Sclerotinia sclerotiorum γ -Glutamyl Transpeptidase (Ss-Ggt1) Is Required for Regulating Glutathione Accumulation and Development of Sclerotia and Compound Appressoria

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Transcripts encoding *Sclerotinia sclerotiorum* γ -glutamyl transpeptidase (Ss-Ggt1) were found to accumulate specifically during sclerotium, apothecium, and compound appressorium development in *S. sclerotiorum*. To determine the requirement of this protein in these developmental processes, gene deletion mutants of *Ss-ggt1* were generated and five independent homokaryotic Δ *Ss-ggt1* mutants were characterized. All deletion mutants overproduced sclerotial initials that were arrested in further development or eventually produced sclerotia with aberrant rind layers. During incubation for carpogenic germination, these sclerotia decayed and failed to produce apothecia. Total glutathione accumulation was approximately 10-fold higher and H₂O₂ hyperaccumulated in Δ *Ss-ggt1* sclerotia compared with the wild type. Production of compound appressoria was also negatively affected. On host plants, these mutants exhibited a defect in infection efficiency and a delay in initial symptom development unless the host tissue was wounded prior to inoculation. These results suggest that Ss-Ggt1 is the primary enzyme involved in glutathione recycling during these key developmental stages of the *S. sclerotiorum* life cycle but Ss-Ggt1 is not required for host colonization and symptom development. The accumulation of oxidized glutathione is hypothesized to negatively impact these developmental processes by disrupting the dynamic redox environment associated with multicellular development.

The Leotiomyces fungus *Sclerotinia sclerotiorum* (Lib.) de Bary is a necrotrophic, broad-host-range plant pathogen of global agro-economic importance (Boland and Hall 1994; Bolton et al. 2006; Purdy 1979). To initiate infection, *S. sclerotiorum* directly penetrates the healthy plant cuticle using a series of modified hyphae ranging from single-celled, unmelanized simple appressoria to multicellular compound appressoria, variably termed complex appressoria, secondary appressoria, lobate appressoria, multihyphal appressoria, infection cushions, and appressorial masses (Akutsu et al. 1983; Emmett and Parbery 1975; Purdy 1958; Tariq and Jeffries 1984). These compound appressoria arise from saprotrophic hyphae, adhere to the host

surface, and grow through the healthy plant cuticle (Lumsden and Dow 1973). The microscopic features and developmental course of compound appressoria development in Sclerotiniaceae species has been documented on a number of hosts and artificial surfaces (Abawi et al. 1975; Akutsu et al. 1983; Backhouse and Willetts 1987; Cruickshank and Wadea 1992; Purdy 1958; Tariq and Jeffries 1984). Studies have further revealed involvement of nutrients (Cruickshank and Wadea 1992), surface hardness (Tariq and Jeffries 1984), and, more recently, cAMP signaling (Jurick and Rollins 2007) as regulators of compound appressorium development and complexity. Systematic studies to identify endogenous genetic regulators of compound appressoria development have not been reported.

Following successful cuticle penetration, subcuticular vesicles form and give rise to inter- and intracellular infectious hyphae which cause water soaking, necrosis, and complete collapse of host cells. Following host colonization, sclerotia form on the surface of or within the dead plant tissue (Abawi et al. 1975; Lumsden and Dow 1973). The sclerotium is formed from aggregates of pseudoparenchymatous hyphae with a continuous, generally single-cell layer of external, melanized cells. As long-term survival structures, sclerotia maintain viability of the fungus through environmental temperature extremes, low moisture, and high UV, and provide protection from microbial degradation and insect predation. Sclerotia formation is a crucial stage in the life cycle of *S. sclerotiorum*; therefore, numerous studies have identified molecules and signal transduction pathways involved in sclerotial initiation and development (Chet and Henis 1975; Erental et al. 2007). Among these, oxidative stress is reported as a common factor linked to sclerotial biogenesis in many sclerotium-forming fungi (Georgiou et al. 2006). In particular, accumulation of reactive oxygen species (ROS) and consequent lipid peroxidation are known to increase during sclerotial differentiation (Georgiou 1997; Papapostolou and Georgiou 2010). Consistent with a requirement for ROS accumulation to initiate and promote sclerotial development, scavengers of ROS inhibit sclerotial formation in *S. sclerotiorum*, *S. minor*, *Sclerotium rolfsii*, and *Rhizoctonia solani* (Georgiou 1997; Georgiou et al. 2006; Patsoukis and Georgiou 2008). The effect of oxidative stress on sclerotial initiation and involvement of thio-redox dynamics reported by Patsoukis and Georgiou (2007, 2008) further indicate significant involvement of thiols, including glutathione (GSH), in sclerotial development.

Reduced GSH is a major and ubiquitous antioxidant functioning to combat oxidative stress in animal and plant cells

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(May et al. 1998; Meister and Anderson 1983). In the presence of ROS, GSH can be oxidized to glutathione disulfide (GSSG) in oxidation-reduction reactions catalyzed by glutathione peroxidase. GSSG can, in turn, be reduced to reform GSH by glutathione reductase (Blokhina et al. 2003). GSH, GSSG, and GSSG/GSH ratios are widely reported to influence cell proliferation and differentiation in animal models (Franco and Cidlowski 2009; Krance et al. 2010; May et al. 1998; Pallardó et al. 2009). In filamentous fungi, a transient spike in oxidation known as the hyperoxidant state is associated with and hypothesized to play a functional role in the promotion of multicellular developmental transitions (Aguirre et al. 2005; Hansberg and Aguirre 1990; Scott and Eaton 2008).

Among the factors regulating GSH accumulation, γ -glutamyl transpeptidase (Ggt) (EC 2.3.2.2) catalyzes the transfer of a γ -glutamyl moiety of GSH, GSSG, and other γ -glutamyl compounds to amino acids, small peptides, or water (Meister and Anderson 1983; Tate and Meister 1981). The transfer of a γ -glutamyl moiety is an essential first step in GSH metabolism and recycling and an important factor in maintaining redox homeostasis in the cellular environment (Zhang and Forman 2009). Characteristics of Ggt function and regulation in filamentous fungi have not been established but the encoded

enzymes are common to fungal genomes, including three predicted Ggt-encoding genes in *S. sclerotiorum* (*Ss-ggt1*, SS1G_14127.3; *Ss-ggt2*, SS1G_10940.3; and *Ss-ggt3*, SS1G_05330.3). Among these, *Ss-ggt1* was determined based on expressed sequence tag (EST) frequency (Amselem et al. 2011) to be transcriptionally upregulated during sclerotial initiation and compound appressorium development. The evidence for the regulation of the redox environment via thiols in sclerotia development (Patsoukis and Georgiou 2007, 2008) lead us to hypothesize that GSH recycling plays an important functional role in the development of sclerotia and other multicellular developmental stages. To test this hypothesis, we created gene deletion *Ss-ggt1* mutants and determined the effects of *Ss-Ggt* loss of function on sclerotial and compound appressorium development and the host-pathogen interaction.

RESULTS

Expression of *Ss-ggt* paralogs.

Queries of the *S. sclerotiorum* genome sequence revealed three genes predicted to encode Ggt activity: SS1G_14127.3 (*Ss-ggt1*), SS1G_10940.3 (*Ss-ggt2*), and SS1G_05330.3 (*Ss-ggt3*). Homologs of all three *Ss-ggt* genes are conserved among plant, animal, and bacterial species. Analysis of the predicted protein sequences curated at the Fungal Secretome KnowledgeBase (Lum and Min 2011) predicts secretion signals and extracellular, non-membrane localization for *Ss-Ggt1* and *Ss-Ggt2* and cytoplasmic localization for *Ss-Ggt3*. *Ss-Ggt1* and *Ss-Ggt2* share 47% amino acid identity and 62% similarity. The identity and similarity between *Ss-Ggt3* and both *Ss-Ggt1* and *Ss-Ggt2* is approximately 24 and 40%, respectively. RNAs extracted from several stages of the *S. sclerotiorum* development were used for Northern hybridization with coding sequence probes from each *Ss-ggt* gene to assess expression during different stages of the lifecycle. This analysis indicated that *Ss-ggt1* transcript accumulation is very high during sclerotium, compound appressorium, and apothecium development but only weakly expressed or undetectable during vegetative mycelial growth and plant colonization (Fig. 1). Peak expression of *Ss-ggt1* was observed during stage 4 (the consolidation stage) of sclerotial development (Fig. 1 and data not shown). Analysis of *Ss-ggt2* transcripts revealed the accumulation of two distinct size isoforms (Fig. 1). A high accumulation level of the small isoform was observed in vegetative mycelia and high levels of both isoforms were observed during apothecial development. Low levels of both isoforms were observed in developing sclerotia and low levels of only the small isoform were observed in compound appressoria and colonized tomato leaves. Very low, constitutive accumulation of *Ss-ggt3*

Fig. 1. Northern hybridization analysis of *Sclerotinia sclerotiorum* γ -glutamyl transpeptidase *Ss-ggt1*, *Ss-ggt2*, and *Ss-ggt3* transcript accumulation in different stages of wild-type development (My, mycelial suspension culture; CA, compound appressoria; S4, stage 4 consolidating sclerotia; A, developing apothecia; IT, infected tomato leaves; T, healthy tomato leaves).

Fig. 2. A, "Split-marker" strategy used for creating *Sclerotinia sclerotiorum* γ -glutamyl transpeptidase (*Ss-ggt1*) deletion mutants and B, Southern hybridization for screening homokaryotic *Ss-ggt1* gene knockout mutants (KO). Left panel, probe 2 hybridization of total genomic DNA double digested with *SpeI* and *SacI*. The wild type (WT) hybridizes with a 5.2-kb band, transformants with a homologous integration at the *Ss-ggt1* locus hybridize with a 2.4-kb gene replacement band (KO), both bands hybridize with heterokaryotic (Ht) transformants, and bands of 5.2 kb and random sizes hybridize in ectopic (Ect) transformants. Right panel, probe 1 (coding sequence) hybridization of total genomic DNA double digested with *SpeI* and *SacI*. Note no hybridization *Ss-ggt1* coding sequences with homokaryotic KO transformants and weak hybridization with the Ht transformants.

transcript was observed in all of the fungal RNA samples (Fig. 1). No cross hybridization with uninoculated tomato leaves was observed with any of the three gene probes. The specific and high level of *Ss-ggt1* transcript accumulation during sclerotium, compound appressorium, and apothecium development suggested a role for Ss-Ggt in these important multicellular differentiation processes. On this basis, *Ss-ggt1* was chosen for further functional characterization. *Ss-ggt1* gene knockout strains were generated and effects of gene deletion on sclero-

tial development and germination as well as development of compound appressoria and pathogenicity were investigated.

Deletion of the *Ss-ggt1* gene.

The strategy employed for *Ss-ggt1* deletion is described below and is illustrated in Figure 2. The 3'-untranslated region (UTR) sequence and partial *Ss-ggt1* coding sequence were used as probes in Southern hybridization to screen for homokaryotic *Ss-ggt1* knockout mutants. In all, 5 of 22 hygromycin-resistant transformants were identified as homokaryotic *Ss-ggt1*-deletion $\Delta Ss-ggt1$ mutants (Fig. 2). Northern hybridization also indicated that there is no accumulation of *Ss-ggt1* transcripts in $\Delta Ss-ggt1$ sclerotia (data not shown). We characterized the phenotypes of all five of these independent gene deletion mutants relative to the wild type (WT) and an ectopic strain (ectopic).

Physiological effects of *Ss-ggt1* deletion.

To test whether deletion of *Ss-ggt1* reduces total Ggt activity and alters the accumulation of total GSH (GSH+GSSG) in developing sclerotia, stage 4 to 6 (consolidating to mature) sclerotia from the WT, ectopic, and $\Delta Ss-ggt1$ mutants were assayed for Ggt activity and GSH+GSSG concentration. As hypothesized, all five independent $\Delta Ss-ggt1$ mutants lost Ggt activity (Fig. 3A) and accumulated approximately 10-fold higher GSH+GSSG in stage 4 to 6 sclerotia relative to the WT and ectopic strain (Fig. 3B) (e.g., 9-day stage 6 sclerotia of the KO mutants averaged 1.3 ± 0.2 μmol GSH+GSSG per g of dry weight versus an average of 0.1 ± 0.01 μmol GSH+GSSG per g of dry weight for the WT and ectopic). At all tested stages of sclerotial development, GSH+GSSG accumulation was significantly higher (Tukey's range test, $\alpha = 0.05$) in the mutants relative to the WT and ectopic. Additionally, H_2O_2 concentration was both quantitatively (approximately 15-fold; WT 2.7 ± 1.9 μmol H_2O_2 per g of dry weight; ectopic 2.2 ± 1.3 μmol H_2O_2 per g of dry weight; KO1-5 average 37 ± 12 μmol H_2O_2 per g of dry weight) and qualitatively higher in mature (stage 6) $\Delta Ss-ggt1$ sclerotia as assessed by the 3,3'-diaminobenzidine (DAB) treatment of sliced sclerotia (Fig. 3C and D).

Effects of *Ss-ggt1* deletion on development.

The *Ss-ggt1* deletion affects both the timing of sclerotial initiation and the maturation of sclerotia but not the rate of hyphal growth. The WT and ectopic strain grown on potato dextrose agar (PDA) form stage 5 to 6 (pigmented and mature) sclerotia between 5 to 7 days after inoculation while $\Delta Ss-ggt1$ sclerotia remain in stage 4 to 5 (consolidating and pigmented) at this time (Fig. 4A). Moreover, from qualitative observations, $\Delta Ss-ggt1$ mutants produce greater numbers of sclerotial initials and condensing sclerotia (stages 1 to 2) than the WT and ectopic strain (Fig. 4A); however, most of these initials eventually senesce, such that the number of $\Delta Ss-ggt1$ sclerotia formed is not noticeably different from the WT or ectopic strain by 9 to 10 days.

In addition to the timing of sclerotial development, *Ss-ggt1* deletion also affects sclerotial tissue organization. Under the conditions of our assays, mature WT sclerotia generally contain a melanized rind composed of a single-cell layer. Mature sclerotia (stage 6) from $\Delta Ss-ggt1$ produced a thickened, multicellular, disorganized, melanized rind (Fig. 4B). Unlike the firm rind of WT sclerotia, the rind and cortex of fully mature $\Delta Ss-ggt1$ sclerotia could be easily peeled away from the medulla when harvested on or after 20 days from the date of plate inoculation (Fig. 4A). Microscopic examination of thin sections determined that the cortex layer of mature sclerotia of the mutants showed partial separation from the medulla. These mutant sclerotia were still able to myceliogenically germinate

Fig. 3. Measurements of γ -glutamyl transpeptidase (Ggt) activity, glutathione (GSH) plus glutathione disulfide (GSSG), and hydrogen peroxide from sclerotia of wild type (WT), ectopic (Ec), and $\Delta Ss-ggt1$ knockout strains (KO1 to KO5). **A**, Ggt enzymatic activity was directly measured from sclerotial extracts. **B**, Total GSH (GSH+GSSG) accumulation in stage 4 to 6 (consolidating to mature) sclerotia. **C**, Hydrogen peroxide accumulation in stage 6 mature sclerotia. **A** to **C**, Means and standard deviation values (1 SD) of three independent biological replications are shown. In **B**, the WT and ectopic values are significantly different from the five *Ss-ggt1* KO mutants at all three time points. In **C**, bars not connected with the same letter are significantly different. Statistical inference was determined by Tukey's range test ($\alpha = 0.05$). **D**, 3,3'-Diaminobenzidine staining of fresh, sliced stage 6 sclerotia.

on agar media; however, the mature sclerotia of the *Ss-ggt1* knockout mutants collected for carpogenic germination became soft and decayed during the incubation period and failed to germinate into apothecia after multiple independent attempts in which the WT and ectopic strain produced apothecia (results not shown).

Effects of *Ss-ggt1* deletion on compound appressoria and pathogenicity.

An in vitro assay was used to assess compound appressorium production in all strains. The WT and ectopic strain formed compound appressoria abundantly, as was shown macroscopically by aggregated hyphae and pigment accumulation associated with mature, compound appressoria (Fig. 5A). The $\Delta Ss-ggt1$ mutant strains failed to produce compound appressoria (Fig. 5A). To determine whether this developmental defect also occurred in planta, onion epidermal strips were inoculated with mycelial plugs and examined microscopically (Fig. 5B to E). The WT and the ectopic strain (not shown) produced compound appressoria, penetrated the onion epidermis, and produced infectious hyphae (Fig. 5B and D). The mutants initiated compound appressorium development, as shown by the production of hyphal tips that hook and bifurcate in a manner characteristic of compound appressorium initiation (Fig. 5C and E), but did not develop into tightly packed multihyphal compound appressoria. Microscopic examination of individual hyphae on cellophane revealed the same initiation but lack of compact, mature compound appressorium production in the mutant relative to WT (Fig. 5F versus G).

Inoculation of unwounded, detached tomato leaflets with $\Delta Ss-ggt1$ mutants revealed a decrease in infection efficiency as measured by the total number of infected leaflets, 47% ($n = 45$), versus the WT and ectopic control, 100% ($n = 18$), and delayed symptom development (Table 1; Fig. 6). Plants that were wounded prior to inoculation did not show a difference in

infection efficiency: 98% ($n = 45$) for $\Delta Ss-ggt1$ mutants versus 100% ($n = 18$) for WT and ectopic control. Likewise, symptom development with $\Delta Ss-ggt1$ mutants was indistinguishable from the WT when leaflets were wounded prior to inoculation (Table 1; Fig. 6). The ectopic strain consistently appeared slightly more aggressive than other strains, including the WT, but overlapped statistically with the WT or $\Delta Ss-ggt1$ -KO2 in lesion area on wounded leaflets (Table 1).

DISCUSSION

In this study, we have identified Ggt activity encoded by the *Ss-ggt1* gene as a developmentally regulated component of the *S. sclerotiorum* ROS antioxidant system. Sclerotia of loss-of-

Table 1. Mean lesion area in nonwounded and wounded tomato leaflets^w

Strain ^y	Area (cm ²) ^x			
	Unwounded		Wounded	
	24 h	48 h	24 h ^z	48 h
WT	44 a,b	334 a	57	351 a,b
EC	55 a	431 a	93	525 a
KO1	8 b,c	70 b	54	346 a,b
KO2	5 c	87 b	70	447 a,b
KO3	5 c	84 b	44	281 b
KO4	0 c	9 b	59	402 a,b
KO5	0 c	20 b	48	340 a,b

^w Following an analysis of variance (ANOVA) with an overall $P = 0.05$, the Bonferroni (Dunn) t test with $\alpha = 0.05$ was used to detect mean differences. Within columns, mean values with the same letter are not significantly different.

^x Measured across three independent experiments with a total of nine reps per treatment.

^y WT, wild type; EC, ectopic; KO1–KO5 *Sclerotinia sclerotiorum* γ -glutamyl transpeptidase knockout mutants ($\Delta Ss-ggt1$).

^z Mean values were not separable by ANOVA with an overall $P < 0.05$.

Fig. 4. Macro- and microscopic features of *Sclerotinia sclerotiorum* γ -glutamyl transpeptidase ($\Delta Ss-ggt1$) sclerotia. **A**, Upper panel: potato dextrose agar (PDA) plates of the wild type (WT), an ectopic strain (Ec), and $\Delta Ss-ggt1$ knockout mutants (KO1 to KO5) 5 days after inoculation (DAI). Immature sclerotia of $\Delta Ss-ggt1$ mutants are indicated by black arrows. Lower panel: normal mature sclerotia (stage 6) harvested from WT, Ec, and $\Delta Ss-ggt1$ mutants (KO1–KO5) 20 DAI. Note the easily peeled rinds of KO strains. Scale bar in upper panel = 1 cm; scale bar in lower panel = 1 mm. **B**, Micrographs from thin sections of mature (stage 6) sclerotia from WT, Ec, and two representative $\Delta Ss-ggt1$ KO mutants (KO1 and -2).

Fig. 5. Compound appressorium development by wild type (WT), ectopic strain (Ec), and *Sclerotinia sclerotiorum* γ -glutamyl transpeptidase knockout ($\Delta Ss-ggt1$) strains (KO1 to KO5). **A**, Macroscopic assessment of appressorium development on cellophane-overlain potato dextrose agar (PDA) media. Scale bar = 1 cm. Vegetative hyphae placed on **B** to **E**, onion epidermal strips and **F** to **G**, cellophane-overlain PDA media. Compound appressorium and infection hyphae can be distinguished easily in the WT while appressorium initials but no distinct compound appressoria were formed by KO strains. Tissues in **B** were stained with 0.05% trypan blue. Scale bars represent 50 μ m.

function $\Delta Ss-ggt1$ knockout mutants lacked Ggt activity, and hyperaccumulated GSH+GSSG and hydrogen peroxide during development. Phenotypic characterization of these knockout mutants indicates that Ggt1 activity is required for proper formation and function of both sclerotia and compound appressoria. These differing developmental structures play independent yet equally critical functions in the infection cycle of *S. sclerotiorum*: sclerotia for long-term survival and production of ascospore inoculum and appressoria for direct penetration of host tissues.

The most obvious manifestation of *Ss-ggt1* deletion in sclerotia was the thickened, disorganized rind and cortex layers that were easily removed from the underlying medulla tissue. As an apparent consequence of these tissue malformations, these sclerotia were unable to produce apothecia, thus decreasing the fitness and infection potential of these mutants. The lack of functional sclerotia precluded the assessment of *Ss-ggt1*'s role in post-germination apothecium development. Relating specifically to infection, compound appressoria were initiated but not properly formed by these mutants. Loss of compound appressorium development resulted in delayed and inefficient infection. These phenotypic consequences are consistent with a specific role for Ggt1 activity during developmental transitions and the maturation of these multicellular structures.

The phenotype of all five independent *Ss-ggt1* mutants supports the hypothesis that this enzyme plays an important role in balancing redox during sclerotial and compound appressorium development. These mutants overaccumulate GSH+GSSG in sclerotial tissue. The associated hydrogen peroxide overaccumulation is consistent with the known function of Ggt in animal systems in maintaining redox homeostasis in the cellular environment (Zhang and Forman 2009). The lack of glutamyl and cysteinylglycine conjugates available for recycling is expected to result in excessive GSSG accumulation, consistent with our finding of excessive GSH+GSSG accumulation in $\Delta Ss-ggt1$ sclerotia. Whether this accumulation is extracellular or intracellular was not explored in our studies. Despite the prediction of a secretion signal in the *Ss-Ggt* protein, the *Saccharomyces cerevisiae* Ggt protein, *ECM38*, also contains a secretion signal but has been localized to vacuoles (Mehdi et al. 2001). This leaves the possibility of intracellular localization for the *S. sclerotiorum* Ggt1 and Ggt2 open for future analyses. The low level of *Ss-ggt2* expression in sclerotia coupled with the lack of Ggt activity in the $\Delta Ss-ggt1$ mutants suggests that total Ggt activity in WT sclerotia is provided by *Ss-Ggt1*.

Fig. 6. Tomato leaflets infected by wild type (WT) and *Sclerotinia sclerotiorum* γ -glutamyl transpeptidase knockout ($\Delta Ss-ggt1$ KO1). Representative photos that best match the mean lesion area for each time point calculated for the WT and the ectopic control across three experiments and for all five $\Delta Ss-ggt1$ KO mutants are shown. The ruler segment is 1 cm.

Without sufficient GSH reductase activity to compensate for the lack of Ggt activity, an imbalance of the redox state would be expected to result in a persistent oxidative environment. This is consistent with the observation of excessive hydrogen peroxide accumulation observed in mature (stage 6) $\Delta Ss-ggt1$ sclerotia. The *S. sclerotiorum* genome is predicted to encode two GSH reductases but their activities are apparently not sufficient in this context to regenerate levels of GSH required to prevent oxidative buildup in the absence of Ss-Ggt1.

The attenuated virulence observed with the $\Delta Ss-ggt1$ mutants appears to be exclusively related to the associated defect in compound appressorium development. Plants that were wounded prior to infection exhibited no significant difference in infection efficiency or rate of lesion expansion when inoculated with WT or $\Delta Ss-ggt1$ mutants. Furthermore, no differences in host colonization or symptom development were detected among the five independent $\Delta Ss-ggt1$ mutants relative to the WT or ectopic control strain. These observations suggest that Ggt1 activity is dispensable during the colonization phase of *S. sclerotiorum* pathogenesis. If there is a role for Ggt activity during host colonization, it may be provided by Ss-Ggt2 or -3. Transcripts of *Ss-ggt2* accumulated to a low level in infected tomato leaves and, as such, may have a function during *S. sclerotiorum* host colonization. A role for Ggt activity during host colonization could be assessed by functional characterization of the *Ss-ggt2* and -3 genes.

The capacity to regulate the external redox environment has been shown to play important roles in host infection by plant-pathogenic fungi closely related to *S. sclerotiorum*. Hyphae of *Monilinia fructicola* treated with host phenols such as caffeic acid show increases in GSH+GSSG levels and corresponding decreases in electrochemical redox potential (Lee and Bostock 2007). When applied to conidia, both caffeic acid and reduced GSH inhibit appressorium formation without significantly affecting conidial germination. This inhibition suggests that appropriate redox regulation is required for appressorium development in *M. fructicola* (Lee and Bostock 2007) and that GSH may play a role in modulating this regulation. In *Botrytis cinerea*, another closely related necrotrophic pathogen, NADPH oxidases (Nox) encoded by *bcnoxA* and *bcnoxB* are differentially important in the infection and colonization process. *bcnoxB* is required for appressorium development and function whereas *bcnoxA* is required for post-penetration secondary lesion expansion (Segmüller et al. 2008). *bcnoxA* and *bcnoxB* are both required for sclerotium development. Nox enzymes are important endogenous generators of ROS in other fungi as well (Takemoto et al. 2007). In *Magnaporthe grisea*, the accumulation of ROS is pronounced during conidiospore germination and appressorium development. Deletion of NADPH oxidases *nox1* and *nox2* in *M. grisea* demonstrate that neither is required for appressorium development but both are required for appressorium penetration and in planta colonization, even when wound inoculated (Egan et al. 2007). Ss-Ggt1 in *S. sclerotiorum* appears to function as a modulator of redox homeostasis, potentially acting in concert with NADPH oxidases. Similar to *bcnoxB*, Ss-Ggt1 activity is restricted to specific developmental transitions. Ss-Ggt1 could potentially function downstream of or cooperatively with the *S. sclerotiorum* equivalent of BcNoxB as a means of balancing or eliminating the hyperoxidant environment during the normal course of development to ensure the transient nature of the hyperoxidant state. This is consistent with both gene mutations affecting sclerotial and compound appressorium development. These findings suggest that Ss-Ggt1 is not a general life-cycle redox regulator but has activity associated with developmental processes.

The data from this study support the hyperoxidant hypothesis of microbial cellular differentiation (Aguirre et al. 2005;

Hansberg and Aguirre 1990) and suggest that Ggt is one of the factors necessary to modulate the rapid accumulation of ROS and ensure the transient nature of the hyperoxidant state associated with development. The requirement for Ss-Ggt1 in both sclerotial and compound appressorium development may have a basis in the common requirement for cellular adhesion and melanin accumulation in both structures. In *Neurospora crassa*, a peak in extracellular accumulation of GSSG is associated with the redox imbalance following exposure of hyphae to an air interface (Toledo et al. 1991). This hyperoxidant state is concurrent and seemingly causal to hyphae-hyphae adhesion (Toledo et al. 1991). In both sclerotia and compound appressorium development, hyphae self-adhere to form their multicellular forms. In the $\Delta Ss-ggt1$ mutants, the increased occurrence of sclerotial initials may be the consequence of an increase in cell adhesion when sclerotial development initiates. As development proceeds, a disruption in cell-cell adhesion is localized to the outer layers of the mature sclerotia. This can be observed in the microscopic sections of sclerotia and by the ease of rind detachment when manipulated by hand. One interpretation of these observations is that the inability to decrease GSSG levels results in a hyperadhesion. This may be followed by an inability to coordinate spatial development of the outermost air-exposed layers due to a failure to establish a final reducing environment required for maturation. Similarly, the developmental defect observed in compound appressoria occurs after initiation. The characteristic hooking and bifurcation of the hypha tip occurs but is followed by further uncontrolled branching and a lack of melanin accumulation, suggesting that the final coordination of developmental maturation may also require a return to a more reducing environment.

In conclusion, these findings implicate Ss-Ggt1 and its substrates GSH and GSSG, as functioning in sclerotial and compound appressorium development. GSH, GSSG, or GSH/GSSG ratios may function as signals directly influencing signal pathways mediating sclerotium and compound appressorium development. Additionally, the redox state affected by GSH/GSSG imbalance may have direct effects on protein and non-protein thiols promoting cell adhesion and other biochemical changes required for sclerotium and compound appressorium formation. Potent inhibitors of Ggt activity have been developed recently (Han et al. 2007) and may have applications in disease control based on our findings that Ggt is required for two essential stages of the disease cycle. Further characterization of the generators and modulators of the redox systems may provide additional targets for interference in the life cycle and infectious development of this and related phytopathogenic fungi.

MATERIALS AND METHODS

Culture growth.

S. sclerotiorum WT strain 1980 was cultured on PDA plates. PDA cultures of $\Delta Ss-ggt1$ mutants (KO1 = Ss-ggt1-3.10, KO2 = Ss-ggt1-2.3, KO3 = Ss-ggt1-2.16, KO4 = Ss-ggt1-3.12, and KO5 = Ss-ggt1-3.26) and ectopic strain 3.29 (ectopic) were amended with hygromycin (final concentration = 100 µg/ml). Sclerotium development stages (S1-S6) previously described by Li and Rollins (2009) were used to define the developmental stages of sclerotia in this study. These stages are S1 = initiation, S2 = condensation, S3 = enlargement, S4 = consolidation, S5 = pigmentation, and S6 = maturation.

Generation of Ggt gene deletion mutants ($\Delta Ss-ggt1$).

A split-marker gene replacement method (Fairhead et al. 1996; Fu et al. 2006) was used to obtain multiple, independent *Ss-ggt1* (SS1G_14127.3) gene-replacement knockout mutants.

The strategy for gene replacement is shown in Figure 2. A plasmid containing a hygromycin resistance cassette flanked with approximately 1.2 kb of 5'- and 3'-UTR sequence from the WT *Ss-ggt1* locus (ggt-5'+hph+3') was constructed based on the method described by Li and Rollins (2010). Primer pairs 5'ggtMu-L1 (5'-TTCAAAAGGGCTGAGTGTGA-3') and 5'ggtMu-R1 (5'-AGGCGCGCCCAACCCGGGAGAATGAGT TA-3'), and 3'ggtMu-L1 (5'-AGGCGCGCCGGGGTTTAAAT CTAGGATACGG-3') and 3'ggtMu-R1 (5'-GAAAGGTGGTG GACTTTTGA-3') were used for polymerase chain reaction (PCR) to acquire 5'- and 3'-UTR amplicons, respectively, of the *Ss-ggt1* locus from WT *S. sclerotiorum* genomic DNA. *AscI* restriction endonuclease recognition sites (underlined) were incorporated into the primers to facilitate cloning. The detailed steps for construction of the ggt-5'+hph+3' construct followed the previously described procedure (Li and Rollins 2010). Two "split" fragments (5'-UTR joined with the 5' *hph* sequence "hy" and the second overlapping *hph* "yg" fragment joined with the 3'-UTR) (Fig. 2) were transformed into WT protoplasts. These fragments were derived from amplification of ggt-5'+hph+3' using primer pairs ggtL (5'-TTCAAAAGGG CTGAGTGTGA-3') and hy (5'-AAATTGCCGTCAACCAAG CTC-3') and yg (5'-TTTCAGCTTCGATGTAGGAGG-3') and ggtR (5'-GAAAGGTGGTGGACTTTTGA-3'). Transformation of WT protoplasts was done according to the method described by Rollins (2003). Constructed plasmids were all transformed into *Escherichia coli* DH5 α for propagation. Plasmid isolation, enzyme digestion, gel electrophoresis, DNA fragment purification, and ligation were conducted using standard procedures (Sambrook and Russell 2001) or according to the manufacturer's instruction.

Nucleic acid isolation and hybridization.

Genomic DNA and RNA were isolated, DNA digested, and gel electrophoresis performed according to standard procedures (Sambrook and Russell 2001). Southern hybridizations were conducted according to the descriptions of Rollins (2003). Restriction endonuclease sites and probes for Southern hybridization are shown in Figure 2. Nonradioactive Northern hybridizations were carried out according to the procedures reported by Solanas and associates (2001). Primer pairs (number 137) ggtCDSprob-L (TGCAAGAATGGAGCTGTTG) and (number 138) ggtCDSprob-R (5'-ATGGTCCCATTGCTTT CTG-3') for *ggt1*, (number 781) ggt2CDSprobe-L (5'-TAG GCG GAG GTG GTT TTA TG-3') and (number 782) ggt2CDSprobe-R (5'-ACG AGC ACC AGG ACG AAC AT-3') for *ggt2*, and (number 783) ggt3CDSprobe-L (5'-ACA CCG GAA AAA TCG CTG AA-3') and (number 784) ggt3CDSprobe-R (5'-CCT TTT ACC AGG AGC GAT GA-3') for *ggt3* were used to synthesize Northern hybridization probes by PCR. Each PCR reaction mixture contained 10 $\times$ digoxigenin (DIG) labeling mixture from DIG High Prime DNA labeling and detection starter kit I Roche, Indianapolis, IN, U.S.A.), 2.5 mM MgCl₂, 0.2 mM dNTPs, 1.6 μ M forward primers, 1.6 μ M reverse primers, 10 $\times$ *Taq* buffer, 0.65 U of *Taq* polymerase (New England Biolabs, Inc., Ipswich, MA, U.S.A.), 100 ng of DNA template, and H₂O made up to the final volume of 25 μ L. The PCR program was 95°C for 4 min; 35 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 1.5 min; followed by 72°C for 10 min. Labeling efficiency was determined as described in the instruction manual (Roche). Final concentration of probe in hybridization buffer was 25 ng/ml.

GSH+GSSG accumulation assay.

Stage 4 to 5 sclerotia and stage 6 sclerotia from WT, ectopic, and *Ss-ggt1* knockout strains were harvested from PDA plates with forceps. Lyophilized sclerotia (100 mg) from each strain

were weighed and homogenized for GSH+GSSG extraction. GSH extraction and determination were conducted using the ApoGSHTM glutathione colorimetric detection kit (Biovision Research Products, Mountain View, CA, U.S.A.) following the manufacturer's instruction.

Enzyme extraction and Ggt activity assay.

To obtain protein extracts for Ggt activity measurement, lyophilized sclerotia from WT, ectopic, and knockout strains were homogenized in protein extraction buffer as previously described (Li and Rollins 2009). Ggt activity was measured spectrophotometrically based on a modified protocol of Ikeda (2000). Ggt reaction mixture (100 μ L) was added to wells of a 96-well microtiter plate containing 100 mM Tris-HCl (pH 8.0), 1 mM γ -GPNA, 20 mM glycylglycine, and 10 μ L of total protein extract. The plate was incubated at 37°C during plate reading. Absorbance values were recorded every 30 s for 10 min at 410 nm using a Bio-Rad microplate reader Model 680 with Microplate Manager Version 5.2 Software (Bio-Rad, Hercules, CA, U.S.A.). Ggt activity was calculated using the equation described by Huseby and Strömme (1974).

Hydrogen peroxide detection.

To detect hydrogen peroxide accumulation in sclerotia, sclerotia were weighed, sectioned (approximately 30 μ m), and immersed into freshly prepared DAB (D5637; Sigma-Aldrich, St. Louis) solution at 1.25 mg mL⁻¹. DAB was dissolved in H₂O and adjusted to pH 3.8 with HCl prior to use. Submerged sclerotia slices were infiltrated under vacuum for 5 min and then shaken at 50 rpm for 4 h. Photographs were taken for a qualitative assessment of DAB staining. To quantitatively determine H₂O₂ concentrations, DAB solution absorbance was measured at 450 nm. Concentration values were extrapolated from a standard curve produced from known concentrations of H₂O₂ incubated for the same time period in DAB solution.

Compound appressorium and plant infection assays.

The production of compound appressoria was assayed in vitro using a cellophane-media overlay technique. A single sheet (9 cm diameter) of autoclaved cellophane (Bio-Rad) was overlain on PDA. Sterile deionized water (500 μ L) was applied to the cellophane surface and spread with a glass rod until a smooth surface was achieved and all the water was absorbed. Hyphae-colonized agar plugs (0.5 cm diameter) were taken from the expanding margin of PDA cultures and placed hyphae-side down on the cellophane in evenly spaced rows. Plates were incubated on the lab bench (approximately 22°C) under ambient fluorescent light conditions (approximately 2.8 μ mol m⁻² s⁻¹, 12 h per day). Following a 48-h incubation period, the bottoms of the plates were photographed.

Inoculation of unwounded tomato leaflets was conducted as previously described (Rollins 2003). For the wounded treatment, a 0.5-cm cut was made through the abaxial and adaxial surface of each leaflet with a scalpel blade and the inoculum plug placed directly on top of the cut. Each leaflet was digitally photographed at 24 and 48 h postinoculation. The images were imported to the Spot Advanced Software program (Diagnostic Instruments, Sterling Heights, MI, U.S.A.) and lesion areas calculated by tracing the necrotic area, calculating the area, and subtracting the inoculum plug area. The lesion area for each fungal strain in wounded and unwounded treatments was calculated for 24 and 48 h postinoculation time points. An analysis of variance was performed with the mean lesion areas from each treatment time point; a *P* value < 0.05 was considered significant. Differences in lesion area among strains within a given treatment time point was determined by the Bonferroni (Dunn) *t* test (α = 0.05). Each experiment included

three leaflets inoculated with each fungal strain and the experiment was repeated three times. Results of a representative replicate from one experiment most closely matching the mean lesion area of the WT and the *Ss-ggt1* KO mutants across all experiments are shown in Figure 6.

Apothecia production.

To test for carpogenic germination, mature sclerotia of $\Delta Ss-ggt1$ mutants and WT were produced, surface sterilized, and incubated as previously described (Li and Rollins 2010).

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LITERATURE CITED

- Abawi, G. S., Polach, F. J., and Molin, W. T. 1975. Infection of bean by ascospores of *Whetzelinia sclerotiorum*. *Phytopathology* 65:673-678.
- Aguirre, J., Riosmomborg, M., Hewitt, D., and Hansberg, W. 2005. Reactive oxygen species and development in microbial eukaryotes. *Trends Microbiol.* 13:111-118.
- Akutsu, K., Ko, K., and Misato, T. 1983. Nuclear behavior from conidial germination to formation of the secondary appressoria of *Botrytis cinerea*. *Ann. Phytopathol. Soc. Jpn.* 49:361-366.
- Amselem, J., Cuomo, C. A., van Kan, J. A. L., 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., Quévillon, 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., Collémar, J., Cotton, P., Danchin, E. G., Da Silva, C., Gautier, A., Giraud, C., Giraud, T., Gonzalez, C., Grossetete, S., Güldener, 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., Rascle, C., Schumacher, J., Séguen, 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.
- Backhouse, D., and Willetts, H. J. 1987. Development and structure of infection cushions of *Botrytis cinerea*. *Trans. Br. Mycol. Soc.* 89:89-95.
- Blokhina, O., Virolainen, E., and Fagersted, K. V. 2003. Antioxidants, oxidative damage and oxygen deprivation stress: A review. *Ann. Bot.* 91:179-194.
- Boland, G. J., and Hall, R. 1994. Index of plant hosts of *Sclerotinia sclerotiorum*. *Can. J. Plant Pathol.* 16:93-108.
- Bolton, M. D., Thomma, B. P. H. J., and Nelson, B. D. 2006. *Sclerotinia sclerotiorum* (Lib.) de Bary: Biology and molecular traits of a cosmopolitan pathogen. *Mol. Plant Pathol.* 7:1-16.
- Chet, I., and Henis, Y. 1975. Sclerotial morphogenesis in fungi. *Annu. Rev. Phytopathol.* 13:169-192.
- Cruickshank, R. H., and Wade, G. C. 1992. Production of appressoria by *Monilinia fructicola*. *Mycol. Res.* 96:425-428.
- Egan, M. J., Wang, Z., Jones, M. A., Smirnov, N., and Talbot, N. J. 2007. Generation of reactive oxygen species by fungal NADPH oxidases is required for rice blast disease. *Proc. Natl. Acad. Sci. U.S.A.* 104:11772-11777.
- Emmett, R. W., and Parbery, D. G. 1975. Appressoria. *Annu. Rev. Phytopathol.* 13:147-167.
- Erental, A., Harel, A., and Yarden, O. 2007. Type 2A phosphoprotein phosphatase is required for asexual development and pathogenesis of *Sclerotinia sclerotiorum*. *Mol. Plant-Microbe Interact.* 20:944-954.
- Fairhead, C., Liorente, B., Denis, F., Soler, M., and Dujon, B. 1996. New vectors for combinatorial deletions in yeast chromosomes and for gap-repair cloning using 'split-marker' recombination. *Yeast* 12:1439-1458.
- Franco, R., and Cidlowski, J. A. 2009. Apoptosis and glutathione: Beyond an antioxidant. *Cell Death Differ.* 16:1303-1314.
- Fu, J., Hettler, E., and Wickes, B. L. 2006. Split marker transformation increases homologous integration frequency in *Cryptococcus neoformans*. *Fungal Genet. Biol.* 43:200-212.
- Georgiou, C. D. 1997. Lipid peroxidation in *Sclerotium rolfsii*: A new look into the mechanism of sclerotial biogenesis in fungi. *Mycol. Res.* 101:460-464.
- Georgiou, C. D., Patsoukis, N., Papapostolou, L., and Zervoudakis, G. 2006. Sclerotial metamorphosis in filamentous fungi is induced by oxidative stress. *Integr. Comp. Biol.* 46:691-712.
- Han, L., Hiratake, J., Kamiyama, A., and Sakata, K. 2007. Design, synthesis, and evaluation of gamma-phosphono diester analogues of glutamate as highly potent inhibitors and active site probes of gamma-glutamyl transpeptidase. *Biochemistry* 46:1432-1447.
- Hansberg, W., and Aguirre, J. 1990. Hyperoxidant states cause microbial cell differentiation by cell isolation from dioxygen. *J. Theor. Biol.* 142:201-221.
- Huseby, N. E., and Stromme, J. H. 1974. Practical points regarding routine determination of gamma-glutamyl transferase (gamma-GT) in serum with a kinetic method at 37 degrees C. *Scand. J. Clin. Lab Invest.* 34:357-363.
- Ikeda, Y. 2000. γ -Glutamyl transpeptidase. Pages 86-87 in: *Experimental Protocols for Reactive Oxygen and Nitrogen Species*. N. Taniguchi and J. M. C. Gutteridge, eds. Oxford University Press, U.S.A., New York.
- Jurick, W. M., and Rollins, J. A. 2007. Deletion of the adenylate cyclase (*sac1*) gene affects multiple developmental pathways and pathogenicity in *Sclerotinia sclerotiorum*. *Fungal Genet. Biol.* 44:521-530.
- Krance, S. M., Keng, P. C., Palis, J., and Ballatori, N. 2010. Transient glutathione depletion determines terminal differentiation in HL-60 cells. *Oxid. Med. Cell Longev.* 3:53-60.
- Lee, M.-H., and Bostock, R. M. 2007. Fruit exocarp phenols in relation to quiescence and development of *Monilinia fructicola* infections in *Prunus*: A role for cellular redox? *Phytopathology* 97:269-277.
- 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.
- Lum, G., and Min, X. J. 2011. FunSecKB: The fungal secretome knowledge-base database. *J. Biol. Database Curat.* doi:10.1093/database/bar001. Published online.
- Lumsden, R. D., and Dow, R. L. 1973. Histopathology of *Sclerotinia sclerotiorum* infection of bean. *Phytopathology* 63:708-715.
- May, M. J., Vernoux, T., Leaver, C., Van Montagu, M., and Inzé, D. 1998. Glutathione homeostasis in plants: Implications for environmental sensing and plant development. *J. Exp. Bot.* 49:649-667.
- Mehdi, K., Thierie, J., and Penninckx, M. 2001. γ -Glutamyl transpeptidase in the yeast *Saccharomyces cerevisiae* and its role in the vacuolar transport and metabolism of glutathione. *Biochem. J.* 359:631-637.
- Meister, A., and Anderson, M. E. 1983. Glutathione. *Annu. Rev. Biochem.* 52:711-760.
- Pallardó, F. V., Markovic, J., García, J. L., and Viña, J. 2009. Role of nuclear glutathione as a key regulator of cell proliferation. *Mol. Aspects Med.* 30:77-85.
- Papapostolou, L., and Georgiou, C. D. 2010. Hydrogen peroxide is involved in the sclerotial differentiation of filamentous phytopathogenic fungi. *J. Appl. Microbiol.* 109:1929-1936.
- Patsoukis, N., and Georgiou, C. D. 2007. Effect of glutathione biosynthesis-related modulators on the thiol redox state enzymes and on sclerotial differentiation of filamentous phytopathogenic fungi. *Mycopathologia* 163:335-347.
- Patsoukis, N., and Georgiou, C. D. 2008. Thiol redox state and related enzymes in sclerotium-forming filamentous phytopathogenic fungi. *Mycol. Res.* 112:602-610.
- Purdy, L. H. 1958. Some factors affecting penetration and infection by *Sclerotinia sclerotiorum*. *Phytopathology* 48:605-609.
- Purdy, L. H. 1979. *Sclerotinia sclerotiorum*: History, diseases and symptomatology, host range, geographic distribution, and impact. *Phytopathology* 69:875-880.
- Rollins, J. A. 2003. The *Sclerotinia sclerotiorum pac1* gene is required for sclerotial development and virulence. *Mol. Plant-Microbe Interact.* 16:785-795.
- Sambrook, J., and Russell, D. W. 2001. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Scott, B., and Eaton, C. J. 2008. Role of reactive oxygen species in fungal cellular differentiations. *Curr. Opin. Microbiol.* 11:488-493.
- Segmüller, N., Kokkelink, L., Giesbert, S., Odinius, D., van Kan, J., and Tudzynski, P. 2008. NADPH Oxidases are involved in differentiation and pathogenicity in *Botrytis cinerea*. *Mol. Plant-Microbe Interact.* 21:808-819.
- Solanas, M., Moral, R., and Escrib, E. 2001. Improved non-radioactive Northern blot protocol for detecting low abundance mRNAs from mam-

- malian tissues. *Biotechnol. Lett.* 23:263-266.
- Takemoto, D., Tanaka, A., and Scott, B. 2007. NADPH oxidases in fungi: Diverse roles of reactive oxygen species in fungal cellular differentiation. *Fungal Genet. Biol.* 44:1065-1076.
- Tariq, V. N., and Jeffries, P. 1984. Appressorium formation by *Sclerotinia sclerotiorum*: Scanning electron microscopy. *Trans. Br. Mycol. Soc.* 82:645-651.
- Tate, S. S., and Meister, A. 1981. γ -Glutamyl transpeptidase—catalytic, structural and functional aspects. *Mol. Cell. Biochem.* 39:357-368.
- Toledo, I., Noronha-Dutra, A. A., and Hansberg, W. 1991. Loss of NAD(P)-reducing power and glutathione disulfide excretion at the start of induction of aerial growth in *Neurospora crassa*. *J. Bacteriol.* 173:3243-3249.
- Zhang, H., and Forman, H. J. 2009. Redox regulation of gamma-glutamyl transpeptidase. *Am. J. Respir. Cell Mol. Biol.* 41:509-515.

AUTHOR-RECOMMENDED INTERNET RESOURCE

Broad Institute *S. sclerotiorum* genome sequence database:
www.broad.mit.edu/annotation/genome/sclerotinia_sclerotiorum/FeatureSearch.html