conservation of structure and diversification of function during evolution of the cupin superfamily. *Microbiol. Mol. Biol. Rev.* **64**, 153–179.
- Dutton, M.V. and Evans, C.S. (1996) Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. *Can. J. Microbiol.* **42**, 881–895.
- Dutton, M.V., Kathiara, M., Gallagher, I.M. and Evans, C.S. (1994) Purification and characterization of oxalate decarboxylase from *Coriolus versicolor*. *FEMS Microbiol. Lett.* **116**, 321–325.
- Eerrakhi, R., Meimoun, P., Lehner, A., Vidal, G., Briand, J., Corbineau, F., Rona, J.P. and Bouteau, F. (2008) Anion channel activity is necessary to induce ethylene synthesis and programmed cell death in response to oxalic acid. *J. Exp. Bot.* **59**, 3121–3129.
- Escutia, M.R., Bowater, L., Edwards, A., Bottrill, A.R., Burrell, M.R., Polanco, R., Vicuña, R. and Bornemann, S. (2005) Cloning and sequencing of two *Ceriporiopsis subvermispora* bicupin oxalate oxidase allelic isoforms: implications for the reaction specificity of oxalate oxidases and decarboxylases. *Appl. Environ. Microbiol.* **71**, 3608–3616.
- Godoy, G., Steadman, J.R., Dickman, M.B. and Dam, R. (1990) Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on *Phaseolus vulgaris*. *Physiol. Mol. Plant Pathol.* **37**, 179–191.
- Guimaraes, R.L. and Stotz, H.U. (2004) Oxalate production by *Sclerotinia sclerotiorum* deregulates guard cells during infection. *Plant Physiol.* **136**, 3703–3711.
- Heller, A. and Witt-Geiges, T. (2013) Oxalic acid has an additional, detoxifying function in *Sclerotinia sclerotiorum* pathogenesis. *PLoS ONE*, **8**, e72292.
- Hofman, T.W. and Jongebloed, P.H.J. (1988) Infection process of *Rhizoctonia solani* on *Solanum tuberosum* and effects of granular nematicides. *Neth. J. Plant Pathol.* **94**, 243–252.
- de Hoogt, R., Luyten, W., Contreras, R. and De Backer, M. (2000) PCR- and ligation-mediated synthesis of split-marker cassettes with long flanking homology regions for gene disruption in *Candida albicans*. *Biotechniques*, **28**, 1112–1116.
- Hu, X., Bidney, D.L., Yalpani, N., Duvick, J.P., Crasta, O., Folkerts, O. and Lu, G. (2003) Overexpression of a gene encoding hydrogen peroxide-generating oxalate oxidase evokes defense responses in sunflower. *Plant Physiol.* **133**, 170–181.
- Kabbage, M., Williams, B. and Dickman, M.B. (2013) Cell death control: the interplay of apoptosis and autophagy in the pathogenicity of *Sclerotinia sclerotiorum*. *PLoS Pathog.* **9**, e1003287.
- Khuri, S., Bakker, F.T. and Dunwell, J.M. (2001) Phylogeny, function, and evolution of the cupins, a structurally conserved, functionally diverse superfamily of proteins. *Mol. Biol. Evol.* **18**, 593–605.
- Kim, K.S., Min, J.Y. and Dickman, M.B. (2008) Oxalic acid is an elicitor of plant programmed cell death during *Sclerotinia sclerotiorum* disease development. *Mol. Plant-Microbe Interact.* **21**, 605–612.
- Kück, U. and Hoff, B. (2006) Application of the nourseothricin acetyltransferase gene (*nat1*) as dominant marker for the transformation of filamentous fungi. *Fungal Genet. Newsl.* **53**, 9–11.
- Lamp, N., Alkan, N., Davydov, O. and Fluhr, R. (2013) Set-point control of RD21 protease activity by AtSerpin1 controls cell death in *Arabidopsis*. *Plant J.* **74**, 498–510.
- Li, M. and Rollins, J.A. (2009) The development-specific protein (Ssp1) from *Sclerotinia sclerotiorum* is encoded by a novel gene expressed exclusively in sclerotium tissues. *Mycologia*, **101**, 34–43.
- Li, M. and Rollins, J.A. (2010) The development-specific ssp1 and ssp2 genes of *Sclerotinia sclerotiorum* encode lectins with distinct yet compensatory regulation. *Fungal Genet. Biol.* **47**, 531–538.
- Li, M., Liang, X. and Rollins, J.A. (2012) *Sclerotinia sclerotiorum* gamma-glutamyl transpeptidase (Ss-Ggt1) is required for regulating glutathione accumulation and development of sclerotia and compound appressoria. *Mol. Plant-Microbe Interact.* **25**, 412–420.
- Liang, X., Liberti, D., Li, M., Kim, Y.-T., Hutchens, A., Wilson, R. and Rollins, J.A. (2014) Oxaloacetate acetylhydrolase gene mutants of *Sclerotinia sclerotiorum* do not accumulate oxalic acid but do produce limited lesions on host plants. *Mol. Plant Pathol.* doi:10.1111/mpp.12211.
- Liberti, D., Rollins, J.A. and Dobinson, K.F. (2013) Peroxisomal carnitine acetyl transferase influences host colonization capacity in *Sclerotinia sclerotiorum*. *Mol. Plant-Microbe Interact.* **26**, 768–780.
- Livingstone, D.M., Hampton, J.L., Phipps, P.M. and Grabau, E.A. (2005) Enhancing resistance to *Sclerotinia minor* in peanut by expressing a barley oxalate oxidase gene. *Plant Physiol.* **137**, 1354–1362.
- Lumsden, R.D. and Dow, R.L. (1973) Histopathology of *Sclerotinia sclerotiorum* infection of bean. *Phytopathology*, **63**, 708–715.
- MacLellan, S.R., Helmann, J.D. and Antelmann, H. (2009) The Yvri alternative σ factor is essential for acid stress induction of oxalate decarboxylase in *Bacillus subtilis*. *J. Bacteriol.* **191**, 931–939.
- Magro, P., Marciano, P. and Lenna, P. (1988) Enzymatic oxalate decarboxylation in isolates of *Sclerotinia sclerotiorum*. *FEMS Microbiol. Lett.* **49**, 49–52.
- Mäkelä, M., Hildén, K. and Lundell, T. (2010) Oxalate decarboxylase: biotechnological update and prevalence of the enzyme in filamentous fungi. *Appl. Microbiol. Biotechnol.* **87**, 801–814.
- Mäkelä, M.R., Sietio, O.-M., de Vries, R.P., Timonen, S. and Hildén, K. (2014) Oxalate-metabolising genes of the white-rot fungus *Dichomitus squalens* are differentially induced on wood and at high proton concentration. *PLoS ONE*, **9**, e87959.
- Martinez, D., Challacombe, J., Morgenstern, I., Hibbett, D., Schmolli, M., Kubicek, C.P., Ferreira, P., Ruiz-Duenas, F.J., Martinez, A.T., Kersten, P., Hammel, K.E., Wymelenberg, A.V., Gaskell, J., Lindquist, E., Sabat, G., BonDurant, S.S., Larrondo, L.F., Canessa, P., Vicuna, R., Yadav, J., Doddapaneni, H., Subramanian, V., Pisabarro, A.G., Lavin, J.L., Oguiza, J.A., Master, E., Henrissat, B., Coutinho, P.M., Harris, P., Magnuson, J.K., Baker, S.E., Bruno, K., Kenealy, W., Hoegger, P.J., Kües, U., Ramaiya, P., Lucas, S., Salamov, A., Shapiro, H., Tu, H., Chee, C.L., Misra, M., Xie, G., Teter, S., Yaver, D., James, T., Mokrejs, M., Pospisek, M., Grigoriev, I.V., Brettin, T., Rokhsar, D., Berka, R. and Cullen, D. (2009) Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. *Proc. Natl. Acad. Sci. USA*, **106**, 1954–1959.
- Micales, J.A. (1995) Oxalate decarboxylase in the brown-rot wood decay fungus *Postia placenta*. *Mater. Org.* **29**, 177–186.
- Micales, J.A. (1997) Localization and induction of oxalate decarboxylase in the brown-rot wood decay fungus *Postia placenta*. *Int. Biodeterior. Biodegrad.* **39**, 125–132.
- Noyes, R.D. and Hancock, J.G. (1981) Role of oxalic acid in the *Sclerotinia* wilt of sunflower. *Physiol. Plant Pathol.* **18**, 123–132.
- Purdy, L.H. (1958) Some factors affecting penetration and infection by *Sclerotinia sclerotiorum*. *Phytopathology*, **48**, 605–609.
- Requena, L. and Bornemann, S. (1999) Barley (*Hordeum vulgare*) oxalate oxidase is a manganese-containing enzyme. *Biochem. J.* **343**, 185–190.
- Rittenour, W.R. and Harris, S.D. (2010) An *in vitro* method for the analysis of infection-related morphogenesis in *Fusarium graminearum*. *Mol. Plant Pathol.* **11**, 361–369.
- Rollins, J.A. (2003) The *Sclerotinia sclerotiorum* pac1 gene is required for sclerotial development and virulence. *Mol. Plant-Microbe Interact.* **16**, 785–795.
- Rollins, J.A. and Dickman, M.B. (2001) pH signaling in *Sclerotinia sclerotiorum*: identification of a pacC/RIM1 homolog. *Appl. Environ. Microbiol.* **67**, 75–81.
- Schütte, H., Flossdorf, J., Sahn, H. and Kula, M.-R. (1976) Purification and properties of formaldehyde dehydrogenase and formate dehydrogenase from *Candida boidinii*. *Eur. J. Biochem.* **62**, 151–160.
- Sharman, S. and Heale, J.B. (1977) Penetration of carrot roots by the grey mould fungus *Botrytis cinerea* Pers. ex Pers. *Physiol. Plant Pathol.* **10**, 63–71.
- Shen, Y.-H., Liu, R.-J. and Wang, H.-Q. (2008) Oxalate decarboxylase from *Agrobacterium tumefaciens* C58 is translocated by a twin arginine translocation system. *J. Microbiol. Biotechnol.* **18**, 1245–1251.
- Shimazono, H. (1955) Oxalic acid decarboxylase, a new enzyme from the mycelium of wood destroying fungi. *J. Biochem.* **42**, 321–340.

- Solanas, M., Moral, R. and Escrib, E. (2001) Improved non-radioactive Northern blot protocol for detecting low abundance mRNAs from mammalian tissues. *Biotechnol. Lett.* **23**, 263–266.
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**, 2731–2739.
- Tariq, V.N. and Jeffries, P. (1984) Appressorium formation by *Sclerotinia sclerotiorum*: scanning electron microscopy. *Trans. Br. Mycol. Soc.* **82**, 645–652.
- Warwar, V., Oved, S. and Dickman, M.B. (2000) Antisense expression of the calmodulin gene from *Colletotrichum trifolii* impairs prepenetration development. *FEMS Microbiol. Lett.* **191**, 213–219.
- Watanabe, T., Hattori, T., Tengku, S. and Shimada, M. (2005) Purification and characterization of NAD-dependent formate dehydrogenase from the white-rot fungus *Ceriporiopsis subvermispora* and a possible role of the enzyme in oxalate metabolism. *Enzyme Microb. Technol.* **37**, 68–75.
- Williams, B., Kabbage, M., Kim, H.J., Britt, R. and Dickman, M.B. (2011) Tipping the balance: *Sclerotinia sclerotiorum* secreted oxalic acid suppresses host defenses by manipulating the host redox environment. *Plos Pathog.* **7**, e1002107.

SUPPORTING INFORMATION

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

Fig. S1 Compound appressorium development (top) and compound appressorium-mediated cuticle penetration (bottom) in *Sclerotinia sclerotiorum*. Following saprotrophic growth, contact with a physical surface induces the hyphal tip to differentiate a compound appressorium through continuous tip swelling and branching. Each compound appressorium can initiate multiple independent penetrations. Scale bars: 100 μ m.

Fig. S2 The *Sclerotinia sclerotiorum* genome encodes two putative oxalate decarboxylases. (A) Protein domain structure. aa, amino acids; SP, signal peptide. (B) Multiple sequence alignment. Sequences were retrieved from GenBank with the following accession numbers: AAK88679.1 (*Agrobacterium tumefaciens* OxDc), CAB15314.1 (*Bacillus subtilis* OxDc), AAQ67425.1 (*Trametes versicolor* OxDc), CAV19809.1 (*Dichomitus squalens* OxDc), CAG34243.2 (*Ceriporiopsis subvermispora* OxDc), AAA20245.1 (*Hordeum vulgare* OxDc). Proton donor sites (corresponding to Glu¹⁶² and Glu³³³ in the *Bacillus subtilis* OxDc) are marked with an asterisk. Amino acid residue keys: grey shade, 100% identity; red, Mn²⁺ ion-binding site; underline, 'lid motif'; number, omitted amino acids. OxDc, oxalate decarboxylase; OxDc, oxalate oxidase.

Fig. S3 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of *Ss-odc2* transcript accumulation at different developmental stages and in response to oxalic acid (OA) treatment. The $\Delta\Delta C_t$ method was used to obtain relative expression values, and expression fold changes relative to mycelia were plotted. Data represent the mean + standard deviation from three independent experimental replicates. M, mycelia [4-day-old stationary potato dextrose broth (PDB) culture]; M+OA, mycelia with OA (4-day-old stationary PDB + 20 mM OA culture); CA, compound appressoria (48 h post-induction on cellophane); S, sclerotia at developmental stage

3–4; A, apothecia at developmental stage 4–5; IL, infected tomato leaves (2 days post-inoculation).

Fig. S4 Expression specificity of the *Ss-odc2* gene determined by endogenous promoter-driven green fluorescent protein (GFP) expression. GFP expression driven by the constitutive *oliC* promoter was used as a control. PDA, potato dextrose agar. Scale bars: 200 μ m.

Fig. S5 Construction and verification of gene knockout mutants of *Ss-odc1* and *Ss-odc2*. (A) *Ss-odc1*. Genomic DNAs were digested with *EcoRV* before Southern hybridization. (B) *Ss-odc2*. Genomic DNAs were digested either with *EcoRI* before being hybridized with probe 1, or double digested with *PstI* and *XhoI* before being hybridized with probe 2. WT, wild-type.

Fig. S6 Growth and development of $\Delta ss-odc1$ and $\Delta ss-odc2$ mutants. (A) Radial growth rate. Each point represents the mean $\pm$ standard deviation from four colony replicates. (B) Colony morphology, sclerotium and apothecium development. Potato dextrose agar (PDA) cultures were photographed at 10 days post-inoculation and apothecia were photographed following 1 month of sclerotia incubation at 15 °C. WT, wild-type.

Fig. S7 Effect of *Ss-odc1* and *Ss-odc2* gene deletions on compound appressorium development and virulence. (A) Compound appressoria (darkly pigmented) produced by mycelial plugs on parafilm (3 days post-inoculation). (B) Infected soybean leaves (2 days post-inoculation). WT, wild-type.

Fig. S8 Virulence assay for $\Delta ss-odc2$ and control strains on different hosts. (A) Relative lesion sizes on detached tomato leaf [3 days post-inoculation (dpi)], detached bean leaf (3 dpi) and detached bean petiole (2 dpi). The bar plots represent means + standard deviations based on 10, eight and eight independent inoculation replicates for tomato leaf, bean leaf and bean petiole, respectively. Bars with different letters are statistically significantly different ($P < 0.05$) as determined by one-way analysis of variance (ANOVA) followed by a *post-hoc* Tukey honestly significant difference (HSD) analysis. (B) Celery stalk infection (3 dpi). WT, wild-type; KO1–KO4, independent $\Delta ss-odc2$ mutants; Ect, ectopic strain; Com, complementation strain.

Fig. S9 The $\Delta ss-odc2$ mutant is inefficient in primary lesion establishment. Detached soybean leaves collected 14 and 24 h post-inoculation were cleared and stained with trypan blue to visualize fungal tissue and dead host cells. Arrowheads indicate compound appressoria formed by the $\Delta ss-odc2$ mutant on the leaf surface. Ect, ectopic strain; Com, complementation strain; WT, wild-type.

Fig. S10 Inoculum with elevated nutrients did not increase the penetration efficiency of the $\Delta ss-odc2$ mutants. The top halves of the leaves were inoculated with the wild-type (WT) and the bottom halves with $\Delta ss-odc2$. Typical infections (circles) with inoculum plugs removed were photographed at 24 h post-inoculation (top panel) and leaves were cleared and stained with trypan blue (bottom panel). Note the inefficient primary

lesion formation of the $\Delta ss-odc2$ mutant from $2 \times$ potato dextrose agar (PDA) inoculum despite the presence of abundant fungal tissue (surface hyphae and appressoria).

Fig. S11 Quantification of oxalic acid (OA) accumulation in culture. (A) Potato dextrose agar (PDA) medium. (B) Stationary potato dextrose broth (PDB) medium. (C) Mycelial PDA plugs induced for compound appressorium development. For (A–C), the bar plots represent means + standard deviations of three independent biological replicates. Bars with different letters are statistically significantly different ($P < 0.05$) as determined by one-way analysis of variance (ANOVA) followed by a *post-hoc* Tukey honestly significant difference (HSD) analysis. WT, wild-type; Ect, ectopic; Com, complementation.

Fig. S12 *Ss-odc2* overexpression in *Sclerotinia sclerotiorum*. (A) Schematic representation of the gene overexpression cassette and

validation of the transformant by reverse transcription-polymerase chain reaction (RT-PCR). (B) Oxalic acid (OA) accumulation kinetics in potato dextrose broth (PDB) shaking culture. The bar plots represent means + standard deviations based on three independent replicates. (C) OA-degrading activity assay of mycelial protein extract. OA at a final concentration of 10 mM was added to 100- μ L protein extracts. Following 30 min of incubation, OA levels were qualitatively indicated by a decrease in blue colour (based on an enzymatic OA quantification kit). 1 μ L of 10 M NaOH was added to the quenched control reaction prior to incubation. (D) Virulence assay on detached soybean leaflets (3 days post-inoculation). (E) OA accumulation in infected soybean leaflets (3 days post-inoculation). WT, wild-type.

Table S1 Polymerase chain reaction (PCR) primers used in this study.