CHARACTERIZATION OF GENES REGULATED DURING SCLEROTIAL DEVELOPMENT IN THE FUNGAL PLANT PATHOGEN *Sclerotinia sclerotiorum* (Lib.) de Bary

By

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LIST OF ABBREVIATIONS

AFS	antifungal substances
BCIP	5-bromo-4-chloro-3-indolyl phosphate
Cys	Cysteine
CWDEs	cell wall degrading enzyme
EST	expressed sequence tag
GFP	green fluorescent protein
GGT or γ-GT	gama-glutamyltranspeptidase
GO	gene ontology
GSH	glutathione
MAPK	mitogen-activated protein kinase
MIPS	Munich information center for protein sequence
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate,
NBT	nitroblue tetrazolium
PCR	polymerase chain reaction
PGs	polygalacturonases
PKA	cAMP-dependent protein kinase A
PVDF	poly (vinylidene fluoride)
ROS	reactive oxygen species ROS
RT-PCR	reverse transcription polymerase chain reaction
qPCR	quantitative polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
ssp1	sclerotia specific protein gene
Ssp1	sclerotia specific protein encoded by *ssp1*
Ss_ssp1	*S. sclerotiorum ssp1* gene

Ss_Ssp1	*S. sclerotiorum* sclerotia specific protein encoded by *Ss_ssp1*
Ss_ggt1	*S. sclerotiorum* gama-glutamyltranspeptidase 1 gene
Ss_Ggt1	*S. sclerotiorum* gama-glutamyltranspeptidase encoded by *Ss_ggt1*
STEM	short-time series miner
WT	wild type
∅	diameter

Abstract of Dissertation Presented to the Graduate School
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Sclerotinia sclerotiorum (Lib.) de Bary is a devastating fungal phytopathogen with a broad host range and global distribution. The resting structures produced by this fungus, sclerotia, are crucial for survival in harsh environments and further dissemination of the fungus when environmental conditions become conducive. Sclerotia can germinate as hyphae to initiate disease directly or germinate as fruiting bodies, apothecia, to produce forcibly-discharged ascospores that act as a dispersible inoculum source. To begin a molecular genetic dissection of sclerotial developmental regulation, the first gene I chose for investigation was *Ss_ssp1*. The protein encoded by this gene was previously described as the major storage protein present in mature sclerotia of *S. sclerotiorum*. I found that *ssp1* transcripts specifically accumulated in all stages of sclerotial development with peak levels in stage IV sclerotia. In contrast with the sclerotia-restricted spatial accumulation of *ssp1* transcripts, Ssp1 protein accumulation was detected in all sclerotial and apothecial stages. Immunolocalization suggests the release of Ssp1 from sclerotial protein bodies and the relocation to apothecia during carpogenic germination. Contrary to our original hypothesis, *Ss_ssp1* deletion does not distinctively affect sclerotial development or carpogenic germination. However, the upregulation of the *Ss_ssp1* paralog,

Ss_ssp2 and another 16kDa major protein in deletion mutants indicates a possible functional redundancy and compensatory role for the Ss_Ssp1 homolog and other sclerotia-accumulating proteins. To comprehensively investigate genes involved in sclerotial development, transcriptome profiling during sclerotial initiation was conducted using a genomic, long oligomer microarray. When compared to gene expression during hyphal growth, 15% of the genes from the *S. sclerotiorum* genome were differentially expressed (up- or down-regulated) during sclerotial initiation. Additionally, 14% or the orphan ESTs examined are predicted to be newly discovered genes on the basis of my microarray analysis and annotation. The gene encoding a gamma-glutamyl transpeptidas (*Ss_ggt*) was one of the genes whose expression was markedly upregulated during sclerotial initiation by microarray analysis. Gene deletion mutant of *Ss_ggt* resulted in distinct morphological aberrations in sclerotial morphology. In mature dry sclerotia, the cortex layer was thickened and easily peeled away with the rind from the medulla. Sclerotia of the *Ss_ggt* deletion mutant failed to carpogenically germinate into apothecia due to an internal breakdown of the interior sclerotial tissue during the carpogenic germination incubation period. This phenotype is attributed to poor environmental protection of the medulla, allowing the cortex to easily be separated from the rind outerlayer.

CHAPTER 1
LITERATURE REVIEW

Introduction of *Sclerotinia sclerotiorum* (Lib.) de Bary

Taxonomy

Sclerotinia sclerotiorum (Lib.) de Bary is the type species for the filamentous fungal genus *Sclerotinia* in the family Sclerotiniaceae in the order Helotiales of the phylum Ascomycota. *S. minor* Jagger and *S. trifoliorum* Eriks are the only other valid species within this genus. The earliest description of *S. sclerotiorum* came from Libert (1837), who placed it within the Pizizomycetes as *Peziza sclerotiorum*. Since, the taxonomic placement of this species has been revised several times (Wakefield, 1924). The current name, *Sclerotinia sclerotiorum* (Lib.) de Bary which was first used by Heinrich Anton de Bary in 1884 was accepted as the conserved name in 1979 (Kohn, 1979) and approved by the International Botanical Congress in 1981.

The family Sclerotiniaceae was erected by Whetzel (1945) as a group of fungi which produce stromata capable of germinating as stipitate apothecia bearing inoperculate asci and also produce globose, hyaline phialomicroconidia/spermatia on stromata and mycelia. The most important feature of members in this family is the ability to form stromata, compact masses of hyphae with a distinct medulla tissue layer contained within a continuous or discontinuous melanized rind.

Economic Significance

S. sclerotiorum is a necrotrophic plant pathogen that can cause disease on more than 400 plant species especially dicotyledonous crops (Boland and Hall, 1994). There are greater than 60 names used to refer to the plant diseases caused by this fungus including “white mold”, “watery soft rot”, “stem rot” and “lettuce drop” (Purdy, 1979). *S. minor* and *S. trifoliorum* are also able to infect and overlapping range of crops. However, *S. minor* is of agro-economic concern primarily

on lettuce and peanut while *S. trifoliorum* is restricted to cause disease of concern on alfalfa and other forage legumes (Anonymous, 2003). According to the reports of the National Sclerotinia Initiative in 2005, collective annual losses in the United States caused by *S. sclerotiorum* for canola, soybeans, dry beans, sunflowers and pulse crops have been as high as 250-280 million US dollars in a single year (Anonymous, 2005a; Anonymous, 2005b), and it "continues to be a very difficult pathogen to control" (Anonymous, 2003). *S. sclerotiorum* is one of the most devastating and cosmopolitan plant pathogens of agriculture crops.

Life Cycle

The long-term persistence of *S. sclerotiorum* in agriculture and the difficulties associated with *Sclerotinia* disease control can be partially attributed to a special survival mechanism in disease life cycle, sclerotial development. A sclerotium is a compact multihyphal aggregate (medulla) enclosed within a melanized outer-layer (rind). Ecologically, the sclerotium is a crucial resting structure for long-term survival. Sclerotia can remain dormant and retain their viability for many years, documented up to eight years (Adams and Ayers, 1979), if the environmental conditions are unfavorable for germination. Epidemiologically, because *S. sclerotiorum* does not produce macroconidia (i.e. it has no functional asexual conidia.) and the microconidia (spermatia) produced on the surface of mature sclerotia and some hyphae are non-propagative, sclerotia also play a key role for further asexual reproduction and sexual reproduction to reinitiate the disease cycle. Under conditions of mild temperature (10-20°C) and adequate moisture, sclerotia can either germinate into apothecia (carpogenic germination) or mycelia (myceliogenic germination) (Pohronezny and L.H., 2002).

Mycelia from sclerotia can directly infect plant tissues usually from sensitive roots or wilted leaves of crops such as lettuce in contact with the soil. The most important diseases caused by myceliogenic germination are sunflower wilt and Sclerotinia rot of carrots (Bardin and

Huang, 2001; Holley and Nelson, 1986). Storage rot can also occur due to the further mycelial invasion after harvest in carrots and snap beans (Lumsden, 1979).

Most diseases caused by *S. sclerotiorum* are initiated by ascospores (Abawi and Grogan, 1979). An apothecium is a fertile structure which consists of a central stipe originating from the sclerotium medulla and a cupulate disc differentiated from the tip of stipe. The upper layer of the disc, the hymenium, produces millions of asci containing eight binucleate ascospores per ascus. When fully mature, ascospores are forcibly discharged from the apothecium surface into the atmosphere continuously for several days. Discharged ascospores have to germinate and grow saprotrophically on senescent or necrotic tissues before hyphae are competent to infect a healthy host plant (Abawi and Grogan, 1979; Lumsden, 1979). This adaptation usually takes place on senescent flower petals or detached leaves that can provide primary nutrients for ascospore germination as well as chances for ascospore-germinated mycelia to contact and invade healthy tissue (Inglis and Boland, 1990; Turkington and Morrall, 1993).

Infectious hyphae penetrate healthy host tissue through the cuticle by forming multicellular melanized infection cushions (Lumsden and Dow, 1973) or directly enter plant tissue through open stomata via deregulating guard cells with secreted oxalic acid (Guimarães and Stotz, 2004). Further hyphal growth and ramification leads to the complete collapse of host cells and eventually sclerotial formation on the surface of or within the dead plant tissue.

Pathogenicity Factors and Disease Control

S. sclerotiorum is a necrotrophic plant pathogen. As such, it kills host cells in advance of colonization. It exhibits little tissue specificity within a host and under conducive conditions infections give rise non-delimited host tissue maceration. Cell wall degrading enzymes (CWDEs) and the secondary metabolite produced by this fungus, oxalic acid, are thought to be the main factors facilitating hyphal penetration and colonization of hosts. Since pectin is a major

constituent of the dicotyledonous plant cell wall, various pectinolytic enzymes (primarily polygalacturonases, PGs) secreted by *S. sclerotiorum* are the most comprehensively studied CWDEs though other non-pectinolytic CWDEs have also been characterized in this fungus (Bolton et al., 2006). Physiological evidence has indicated that these CWDEs might work collaboratively and synergistically with oxalic acid to function in a dynamic pH ambient environment and respond to various carbon/nitrogen sources or even host-specific factors to bring about symptom development.(Alghisi and Favaron, 1995; Bateman and Beer, 1965; Kars et al., 2005; Kasza et al., 2004). For example, *S. sclerotiorum* is able to secret several different molecular forms (isozymes) of PGs that exhibit similar enzymatic activities. Some have different isoelectric points with a similar molecular weights due to the differential glycosylation (Fraissinet-Tachet et al., 1995). Another example comes from a closely related necrotrophic plant pathogen *Botrytis cinerea* (Kars et al., 2005). Five endoPGs recently found in this fungus display different biochemical properties and necrotizing activity on different hosts. This variability is speculated to confer flexibility and adaptability to a pathogen with such a broad host range (Bolton et al., 2006). The production and secretion of oxalic acid is a factor thought to be critical for pathogenicity of *S. sclerotiorum*. Evidence for such involvement was first provided by de Bary (1887) by recovery of oxalic acid from *S. sclerotiorum*-infected tissue and the demonstration that exogenously applied oxalic acid killed host cells. Numerous contemporary studies have strengthened this correlation by associating concentrations of oxalate in the host with the extent of symptom development (Marciano et al., 1983; Maxwell and Lumsden, 1970) and by reproducing disease-like symptoms by direct injection of oxalate into plants (Maxwell and Lumsden, 1970; Noyes and Hancock, 1981). Using a UV mutagenesis approach, Godoy *et al.* (1990) demonstrated that oxalic acid deficient mutants lost pathogenicity whereas a strain

reverting to oxalate production also regain pathogenicity. A number of mechanisms by which oxalic acid aids in pathogenicity having been proposed, reviewed by Bolton *et al.* (2006). These can be summarized as: i) provide favorable acidic pH environment for optimum CWDE activities (Favaron et al., 2004) or pH-regulated genes necessary for the pathogenesis (Kim et al., 2007); ii) suppress oxidative burst initiating plant defense response (Cessna et al., 2000); iii) facilitate hyphal penetration by deregulating guard cell functions to induce stomatal opening (Guimarães and Stotz, 2004). One point that should be emphasized here is that oxalic acid appears to be a primary physiological determinant of pathogenicity; but, oxalic acid also plays an important regulatory role as well. The full virulence of *S. sclerotiorum* relies on the correct regulation of genes in response to the ambient pH. Current evidence for this is that Pac1 activating mutants can constitutively accumulate oxalic acid under both low and high pH but exhibit an attenuated virulence phenotype (Kim et al., 2007). Thus, oxalate is required for disease development but must be produced in the proper time and amount to be fully virulent.

Controlling plant diseases caused by *S. sclerotiorum* remains a challenge for modern agriculture. The primary reason that this disease has not been effectively managed is the lack of major simply inherited resistance in susceptible crops. After oxalic acid was described as a determinant of pathogenicity (Godoy et al., 1990), a number of independent labs worked to incorporate oxalic acid-degrading enzymes into important crop plants including soybean, sunflower and peanut. A number of these transgenic lines have shown increased resistance to *Sclerotinia spp.* (Donaldson et al., 2001; Hu et al., 2003; Kesarwani et al., 2000; Livingstone et al., 2005). However, in the only field-tested lines, the introduction of oxalate oxidase does not increase the seed output of transgenic sunflowers compared to wild-type (Burke and Rieseberg, 2003) though it does reduce disease severity in some environments. The common control

methods for *Sclerotinia* diseases continue to be the application of fungicides for most *Sclerotinia* host plants (Mueller et al., 2002) or crop rotation for certain crops such as sunflower where inoculum densities in the soil play a primary role in disease development. The possibility of biological control for *Sclerotinia* diseases has also been investigated in a number of studies. Some mycoparasites such as *Coniothyrium minitans* and *Sporidesmium sclerotivorum* specifically attack and degrade sclerotia. Both fungi have been commercially applied for *Sclerotinia* disease control (del Rio et al., 2002; Li et al., 2006; Partridge et al., 2006). *C. minitans* appears to secrete an antifungal substance (AFS) that inhibits hypal growth or mycelial germination of sclerotia. The nature of this AFS has been investigated recently (McQuilken et al., 2003; Yang et al., 2007). One purified AFS activity was identified as macrosphelide A (McQuilken et al., 2003). The research on mycoparasites and their effective antifungal substances will continue to be an active area of research as an alternative to chemical control methods.

Why Study Sclerotial Development

Most new infections of *Sclerotinia* diseases are initiated by ascospores yet we are unable to neglect the significance of sclerotial development in the cycle. Not only is the development of the ascospore-bearing apothecium dependent on sclerotia, but also, sclerotia themselves can initiate diseases on some hosts via myceliogenic germination. Sclerotial survival is a key link in the life cycle of *S. sclerotiorum* maintaining viability of the fungus under conditions in which hosts plants do not exist or under which disease development is not conducive. As such, the possibility to control *Sclerotinia* diseases by suppressing sclerotial formation or germination would eliminate new infections. In the laboratory, *S. sclerotiorum* stains have been observed that are attenuated in their virulence or that have lost altogether their ability to cause disease. Interestingly, these same strains have lost their capacity to produce normal sclerotia (Erental et

al., 2007; Godoy et al., 1990; Jurick and Rollins, 2007; Rollins, 2003). Understanding the relationship between pathogenicity and sclerotial development may assist us in finding better ways to manage Sclerotinia diseases and give us some new insights to the physiological and genetic links between fungal development and pathogenicity.

Sclerotial Development

Sclerotial Structure and Evolution

True sclerotia are produced by species within and outside of the Sclerotiniaceae. These stromata, compact masses of vegetative hyphae that commonly support the development of asexual or sexual reproductive organs, are all categorized as sclerotia based on several common features. First, functionally they are all quiescent resting structures that maintain the viability of the fungus under harsh environment conditions. Second, phenotypically all of them can be easily detached from the substratum at maturity while substratal stromata closely connect with the remains of the tissue and are difficult to separate from host tissue at maturity (Willetts and Bullock, 1992). Third, anatomically and histochemically they have similar ultrastructures.

Three continuous layers can be distinguished in tuberoid and plano-convexoid sclerotia: rind, cortex and medulla. The rind, one to several cells deep, is the outer-most layer composed of thickened, pigmented, parenchyma-like cells. Pigmentation of the rind results from the deposition of melanin in the cell wall and is thought to play a key role in protection from environmental stress in many fungal tissues (Bell and Wheeler, 1986; Henson et al., 1999). Melanin deposited in the sclerotial rind decreases the permeability of sclerotia so that water and nutrients can be retained and the mature sclerotium can be protected from chemicals, radiation or other environmental factors that may otherwise permeate into the sclerotium (Young and Ashford, 1992). Melanin is also thought to protect the sclerotium from biological degradation because this heterogeneous phenolic polymer in the fungal walls can inhibit the polysaccharidase

secreted by antagonistic microorganisms (Willetts, 1971). Not all sclerotia form a discrete rind (Willetts and Bullock, 1992), but all true sclerotia formed by members of the Sclerotiniaceae do contain this layer.

The cortex layer is position to the interior of the rind and this dense tissue layer separates the rind from central mass (medulla) of the sclerotium. The thickest cortex among the Sclerotiniaceae is up to six cells thick (Kohn and Grenville, 1989a; Kohn and Grenville, 1989b). Its development appears to be related to the oxygen concentration and availability of nutrients around the sclerotia during development though the thickness of this layer differs between different species and even within the species is not always morphologically distinguishable (Willetts and Bullock, 1992).

The medulla is the interior-most layer and occupies the greatest volume of a sclerotium. It consists of an aggregation of hyphal tissue embedded within an extracellular matrix. This matrix is composed of polysaccharide secreted by the hyphae and appears to play several important functions. It is presumed to play roles in sclerotial morphogenesis, protection of sclerotia from dehydration, facilitation of water uptake and provision of energy from a large reserve of carbohydrates (Willetts, 1971; Willetts and Bullock, 1992). Both of cortex and medulla accumulate nutrient reserves. Histochemical analyses show less abundant storage bodies in cortex than in medulla. But the composition of these reserves, glycogen, protein, polyphosphate and lipid within these two layers are very similar besides this small difference.

Besides members of the Sclerotiniaceae, a number of taxonomically diverse species in both the Ascomycota and the Basidiomycota produce sclerotia. *Claviceps purpurea* is one such ascomycete that produces sclerotia and has received significant attention due to the ergot alkaloids produced within them. Additionally, species of *Verticillium*, *Aspergillus* and

Penicillium (anamorphs of ascomycetes) also produce sclerotia. In the Basidiomycota, some plant pathogens such as *Typhula* spp. (snow mould on turfgrass) and *Sclerotium* spp. (typically *Scleortium rolfsii*) form sclerotia. These phylogenetically distantly related species all evolved the ability to produce sclerotia, which play the similar roles in the life cycle of these diverse species. The patchy appearance of this character within the fungal phylogenetic tree supports the hypothesis that sclerotia are the result of convergent evolution (Willetts, 1972). Willetts also proposed that sclerotia of different fungal species originated from different fungal tissues and that a variety of degenerated or aborted structures might be the developmental origin of sclerotia. These include undifferentiated conidiophores or conidial masses from ascomycetes, sterile basidiocarps from basdiomycetes, and perithecia or cleistothecia from deuteromycetes (Willetts, 1972). Though the evidence is still limited, some investigations from Sclerotiniaceae species suggested that true sclerotia (tuberoid sclerotia and plano-convexoid sclerotia) produced by these species were derived from sporogenous tissue (Willetts, 1997). One supporting observation is that *Monilinia* spp., which produce substratal stromata, will produce some small black outgrowths on the surface of infected fruits when conidia differentiation is inhibited under high humidity (Willetts, 1997; Willetts and Bullock, 1992). These are anatomically and histochemically similar to tuberoid sclerotia of *Sclerotinia* spp. Willetts suggested that the structure of the tuberoid sclerotium evolved from a pathway in common with a conidial chain developmental pathway in that these black outgrowths are terminally aggregated (Willetts, 1997; Willetts and Bullock, 1992). Another point of evidence is that chain-like structures similar in appearance to chains of monilioid maroconidia were found in sclerotial initials and at the margins of cultures of *S. sclerotiorum* (Jayachandran, 1982). Jayachandran (1982) also observed "*Cristulariella*-like multicellular anamorphs" present in cultures. These structures may be the

vestigial macroconidia and multihyphal reproductive anamorphs produced by the progenitor of *S. sclerotiorum* (Jayachandran, 1982; Willetts, 1997). The modern species producing tuberoid sclerotia have completely lost their ability to produce conidia. For species like *Botrytis* spp. producing plano-convexoid sclerotia, the conidial state is not completely replaced by sclerotia; however; they do not produce sclerotia and conidia at the same time. Usually the environmental conditions suitable to induce conidia formation inhibit sclerotial development and vice versa (Coley-Smith, 1980). This suggests that the primordia are indeterminant and will form plano-convexoid sclerotia or conidia depending on the environment in which they initiate and develop. The sclerotia produced by *Monilinia* ssp. in Sclerotiniaceae are not considered true sclerotia but pseudosclerotia (Batra, 1991) since they are simply interwoven randomly growing vegetative hyphae within the host and can not be detached from the host at maturity. However, the resting structures produced by the species outside of Sclerotiniaceae are considered true sclerotia based on the similarities in sclerotial development, ultrastructure and roles in fungal life cycles (Chet and Henis, 1975; Luttrell, 1980; Punja, 1985; Zarani and Christias, 1997). The evolutionary origins of sclerotia remain obscure, but the hypothesis made by Willetts may be testable as the core regulatory components of these different developmental pathways become known.

Sclerotial Developmental Stages

Apart from the common features above shared by true sclerotia, sclerotia also have a similar developmental course. Townsend and Willetts (1954) described sclerotial development as a three-step sequential event: i) initiation ii) development and iii) maturation. This characterization has been widely used by other researchers. In culture, initiation of sclerotial development is marked by the appearance of sclerotial initials, white, aerial, fluffy hyphal aggregates, usually observed near the edge of agar medium once vegetative mycelia has covered the plate surface and hyphal extension is complete. These sclerotial initials further aggregate and

grow to a larger size and accumulate clear or yellow exudates on their surfaces. Finally, sclerotia will enter the maturation stage through surface delimitation, melanization, consolidation and exudate evaporation.

Factors Involved in Sclerotial Development

Environmental factors

There are many environmental factors that can affect sclerotial development. Chet and Henis (1975) reviewed various factors influencing sclerotial development and later other researchers also discussed these general factors and added others (Le Tourneau, 1979; Willetts and Bullock, 1992). Some factors have been accepted widely: 1) light conditions can influence sclerotial initiation and further sclerotial development although whether this is solely attributable to photo-oxidation or if specific photoreceptors may play a role is not yet understood yet; 2) temperature affects sclerotial maturation and pigmentation; 3) an alkali pH environment inhibits sclerotial development; 4) oxygen is required for sclerotial imitation; 5) physical and chemical barriers that inhibit polar elongation of hyphae induce sclerotial initiation; 6) staling products secreted by sclerotia-producing fungi or secondary metabolites produced by other microorganisms can induce sclerotial development; 7) various nutrients affect sclerotial development such as carbon/nitrogen source, lipids, minerals, vitamins and sulfur-containing compounds. These general factors that affect sclerotial development have been known for many years. The molecular mechanisms underlying their influence have begun to be investigated only very recently. Current advancements in the understanding of the molecular regulators of sclerotial development are described below.

Oxalic acid

In the early 1990's, oxalic acid biosynthesis was demonstrated to be related to sclerotial formation. Godoy *et al.* (1990) screened colonies derived from UV-irradiated ascospores for loss

of oxalic acid production. Four independent mutants that lost the ability to produce oxalic acid and cause disease, were also unable to form sclerotia. A revertant mutant which regained oxalic acid production was also restored for the ability to produce sclerotia. This indicated that, oxalic acid, which accumulates to high concentrations in media accompanying mycelial growth is required for sclerotial development. Later observations determined that these mutants produce numerous spermatia on young mycelia, which are only sporadically found on old mycelia and mature sclerotia of the wild type (Rollins, unpublished results). There appears to be a linkage between these developmental pathways that is influenced by oxalic acid biosynthesis. Whether this role is strictly metabolic or whether oxalic acid influences these developmental pathways as a component of the growth environment will require further investigation to elucidate.

Ambient pH

One way in which oxalic acid may influence sclerotial development is by lowering the ambient pH environment. As mentioned above, the ambient pH environment is one of the general factors affecting sclerotial development. Rollins and Dickman (2001) reported that neutral ambient pH negatively regulates and an acidic ambient pH positively regulates sclerotial development. They also found that Pac1 acts as a pH-responsive transcription factor in *S. sclerotiorum* and loss-of-function Pac1 replacement mutants display an aberrant sclerotial development and maturation phenotype (Rollins, 2003). This indicates that a pH-sensing signal transduction pathway is involved in sclerotial development. Despite these observations, simply adding oxalic acid to growth medium at low pH is not sufficient to complement the sclerotial defects of various sclerotia minus strains. This indicates that the association is dynamic and may have a basis in general metabolism.

Oxidative stress

Chet and Henis (1975) first put forward the idea that O_2 is required for sclerotial development. The role of oxygen in sclerotial development, however has not been well understood. Recent observations indicate that oxygen is needed to provide a hyperoxidant state of oxidative stress to initiate sclerotial development. Georgiou (1997) first determined that sclerotial biogenesis in *S. rolfsii* is associated with an increase of lipid peroxidation. Lipid peroxides increase before the initiation of sclerotia and reach a maximum when sclerotial initials and early developing sclerotia are formed. Additionally, the number of sclerotia is positively related to the level of lipid peroxidation in mycelia. Later, this same group determined that a series of hydroxyl radical scavengers as well as certain endogenous antioxidants had a negative effect on sclerotial metamorphosis (Georgiou and Petropoulou, 2001a; Georgiou and Petropoulou, 2001b; Georgiou et al., 2001; Georgiou and Zees, 2002; Patsoukis and Georgiou, 2007). These investigations suggest that oxidative stress triggers mycelial differentiation that initiates and propagates sclerotium development (Georgiou et al., 2006).

cAMP and signaling pathway

Rollins and Dickman (1998) found that increases in endogenous and exogenous 3',5'cyclic adenosine monophosphate (cAMP) levels inhibit sclerotial development. Recently, Jurick and Rollins (2007) also demonstrated that low levels of endogenous cAMP production via deletion of the adenylate cyclase-encoding gene, *sac1*, lead to abnormalities in sclerotial formation as well as hyphal growth. The cAMP-dependent protein kinase A (PKA) is an important recipient of the second messenger, cAMP, in cAMP-dependent signal transduction pathway. PKA activity has been shown to increase during sclerotial development but remains at low levels in a non-sclerotia-producing mutant of *S. sclerotiorum* (Harel et al., 2005). However, deletion mutants of PKA catalytic subunit encoding gene, *pka1*, did not exhibit aberrant sclerotial formation or

abolishment of sclerotial initiation (Jurick et al., 2004). After investigating Pka1 orthologs in other filamentous fungi, these authors hypothesized the existence of another PKA-encoding gene in the *S. sclerotiorum* genome. This hypothesis has been validated by the sequencing of the *S. sclerotiorum* genome <http://www.broad.mit.edu/annotation/genome/sclerotinia_sclerotiorum/Home.html>. Their findings suggest that the second PKA gene might contribute full PKA activity in sclerotial development or there is another PKA-independent signaling pathway existing in sclerotial development. Later, Chen and Dickman (2005) reported that a Ras/MAPK pathway is required for sclerotial development and that this pathway is negatively regulated by another small GTPase, Rap-1, in a novel cAMP-dependent but a PKA-independent pathway. Chen *et al.* (2004) provided evidence that this cAMP regulation operates antagonistically to ERK-type mitogen-activated protein kinase (MAPK) and G-protein signaling. They isolated a highly conserved Smk1-MAPK-encoding gene, *smk1*, from *S. sclerotiorum*. Inhibition of *smk1* transcript accumulation via antisense expression blocked sclerotial maturation (Chen et al., 2004). In addition, *smk1* shows positive pH-responsive regulation during sclerotial development and its transcription can be inhibited by addition of cAMP. Results from this study suggest that a cAMP-dependent pathway might stimulate filamentous growth by inhibition of a *smk1*-dependent pathway. Erental *et al.* (2007) additionally showed that interference with protein phosphatase 2A (PP2A) activity via antisense suppression of the regulatory B subunit affected sclerotial maturation. Furthermore, PP2A activity was shown to be dependent on Smk1 and NADPH oxidase functions suggesting interconnections between phosphatases, MAPKs and reactive oxygen signaling which is known to be an important regulator of sclerotial development (Georgiou et al., 2006).

Light regulation

Light has been previously reported to affect sclerotial morphogenesis; however, the role of light in this process remains poorly understood. One possible function of light is to produce oxidative stress by triggering photosensitization reactions since light can reduce flavins and flavinoproteins which can react with molecular oxygen to produce reactive oxygen species (ROS) such as hydrogen peroxide, superoxide radicals, hydroxyl radicals and singlet oxygen (Georgiou et al., 2006). Miller and Limberta (1977) found that light exposure lead to melanin accumulation in sclerotia of *S. rolfsii* via induction of tyrosinase and also that light exposure cultures induced more sclerotia than cultures left in the dark. Melanin is known as a free radical scavenger which indirectly indicates that maturation of sclerotia may occur as a protective response to oxidative stress generated by light exposure or other sources of ROS. A gene involved in light induction of sclerotial formation is found recently in *Aspergillus parasiticus*. The velvet gene, *veA* was originally described in *A. nidulans* as a light-dependent negative regulator of asexual sporulation and a positive regulator of the sexual cleistothecial development in the dark (Kim et al., 2002). Calvo e*t al.* (2004) found that the ortholog of this gene in *A. parasiticus* is also required for sclerotial formation and the deletion of *veA* from wild type lead to the blockage of sclerotial production even under the optimal condition for wild type to form sclerotia.

Storage proteins

In addition to environmental factors and components of signal transduction pathways mentioned above, other gene products that may offer insight into sclerotial developmental regulation are proteins highly and specifically expressed during sclerotia development. Petersen *et al.* (1982) and Russo *et al.* (1982) isolated a 36kDa protein (Ssp) from sclerotia of three *Sclerotinia* species. These proteins were not detected in vegetative hyphae but made up

approximately 35 to 40% of the total soluble sclerotial proteins. Russo and Van Etten (1982) purified Ssp from S. *sclerotiorum* and analyzed its biochemical characteristics. They demonstrated that Ssp protein consisted of three charge isomers, with one isomer making up 80 to 90% of the total. Using TEM and immunolocalization, they also demonstrated that Ssp accumulated in membrane-bound, organelle-like structures which resemble protein bodies found in seeds of many higher plants (Russo and Van Etten, 1985). The functional relationship between Ssp accumulation and sclerotial development has not been previously investigated.

Genetic Approaches to Investigating Sclerotial Development in *S. sclerotiorum*

Comprehensive Transcript Profiling by Microarray Analysis

Though some advancements on the molecular mechanisms regulating sclerotial development have been achieved, a more comprehensive and systematic study utilizing genomic and functional analyses should be carried out to gain new insights. As an important plant pathogen with broad host-range, the *S. sclerotiorum* genome was sequenced via a whole genome shotgun sequencing approach with an average of 8X coverage. The genome assembly was released late in 2005 (http://www.broad.mit.edu/annotation/genome/sclerotinia_sclerotiorum/Home.html). According to the current automated annotation using a combination of FGENESH and GENEID and comparing predicted genes to expressed sequence tags (EST) data, there are 14,522 predicted genes residing in the ~38Mb genome. Further analysis of genome data revealed that 1) the gene density average is one gene for every 2,643bp of nucleotide sequence; 2) the average gene length is 1,067bp; and 3) the average intergenic length is 974bp. Some misannotations have been found in predicted gene sequences but an optical map has validated the assembly and placed the genome into predicted chromosomal units. This genome data can be used as a resource for DNA microarray analysis so that we can initiate whole genome transcript profiling in sclerotial

development and compare it to other developmental stages. In this dissertation, I have obtained transcript profiles comparing the stage of sclerotial initiation with vegetative growth using competitive hybridization microarray analyses. This method has been successfully applied onto other several filamentous fungi for developmental studies (Kasuga et al., 2005; Nowrousian et al., 2005; Qi et al., 2006). We believe the application of microarray analysis to sclerotial development in *S. sclerotiorum* will help us to understand the genes and signal transduction pathways regulated in this process more comprehensively and systematically.

Deletion of Candidate Genes Involved in Sclerotial Development

Characterizing the roles of specific genes in sclerotial development is another goal of my dissertation research. The first candidate I choose to focus on is the *ssp1* gene encoding Ssp, the novel protein that specifically and highly accumulates in mature sclerotia but not in vegetative hyphae. Although this protein was first described 26 years ago (Russo et al., 1982), the function of Ssp in sclerotial development is still unknown. The tight relationship between Ssp expression and sclerotial development gave rise to my interest in characterizing this gene. Another gene I characterized is *ggt*. This gene encodes a γ-glutamyltranspeptidase (GGT). I chose this gene due to its high up-regulation during sclerotial initiation vs. vegetative hyphal growth in a small-scale cDNA microarray hybridization performed previously. This up-regulation was validated using the whole genome microarrays. I investigated the role of GGT in sclerotial development by comparing the phenotype of *S. sclerotiorum* wild type strain and *ggt* knock-out mutant stains. Other genes exhibiting differential expression by microarray analysis were characterized bioinformatically and provide a valuable resource for future functional analyses.

CHAPTER 2
REGULATION, ACCUMULATION AND TRANSLOCATION OF A SCLEROTIA DEVELOPMENT-SPECIFIC PROTEIN (SSP1) IN *Sclerotinia sclerotiorum*

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is an important plant pathogen that annually causes substantial world-wide losses in crop production (Bolton et al., 2006; Purdy, 1979). The persistent infection capability of *S. sclerotiorum* is due in large part to the formation and long-term survival of sclerotia. These multihyphal, dark, hard, tuberoid resting structures are capable of reproducing the fungus vegetatively and meiotically. Meiosis-related development is initiated within the sclerotium with the formation of apothecial primordia. The sclerotium serves as the sole source of nutrients during this stage and during subsequent stages of apothecial development and ascospore production. As such, the sclerotium ensures that new infections will occur locally as well as on hosts both temporally and spatially separated from the point of initial infection.

Mechanisms regulating sclerotial development have been widely investigated in *S. sclerotiorum*. Several recent articles have reported on molecular signaling pathways and genes related to sclerotial development. One such pathway is the ambient pH signaling pathway. Neutral ambient pH negatively regulates sclerotial development and acidic ambient pH positively regulates sclerotial development (Rollins and Dickman, 2001). Pac1 acts as a pH-responsive transcription factor in *S. sclerotiorum* and loss-of-function Pac1 replacement mutants display an aberrant sclerotial development and maturation phenotype (Rollins, 2003). Rollins and Dickman (1998) also found that endogenous and exogenous increases in 3',5'cyclic Adenosine monophosphate (cAMP) levels inhibit sclerotial development. Recently, the deletion of the adenylate cyclase-encoding gene, *sac1*, was demonstrated to lead to abnormalities in sclerotial formation as well as hyphal growth (Jurick and Rollins, 2007). cAMP-dependent protein kinase A (PKA) activity increases during sclerotial development but remains at low

levels in a non-sclerotia-producing mutant of *S. sclerotiorum* (Harel et al., 2005). However, a deletion mutant of PKA subunit gene *pka1* did not show aberrant sclerotial formation or complete abolishment of sclerotial biogenesis (Jurick et al., 2004) likely due to the function of another PKA homolog in *S. sclerotiorum* genome or to a PKA-independent signaling pathway functioning during sclerotial development. Later, Chen and Dickman illustrated (2005) that a Ras/MAPK pathway is required for sclerotial development and this pathway is negatively regulated by another small GTPase, Rap-1, in a novel cAMP-dependent but a PKA-independent pathway. They also provided evidence that this cAMP regulation operates antagonistically to ERK-type mitogen-activated protein kinase (MAPK), Smk1, and G-protein signaling (Chen et al., 2004). Inhibition of *smk1* transcript accumulation through antisense expression blocked sclerotial maturation (Chen et al., 2004). In addition, *smk1* shows positive pH-responsive regulation during sclerotial development and its transcription can be inhibited by addition of cAMP. Results from this study suggest that a cAMP-dependent pathway might stimulate filamentous growth by inhibition of a *smk1*-dependent pathway. Erental *et al.* (2007) additionally showed that interference with protein phosphatase 2A (PP2A) activity via antisense suppression of the regulatory B subunit affected sclerotial maturation. Furthermore, PP2A activity was shown to be dependent on Smk1 and NADPH oxidase functions suggesting interconnections between phosphatases, MAPKs and reactive oxygen signaling which is known to be an important regulator of sclerotial development (Georgiou et al., 2006). The velvet gene, *veA* was originally characterized in *Aspergillus. nidulans* as a positive regulator of cleistothecial production and a negative regulator of asexual sporulation (Kim et al., 2002) has recently been shown to be required for sclerotial formation in *Aspergillus parasiticus* (Calvo et al., 2004). Deletion of *veA*

from the wild type leads to lockage of sclerotial production even under the optimal condition for wild type sclerotia formation (Calvo et al., 2004).

In addition to the above mentioned genes and signal transduction pathways, genes and proteins that are highly and specifically expressed during sclerotia development may offer insight into sclerotial developmental regulation. In 1982, Petersen *et al*. (1982) isolated a 36kDa protein (Ssp) from the sclerotia of three *Sclerotinia* species. These proteins were not detected in vegetative hyphae but made up approximately 35 to 40% of total soluble sclerotial proteins (Petersen et al., 1982). Russo and Van Etten (1985) purified Ssp from *S. sclerotiorum* and analyzed its cytochemical characteristics. They demonstrated that Ssp protein consisted of three charge isomers, with one isomer making up 80 to 90% of the total. Using TEM and immunolocalization, they also demonstrated that Ssp accumulated in membrane-bound, organelle-like structures which resemble protein bodies found in seeds of many higher plants (Russo and Van Etten, 1985).

Recently we identified a cDNA clone (*Ss_ssp1*) encoding the Ssp1 protein from *S. sclerotiorum*. Using this sequence we were able to identify homologous sequences from other closely related fungi. Due to the similar cytological features of Ssp1-containg protein bodies and its comparable tissue-specific expression reminiscent of plant seed storage proteins (Guerche et al., 1990; Higgins, 1984), we investigate its tissue-specific expression and further try to determine if *Ss_ssp1* transcript accumulation can be used as a biomarker for sclerotial initiation and development in *S. sclerotiorum* and other determinant sclerotial-forming fungi. There have been several examples of plant storage proteins used to explore temporal and spatial gene regulation in plants(Conceicao and Krebbers, 1994) and *Ss_ssp1* may be used likewise for fungi. Our results demonstrate tight tissue-specific transcriptional and translational regulation of *ssp1*

and massive, developmentally-triggered protein translocation. Furthermore, the magnitude of its tissue-specific expression suggests promising potential for biotechnology application involving heterologous protein production.

Materials and Methods

Fungal Cultures and Tissue Collection

The *S. sclerotiorum* wild type "1980" isolate and a sclerotia minus mutant (A1) derived from this isolate were previously described by Godoy *et al.* (1990). Natural sclerotia minus isolates of *S. sclerotiorum*, LMK28 and LMK44, were obtained from Dr. Linda Kohn (University of Toronto, Toronto, Canada).All isolates and the A-1 strain were maintained and propagated on potato dextrose agar (PDA) (Difco, MI, U.S.A.) plates at room temperature. Mutants, Δ*pac*1 (Rollins, 2003) and Δ*snf*1 (Hutchens, 2005) forming aberrant sclerotia, were maintained and propagated on PDA containing 100µg/ml hygromycin. To harvest the mycelia and aberrant sclerotia from agar plates, cellophane was overlaid on the medium before transferring mycelial plugs onto the media. Therefore the mycelia or aberrant sclerotia tightly adhering with mycelia can be easily peeled off from the film without agar. Liquid shake cultures of wild type mycelia were obtained as previously described by Rollins (2003).

Apothecia Induction and Ascospore Collection

Mature sclerotia for apothecia induction were produced from *Sclerotinia* cultures growing on autoclaved diced potatoes at room temperature. To produce apothecia, mature sclerotia are washed with repeated changes in running water gently to avoid breaking sclerotia. Clean sclerotia were surface sterilized by immersion in 0.5% bleach for 5 min and then rinsed with sterile water for 5 min. After 3 rinses, sclerotia were dried in an air flow hood on sterile paper towels for 8 hours. Dried sclerotia were placed on the surface of autoclaved water-saturated vermiculite in glass petri dishes (10cm ∅). The freeze-thaw method of Russo *et al.* (1982) was

used to condition sclerotia for carpogenic germination. Plates were placed at –20°C for 24 hours and then at room temperature for 24 h for 3 cycles. After the third cycle, plates were moved to a 15°C incubator with constant lighting, using fluorescent, cool white bulbs. Mature apothecia usually developed in 4-5 weeks. Ascospores were harvested from mature apothecia through a vacuumed funnel assembly previously described by Steadman (Steadman and Cook, 1974).

Identification of Ssp1-encoding Sequences

Total soluble protein was extracted from mature, PDA-grown sclerotia as described below. Twenty micrograms of soluble protein were denatured and separated in multiple lanes of a 12% SDS-PAGE gel. The major protein band migrating at 36kD was excised from the gel, trypsin hydrolyzed, purified by reversed-phase HPLC and subjected to internal Edman sequence analysis using an Applied Bioystems (ABI) 494 gas-phase/pulsed-liquid Procise-HT sequencer at the University of Florida Protein Chemistry core facility. The sequences of five internal peptide fragments ranging in size from 8 to 16 amino acid residues were obtained. These sequences were used to query a small collection (164 clones) of translated EST sequences (unpublished data) derived from a cDNA library prepared from polyA+ RNA isolated from sclerotial initials (Rollins and Dickman, 2001). One translated EST sequence matched all five peptide fragments with 75-100% identity. This clone was fully sequenced on both strands and represents the full length *Ss_ssp1* coding sequence.

Genomic DNA Cloning

The Universal GenomeWalker Kit (BD Biosciences Clontech Inc., CA, USA) was used to amplify 5' upstream genomic sequence of *Ss_ssp1* gene according to the manufacturer's direction. To construct GenomeWalker libraries, lyophilized mycelia from liquid shake cultures of *S. sclerotiorum* were used to isolate total genomic DNA with high purity and high molecular weight based on the method previously described by Yelton *et al.* (1984) for *A. nidulans*. Gene

specific primer (sspGSP1) (5'-CGAATTTCGACGATGCCCATCTTGCCAT-3') was designed based on the sequence of *Ss_ssp1* acquired from cDNA clone pSSPEST6.3. The forward primer was adaptor primer 1 (AP1) (5'-GTAATACGACTCACTATAGGGC-3') provided by the GenomeWalker kit. Genome walking PCR program consisted of 7 cycles of 2s at 94ºC and 3min at 72ºC, and 32 cycles of 2s at 94ºC, 3min at 67ºC, and followed by 4min at 67ºC. The acquired 2kb PCR product was purified and cloned into TOPO plasmid (Invitrogen, CA, USA). *E. coli* strain DH5α was used to propagate plasmids.

Multiple Sequence Alignment and Phenogram Construction

The amino acid sequences for Ss_Ssp1 (SS1G_14065.1), Bc_Ssp1 (BC1G_03185.1), and Ss_Ssp2 (SS1G_12133.1) were derived by translation of the coding sequences deposited in GenBank. Translated amino acid sequences for St_Ssp1 and Sm_Ssp1 were derived from sequenced cDNA sequences. The *A. oryzae* AO090038000546 (Ao_SspB) sequence was initially identified via a BlastP query of the NCBI non-redundant protein sequence database. Comparisons using the *Sclerotinia* and *Botrytis* sequences indicated that the three prime portion of the *Botrytis* gene was misannotated. The sequence was re-annotated based on nucleotide and amino acid multiple sequence alignments using ClustalX (Thompson et al., 1997) to include a third intron and a fourth exon. The *A. flavus* sequences (AFL2G_10697.2 (Af_SspA) and AFL2G_07878.2 (Af_SspB) were identified by BlastP query of translated transcripts in the *Aspergillus* Comparative Genomes Database (https://www.broad.mit.edu:443/annotation/genome/aspergillus_group/MultiHome.html). The *A. oryzae* Ao_SspA sequence was identified by tblastn query of the Aspergillus comparative genomic sequences database using the Ss_Ssp1 sequence. Annotation of intron and exon junctions was performed via multiple sequence alignments with the other sequences. Amino acid sequences were aligned using ClustalX (Thompson et al., 1997) and aligned sequences were

edited manually in MacClade version 4.06 (Maddison and Maddison, 2003). An unrooted neighbor-joining tree was constructed using PAUP* 4.0b10 (Swofford, 2002).

Northern Hybridization Analysis

Total RNA used for Northern blots was isolated with Trizol reagent (Gibco BRL, MD, USA) according to the manufacturer's instructions. DNA/RNA transfer and hybridization analysis were conducted by previously reported procedures (Rollins, 2003). High stringency hybridization and membrane washing were at 65ºC, while low stringency hybridization and membrane washing were conducted at 55ºC. A fragment of the *ssp1* coding sequence digested from purified plasmid pEST6.3 by *Xba*I and *Pst*I was used as the probe for both DNA and RNA hybridization.

Protein Extraction and Hybridization

Lyophilized mycelia from liquid shake culture, sclerotia and apothecia in different developmental stages were used to extract total soluble protein as described by Jurick *et al.* (2004). The only modification was the elimination of leupeptin, aproteinin and sodium fluoride from the protein extraction buffer. To extract soluble protein from ascospores, 500 µl of 0.5mm glass beads were deposited in a tube containing approximately 5×10^7 lyophilized ascospores scraped from filter paper. Beadbeating (Biospec Products Inc., OK, USA) was used to homogenize ascospores using three 30sec pulses. Before each cycle, the spore tube was placed into liquid nitrogen for 1min. 500µl of protein extraction buffer was added to the homogenized spores. After incubating the homogenized spores with extraction buffer on ice for 30min, the mixture was centrifuged at 4ºC for 30min and the supernatant transferred to a new tube and stored at -20ºC. Primary antibody raised against Ss_Ssp1 was acquired from Van Etten's lab (University of Nebraska, NE, USA). For Western blots, 20µg of extracted soluble proteins was separated by12% SDS-PAGE and then transferred on PVDF membrane (Bio-Rad, CA, USA).

After semi-dry electroblotting, the membrane were treated with PBST (80mM Na2HPO4, 20mM NaH2PO4, 100mM NaCl, 0.1% Tween-20) containing 5% non-fat dry milk overnight at 4ºC and then incubated with primary antibody for 1hr at room temperature. The secondary antibody was goat anti-rabbit Ig-conjugated alkaline phosphatase (Bio-Rad, CA, USA). The blots were developed with substrate buffer containing 0.1% NBT and 0.1% BCIP.

Two-step Semiquantitative RT-PCR and Quantitative RT-PCR (qPCR)

Five micrograms of total RNA were used as templates to synthesize the first strand cDNA using Superscript II (Invitrogen, CA, USA) reverse transcriptase. Reverse transcription reaction mixture includes 1µl of Superscript II reverse transcriptase, 4µl of 5x first strand buffer (Invitrogen, CA, USA), 4µl of 25mM $MgCl_2$, 2µl of 0.1mM DTT, 1µl of RNase inhibitor (Invitrogen, CA, USA), 1µl of 10mM dNTPs, 1µl of 0.5µg/ul oligo (dT), 5µg of total RNA and made up to a final volume of 20µl with RNase-free water. The reactions of *in vitro* reverse transcription were performed at 42ºC for 50min, then terminated by incubating the reaction mixture at 70ºC for 15min. RNaseH, 1µl, was used in a final step to degrade RNA templates. Two microliters of the 20µl reverse transcription reactions were used as templates for PCR. The PCR reaction mixture included 0.3µl 5U/µl Taq DNA polymerase , 5µl of 10x Mg-free buffer, 2.5µl of 25mM $MgCl_2$, 4µl of 2mM dNTPs, 1µl of 2mM primer, 2µl of undiluted or diluted RT-reactions and made up to a final volume of 50µl with double-distilled sterile water. The thermocycle program consisted of 4min at 94ºC, 30 cycles of 1min at 94ºC, 1min at 55ºC and 1min at 72ºC, and followed by 7min at 72ºC. Primer pair, SspRT-R (5'-TTGAACCTTGTCTTTCGGAATGAAG-3') and sspRT-F (5'-TCTCTTCTTACCACGGAGCTTGCTTG-3'), were used to amplify 680bp fragment in semiquantitative PCR products. A 338bp amplicon derived from Histone H3 SS1G_09608.1 (GenBank ID for CoreNucleotide sequence: XM_001589836), amplified using primer pair of

H3-F2 (5'-TCATCAATCCACAACAACCAC-3') and H3-R1 (5'-AGAGCACCAATAGCGGAAGA-3'), was used as a normalization control. To determine the absolute concentration of *Ss_ssp1* transcripts in different developmental stages, Bio-Rad qPCR cycler was used to perform qPCR and quantify values. A 10x dilution series of plasmid pSSP6.3 was used to construct a standard curve for qPCR. Primers, qPCR-F (5'-GTTCACAATGGGCATACTTTTCAGG-3') and sspRT-R, were used with diluted RT reactions from RNA of different sclerotia developmental stages to amplify a 250bp amplicon. The qPCR program consisted of 2min at 50ºC, 15sec at 95ºC and followed by 40 cycles of 15sec at 94ºC, 20sec at 56ºC and 30 sec at 70ºC. To investigate the transcriptional pattern of *ssp2*, the homologue of *ssp1*, in different developmental stages, primer pair of ssp2-F (5'-GTACCTCTGCGCCTGATGATA-3') and ssp2-R (5'- TATTTCCATTGAACGCTCCAC-3') were used to amplify a 363bp amplicon.

Tissue Fixation, Embedding and Sectioning

Fresh mature sclerotia and carpogenically-germinated sclerotia with apothecia were harvested, fixed and embedded using the method previously described by Kladnik *et al.*(2004). Embedded samples were sectioned (3µm) using a rotary microtome HM325 (Richard-Allan Scientific, MI, USA) and mounted on ProbeOne Plus Microscope Slides (Fisher Scientific, USA) in cytoseal (Richard-Allan Scientific, PA, USA).

Immunolocalization

Ready to use sections were dewaxed in Histoclear (National Diagnostics, GA, USA) and rehydrate in ethanol series and then hybridized with primary Ab described above for Western blots. Primary Ab was diluted (1:7500) in PBS with 10% goat-serum after the sections were blocked in PBS with 10% goat-serum for 20min. After an overnight incubation in a humidified chamber at 4 ºC, the slides were rinsed and incubated with PBS twice for 5min each time. The

same secondary Ab (1:500) used in previous Western blots was used to conjugate with the Ssp1 Ab for 30min at RT. Histochemical detection was performed using NBT and BCIP substrate as described for Western blots for 1hr. The slides were dehydrated in the graded ethanol/water again and mounted in cytoseal for light microscopic observation.

Constructing *ssp1* Promoter-driven GFP Expression System Using

ToxA promoter-driven GFP expression vector pCT-73 (Andrie et al., 2005) was obtained from L. Ciuffetti's lab (Oregon State University, OR, USA). pCT-73 was modified by replacing the ToxA promoter with a ~1kb fragment containing the in-frame sequence of *Ss_ssp1* encoding the first 6 codons of *Ss_ssp1* and 1085bp 5' upstream of the start codon. A 1.4kb fragment containing a TrpC promoter driving hygromycin phosphotransferase (Carroll et al., 1994) gene expression from pCT-74 (Andrie et al., 2005) was digested with *Sal*I and cloned into the modified pCT-73 (pCT73-Pssp1) to make an *Ss_ssp1* promoter-driven GFP expression system, pCT74-Pssp1. The final construct is shown in Figure 2-10.

Results

Macroscopic Refinement of Sclerotial Developmental Stages

As a prerequisite to our studies, we sought to standardize the description of macroscopic sclerotial development in culture based on invariable features appearing in each developmental stage. We refined descriptions of the sclerotial developmental process previously described as a three step process i) initiation, ii) development and iii) maturation (Townsend and Willetts, 1954; Willetts and Bullock, 1992). From observations made under a variety of *in vitro* growth conditions, six distinct, sequential stages have been defined for this study: I) multihyphal aggregation, II) exudation and condensation, III) enlargement, IV) consolidation, V) pigmentation, and VI) maturation (Figure 2-1). An important caveat to defining stages of sclerotial development is that sufficient hyphal growth is a prerequisite for sclerotial formation.

Evidence for this is observed in reports of gene deletion mutants that affect hyphal and colony vigor and also influence the number, size and morphology of sclerotia (Jurick and Rollins, 2007; Rollins, 2003). Secondly, sclerotia do not form in submerged cultures but do form rapidly if hyphae are moved to conditions with a hyphal-air interface (Hadar et al., 1981). Hence, hyphal growth should be considered a prerequisite but not a committed step in sclerotial development. We designate vegetative hyphal growth before sclerotial formation as stage 0 of sclerotial development (Figure 2-1). In the second stage of development, vegetative hyphae begin to aggregate and form white aerial sclerotial initials. We designate sclerotia in this stage as stage I sclerotia or sclerotial initials (Figure 2-1). In the next stage, stage II (condensation and exudation), sclerotial initials simultaneously condense and increase in size. Small amounts of exudates can be seen on the surface of aerial hyphae at this stage and discrete delimited larger white aggregates are observed (Figure 2-1). In the enlargement stage (stage III), the main feature is that the size of sclerotia increase at their highest rate and large amounts of exudates are observed. Sclerotia at this stage still exhibit a white hyphal surface (Figure 1). In the consolidation stage (stage IV), the sclerotial color is buff and a delimited surface becomes visible while a small increase in size occurs (Figure 1). Stage V sclerotia are pigmented sclerotia as melanin accumulates during this period and clear or lightly pigmented exudates still exist on the sclerotial surface (Figure 2-1). The last stage (stage VI) of sclerotial development is maturation. In this period, sclerotia grow to full size and have a dark hard surface lacking exudates and sometimes are covered with a thin layer of hyphae (Figure 2-1). The findings on *ssp1* transcript and Ssp1 protein accumulation in this study are based on this scheme of sclerotial developmental stages. Our results demonstrate tight tissue-specific transcriptional and translational regulation of *ssp1* and massive, developmentally-triggered protein translocation.

Gene Sequence and Computational Analysis

The partial cDNA sequence and the full length genomic sequence of the *ssp1* gene obtained before the release of the *S. sclerotiorum* genome sequence is identical with the sequence of predicted gene SS1G_14065.1 (*Ss_ssp1*) in the genome database (http://www.broad.mit.edu/annotation/fungi/sclerotinia_sclerotiorum/). The mRNA splice sites were determined by comparing the cDNA and genomic sequences and confirmed the predicted 4 exons and 3 intron structures. The joined *Ss-ssp1* exons are predicted to encode a 34.9 kDa novel protein with 311 amino acid residues, which is very close to the 36.1 kDa estimated by SDS-PAGE (Russo et al. 1982). Homologues of Ssp1 were found both in *S. sclerotiorum* and *Botrytis cinerea* by BLASTp queries of the Broad Institute fungal genome databases. Predicted gene SS1G_12133.1 (*Ss_ssp2*) in the *S. sclerotiorum* database and BC1G_03185.1 (*Bc_ssp1*) in *B. cinerea* database displayed high similarity to *ssp1* (49% and 81% identity respectively) (Figure 2). DNA sequences for *Ss_ssp1* orthologues in *Sclerotinia trifoliorum* and *Sclerotinia minor* were also acquired by amplifying genomic DNA using primers designed to conserved sequences flanking the *Ss_ssp1 and Bc_ssp1* coding sequences. The *S. trifoliorum* ortholog of *ssp1 (St_ssp1)* shares 92% and *S. minor (Sm_ssp1)* shares 91% amino acid identity with *Ss_ssp1*. BlastP queries of the NCBI non-redundant protein sequences also revealed significant homology (e^{-24}) to one other sequence in the database other than Bc_Ssp1 (e^{-151}) and Ssp2 ($2e^{-95}$). This sequence (GenBank accession No.: BAE64317.1) is from *Aspergillus oryzae* and is annotated as an unnamed protein product. Additional BLAST queries to the *Aspergillus* Comparative Genomes database (https://www.broad.mit.edu:443/annotation/genome/aspergillus_group/MultiHome.html) identified three additional *ssp1*-related sequences. Two of these sequences were from *A. flavus* (AFL2G_10697.2; designated *Af_sspA* and deposited in GenBank after correcting for a proposed

intron as Accession######); AFL2G_07878.2 (designated *Af_sspB* and deposited in GenBank under Accession ######), and one sequence (supercontig 18.1 nucleotides 1658493-1659588 +) in addition to the previously identified GenBank BAE64317.1 sequence (AO090038000546) were identified from *A. oryzae*. The intron-exon structure of these sequences was predicted from multiple sequence alignments. The supercontig 18.1 sequence was designated *Ao_sspA* and deposited in GenBank under accession #####. The BAE64317.1/AO090038000546 sequence was edited for a predicted intron and included additional 3' coding sequence. This sequence was designated *Ao_sspB* and deposited in Genbank under accession #####. Multiple sequence alignment of Ssp1 homologs and an unrooted neighbor-joining tree are shown in Figure 2-2 and Figure 2-3 respectively.

No conserved domains were found in Ss_Ssp1 using biotools including InterPro Scan, Pfam, Smart and ProSite Scan, whereas a mini-motif of a potential C-terminal sorting signal VXPX was found in the C terminus of all *Sclerotinia* spp. homologs by MnM (Balla et al., 2006) (http://mnm.engr.uconn.edu) (Figure 2-2). This is consistent with the observation that Ss-Ssp1 is deposited in protein bodies of mature sclerotia enclosed within a cell membrane (Russo and Van Etten, 1985). This putative sorting signal is also found in the C terminus of Af/Ao_SspA but not in Bc_Ssp1 and Af/Ao_SspB. The similarities of sequences ~500bp 5' upstream from the start codon of *Ss_ssp1* and its homologues were also compared. The TATA box, general transcriptional binding site, was found in all 5 sequences at ~100bp upstream of the start codon. A binding site perfectly matching the consensus for binding AbaA (5'-CATTCT-3'), a known regulator of asexual development in *Aspergillus nidulans* (Andrianopoulos and Timberlake, 1994), was also found in all *ssp1* orthologues as well as in *ssp2*. Additionally, an 8-bp sequence (5'-TGGCGGCT-3') which shares 8-of-9 identical nucleotides with the 9-bp palindromic

sequence (5'-TCGGCGGCT-3') of the CAR1 repressor (UME6/CAR80) (Strich et al., 1994) binding site ($URS1_C$) was found in the *Sclerotinia* spp. *Ss_ssp1* orthologs but not in *Bc-ssp1* or in *Ss_ssp2*.

Ssp1 Accumulation at Different Developmental Stages

Total soluble proteins extracted from 14 unique stages of the asexual and sexual life cycles were used for protein Western Blots. Equal amounts (~20µg) of total protein were loaded in each lane and polyclonal Ss_Ssp1 antibody was used to determine the presence of Ss_Ssp1 in distinct developmental stages. Detection of Ss_Ssp1 indicated that the protein accumulated throughout sclerotial and apothecial developmental stages but not in any mycelia stage (Figure 2-4) or in ascospores (results not shown).

Developmental Accumulation of *ssp1* Transcripts

Total RNA isolated from the same diverse developmental tissues as before was used for Northern analysis and semiquantitative RT-PCR (Figure 2-4). Northern hybridization analysis failed to detect *Ss_ssp1* transcripts in any apothecial stage but transcript accumulation was detectable in sclerotial initials, peaking in stage III and IV sclerotia and at the lowest level in mature (stage VI) sclerotia. RT-PCR revealed the same pattern and relative abundance observed with Northern hybridization.

Quantitative RT-PCR

qPCR was employed to quantitatively determine the concentration of *Ss_ssp1* transcripts and relative *Ss_ssp1* transcript levels during different stages of development. Results indicated that stage IV sclerotia had the highest transcript accumulation level among investigated stages (1.25E+10 copies/5µg total RNA) while mycelia had the lowest transcript level (6.08E+4 copies/5µg total RNA). This represents a dramatic 2×10^5 fold difference between minimum and maximum detected *Ss_ssp1* transcripts. The relative concentrations of *Ss_ssp1* transcripts in

other stages compared to *Ss_ssp1* transcripts in mycelial stage were obtained by dividing copy numbers of *Ss_ssp1* transcripts in other stages with copy number of *Ss_ssp1* transcripts in the mycelial stage (Figure 2-5). The pattern of *Ss_ssp1* transcript accumulation levels obtained by qPCR is very similar qualitatively with that obtained by Northern analysis. The difference is that extremely low levels of *Ss_ssp1* transcription can be detected in the mycelial stage and the etiolated stipe stage by qPCR while they can not be detected by Northern hybridization or semiquantitative RT-PCR.

Detection of *Ss_ssp1* Transcripts as a Biomarker of Sclerotial Development

Various mutations and physiological treatments have been shown previously to inhibit or perturb sclerotial development. Since *Ss_ssp1* transcripts could not be detected in any stage of mycelial growth via Northern hybridization but were readily detected in early stages of sclerotial development, we sought to determine if *Ss_ssp1* transcript accumulation could be used as a biomarker of sclerotial initiation. To determine this, we examined *Ss_ssp1* expression in a variety of mutants, natural sclerotia-minus isolates and the wild type under conditions designed to inhibit sclerotial development. The result indicated that accumulation of *Ss_ssp1* transcripts was undetectable by semiquantitative RT-PCR at any stage of growth or development in A-1 (an oxalate minus, sclerotia minus UV mutant), LMK28 and LMK44 (natural sclerotia minus isolates) and wild-type hyphae incubated under aerial stationary culture conditions at pH7. Relative to transcript accumulation in sclerotial initials, significantly reduced levels of *Ss_ssp1* transcript were present in the aberrant sclerotia produced by Δ*snf*1 and Δ*pac*1 mutants and in wild-type hyphae treated with 2.5mM caffeine, a concentration that partially inhibits sclerotial initiation (Figure 2-6).

Accumulation of *ssp1* in Other Sclerotia-forming Species

Ssp1 proteins are also present in other determinate sclerotia-forming Sclerotiniaceae species (Novak and Kohn, 1991; Petersen et al., 1982). We sought to determine if the gene encoding these proteins were regulated similarly to *Ss_ssp1*. Therefore, total RNA from one isolate of *S. minor*, one isolate of *S. trifoliorum* and one isolate of *B. cinerea* were used in Northern hybridization with the *Ss_ssp1* coding sequence. Under high stringency hybidization conditions, the *Ss_ssp1* probe successfully hybridized with total RNA isolated from stage IV sclerotia from both of *S. minor* and *S. trifoliorum* but failed to hybridize with total RNA isolated from hyphae of either species (Figure 2-7). Under low stringency hybridization conditions, the *Ss_ssp1* probe hybridized with total RNA isolated from developing sclerotia of *B. cinerea* but not with total RNA isolated from *B. cinerea* hyphae (Figure 2-7). This indicates that Ssp1 proteins in other determinate sclerotia-forming species are related at the sequence and regulatory levels and may perform a conserved function.

***Ss_ssp2* Transcript Accumulation Pattern**

Semiquantitative RT-PCR indicated that *Ss_ssp2* transcripts specifically accumulated in developing apothecia rather than in vegetative mycelia or sclerotia initials. (Figure 2-8). When searching the ESTs frequency for *Ss_ssp1* and *Ss_ssp2* in the data of *S. sclerotiorum* EST collections (ESTs are available from three cDNA libraries made from mycelia, sclerotial initials and developing stipes respectively in the Broad Institute database), we found 33 EST clones of *Ss_ssp1* in the sclerotial initials EST collection and no sequence from the other two EST collections (mycelia and etiolated stipes). Two *Ss_ssp2* ESTs were found in the developing stipes collection but none from the other two ESTs collections. This is consistent with RT-PCR results which suggested that expression of *Ss_ssp1* and *Ss_ssp2* have differing tissue specificities. This discovery of a related protein with apothecia-specific expression leads us to investigate whether

our antibody detection of Ss_Ssp1 in apothecia was actually cross reactive with Ss_Ssp2. For this, we isolated the Ss_Ssp1 bands from SDS-PAGE separated protein samples of mature sclerotia and developing apothecia, and subjected them to trypsin digestion and tandem mass spectrometry analysis. The obtained peptide fragment profile identified Ss_Ssp1 as the dominant constituent of the band obtained both from sclerotia and from apothecia (results not shown).

The Ss_Ssp1 Immunolocalization

Following the work of Russo and Van Etten (1985), we used immunohistochemistry to visualize protein bodies in mature sclerotia (Figure 2-9). We further observed that in carpogenically-germinated sclerotia, protein bodies were absent in the sclerotial region interior to the germination point resulting in a distinct clear zone in the medulla. This indicated that Ss_Ssp1 was being released from protein bodies and being metabolized or translocated into apothecia. Distinct visualization of Ss_Ssp1 in apothecia stipes or discs as expected based on the ability to detect Ss_Ssp1 with western blots from apothecial proteins was not obtained. Yet, when Western blots with proteins from carpogenically-germinating sclerotia were examined, no indications of digested Ss_Ssp1 were observed. Possibly, Ss_Ssp1 is being solubilized in germinating sclerotia and is much less concentrated in apothecia than it is in sclerotia or perhaps interactions with other proteins in the apothecia tissue prevent its visualization by immunolocalization.

Ss_ssp1 Promoter as a Tool for Heterlogous Protein Expression

The 1085 bp sequence upstream of the Ss_*ssp1* coding sequence was used to drive the expression of green fluorescent protein. GFP accumulation was detected only in sclerotia and not in hyphae or apothecia (Figure 2-11). The observations corroborate our findings that *Ss_ssp1* is only transcribed to significant levels during sclerotial development. The lack of even diffuse GFP visualization further suggests that specific residues of Ss_Ssp1, not included in our GFP

construct necessary for the translocation of protein from the sclerotium to the apothecium. The demonstrated tissue specificity of the ~1kb promoter fragment suggests that it can be used to heterologously express other proteins in *S. sclerotiorum* to high levels without concern for disrupting normal hyphae growth. Additional promoter truncations should reveal minimum requirement for high levels of tissue-specific expression.

Discussion

Ss_Ssp1, a 34.9 kDa protein, accumulates in membrane-bond protein bodies to comprise the major proportion of total soluble proteins in mature sclerotia. These characteristics are very similar to those of plant storage proteins (Shewry et al., 1995). Plant storage proteins include seed storage proteins and vegetative storage proteins. Seed storage proteins accumulate to high levels in late stages of seed development and are degraded during plant seed germination, and the resultant derived amino acids presumably serve as a source of nitrogen and carbon skeletons for the synthesis of new proteins required for germination (Larkins, 1981; Muntz, 1998). The most well studied seed storage proteins are cereal and maize seed storage proteins (Coleman and Larkins, 1999; Shewry and Halford, 2002). These proteins are known to be processed by the secretory pathway and deposited in discrete protein bodies (Muntz, 1998) though some (e.g., globulins and prolamins) do not have distinct sequence signal conferring vacuolar targeting (Kermode and Bewley, 1999). A short potential signal peptide (VXPX) is found at C-terminus of *Sclerotinia* ssp. Ssp1 homologs and Af/Ao_SspA. It is difficult to conclude that this is a true sorting signal or if there are other segments within the protein or if associated tertiary structure of the mature protein acts as a signal for its deposition in protein bodies as has been hypothesized as a sorting mechanism of seed storage proteins without distinct cleavable signal domain at N-terminus (Shewry et al., 1995). Most seed storage proteins are thought to be nutritional reservoirs

for subsequent seed germination but not all of them are required for normal seed development and germination (Kriz and Wallace, 1991).

Vegetative storage proteins comprise the second class of plant storage proteins. These storage proteins specifically accumulate in vegetative tissues including leaves, stems and tubers. They exhibit more diverse biological functions compared to seed storage proteins. The most widely studied of these vegetative storage proteins are patatin and its orthologs. These are soluble proteins and accumulate to high levels in potato or other tuber-forming plants (Shewry, 2003). They possess different enzymatic activities including phospholipase (Hirschberg et al., 2001), acidic ß-1,3-glucanase (Tonon et al., 2001), antioxidant (Hou et al., 2001), and carbonic anhydrase (Hou et al., 1999) activities with roles in protecting tubers from pests, pathogens and abiotic stresses (Shewry, 2003).

Similar storage proteins and tissue specific proteins are also found in fungi. In the late 1970s and the early 1980s, Van Etten *et al.* (1979) and Peterson *et al.* (1983) reported a major protein, muiridin, which accumulates in dormant spores of *Botrydiplodia theobromae* but is not present or present in very low amounts in vegetative hyphae. The degradation of muiridin is tightly related to subsequent spore germination. Nowrousian *et al*. (2007) recently found an abundant perithecial protein (App) that is specifically expressed in perithecia of *Sordaria macrospore* and *Neurospora* crassa but not present in hyphal tissue. App is not required for the fertility in either species and there are no distinct differences in perithecial morphology or developmental timing between Δ*app* and wild type strains. Likewise, the spore-specific protein (*ssp1*) from *Ustilago maydis*, which shares homology with other fungal oxygenases, is highly expressed in mature teliospores, but disruption of this gene has no obvious phenotype (Huber et al., 2002). This provides a caution that some fungal tissue specific protein might not be required

for normal function of those tissues, even though they are highly temporally and spatially regulated during development, and like some plant seed storage proteins not necessarily required for normal development and germination.

In immunolocalization assays of germinated sclerotia, a clear region near the apothecial germination point suggests to me that Ss_Ssp1 may be utilized during apothecial germination. However, we did not observe degradation of Ss_Ssp1 in germinated sclerotia or in apothecia by Western analysis. We did observe the reduction of Ss_Ssp1 in carpogenically-germinated sclerotia compared to ungerminated sclerotia as previously reported (Russo et al., 1982), but significantly, we also observed high levels of Ss_Ssp1 in apothecia, a previously undescribed phenomenon. This apothecial-accumulation of Ss_Ssp1 protein occurs with approximately 10^4-fold lower *ssp1* transcript accumulation relative to stage IV sclerotia. This suggests that the majority of the Ss_Ssp1 protein present in apothecia is the result of solubilization and translocation of Ss_Ssp1 from sclerotia to apothecia. Based on these observations, we can not conclude that Ss_Ssp1 functions as a nutrient source for apothecia germination analogous to plant seed storage proteins. Whether Ss_Ssp1 plays a role in carpogenic germination or myceliogenic germination or fulfills some other biologically relevant role will require mutational and phenotypic analyses.

Levels of *Ss_ssp1* transcript not only differed during sclerotial development but differences were also observed in various strains with aberrant sclerotial development. In our investigations, strains and isolates of S. *sclerotiorum* with aberrant sclerotia (*Δpac1, Δsnf1*), with few sclerotial initials (caffeine treated WT) or without sclerotia (A-1, LMK-28, LMK-44) have either less *Ss_ssp1* transcripts than wild type sclerotial initials, or fail to accumulate detectable *Ss_ssp1* transcripts.

Based on Blast queries of the NCBI non-redundant databases and public fungal genome databases, Ssp1-encoding genes are absent from most fungal lineages. The exceptions we have found include closely related Sclerotiniaceae species (*S. trifoliorum*, *S. minor*, and *B. cinerea*) and surprisingly, the distantly related Eurotiomycetes *A. flavus* and *A. oryzae*. A character that unites these *Aspergillus* spp. with the Sclerotiniaceae species is the production of determinate sclerotia. To our knowledge, *S. sclerotiorum*, *B. cinerea*, *A. flavus* and *A. oryzae* are the only sclerotia-producing fungi whose genomes have been sequenced. The availability of sequenced genomes from eight varying *Aspergillus* species allowed us to comprehensively search for *ssp*-homologous sequences in closely related sclerotia-producing and non-producing *Aspergillus* species. The presence of these sequences only within sclerotia-producing *Aspergillus* spp. genomes further suggests a functional relationship between sclerotia production and Ssp1. Determining whether these *ssp* homologs are truly associated with sclerotial development in *A. flavus* and *A. oryzae*, whether other taxonomically diverse sclerotia-producing fungi have these genes, and whether indeterminate stroma-producing Sclerotiniaceae encode these genes, may provide valuable insights into the evolution of fungal sclerotia.

The absence of an *ssp2* ortholog from the genome of both sequenced isolates of *B. cinerea* was initially surprising given the presence of two *ssp*-related sequences in both *A. flavus* and *A. oryzae*. These Aspergillus sequences are obviously related to Ssp1 and Ssp2 based on the low BLASTP e value scores (e^{-60} to e^{-31}) and the conserved positions of three introns. The orthologous relationships among the Ss_*ssp1*, Ss_*ssp2* and the Af/Ao_sspA and Af/Ao_sspB genes is not clear based on sequence homology. Best bi-directional BLAST queries of the *A. flavus*, *A. oryzae*, and S. *sclerotiorum* genomes indicate that *ssp1* is the best Sclerotinia gene match for both Af/Ao_sspA and Af/Ao_sspB. The percent identity and similarity in global amino

acid alignments also bears this out with Ss_Ssp1 sharing 38% identity to Af/Ao_SspA and 32% similarity to Af/Ao_SspB compared to Ss_Ssp2 sharing 33% identity to Af/Ao_SspA and 25% similarity to Af/Ao_SspB. The neighbor joining phylogram further substantiates this observation and indicates that Ss_Ssp1 shares a more common evolutionary history with Ss_Ssp2 than with either Aspergillus gene but also that Ss_Ssp1 is more closely related to both Af/Ao_SspA and Af/Ao_SspB than is Ss_Ssp2. The high degree of Ssp1-Ssp2 divergence suggest that the presence of both genes in the *S. sclerotiorum* genome is the result of an ancient duplication and subsequent loss from the *B. cinerea* genome, or a more recent duplication in Sclerotinia and strong diversifying selection for function and regulation. Determining if the Aspergillus *ssp* gene duplication was independent of the Ss_*ssp* duplication event or the same duplication event with differing selection pressure may provide insight into the biological function of these proteins and whether the original function has been split or if new functions have been selected.

In other studies, Ssp protein accumulation in several genera and species of the Sclerotiniaceae including both sclerotia-forming and substratal stromata-forming species by immunoblot and ELISA analysis using anti-Ssp1 antibodies was investigated (Kohn and Grenville, 1998; Novak and Kohn, 1991). These studies revealed antigenically reactive Ssp1 proteins present in total sclerotial proteins from all sclerotial species but not in substratal stromatal species. With the *Ss_ssp1* gene sequence now in hand, the major stroma-specific protein-encoding genes can be investigated. Are Ssp proteins from sclerotia- and substratal stroma-forming Sclerotiniaceae merely antigenically unique or have they also arisen from evolutionarily distinct progenitors as well? Our inability to PCR amplify homologous sequences from the substratal-forming species *S. homoeocarpa* using primers effective for other Sclerotinia spp. and *B. cinerea* suggests that the *ssp* genes from these substratal stroma species are

phylogenetically unique. The specificity of Ss_*ssp1* transcript accumulation during sclerotial development can be utilized as biomarker of sclerotial initiation and development and may help to elucidate the evolutionary origins of sclerotia and other stroma. Unexpectedly, the homologous *ssp2* gene has an expression pattern quite distinct from *ssp1*. This paralog is specifically and highly transcribed throughout apothecial developmental but not in sclerotia or other stages of the life cycle. Determining what regulatory elements result in these very different expression patterns of two similar genes may shed insight into the different regulatory pathways functioning in these unique multicellular developmental programs.

Ss_ssp1 should be a valuable gene for understanding spatial and temporal-specific gene expression in fungi. *In silico* promoter analysis found a potential CAR1 repressor (UME6/CAR80) binding site ($URS1_C$) in the upstream of all *Sclerotinia* spp. *ssp1* orthologs. CAR1 encodes an arginase that participates the first committed step of arginine degradation (Middelhoven, 1964). And the disruption of CAR1 enhanced freeze tolerance of *Sacchromyces cerevisiae* (Shima et al., 2003). URS1 does not only function to repress CAR1 expression, it has also been found in the promoters of a wide variety of yeast genes including most early meiotic gene as well as some nonmeiotic genes like CAR1 (GailusDurner et al., 1997). The current findings indicated that this binding site may interact with an Ume6 ortholog. Other proteins like replication protein A may also be able to interact with this *cis* element and activate gene transcription (GailusDurner et al., 1997). Deletion and site-directed mutagenesis experiments on the ~1kb 5' region upstream of *ssp1* to identify core promoter elements sufficient to initiate transcription of introduced GFP in a sclerotial-specific manner are planned. Using this construct to investigate tissue specific expression in other sclerotial forming and non-forming fungi may also provide insight into the origins of sclerotial regulation. The study of Ssp1 regulation may

not only help to better understand gene regulations in fungi but also might be of industrial interest as a system for heterologous eukaryotic protein expression. It may be feasible to produce proteins within sclerotia using readily available agriculture waste products as a substrate and obtain large quantities of concentrated protein in a readily harvested sclerotial packet within a period of ten days from inoculation to harvest. This would represent a unique eukaryotic protein expression tool in which protein-rich tissue could be readily produced and purified in a rapid and easily manipulated system.

Figure 2-1. Sclerotia developmental stages. 0: Stage 0 (vegetative hyphal growth); I: Stage I (initiation); II: Stage II (condensation); III: Stage III (enlargement); IV: Stage IV (consolidation); V: Stage V (pigmentation); VI: Stage VI maturation.

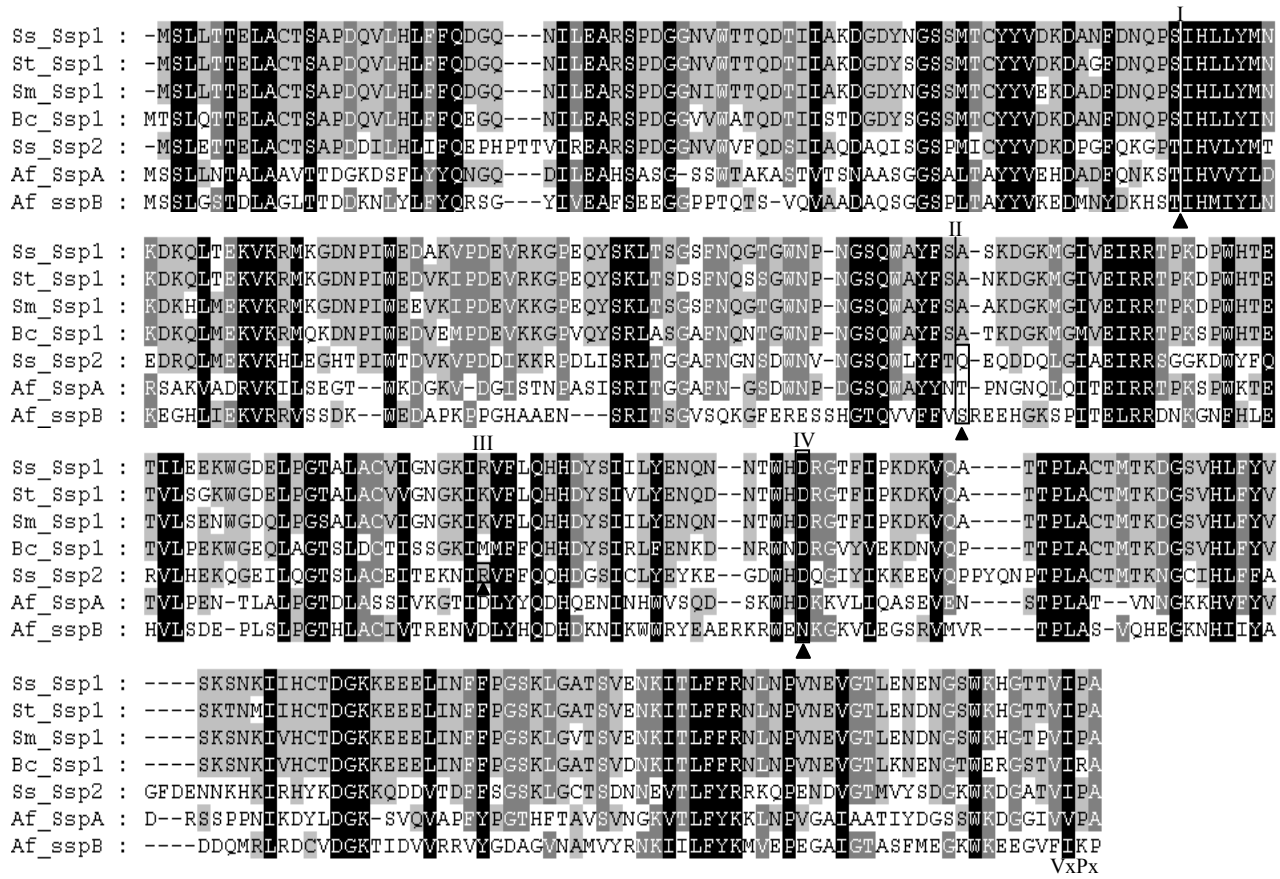


Figure 2-2. Multiple sequence alignment of Ssp1 homologs from *S. sclerotiorum* (Ss), *S. minor* (Sm), *S. trifoliorum* (St), *B. cinerea* (Bc) and *A. flavus* (Af). Conserved amino acid residues were shaded in the following manner: black = 100% identity or conserved substitution, grey > 80%identity or conserved substitution, light grey >60% identity or conserved substitution, white ≤ 60% identity or conserved substitution. Amino acid residues are considered to be conserved substitution in the same site as following: D=N, E=Q, S=T, K=R, F=Y=W, L=I=V=M. The positions of joint exons are labeled with solid triangles. The introns either are present between codons of two residues separated by vertical lines or reside within the codon of the boxed residues. Intron no. is shown above the intron labels. Intron I, II and IV are present in all homologous sequences and Intron III is only present in Ss_Ssp2. The putative sorting signal peptide of Ssp1, 'VxPx' is shown at N-terminus.

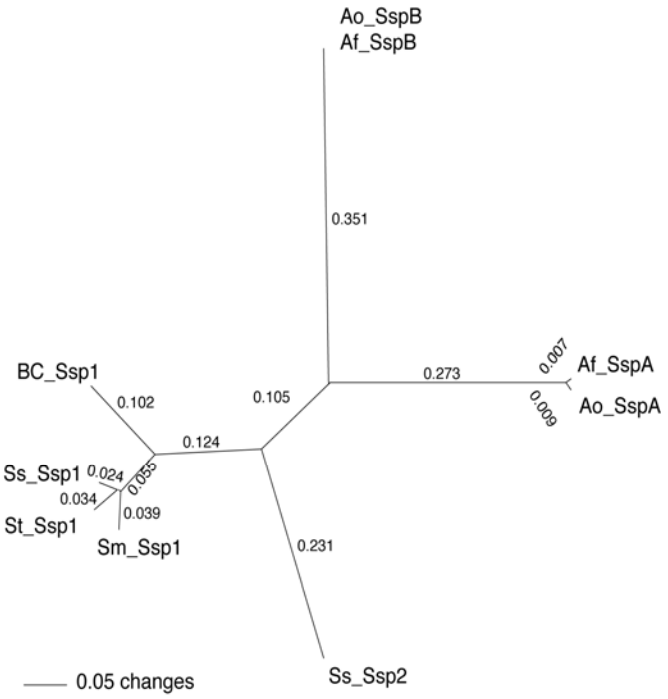


Figure 2-3. Phylogram of Ss_Ssp1 homologs from *S. sclerotiorum* (Ss), *S. minor* (Sm), *S. trifoliorum* (St), *B. cinerea* (Bc), *A. flavus* (Af) and *A. oryzae* (Ao). Unrooted neighbor-jointing phylogenetic tree constructed based on the multiple sequence alignment. Ao_SspA and Ao_SspB were added into the alignment and phylogram.

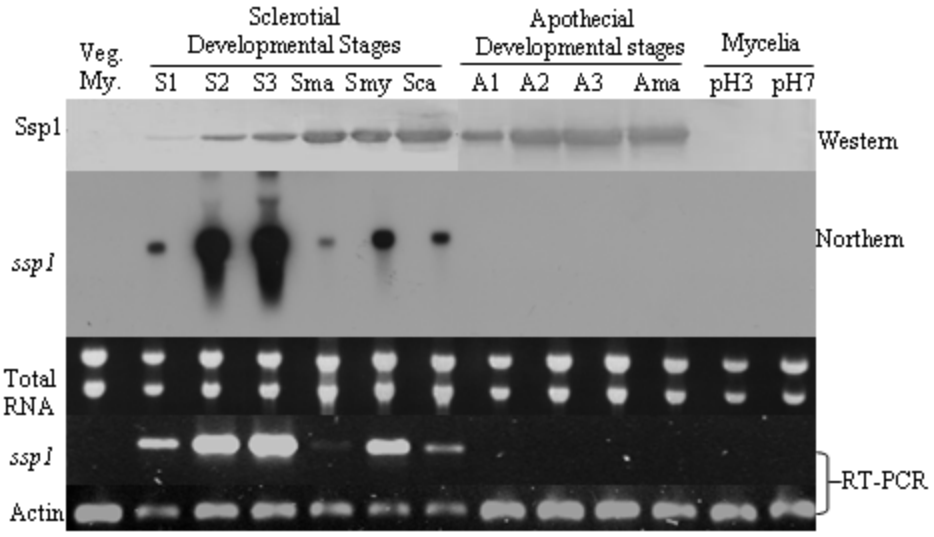


Figure 2-4. Western blot, Northern blot and Semiquantitative RT-PCR analysis for Ssp1 protein accumulation and transcripts accumulation in different developmental stages. (S1= stage I sclerotia, S2=stage III sclerotia, S3=stage IV sclerotia, Sma=mature sclerotia, Smy=myceliogenically germinated sclerotia, Sca= carpogenically germinated sclerotia, A1= etiolated stipes, A2= differentiating tipes, A3= expanded apothecia, Ama=mature apothecia, pH3=mycelial suspension culture in pH3 medium for 4 hours, pH7=mycelia suspension culture in pH7 medium for 4 hours).

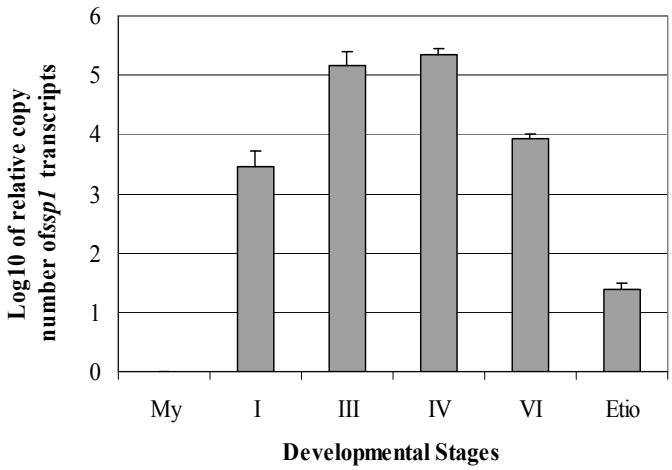


Figure 2-5. Quantitative RT-PCR results. The X axis represents the *ssp1* transcripts in different developmental stages (My= mycelia, I= stage I sclerotia, III= stage III sclerotia, IV= stage IV sclerotia, VI= stage VI sclerotia, Etio= Etiolated stipes). The Y axis represents the LOG10 values of *ssp1* transcript copy number in each developmental stages divided by the copy number of *ssp1* transcripts in Mycelial stage.

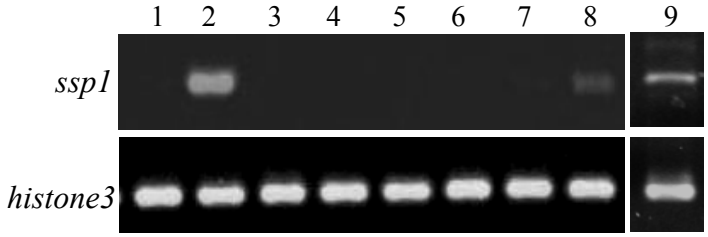


Figure 2-6. Semi-quantitative RT-PCR of *ssp1* transcriptional products in different *S. sclerotiorum* isolates. (Lane1: vegetative mycelia of WT 1980 isolate, Lane2: mycelia of WT with sclerotial initials, Lane3: LMK44, Lane4: LMK28, Lane5: A-1, Lane6: mycelia of WT 1980 isolate under pH7, Lane7: mycelia of WT 1980 isolate treated with 2.5mM caffeine, Lane8: Δ*snf1* stage IV sclerotia, Lane9: Δ*pac1* stage IV sclerotia)

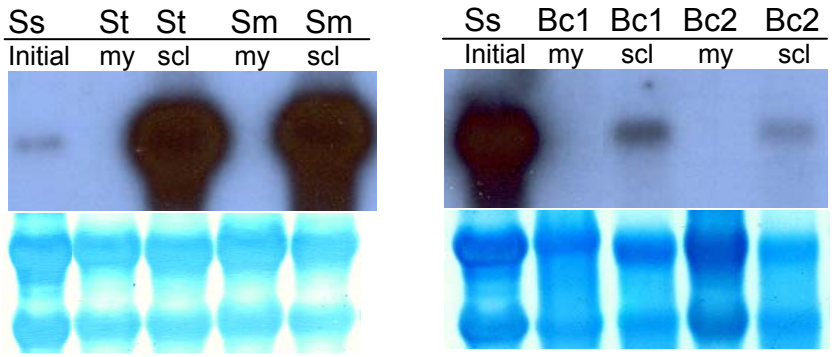


Figure 2-7. Northern hybridization for *ssp1* transcription in other sclerotial forming species. Total RNA isolated from *S. trifoliorum* and *S. minor* strain was labeled as 'St' and 'Sm' respectively. Total RNA isolated from two *B. cinerea* strains was labeled as Bc1 and Bc2. Total RNA isolated from vegetative hyphae was labeled as 'my' and total RNA from sclerotia was labeled as 'scl'. Total RNA from *S. sclerotiorum* (labeled as 'Ss') sclerotial initials (labeled as 'initial') was used as a positive control. *ssp1* coding sequence was used as a probe. Hybridization of *ssp1* coding sequence with St/Sm RNA was performed under 65 ºC and hybridization of *ssp1* coding sequence with Bc was performed under 55 ºC.

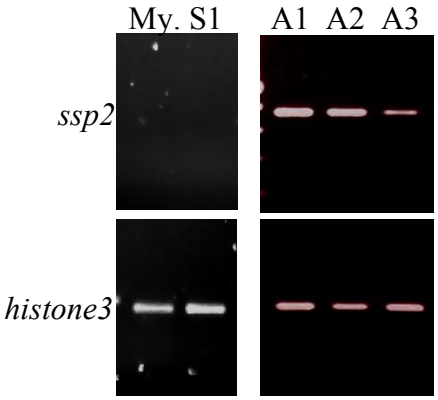


Figure 2-8. Semi-quantitative RT-PCR of *ssp2* transcriptional products in different developmental stages (My. = vegetative mycelia, S1= stage I sclerotia, A1= etiolated stipes, A2= differentiating stipe, A3= expanded apothecia)

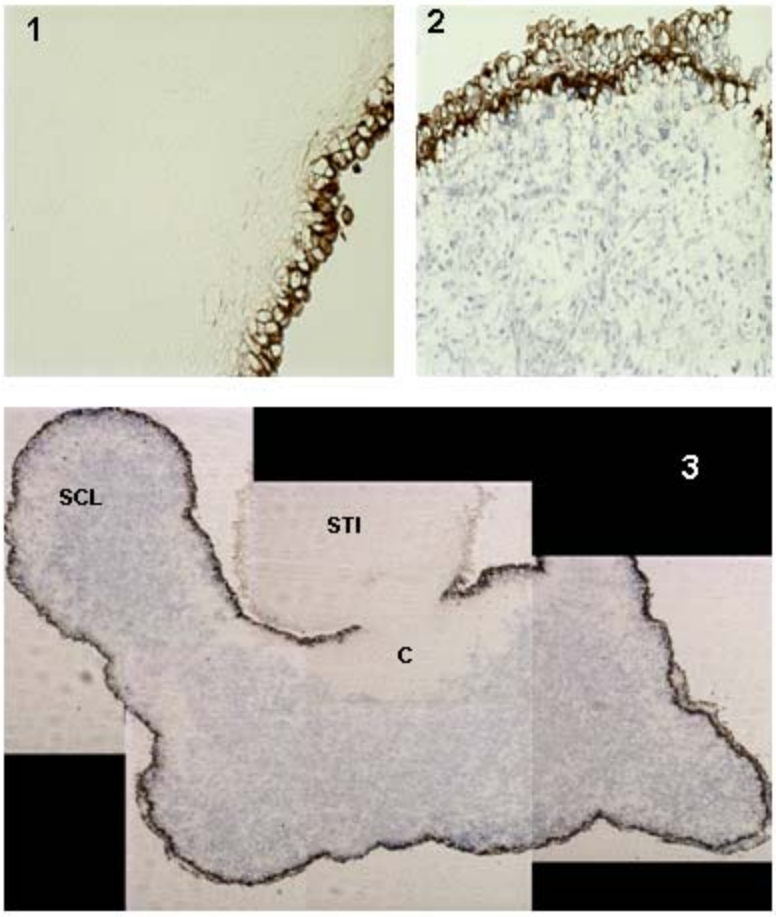


Figure 2-9. Immunolocalization of Ssp1 in mature sclerotium and carpogenic germinated sclerotium with apothecial stipe. 1) bright field micrograph of Preimmune sclerotium with 10× magnification. 2) bright field micrograph of mature sclerotium reacting with Ssp1 Ab with 10× magnification. Blue spots are where Ssp1 protein bodies exist. 3) assembled bright field micrograph of carpogenically germinated sclerotium with apothecial stipe (10× magnification). A clear region (C) around the stipe (STI) germination site can be distinguished with other regions in germinated sclerotia (SCL) showing Ssp1 deposition.

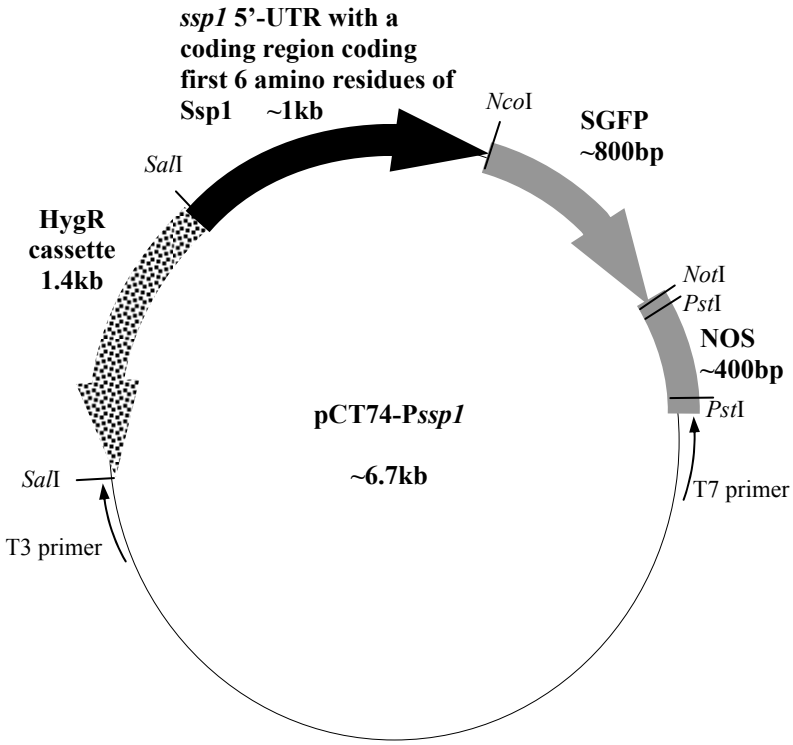


Figure 2-10. A GFP expression vector with ssp1 5'-UTR, pCT74-Pssp1. pCT74-Pssp1 derived from pCT-73 and pCT-74 (from Lynda Ciuffetti, Department of Botany and Plant Pathology, Oregon State University). A 1kb fragment including ssp1 5'-UTR and first 18 bp of ssp1 coding region replaced original ToxA promoter region and was inserted into SalI/NcoI sites of pCT-73. The modified new vector was designated as pCT73-Pssp1. The 1.4 kb SalI fragment containing the hygromycin resistance gene was digested from pCT-74 and was ligated into the SalI site of pCT73-Pssp1. This new vector was designated as pCT74-P*ssp1*.

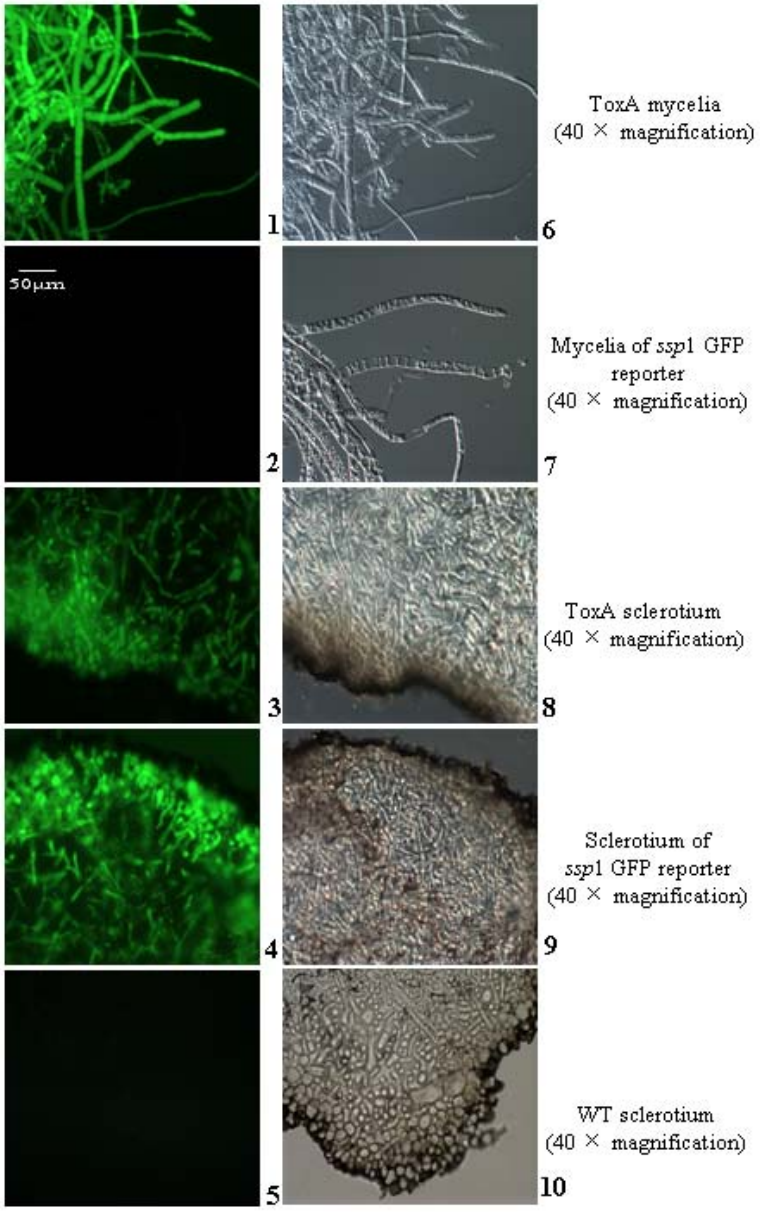


Figure 2-11. Fluorescent micrograph (1-5) and Differential Interference Contrast micrograph (6-10) for GFP expression of pCT74-P*ssp1* in WT vegetative hyphae and mature sclerotia. The vegetative hyphae and mature sclerotia of transformant containing ToxA-driven GFP were used as a positive control since GFP in this transformant can be expressed in any developmental stages in *S. sclerotiorum*. And WT vegetative hyphae and mature sclerotia were used a negative control.

CHAPTER 3
FUNCTIONAL ANALYSIS OF A SCLEROTIA DEVELOPMENT-SPECIFIC PROTEIN (SSP1) IN *Sclerotinia sclerotiorum* BY GENE DELETION

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary in the Sclerotiniaceae family is a devastating plant pathogen capable of infecting more than 400 plant species (Boland and Hall, 1994). *S. sclerotiorum* does not produce macroconidia. Survival and further dispersal rely on the formation of a sclerotium, long-term persistence of this multihyphal structure and appropriately timed germination of it. In the field or under laboratory conditions, various environmental factors can trigger the morphogenesis of this compact, highly melanized, tuber-like hyphal aggregate (Chet and Henis, 1975; Le Tourneau, 1979; Willetts and Bullock, 1992; Willetts and Wong, 1980). A sclerotium can tolerate a range of adverse environmental conditions, such as low temperature, low humidity and UV irradiation, and survive long periods of time in agricultural soils. Survival for periods as long as eight years have been documented (Adams and Ayers, 1979). Under favorable environmental conditions, a dormant sclerotium either germinates and forms vegetative hyphae (myceliogenic germination) or an apothecial fruiting body (carpogenic germination) that produces millions of ascospores. Both types of germination can lead to plant infections, but most *Sclerotinia* diseases are initiated from hyphae produced by ascospores (Bolton et al., 2006).

In the past fifty years, research on sclerotial development has changed from histochemical and ultrastructural analyses to genetic investigations. Some genes and signal transduction pathways involved in sclerotial development have been characterized. An ambient pH signaling pathway (Rollins, 2003; Rollins and Dickman, 2001) and cAMP signal transduction pathways (Chen and Dickman, 2005; Chen et al., 2004; Jurick et al., 2004; Rollins and Dickman, 1998) are the well-documented pathways known to regulate sclerotial development. Investigations of a

mitogen-activated protein kinase (MAPK) in *S. sclerotiorum* (Smk1) revealed that crosstalk between these two pathways (Chen et al., 2004). Oxidative stress is also demonstrated to be an important trigger of sclerotial initiation (Georgiou et al., 2006; Patsoukis and Georgiou, 2007). Protein phosphatase 2A (PP2A) (Erental et al., 2007) was demonstrated to play a role in sclerotial maturation and its activity is regulated by Smk1 and NADPH oxidase suggesting an interaction between phosphatases, MAPKs and reactive oxygen signaling. Another finding, not from *S. sclerotiorum* but from *Aspergillus* spp., has demonstrated that a gene mediating developmental light responses, *veA*, which is required for cleistothecia development in *A. nidulans* is also required for sclerotial development in *A. parasiticus* (Calvo et al., 2004).

Other components that might play a role in sclerotial development or sclerotial function are proteins that specifically and highly accumulate in developing and mature sclerotia but not in other developmental stages. Sclerotial specific protein (SSP), first described by Russo *et al.* (1982; 1985), exhibited a high level of accumulation in mature sclerotial but could not be detected in vegetative hyphae while a much lower amount of SSP was detectable in apothecia. This protein is found not only in *S. sclerotiorum*, but also in other *Sclerotinia* spp. (Petersen *et al.*, 1982). Immunoblots and ELISA analysis using polyclonal anti-SSP antibodies further revealed that SSP homologs are present universally in determinate sclerotia-forming species in the Sclerotiniaceae but not in substratal stroma-forming species within the Sclerotiniaceae or sclerotial forming species outside of the Sclerotiniaceae (Novak and Kohn, 1991). The high level of SSP accumulation in sclerotia of *Sclerotinia* spp. and its specific presence in sclerotia-forming species of the Sclerotiniaceae aroused my interest in this protein.

In Chapter 2, I reported that we designated SSP in *S. sclerotiorum* as Ss_Ssp1. The full length cDNA sequence of the Ss_*ssp1* gene was obtained before the releasing of *S. sclerotiorum*

genomic sequence assembly from the Broad Institute by searching translated EST sequences with short peptide sequences acquired by trypsin-digested-Ss_Ssp1-peptide sequencing. The full length genomic sequence of this locus was obtained through a genome walking strategy. BlastP queries indicated that Ss_Ssp1 is an unknown protein corresponding to locus ID SS1G_14065 in the released *S. sclerotiorum* genome sequence (http://www.broad.mit.edu/annotation/fungi/sclerotinia_sclerotiorum/). A homolog, Ssp2 (SS1G_12133) is also present within the genome. Northern analysis using *ssp1* coding sequence as a probe and semiquantitative RT-PCR indicated that the high transcription of *ssp1* only occurs in sclerotial developmental stages but not in vegetative hyphae or apothecial developmental stages. However, Western hybridizations show proteins accumulation both in sclerotial stages and apothecial stages but not in vegetative growth stage. Immunolocalization assays using anti-SSP antibody show cross-reaction with Ss_Ssp1 in mature sclerotia. But carpogenically germinated sclerotia displayed a clear region in medulla around the germinated point

These observations are consistent with translocation of Ss_Ssp1 protein from the sclerotium to the apothecium. This has led me to hypothesize that Ss_Ssp1 serves as a nutrition reservoir for sclerotial germination. However, the lack of observed Ss_Ssp1 degradation in mature sclerotia or germinated sclerotia does not support this hypothesis. To further clarify the function of Ss_Ssp1 in sclerotial development and function, I created a *Ss_ssp1* deletion mutant and investigate the effect of Ss_Ssp1 loss on sclerotial development and germination.

Materials and Methods

Fungal Cultures and Maintenance

The *S. sclerotiorum* wild type 1980 isolate was maintained and propagated on potato dextrose agar (PDA) (Difco, MI, U.S.A.) plates at room temperature. For *Δssp1* mutants or the

complemented strain, Cssp1, hygromycin (final concentration was 100µg/ml) or bialaphos (final concentration was 5µg/ml) was added to PDA media for maintenance and propagation.

Nucleic Acid Isolation and Hybridization

Genomic DNA and RNA were isolated as previously described (Yelton et al., 1984). Southern and Northern hybridization were done according to the descriptions of Rollins (2003). DNA restriction nuclease used for genomic DNA digestion and restriction sites are shown in Figure 1 as well as probes used for hybridization.

Gene Replacement and Complementation

The *Ss_ssp1* replacement vector was constructed in the manner described by Jurick (2007). Gene specific primer pairs, 5'ssp1MuL1 (5'-CAATGTTGTGTAAGCAGCCTTTAC-3')/5'ssp1MuR1 (5'-AGGCGCGCCCAATCGACTTAAGTATGAGTGTTGG-3') and 3'ssp1MuL1 (5'-AGGCGCGCCGGTTAATAACGAGGAGGAGGA-3')/3'ssp1MuR1 (5'-ATGGGAATAAAGGTGTGATTG-3') were used respectively to amplify 1.2kb 5'-UTR sequence with an *Asc*I restriction site (underlined) at 3'-end and a 1.2kb 3'-UTR with an *Asc*I restriction site at 5'-end (underlined). The two amplicons were cloned into pGEM-T vectors (Promega, WI, USA) and the resulting vectors were designated pSSP1-5' and pSSP1-3' respectively. Both pSSP1-5' and pSSP1-3' were double digested with *Not*I/*Asc*I and fragments separated on a 0.8% agarose TBE gel. The pSSP1-5' 1.2kb fragment containing the *ssp1 5' UTR* and the 4.2kb fragment of pSSP1-3'containg the 3' UTR and vector sequences minus a small portion of the multi cloning site, were ligated to form pSSP1-5'+3'. A hygromycin phosphotransferase (*hph*) cassette containing trpC promoter and terminator was released from the pGEM-HPH vector made by Hutchens (2005) using *Asc*I and ligated into *Asc*I digested pSSP1-5'+3' to give rise to a 7.6kb *ssp1* replacement vector, pSSP1-5'+hph+3'. This construct was used as a template to obtain two *ssp1-hph* hybrid amplicons for DNA transformation. This is

'split-marker' gene replacement system (Fairhead et al., 1996; Fu et al., 2006) to improve homologous recombination efficiency during gene replacement. The strategy for this method is shown in Figure1. Primer pair, spL (5'-CAATGTTGTGTAAGCAGCCTTTAC-3')/hy (5'-AAATTGCCGTCAACCAAGCTC-3') was applied to amplify a 2.5kb fragment containing 5'-UTR with first 1.2kb *hph* coding sequence using pSSP1-5'+hph+3' as a template. Primer pair, yg (5'-TTTCAGCTTCGATGTAGGAGG-3')/spR (5'-ATGGGAATAAAGGTGTGATTG-3') was used to amplify a 2.9kb fragment composed of the second part of *hph* coding sequence joined with the 3'-UTR using pSSP1-5'+hph+3'. The 3' portion of the *hph* coding sequence overlaps with the 5' portion of the *hph* coding sequence in a 741bp region so that homologous recombination can occur in this region to reconstitute a complete *hph* cassette when recombined. DNA transformation of protoplasts was done according to the method previously described by Rollins (2003) For *Δssp1* complementation, 3.4kb amplicon containing full length WT *Ss_ssp1* open reading frame flanked with 5'-UTR and 3'-UTR was inserted into pBARKS1 as described by Jurick and Rollins (2007).and Jurick and Rollins (2007). Shrimp Alkaline Phosphatase (Promega, WI, USA) was used to improve ligation efficiency. Constructed plasmids were all transformed into *E. coli* strain DH5α for propagation and plasmid isolation, enzyme digestion, gel electrophoresis, DNA fragment purification and ligation were conducted using standard procedures (Sambrook and Russell, 2001).

Western Hybridization and Immunolocalization

Lyophilized suspension cultured mycelia, sclerotia and apothecia from the WT isolate, the *Δssp1* mutant and the *Δssp1*complemented strain (Cssp1) were used for total soluble protein extraction as described by Jurick *et al.* (2004). Primary antibody raised from rabbit immunized against denatured Ss_Ssp1 is acquired from J. Van Etten (University of Nebraska, NE, USA) and designated as SspVE-Ab. Other two antibodies acquired from L. Kohn (University of Toronto,

Toronto, Canada) were derived from immunized chicken. The one raised against native Ss_Ssp1 is designated as SspLKn-Ab and another one raised against denatured Ss_Ssp1 is designated as SspLKd-Ab. For Western blots, 20µg of extracted soluble proteins were separated with 12% SDS-PAGE and transferred to PVDF membrane (Bio-Rad, CA, USA) by semi-dry electroblotting. The membranes were treated with PBST (80mM Na2HPO4, 20mM NaH2PO4, 100mM NaCl, 0.1% Tween-20) containing 5% non-fat dry milk overnight at 4ºC and then incubated with primary antibody for 1hr at room temperature. The secondary antibody was goat anti-rabbit Ig-conjugated alkaline phosphatase (Bio-Rad, CA, USA). The blots were developed with substrate buffer containing 0.1% NBT and 0.1% BCIP. For immunolocalization assays, fresh, mature sclerotia from the WT isolate, the *Δssp1* mutant and the Cssp1 strain were harvested, fixed and embedded using the method previously described by Kladnik *et al.* (2004) Embedded samples were sectioned (3µm) using a rotary microtome HM325 (Richard-Allan Scientific, MI, USA) and mounted on ProbeOne Plus Microscope Slides (Fisher Scientific, USA) in cytoseal (Richard-Allan Scientific, PA, USA). Ready to use sections were dewaxed in histoclear (National Diagnostics, GA, USA) and rehydrated in an ethanol series followed by hybridized with primary Ab described above for Western blots. Primary Ab was diluted (1:7500) in PBS with 10% goat-serum after the sections were blocked in PBS with 10% goat-serum for 20min. After an overnight incubation in a humidified chamber at 4ºC, the slides were rinsed and incubated with PBS twice for 5min each time. The same secondary Ab (1:500) used in previous Western blots was used to detect the Ss_Ssp1 Ab for 30min at RT. Histochemical detection was performed using NBT and BCIP substrate as described for Western blots for 1hr. The slides were dehydrated in the graded ethanol/water series again and mounted in cytoseal for light microscopic observation.

Apothecia Production and Acquisition of Ascospore Progeny

Mature sclerotia for apothecia induction were produced from cultures grown on autoclaved smashed potatoes containing 1.5% agar in petri dishes (20cm ∅) at room temperature. Antibiotics were added to this potato agar when needed. To produce apothecia, mature sclerotia were washed with repeated changes in running water gently to avoid breaking sclerotia. Clean sclerotia were surface sterilized by immersion in 0.5% bleach for 5 min and then rinsed with sterile water for 5min. After 3 rinses, sclerotia were dried in a sterile airflow hood on sterile paper towels for 8 hours. Dried sclerotia were placed on the surface of glass petri dishes (10cm ∅) which were covered on the bottom thinly with autoclaved, water-saturated vermiculite. Plates were placed at –20°C for 24 hours and then at room temperature for 24 h for 3 cycles. After the third cycle, plates were moved to a 15°C incubator with constant lighting, using fluorescent, cool white bulbs. Once mature apothecia developed 6-8 weeks, ascospores from the *Δssp1* mutant were harvested using a vacuum funnel assembly previously described by Steadman (1974). To acquire single-ascospore isolates, diluted ascospore suspension was spread onto PDA plates to form into single colonies and then the hyphal tip of each colony was transferred to a new PDA plates with hygromycin to grow into mycelia.

Two-step Semiquantitative RT-PCR

Five micrograms of total RNA were used as templates to synthesize first strand cDNAs using Superscript II (Invitrogen, CA, USA) reverse transcriptase. Reverse transcription reaction mixtures included 1µl of Superscript II reverse transcriptase, 4µl of 5x first strand buffer (Invitrogen, CA, USA), 4µl of 25mM $MgCl_2$, 2µl of 0.1mM DTT, 1µl of RNase inhibitor (Invitrogen, CA, USA), 1µl of 10mM dNTPs, 1µl of 0.5µg/ul oligo (dT), 5µg of total RNA and made up to a final volume of 20µl with RNase-free water. The reactions were performed at 42ºC

for 50min, then terminated by incubating the reaction mixture at 70ºC for 15min. RNaseH, 1µl, was used in a final step to degrade RNA templates for 30min. One microliter of 6x diluted reverse transcription reactions was used as templates for PCR. The PCR reaction mixture included 0.3µl of 5U/µl Taq DNA polymerase, 5µl of 10x Mg-free buffer, 2.5µl of 25mM $MgCl_2$, 4µl of 2mM dNTPs, 1µl of 2mM primer, 2µl of undiluted or diluted RT-reactions and made up to a final volume of 50µl with double-distilled sterile water. The thermocycle program consisted of 4min at 94ºC, 40 cycles of 15sec at 94ºC, 30sec at 56ºC and 30sec at 72ºC, followed by 7min at 72ºC. Primer pair, ssp1qR (5'-TTGAACCTTGTCTTTCGGAATGAAG-3') and ssp1qF (5'-GTTCACAATGGGCATACTTTTCAG -3'), were used to amplify 269bp fragment in semi-quantitative PCR products. Primer pair, ssp2-R (5'-TATTTCCATTGAACGCTCCAC -3') and ssp2-F (5'-GTACCTCTGCGCCTGATGATA-3') were used to amplify a 363bp fragment as an indicator of *ssp2* expression. A 338bp PCR product of Histone H3 SS1G_09608.1 (GenBank ID for CoreNucleotide sequence: XM_001589836), was amplified using primer pair H3-F2 (5'-TCATCAATCCACAACAACCAC-3') and H3-R1 (5'-AGAGCACCAATAGCGGAAGA-3') and used as an expression normalization control.

Results

Deletion of *ssp1* Locus

The split marker-based strategy used for homologous recombination and specific deletion of the *Ss_ssp1* (Δ*Ss_ssp1*) locus is shown in Figure 3-1A. To screen for homokaryotic Δ*Ss_ssp1* deletions within the hygromycin resistant transformants, a 5'-UTR sequence and the partial coding sequence of *Ss_ssp1* were used as Southern hybridization probes with *Xba*I-digested genomic DNA isolated from hygromycin resistant transformants. As shown in Figure 3-1C, the wild type hybridizes with a 4415bp band when the 5'-UTR sequence is used as a probe while the homokaryotic knock-out mutant hybridizes with a 5472bp band due to the replacement of

Ss_ssp1 with the hygromycin cassette. Heterokaryotic transformants show both of 4415bp band and 5472bp band (results are not shown). When using *Ss_ssp1* coding sequences as a probe, there is no band detected from homokaryotic transformants while a 4415bp band is present in the wild type isolate (Figure 3-1B) and a comparatively weaker band of the same size can be seen for heterokaryotic transformants (results are not shown). Based on this Southern analysis, one out of ~80 Δ*Ss_ssp1* transformants proved to be a genetically pure *Ss_ssp1* knock-out mutant. Genetic complementation of this strain was pursued using a vector containing the *Ss_ssp1* coding sequence flanked with 1.2kb of contiguous upstream sequence and 1.2kb of contiguous downstream sequence on a vector containing the *bar* gene for bialaphos selection. Southern hybridization indicated that these sequences were successfully introduced back into the Δ*Ss_ssp1* mutant at multiple loci (Figure 3-1B).

Absence of Ss_Ssp1 in the *Ss_ssp1* Deletion Mutant

Northern hybridization performed with RNA isolated from WT and Δ*Ss_ssp1*mutant sclerotia demonstrated that *Ss_ssp1*transcripts are absent from the Δ*Ss_ssp1*mutant (Figure 3-2A). A Ss_Ssp1 immunolocalization assay using mature sclerotia of the wild type and the Δ*Ss_ssp1*mutant indicated that Ss_Ssp1 fails to accumulate in the Δ*Ss_ssp1*mutant whereas it is readily redetectable deposited in protein bodies of WT mature sclerotia (Figure 3-2B).

Effects of *Ss_ssp1* Deletion on Sclerotial Development and Apothecia Development

Deletion of *Ss_ssp1*from *S. sclerotiorum* does not affect the phenotype of mature sclerotia when grown on PDA plates without hygromycin selection while maturation of most sclerotia from mutants is blocked when grown on PDA with hygromycin selection (Figure 3-3). The Δ*Ss_ssp1*mutant is a pure homokaryotic knock-out based on the above Southern hybridization data. To further validate the purity, single ascospore isolates derived from this homokaryotic mutant were collected and also displayed this phenotype when grown on PDA with or without

hygromycin (results are not shown). Therefore the lack of maturation of Δ*Ss_ssp1*sclerotia on PDA with hygromycin is not due to heterokaryotic impurity conferring partial hygromycin resistance. By some mechanism, hygromycin itself appears to contribute to the formation of immature sclerotia on PDA with hygromycin. In contrast, a WT transformant containing a randomly inserted hygromycin cassette does not display a block in sclerotial maturation on PDA with hygromycin (Figure 3-3) which indicates that the *Ss_ssp1* deletion leads to the failure of maturation for most sclerotia formed on PDA plates with hygromycin. However, the *Ss_ssp1* deletion does not cause a noticeable effect on apothecial development. The mature sclerotia of Δ*Ss_ssp1* still germinated into fertile apothecia (Figure 3-3) though the timing for germination is delayed by approximately five weeks relative to wild type.

Ssp2 and a 15.5kDa Protein are Upregulated in the Δ*Ss_ssp1* Mutant

An interesting phenomenon observed in Δ*Ss_ssp1* mutants is the upregulation of *ssp2* transcript and protein accumulation in Δ*Ss_ssp1* sclerotia. *ssp2* is the only homolog of *Ss_ssp1* in *S. sclerotiorum* genome but its expression pattern varies markedly from that of *ssp1* (Chapter 2). RT-PCR results presented in Chapter 2 revealed that *ssp2* transcripts accumulate preferentially throughout apothecial development but not in sclerotial initials or vegetative hyphae. Transcript accumulation results shown in Figure 3-5 indicate that in WT *ssp2* transcripts are detectable in stage IV sclerotia at a low level compared to apothecia. However the accumulation of *ssp2* transcripts in stage IV Δ*Ss_ssp1* sclerotia increased to a level exceeding that present in WT apothecia based on the semi-quantitative RT-PCR (Figure 3-5). Furthermore, Western hybridization with SspVE-Ab crossreacts with a protein approximately 1-2 kDa larger than Ss_Ssp1in both sclerotia and apothecia of the Δ*Ss_ssp1* mutant and complemented strain (Figure 3-4). This cross reactivity was not observed in western blots of WT sclerotial proteins previously. The observations that the migration of this protein matches that of Ssp2 and given the

level of sequence conservation between Ss_Ssp1 and Ssp2, this antigenically crossreacting protein is likely to be Ssp2. The failure to observe a crossreacting band in WT sclerotial and apothecial Westerns previously is likely due to the low relative level of Ssp2 and masking of crossreactivity due to the abundance of Ss_Ssp1. The combination of Western analysis and RT-PCR results strongly suggests that *ssp2* expression is up-regulated as a result of *Ss_ssp1* deletion. In the complemented strain, *ssp2* expression levels in sclerotia and apothecia are not reduced to wild-type levels although *Ss_ssp1* expression is recovered in mature sclerotia. This might be attributed to a lower level of *Ss_ssp1* expression in this strain due to the random insertion of *Ss_ssp1* during complementation. We also observed that the accumulation of the 15.5kDa protein previously described by Russo *et al.* (1982) is also increased in Δ*Ss_ssp1* mutant and the complemented strain.

Discussion

Previous studies have demonstrated that Ss_Ssp1 accumulates in membrane-bound protein bodies specifically in sclerotia (Russo and Van Etten, 1985). Furthermore I demonstrated in Chapter 2 that Ss_Ssp1 is translocated from the sclerotium to the apothecium, but the *Ss_ssp1* transcript is sclerotial specific. These findings indicate that this protein plays an important role in the function of sclerotia, particularly in the support of apothecial development, yet not necessarily a nutritional role. The deletion of the *Ss_ssp1* gene did not affect the sclerotia development on solid media in the absence of antibiotics. Adding hygromycin to the growth medium, however, resulted in a block in the maturation of most sclerotia without a distinct negative effect on hyphal growth. Although Δ*Ss_ssp1* is resistant to hygromycin via transformation using *hph* as a selection marker, and single ascospore selection and Southern analysis confirm the homokaryotic state of the mutant, hygromycin still affects sclerotia development. This indicates that the deletion of *Ss_ssp1* itself may attenuate the tolerance to

hygromycin specifically during sclerotial development and lead to sclerotia arrested in an immature state. A mechanism for this decreased tolerance in the sclerotial state is unknown. Lack of Ss_Ssp1 does not produce any observed effects on sclerotial germination other than a delay in germination. However, the observation that Ssp2 transcript and protein are both upregulated and another 15.5kDa major protein accumulation is also increased in Δ*Ss_ssp1* sclerotia and apothecia suggests that the Ss_Ssp1 homolog Ss_Ssp2 or other proteins with similar functions might be able to functionally compensate for the absence of Ss_Ssp1. Consequently, the maturation of sclerotia in the *Ss_ssp1* null mutant affected by hygromycin indicates a possible role of Ss_Ssp1 in protecting sclerotia from compounds secreted by other organisms in the environment.

Ss_ssp1 is a development specific gene accumulates to a high level only in certain developmental stages. There are few other examples of genes with development specificity found in fungi, e.g., muiridin described by Van Etten *et al.* (1979) and Peterson *et al.* (1983) which accumulates in dormant spores of *Botrydiplodia theobromae* but is not present or present in very low amounts in vegetative hyphae; an abundant perithecial protein (App) found by Nowrousian *et al.* (2007) which is specifically expressed in perithecia of *Sordaria macrospore* and *Neurospora crassa* but not present in hyphal tissue; and the spore-specific protein (*ssp1*) from *Ustilago maydis* which shares homology with other fungal oxygenases and is highly expressed in mature teliospores (Huber et al., 2002). The gene encoding muiridin and the biological function of this protein have not been characterized. Deletion of App unexpectedly had no effects on the fertility of *S. macrospore* or *N. crassa* and there are no distinct differences in perithecial morphology or developmental timing between Δ*app* and wild type strains

(Nowrousian et al., 2007). Likewise, disruption of *ssp1* in *U. maydis* did not give rise to an obvious phenotype either.

Looking to other biological systems may give us some insight into the functions of highly accumulating, tissue-specific proteins. In planta, the most common development specific proteins are storage proteins. Russo and Van Etten (1982) even borrowed the term 'storage protein' from plant storage protein to refer Ss_Ssp1 as a fungal storage protein since their similar developmental specificity and similar deposition location in cells (protein bodies). The investigations of plant storage proteins are comparatively much more comprehensive and thorough than studies in fungi. These proteins can be categorized as seed storage proteins and as vegetative storage proteins (Shewry et al., 1995). Most plant seed storage proteins do not display biological activities besides the role of a nutritional reservoir (Larkins, 1981). Some are not required for seed germination (Kriz and Wallace, 1991). But there are some storage proteins, especially tuber storage proteins (Flores et al., 2002; Shewry, 2003), which do show specific biological activities. Ss_Ssp1 does not show any sequence similarity or structural similarity with any described plant storage protein. This suggests a very different evolutionary origin for fungal storage proteins and potentially differing functions. More examples of fungal development-specific genes should help to determine if functional parallels can be drawn between storage proteins in plants and fungi and determine if some functions are common to both.

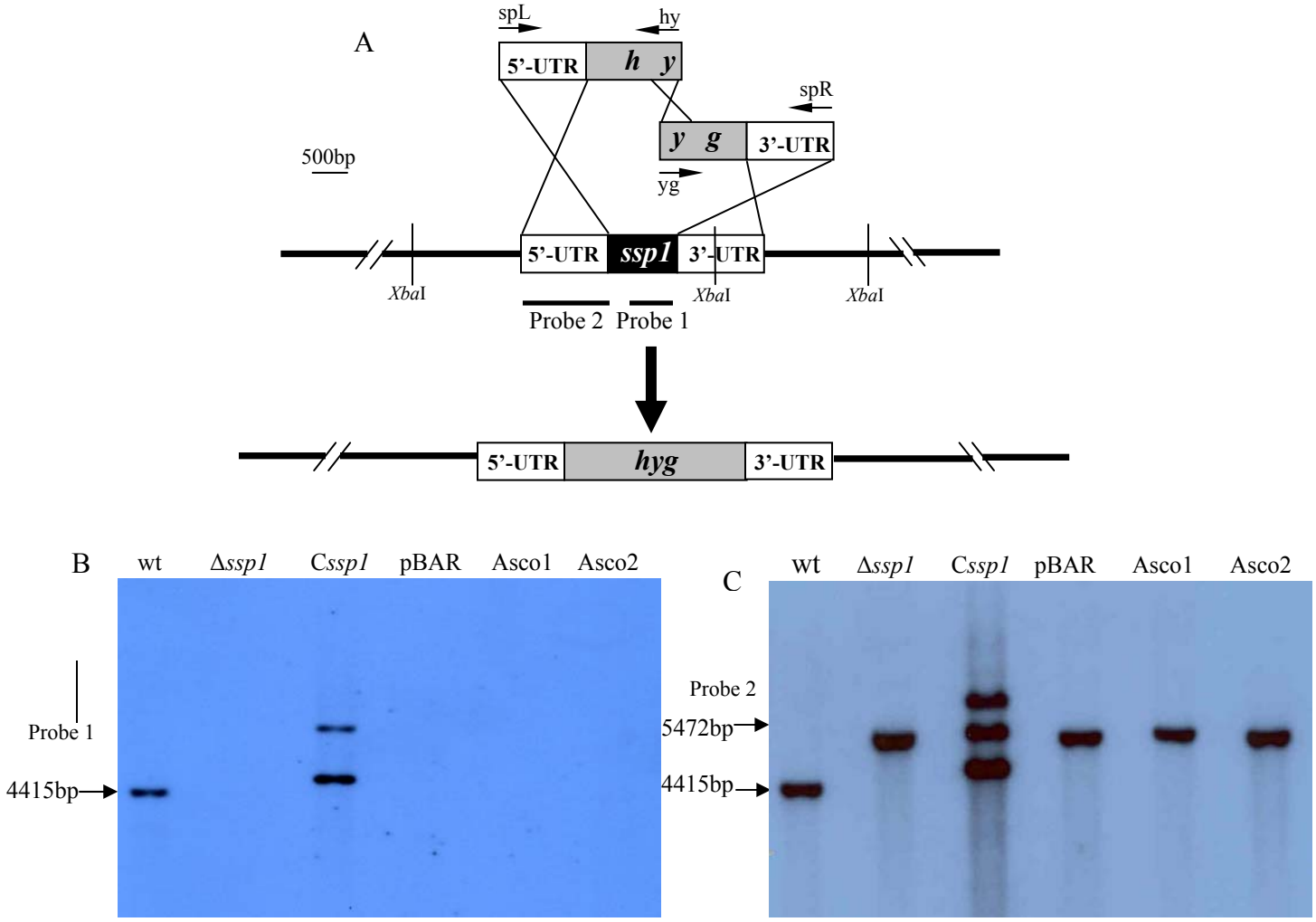


Figure 3-1. A 'Split-marker' strategy used for *Ss_ssp1* replacement and identification. A) The strategy used for *Ss_ssp 1*replacement shows the recombination events and the resulting Δ*Ss_ssp1* deletion. B) and C) Southern hybridization with probe1 and probe2 to confirms the gene replacement and genetic purity of Δ*Ss_ssp1*and two single-ascospore isolates (Asco1 & Asco2). The reintroduction of the *Ss_ssp1* gene into Δ*Ss_ssp1* to create a complemented strain (Cssp1) was also confirmed. Genomic DNAs from each strain were digested with *Xba*I.

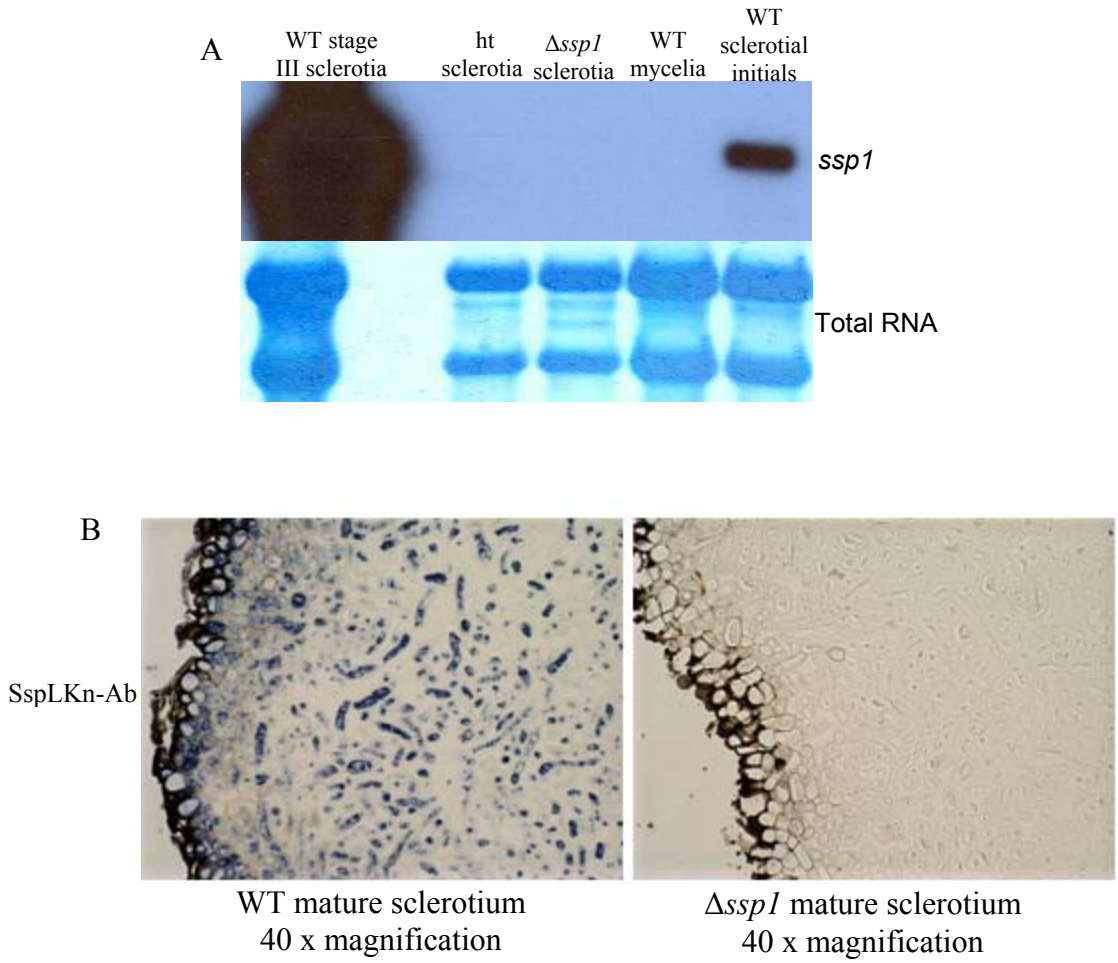
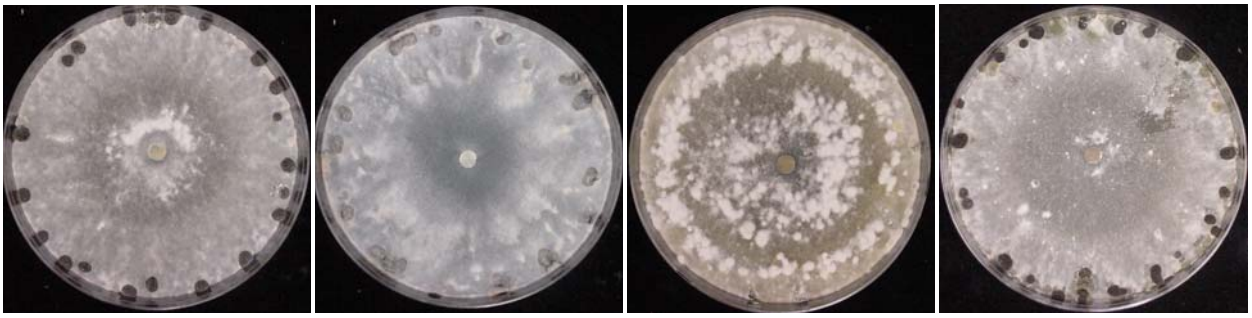
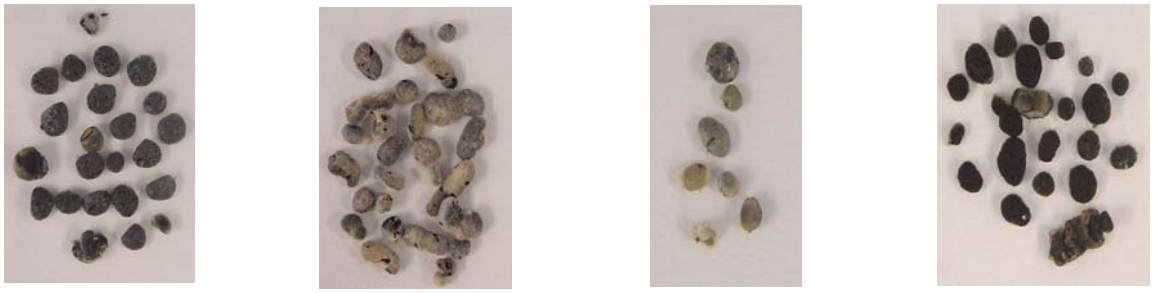
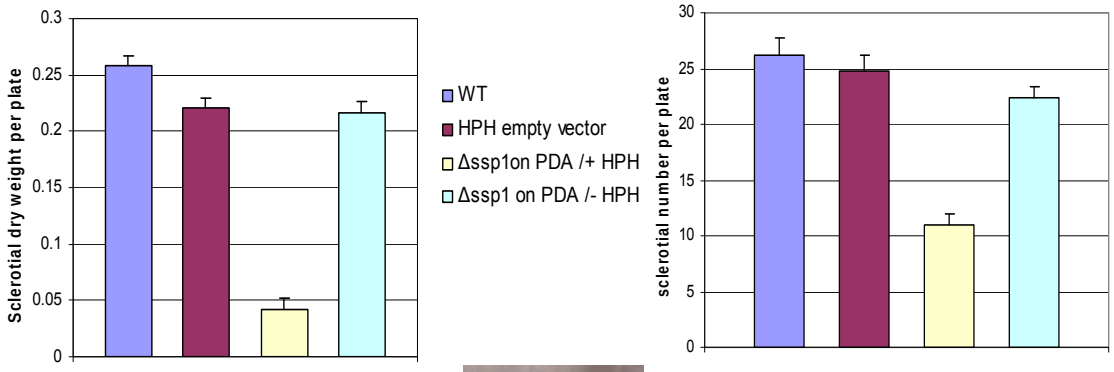


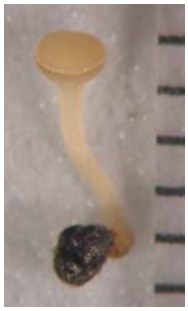
Figure 3-2. Transcript and protein accumulation of *Ss_ssp1* in Δ*Ss_ssp1* sclerotia. A) Northern hybridization for the purified Δ*Ss_ssp1* mutant and a heterokaryotic (ht) mutant stage IV sclerotia and wild type tissues. B) Immunolocalization of wild type mature sclerotium and a Δ*Ss_ssp1* mature sclerotium.





WT on PDA /-HPH | WT-*hph* on PDA /+ HPH | *Δssp1* on PDA /+ HPH | *Δssp1* on PDA /- HPH





Δssp1 germinated sclerotium with apothecium

Figure 3-3 Phenotype of *ΔSs_ssp1* sclerotia on PDA with and without hygromycin and apothecium of the *Δssp1* mutant.

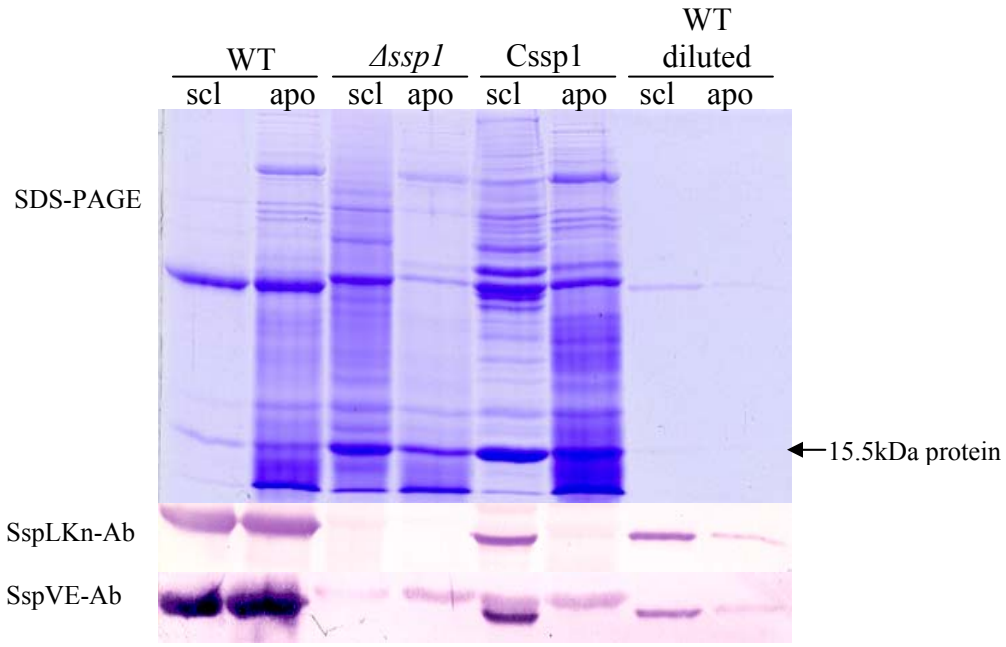


Figure 3-4. SDA-PAGE and Western hybridization with sclerotial and apothecial proteins extracted from wild type, Δ*Ss_ssp1* and Cssp1

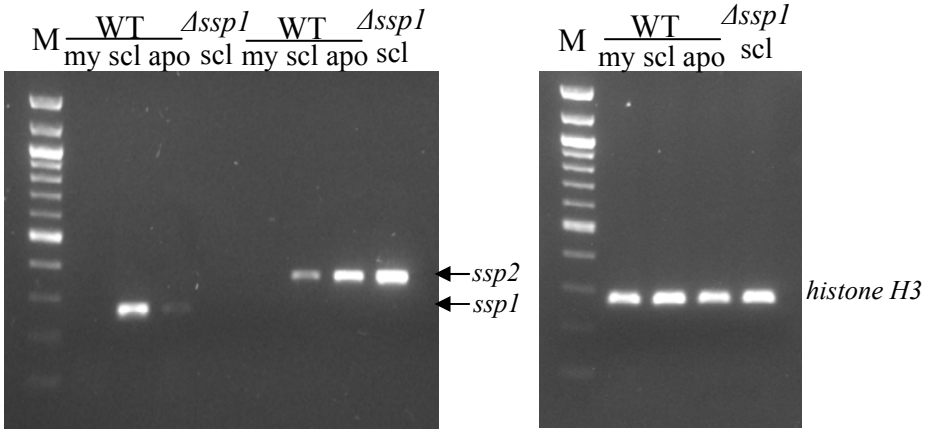


Figure 3-5. RT-PCR detection of *Ss_ssp1* and *Ss_ssp2* transcript accumulation in Δ*Ss_ssp1* stageIV sclerotia. *Ss_ssp1* and *Ss_ssp2* transcript accumulation in WT mycelia (my), StageIV sclerotia (scl) and expanded apothecia (apo) are used for comparison. Histone H3 transcript accumulations in corresponding stages was used for normalization.

CHAPTER 4
TRANSCRIPT PROFILING DURING SCLEROTIAL INITIATION BY LONG-OLIGOMER MICROARRAY ANALYSIS

Introduction

The filamentous fungus, *Sclerotinia sclerotiorum* (Lib.) de Bary, is a necrotrophic plant pathogen with very broad host range (Boland and Hall, 1994; Purdy, 1979). An important characteristic of this fungus is its ability to form a specialized, multihyphal resting structure known as a sclerotium. These macroscopic tuberoid hyphal aggregates are surrounded by a melanized rind and can withstand adverse environmental conditions under which mycelia can not survive. As a long-term survival structure, sclerotia maintain viability of the fungus through winters in temperate climates, in environments with low humidity and in conditions of high temperature and high UV radiation. Upon return of optimal environmental conditions, sclerotia germinate directly as mycelia (myceliogenic germination) or as one or multiple apothecia (carpogenic germination). The millions of ascospores forcibly discharged from a mature apothecium can germinate and grow saprophitically to propagate the fungus at a time and place distant from the original colonization. Hyphae originating from sclerotia or from ascospore-derived, saprophytic mycelia can initiate new infections of plant hosts. Colonization causes cell death and necrosis of tissue. Under favorable environmental conditions the whole plant may become colonized and die. Thus, sclerotium development is a critical stage in the disease cycle: it is essential for long-term survival and supports the production of inoculum. Studies of sclerotial development extend back for many decades with an early focus on microscopic and histochemical analyses (Bullock et al., 1980; Saito, 1974; Willetts and Bullock, 1992; Willetts and Wong, 1971). In recent years, these studies have moved towards dissecting molecular mechanisms involved in regulating sclerotial development (Chen and Dickman, 2005; Chen et al., 2004; Rollins, 2003; Rollins and Dickman, 1998).

Small molecules and signal transduction pathways known to be involved in sclerotial development include: 1) ambient pH and the pH-responsive transcription factor *pac1* (Rollins, 2003; Rollins and Dickman, 2001); cAMP and the adenylate cyclase encoding gene *sac1* (Jurick and Rollins, 2007; Rollins and Dickman, 1998); 3) a PKA-independent but Rap-1 dependent cAMP signal transduction pathway interacting through the map kinase Smk1 (Chen and Dickman, 2005; Chen et al., 2004); 4) oxidative stress (Georgiou et al., 2006; Patsoukis and Georgiou, 2007). 5) protein phosphatase 2A (PP2A) (Erental et al., 2007) associated with Smk1 and NADPH oxidase; 6) *veA*, a gene mediating developmental light responses, associated with *Aspergillus parasiticus* sclerotial development (Calvo et al., 2004). These investigations reveal that interplay exists among different signal transduction pathways. Since regulation of sclerotial morphogenesis requires complex temporal and spatial coordination of multiple genes, investigations of individual genes or molecules do not provide a comprehensive view of the regulatory networks involved in this developmental process. Owing to the availability of the *S. sclerotiorum* genome sequence that was assembled and released by the Broad Institute <http://www.broad.mit.edu/annotation/genome/sclerotinia_sclerotiorum/Home.html>, a full genome, long-oligomer microarray has been developed (Rollins et al. unpublished) that will allow for the comprehensive analysis of differential gene expression during sclerotial development.

Since the first fungal microarray study was reported in the budding yeast *Saccharomyces cerevisiae* in 1997 (DeRisi et al., 1997), microarrays using various platforms and completeness have been successfully constructed for more than 20 species of filamentous fungi. These have been used to identify differentially expressed genes related to metabolism, development, pathogenesis, symbiosis and processes of industrial interest (Breakspear and Momany, 2007).

The earliest microarray studies for fungal development were cDNA microarrays used to identifying light-regulated genes and clock-controlled genes in *Neurospora crassa* (Correa et al., 2003; Lewis et al., 2002; Nowrousian et al., 2003). cDNA microarrays were also used to investigate the expression of genes related to fruiting body development in *Sordaria macrospora* (Nowrousian et al., 2005). Later, long-oligomer microarray technology was applied to identify genes differentially expressed during conidial germination in *N. crassa* (Kasuga et al., 2005) and Affymetrix genechips recently were used to investigate differential expression of genes involved in perithecium development of *Fusarium graminearum* (Hallen et al., 2007). In this chapter, I use a long-oligomer microarray developed by the Rollins lab to identify genes differentially expressed during sclerotial initiation relative to vegetative hyphal growth in order to gain new insights into the number and identity of genes that change their expression when sclerotial development is initiated.

Gene deletion analysis was used to confirm if genes differentially expressed during sclerotial initiation play a role in sclerotial development. I chose a gene encoding γ-glutamyl transpeptidase (γ-GT) which was identified as being upregulated during sclerotial initiation for functional characterization. γ-GT is a ubiquitous enzyme catalyzing the transfer of a γ-glutamyl moiety of glutathione (GSH) and other c-glutamyl compounds to amino acids and peptides (Tate and Meister, 1981). The transfer of a γ-glutamyl moiety from GSH is an essential first step in GSH degradation in mammals. γ-GT homologs in Arabidopsis and yeast display similar enzymatic activity properties, post translational processing and cellular localization to the mammalian enzymes (Mehdi et al., 2001; Storozhenko et al., 2002). Characteristics of γ-GTs function and regulation in filamentous fungi has not been established. There are three γ-GT-encoding genes (SS1G_14127, SS1G_10940 and SS1G_05330) predicted in *S. sclerotiorum*

genome. Previous preliminary cDNA microarray analysis for sclerotial initiation indicated that one of these (SS1G_14127) is among the genes with the highest upregulation during sclerotial initiation compared with vegetative hyphae from liquid shake cultures. I present here that this γ-GT also displays a consistent differential expression pattern when comparing hyphal growth in plate culture versus sclerotia initiation in plate culture using long-oligomer microarray hybridization analysis. I further tested the hypothesis that deletion of the γ-GT-encoding gene (SS1G_14127) will affect sclerotial development and germination.

Material and Method

Culture Growth and Harvesting

S. sclerotiorum wild type strain 1980 was cultured on potato dextrose agar (PDA) plates. An approximately 1cm^2 mycelial plug from the expanding edge of a colony was transferred to a PDA plate overlaid with a cellophane film (Promega, WI, USA). Vegetative hyphae were harvested by peeling the colony from the film before the expanding hyphae reached the edge of the plate, ca. 2 days. To harvest tissue representing sclerotial initials, the colonies were allowed to reach the edge of the plates until sclerotial initials (Stage I sclerotia) scattered on the surface of the plate were obvious before peeling the entire colony from the film, approximately 3 Days. Eight independent biological replications were collected of each tissue type.

Construction of *S. sclerotiorum* Oligonucleotide Microarrays

The construction of the *S. sclerotiorum* genome plus microarray will be described in a publication independent from this dissertation. In brief, two independent 60mer oligonucleotide probes were designed to represent all 14,522 predicted genes from the automated genome annotation <http://www.broad.mit.edu/annotation/genome/sclerotinia_sclerotiorum/Home.html>. In addition, two independent probes representing 1,012 randomly selected reverse complemented predicted gene sequences; 1066 orphan Expressed Sequence Tags (ESTs) that did not match a

predicted gene; 7 genes from host plants, and an additional pool that included only one probe from 7497 orphan ESTs based on a sequence clustering analysis performed at the University of Florida's Interdisciplinary Center for Biotechnology Research bioinformatics core were included. These probes were synthesized *in situ* on glass slides by Agilent Technologies (Santa Clara, CA) using phophoramidite chemistry (Hughes et al., 2001). In addition there were 1,417 probes used as internal quality controls for each hybridization. This probe set, 40,028 independent probes, was printed in a 4x44k format, i.e., 4 arrays per glass slide each containing 40,028 probes.

Total RNA Extraction, Microarray Hybridization and Image Acquisition

Total RNA was extracted from lyophilized tissues of each of the eight vegetative hyphae and the eight sclerotial initial samples described above using TRIzol (Invitrogen, CA, USA) following the manufacturer's instruction. RNA was purified via RNeasy Mini kit (Qiagen, MD, USA) to remove DNA from total RNA based on the manufacturer's protocol. Eight biological replicates were performed for both vegetative hyphae and sclerotial initials. cDNA synthesis, labeling and array hybridization were carried out according to the method described by Ma *et al.* (2006). Images of hybridization arrays were scanned using an Agilent scanner and the raw intensity hybridization signals were quantified using Agilent microarray scanner and feature extraction software.

Data Analysis

Statistical analyses of the hybridization fluorescence intensities were performed by Drs. G. Casella and Jie Yang (University of Florida, Department of Statistics). In brief, signal intensity values for each probe were compared among the biological replications first for probe by treatment interactions. A probability that differences (magnitude or direction of expression) between two probes representing the same gene can be explained by chance was assigned.

Second differential gene expression across all eight biological replications under different treatments for genes with 2 probes was analyzed by an F-Test and the probability of differential expression between treatments for each gene was assigned. A multiple corrections test was run and a Bonferroni cutoff of 0.001 was used to identify genes that were differentially regulated between vegetative hyphae and the sclerotial initial stage. To reduce the number of genes analyzed, we combined genes differentially expressed in this microarray analysis with genes determined to be differentially expressed in another microarray analysis for apothecial development (Rollins et al. unpublished data). The Short-Time Series Expression Miner (STEM) tool (Ernst and Bar-Joseph, 2006) was used to examine the combined data for genes that have peak expression in vegetative hyphae or sclerotial initials and expression levels in apothecia lower than either of these two stages. Expression patterns for these genes were chosen for further clustering analysis via Gene Clustering 3.0 (Eisen et al., 1998). The functional annotations of autocalled genes were obtained from a secure website http://urgi.versailles.inra.fr/pascodb/ developed by the consortium for joint manual annotation of the *S. sclerotiorum* and *Botrytis cinerea* genomes.

Quantitative RT-PCR

5 µg of DNaseI treated total RNA from the same pools used for microarray analysis were combined with 1µl of 0.5µg/ul oligo (dT) in a volume of 10 µl, and incubated for 10min at 65ºC. The sample was cooled on ice for 5min and combined with 4µl of 5x first strand buffer (Invitrogen, CA, USA), 1µl of 100mM $MgCl_2$, 2µl of 0.1mM DTT, 1µl of RNase inhibitor (Invitrogen, CA, USA) and 1µl of 10mM dNTPs. After incubating the mixture at 70 ºC for another 2min, 1µl of Superscript II reverse transcriptase was added to make a final volume of 20 µl for the reverse transcription (RT) reaction. Reactions were performed at 42ºC for 50min, then terminated by incubating the mixture at 70ºC for 15min. RNaseH, 1µl, was used in a final step to

degrade RNA templates. Real time PCR was performed in SmartCycler or SmartCycler II (Cepheid, CA, USA) with qPCR SuperMix for SyberGreen (Bio-Rad) in a volume of 25µl. Oligonucleotide primers used for qPCR are listed in Table 4-2. The thermocycle program was as follows: 4min at 94ºC, 40 cycles of 15sec at 94ºC, 30sec at 56ºC and 30sec at 72ºC, and followed by 7min at 72ºC. Each reaction was carried out in triplicate and mean Ct values of triplicates were used to calculate expression rations according to delta-delta method presented by Perkin Elmer Applied Biosystems (Perkin Elmer, Forster City, CA). The Ct values for the amplicon derived from Histone3 mRNA were used as the normalization reference.

Constructing a γ-GT Gene Deletion Mutant (Δ*ggt*) and its Genetic Complementation

To improve homologous recombination efficiency, a split-marker gene replacement system (Fairhead et al.1996; Fu et al.2006) was used to obtain a *ggt* (SS1G_14127) gene replacement knock-out mutant. The strategy for gene replacement is shown in Figure 4-3. A plasmid containing a hygromycin cassette flanked with ~1.2kb of 5'-UTR and 3'-UTR sequence from the wild type *ggt* locus (ggt-5'+hph+3') was constructed based on the method described by Jurick and Rollins (2007). Primer pairs, 5'ggtMu-L1 (5'-TTCAAAAGGGCTGAGTGTGA-3')/5'ggtMu-R1 (5'-AGGCGCGCCCAACCCGGGAGAATGAGTTA-3') and 3'ggtMu-L1 (5'-AGGCGCGCCGGGGTTTTAATCTAGGATACGG-3')/3'ggtMu-R1 (5'-GAAAGGTGGTGGACTTTGGA-3') were used respectively for PCR to acquire 5'-UTR (attached with *Asc*I (underlined) at 3' end)and 3'-UTR (attached with *Asc*I (underlined) at 5' end) amplicons of *ggt* from wild type *S. sclerotiorum* genomic DNA. The detailed steps for construction of the ggt-5'+hph+3' construct followed the previously described procedure (2007). Two 'split' fragments (5'-UTR attached with first part *hph* sequence '*hy*' and the second part overlapping *hph* sequence '*yg*' attached with 3'-UTR as shown in Figure 4-3) were transformed into wild type protoplasts. These fragments were derived from amplification of ggt-5'+hph+3'

using primer pairs ggtL (5'-TTCAAAAGGGCTGAGTGTGA-3')/hy (5'-AAATTGCCGTCAACCAAGCTC-3') and yg (5'-TTTCAGCTTCGATGTAGGAGG-3')/ggtR (5'- GAAAGGTGGTGGACTTTGGA-3'). Transformation of wild type protoplasts was done according to the method described by Rollins (2003). For *Δggt* complementation, 4.2kb amplicon containing a full length WT *ggt* open reading frame flanked with 5'-UTR and 3'-UTR was amplified and inserted into pBARKS1 with a *bar* gene for bialaphos selection as described by Jurick and Rollins (2007). Shrimp Alkaline Phosphatase (Promega, WI, USA) was used to improve ligation efficiency. Constructed plasmids were all transformed into *E. coli* strain DH5α 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.

Microscopy

For light microscopic observation, fresh mature sclerotia from the WT isolate, the *Δggt* mutant and the genetically complemented strain, Cggt, were harvested, fixed and embedded using the method previously described by Kladnik *et al.*(2004). Embedded samples were sectioned (5μm) using a rotary microtome HM325 (Richard-Allan Scientific, MI, USA) onto ProbeOne Plus microscope slides (Fisher Scientific, USA). Ready to use sections were dewaxed in Histoclear (National Diagnostics, GA, USA) and rehydrate in ethanol series, stained with Amido Black 10 B (Bullock et al., 1980) and mounted in cytoseal (Richard-Allan Scientific, PA, USA).

Apothecia Production for *Δggt*

Mature sclerotia for apothecia induction were produced from cultures grown on autoclaved smashed potatoes with 1.5% agar in petri dishes (20cm ∅) at room temperature. Antibiotics were added to the potato agar when needed. To produce apothecia, mature sclerotia are washed

with repeated changes in running water gently to avoid breaking sclerotia. Clean sclerotia were surface sterilized by immersion in 0.5% bleach for 5 min and then rinsed with sterile water for 5 min. After 3 rinses, sclerotia were dried in hood on sterile paper towels for 8 hours. Dried sclerotia were placed on the surface of glass petri dishes (10cm ∅) containing a layer of autoclaved water-saturated vermiculite. Plates were placed into –20°C for 24 hours and then room temperature for 24h for 3 cycles. After the third cycle, plates were moved to a 15°C incubator with constant lighting, using fluorescent, cool white bulbs.

Results

General Information for Microarray Analysis

As shown in Table 4-1, microarray analysis indicated that 1177 genes out of 14,522 total autocalled genes (8%) were upregulated beyond a two-fold cut-off during sclerotial initiation. Of the 1177 genes, there are 634 (54%) encoding proteins with known putative functions or known conserved domains based on Blast analysis. A total of 973 genes (7%) were downregulated more than two fold during sclerotial initiation with 700 genes among them (72%) encoding proteins with known putative functions or known conserved domains. These differentially expressed genes encoding proteins with predicted functions can be categorized into varied functional groups. The most distinct difference between genes upregulated and genes downregulated in sclerotial initiation was in gene groups involved in protein biosynthesis and mitochondria metabolism. There are 92 genes related to protein biosynthesis downregulated during sclerotial initiation while only 9 genes in this group were upregulated during sclerotial initiation. A similar observation is made for genes involved in mitochondrial metabolism. There are 50 genes related to mitochondria synthesis or transport that are downregulated during sclerotial initiation while only 5 genes in this category are upreglated during sclerotial initiation. The ten genes upregulated in sclerotial initials with highest F-values and highest fold changes are listed in

Table 4-3 and 4-4, respectively. Table 4-5 and 4-6 show downregulated genes in sclerotial initials with highest F-values and highest fold changes. Ten genes exhibiting varying fold expression changes and pattern of expression were chosen for quantitative PCR to determine differential expression by an independent method. These genes all display the same direction and relative magnitude of change by qPCR as observed in the microarray data as shown in Table 4-2.

Discovery of New Genes via Microarray Analysis

1066 orphan ESTs were also investigated by microarray hybridization. Among them, 56 were upregulated and 98 ESTs were downregulated more than two fold during sclerotial initiation. Blast queries with these EST sequences against the *S. sclerotiorum* genome sequence revealed that most orphan ESTs are actually the 5 prime or 3 prime portion of misannotated genes since the ESTs have overlapping sequence homology with predicted genes and usually these overlapping genes share similar differential expression values with these ESTs in microarray data. Still some EST sequences do not overlap with neighboring genes and do not share differential expression values with neighboring genes. Nine ESTs out of the 56 upregulated during sclerotial initiation were categorized as new genes. These are listed in Table 4-7.

STEM (Short-Time Series Expression Miner) Analysis

To better analyze genes involved in sclerotial initiation I chose to focus on genes differentially expressed only during sclerotial initiation but not in apothecial developmental stages for which microarray data was available (Rollins et al, unpublished). This set of microarray data contained gene hybridization fluorescence intensity values of transcripts from dark-germinated apothecial stipes and UV-exposed apothecial stipes. This data set was combined with the current data set so that the genes expressed to a higher level in either of the two stages of apothecial development were removed from the gene list. The remaining genes are specifically upregulated or downregulated in sclerotial initials. Using these criteria, expression

levels of genes in apothecial stages are equal to or lower than the levels in vegetative hyphae. The microarray data acquired by combining the genes differentially expressed during sclerotial initiation and the genes differentially expressed in early apothecial development were input into STEM for clustering. In STEM analysis, the values at one time point (one developmental stage here) are used as a normalization baseline, '0'. The values of other time points are compared to the baseline to obtain the pattern of change across the samples. Patterns representing genes differentially expressed during sclerotial initiation and at a lower basal level in the two apothecia developmental stages are shown in Figure 4-1 and 4-2. These genes were clustered by functional group and heat maps generated as shown in Figure 4-1 and 4-2.

Differential Expression of *ggt* and its Orthologs During Sclerotial Initiation

A cDNA microarray study previously indicated that a gene encoding a gamma-glutamyltranspeptidase (GGT) was highly upregulated during sclerotial initiation (data not shown). This gene is found to be SS1G_14127.1 (*Ss_ggt1*) in the *S. sclerotiorum* genome database at the Broad Institute. Feature searches in *S. sclerotiorum* genome sequence (http://www.broad.mit.edu/annotation/genome/sclerotinia_sclerotiorum/FeatureSearch.html) revealed that two other genes encode GGT paralogs, SS1G_10940 (*Ss_ggt2*) and SS1G_05330.1 (*Ss_ggt3*). Among these three GGT proteins, Ss_Ggt2 and Ss_Ggt1 share 44% similarity while the identity of Ss_Ggt3 with Ss_Ggt1 or Ss_Ggt2 is less than 7%. Homologs of Ss_Ggt1 and Ss_Ggt2 can also be found in Arabidopsis (CAB_79679.1) and other plant and animal species whereas homologs of Ss_Ggt3 only exist in fungal and bacterial species. Long oligomer microarray analysis illustrated that both of *Ss_ggt1* and *Ss_ggt3* are upregulated during sclerotial initiation while *Ss_ggt2* is downregulated (1.5 fold). The level of *Ss_ggt1* transcript during sclerotial initiation is 14 fold higher than in vegetative hyphae. The level of *Ss_ggt3* transcript during sclerotial initiation is 1.5 fold higher than in vegetative hyphae. Northern hybridization

analysis indicated that the level of *Ss_ggt1* transcript accumulation reaches a peak in Stage IV sclerotia as shown in Figure 4-4. Since *Ss_ggt1* was demonstrated to be regulated by sclerotial development via microarray analysis, I investigated if the deletion of *Ss_ggt1* affects sclerotial development or germination.

Deletion of the *Ss_ggt1* gene

The strategy applied for *Ss_ggt1* deletion is described in the Material & Methods and illustrated in Figure 4-3. The 3'-UTR sequences and partial *Ss_ggt* coding sequence were used as probes in Southern hybridization to screen for homokaryotic *Ss_ggt* knock-out mutants. As shown in Figure 4-3, five out of 22 hygromycin-resistant transformants were found to be pure *Ss_ggt1*-deletion mutants. Northern hybridization also indicated that there is no accumulation of *Ss_ggt1* transcripts in Δ*Ss_ggt1* sclerotia (Figure 4-4).

Effects of *Ss_ggt1* Deletion on Sclerotial Development and Germination

The *Ss_ggt1* deletion does not distinctly affect the number or size of sclerotia grown on PDA media compared to wild type. However, this mutation does affect sclerotial tissue organization. Mature sclerotia contain a rind (pigmented outer layer), a cortex (rounded, plectenchyma-cell layer between the outer layer and the medulla layer) and medulla (interwoven internal hyphae). When thin sections of mature sclerotia from Δ*Ss_ggt1* and wild type strain were compared, I found that the cortex layer in the gene deletion mutant is much thicker than wild type. The typical thickness of a WT mature sclerotial cortex usually is 1-3 cells thick while the cortex of Δ*Ss_ggt1* mature sclerotia can reach up to 6-7 cells deep. I also observed the mature sclerotia harvested from another gene deleted mutant, Δ*Ss_ssp1* (chapter 3). The Δ*Ss_ssp1* sclerotial cortex layer is similar to WT sclerotia. The complemented strain of Δ*Ss_ggt1* exhibits a cortex of similar thickness as wild type (Figure 4-5). Interestingly, a thicker cortex does not appear to benefit sclerotial health. Unlike the firm rind of normal WT sclerotia, the rind and

cortex of fully mature Δ*Ss_ggt1* sclerotia can be easily peeled away from the medulla when harvested on or after 20 days from the date of plate inoculation (Figure 4-5). Staining and examination of thin sections determined that the cortex layer of old sclerotia partially separated from the medulla. These mutant sclerotia were still able to myceliogenically germinate on agar media. However, the mature sclerotia of the *Ss_ggt1* knock-out mutant collected from potato media for carpogenic germination failed to germinate into apothecia (results not shown). Instead, they became soft during the incubation for carpogenic germination. This suggests that the separation of rind and cortex from sclerotial medulla impairs sclerotial viability after maturation so that these poorly protected sclerotia are unable to function and develop into apothecia.

Discussion

Owing to the availability of *S. sclerotiorum* genome annotation and utilization of microarray analysis, the genes involved in sclerotial development in *S. sclerotiorum* could be investigated in a large scale for the first time. Initiation is the first committed step in sclerotial development. Therefore, the sclerotial initiation stage was chosen as the first subject for investigation of sclerotial development via microarray analysis. This investigation revealed that large numbers of genes involved in protein biosynthesis and energy metabolism that are very active during vegetative growth, are downregulated during sclerotia initiation. There are also several genes that would normally be thought to be involved in fungal colonization of host plants (e.g., pectinases and polygalacturonases) that are downregulated during sclerotial initiation. These findings are consistent with the fact that sclerotial initiation occurs in the infection cycle after hyphae have successfully colonized plant cells and used up most available nutrients. During this phase, most metabolic pathways used for colonization, energy and protein production are downregulated to adapt to limited nutrients. Conversely, many regulatory proteins such as transcription factors involved in regulation of genes required for sclerotial development, certain

catalytic enzymes involved in further modification of metabolic stores and precursors, proteins required as important reserves in sclerotia, specific transporters for the translocation of reserves as well as proteins with unknown functions are upregulated in this stage. Two regulatory genes (*pac1* (Rollins, 2003) and *sac1* (Jurick and Rollins, 2007)) that were previously demonstrated to affect the maturation of sclerotia are not differentially expressed during sclerotial initiation in this microarray analysis. This may be attributable to their function in late stages of sclerotial development. Genes encoding regulatory proteins such as, STE-like transcription factor (SS1G_07136.1), Glutathione S-transferase (SS1G_10108.1), polyketide synthase (SS1G_07098.1) and monosaccharide transporter (SS1G_06620.1) did show differentially regulation during sclerotial initiation. These genes have not been previously associated with sclerotial developmental regulation and their functional analysis may provide greater insight into signal transduction pathways regulating the initiation transition between hyphal elongation growth and sclerotial initiation. Microarray analysis has also helped us to effectively identify new genes from the set of differentially expressed orphan ESTs. Nine of the 56 ESTs (16%) upregulated in sclerotial initials are identified as new genes which were missed in the automated genome annotation. Extrapolating from this rate of discovery suggests that further analysis of the 1066 expressed ESTs uncovered in this analysis may add more than 100 new genes to the *S. sclerotiorum* genome.

There are three γ-GT homologs present in *S. sclerotiorum* genome.Among these genes, microarray analysis indicated that *Ss_ggt1* is the most highly upregulated during sclerotial initiation. Consequently, *Ss_ggt1* was chosen to be functionally analyzed to determine if it is essential for sclerotial development or function. It has been known that γ-GT in animals are crucial enzymes mediating glutathione (GSH) degradation, reabsorption and transportation of

Cys in the form of GSH. Mutant mice lacking a functional γ-GT die from Cys starvation since the Cys in the form of GSH exported outside cells can not be released and transported into cells again (Lieberman et al., 1996). Therefore, I hypothesized that the loss of function mutant of *Ss_ggt1* will produce unhealthy sclerotia on defined agar media or even on completed agar media due to the Cys starvation, which would result from reduced ability to recycle Cys. We did observe the morphological chage microscopically in knock-out mutant mature sclerotia (thicker cortex layer) compared to wild type sclerotia. The reason for this morphological change in Δ*Ss_ggt1* sclerotia is not fully understood. Our current hypothesis is that this ultrastructure phenotype is still related to Cys deficiency in mutant cells and that increased oxidative stress may result in a thickened cortex tissue layer. The thicker cortex layer in Δ*Ss_ggt1* mature sclerotium did not benefit sclerotial viability. On the contrary, mature sclerotia lacking functional *Ss_ggt1* develop a loose outer layer attached with the medulla such that this outer layer can be easily peeled away from the medulla and the medulla therefore is not well protected from the external environment. A direct effect of this poor protection is that the viability of the sclerotium is weakened and the mutant sclerotia fail to germinate into apothecia. However, the deletion of *Ss_ggt1* does not impair sclerotial initiation. This indicates that *Ss_ggt1* is regulated by sclerotial initiation and the gene upregulation during this stage affects further sclerotial development but not sclerotial initiation.

In the future, more genes regulated during sclerotial initiation will be chosen for functional characterization. How best to choose candidate genes from the pool containing hundreds of genes is an issue. Reducing the range of investigated genes by clustering genes differentially expressed in sclerotial development but not in other developmental stages (such as vegetative growth, apothecial development, ascospore germination) is an effective way to solve this

problem. Through STEM analysis, the genes upregulated in sclerotial initials for further investigation are reduced from 1177 to 201. These 201 genes are those only upregulated in sclerotial initials but not in vegetative growth or apothecial development. The same process was used to filter genes downregulated in sclerotial initials and the gene pool for further investigation was reduced from 973 to 649 genes. That means only less than 40% differentially expressed genes left for further screening.

Table 4-1 Summary of unigenes differentially expressed on S. sclerotiorum microarray

S. sclerotiorum Unigene represented by 2 probes on array	Number of unigenes		
	Represented on array	Upregulated in sclerotial initials more than 2 folds	Downregulated in sclerotial initials more than 2 folds
Autocalled genes	14,523	1177 (8%) (634 with known functions)	973 (7%) (700 with known functions)
Orphan ESTs	1066	56 (5%)	98 (9%)
Reverse complemented autocalled genes	1012	29 (3%)	44 (4%)
Total	16,601	1262 (7.6%)	1155 (6.9%)

Table 4-2 Genes and primer pairs used for quantitative PCR confirmation

Gene ID	Gene Designation	primer sequences (5'-3')	Mean fold change (scl/ veg) acquired from microarray	Relative con. acquired from qPCR (scl/ veg)
SS1G_07095.1	Integral membrane protein	F:CTCGTTGATTTCGCGCTATT R:ATTGCAACGATTCCGAGAGT	13.45	1500
SS1G_07136.1	STE-like transcription factor	F:GCCGTCTGTTCAAGCGTTTA R:CAGATCCATCACCACGATCA	2.19	22.45
SS1G_10108.1	Glutathione S-transferase	F:TCAATGGCTCATGTTCCAAA R:CCAACAAGCCATTGTTTTCC	10.78	373.5
SS1G_11992.1	Lipolytic enzyme G-D-S-L	F:CTTGGTGGTGGTGGAACTG R:GATCCTCCATCGTTGTGACC	9.99	70.35
SS1G_14127.1	Gamma-glutamyltranspeptidase	F:AGCGAAAGTTCGATTTGCTC R:GGAGCCATCTCACGAAAATC	14.62	90.03
SS1G_14065.1	Ss_Ssp1	F:GTTCACAATGGGCATACTTTTCAGC R:TCTCTTCTTACCACGGAGCTTGCTTG	35.5	256.75
SS1G_00263.1	unknown protein	F:CGTCAGCACATCCAGCTCTA R:GGAACACGCCCATAAACATC	-23.59	-264.43
SS1G_05223.1	methyltransferase	F:GCCGGAGATTAAGTGGGAAT R:TCCTCGAGATGCAGATAGCA	-21.86	-23.62
SS1G_05832.1	polygalacturonase	F:TGGCCATGTAGTTTTCAGCA R:AGCGTTTTGGAAGACGAATG	-18.77	-39.61
SS1G_09608.1	Histone H3	F:TACCGAGCATGGTTCGTTTG R:CGAGCCAGCTGAAAACCATT	-0.4	1.8

Table 4-3 Ten genes upregulated in sclerotial initials with highest F-Values

Gene Name	F-Value	Blast Intelligent	Mean of log2VEG/SCL	Folds up
SS1G_07295.1	3974.59	hypothetical protein	-4.36	20.47
SS1G_00274.1	3621.66	covalently-linked cell wall protein	-3.40	10.57
SS1G_03241.1	3443.76	hypothetical protein	-2.44	5.41
SS1G_11285.1	3103.78	cysteine dioxygenase (EC 1.13.11.20) (CDO)	-3.05	8.29
SS1G_00291.1	3015.08	hypothetical protein	-4.40	21.11
SS1G_11992.1	2947.42	rhamnogalacturonan acetylesterase precursor (EC 3.1.1.-) (RGAE)	-3.32	9.97
SS1G_12510.1	2867.92	chitinase	-3.28	9.74
SS1G_07095.1	2459.19	integral membrane protein	-3.75	13.45
SS1G_08163.1	2439.6	predicted protein	-4.08	16.94
SS1G_06620.1	2055.5	monosaccharide transporter	-4.96	31.13

Table 4-4 Genes upregulated in sclerotial initials with highest fold changes

Gene ID	F-Value	Blast Intelligent	Mean of log2VEG/SCL	Folds up
SS1G_04565.1	619.71	metalloexopeptidase	-5.46	44.02
SS1G_05915.1	423.31	mixed-linked glucanase (fragment)	-5.40	41.06
SS1G_04563.1	222.81	phosphatidylserine decarboxylase like protein	-5.31	39.67
SS1G_13613.1	983.44	predicted protein	-5.31	39.58
SS1G_02331.1	466.51	haemolytic enterotoxin precursor	-5.30	39.26
SS1G_03736.1	440.61	plasma membrane ATPase (proton pump)	-5.17	35.92
SS1G_14065.1	994.02	predicted protein	-5.15	35.45
SS1G_05917.1	623.11	hypothetical protein B7A16.100	-4.97	31.36
SS1G_07098.1	982.32	polyketide synthase	-4.96	31.18
SS1G_06620.1	2055.5	monosaccharide transporter	-4.96	31.13

Table 4-5 Genes downregulated in sclerotial initials with highest F-Values

Gene Name	F-Value	Blast Intelligent	Mean of log2VEG/SCL	Folds down
SS1G_00263.1	3937.21	predicted protein	4.56	-23.56
SS1G_10167.1	1473.22	neutral endopolygalacturonase SSPG1D	2.53	-5.77
SS1G_12210.1	1446.23	Aorsin (serine proteinase) precursor	2.72	-6.60
SS1G_05151.1	1221.99	cellobiose dehydrogenase	2.81	-7.03
G787P540RC1.T0	1209.56	SS1G_00215 (predicted protein)	1.18	-2.27
SS1G_02046.1	1145.34	predicted protein	3.18	-9.04
SS1G_01855.1	1123.29	expressed protein	1.41	-2.66
SS1G_08099.1	1073.33	1-acyl-sn-glycerol-3-phosphate acyl transferase 3 (EC 2.3.1.51)	2.55	-5.84
SS1G_13860.1	1063.21	Cellulose (EC 3.2.1.4)	2.20	-4.61
SS1G_05859.1	1018.98	UV-induced protein UVI22	1.28	-2.43

Table 4-6 Genes downregulated in sclerotial initials with highest fold changes

Gene Name	F-Value	Blast Intelligent	Mean of log2VEG/SCL	Folds down
SS1G_00891.1	343.89	endoglucanase III	5.03	-32.65
SS1G_04177.1	254.58	polygalacturonase 5 (*pg5*)	4.81	-28.01
SS1G_07491.1	203.56	hypothetical protein	4.31	-19.82
SS1G_05832.1	327.39	exo-polygalacturonase	4.23	-18.74
SS1G_02690.1	703.52	predicted protein	4.20	-18.36
SS1G_13641.1	346.41	polyketide synthase	4.45	-21.93
SS1G_05223.1	753.75	TRP-1 (methyltransferase)	4.45	-21.92
SS1G_00263.1	3937.21	predicted protein	4.56	-23.56
SS1G_00468.1	246.08	pectin methylesterase	4.40	-21.16
RC_SS1G_05832.1	108.19	antisense SS1G_05832.1 (exo-polygalacturonase)	4.11	-17.30

Table 4-7 New genes found in orphan ESTs via microarray analysis

EST ID	Support for new gene
G787P566FA12.T0	A new gene overlapping with SS1G_08026 or SS1G_ 02831 (1.12392E-20) in different supercontigs (both of them are homologs of BC1G_15966 and no EST support). Reverse orientation
G786P573RC4.T0	New gene 500bp upstream of SS1G_10336. SS1G_10336 is not differentially expressed
G787P552RB2.T0	New gene with partial overlap with SS1G_02236 or misannotated SS1G_02236. SS1G_02236 is not differentially expressed.
G787P562RC7.T0	New gene 100bp upstream of SS1G_12883 (Non-overlapping). No differential expression of SS1G_12883.
Ssc_BI_UF.185.C1___1_nr0nt	A new gene on the antisense strand overlapping with SS1G_10229. SS1G_10229 is not differentially expressed.
Ssc_BI_UF.119.C1___1_nr0nt	New gene 100bp downstream of SS1G_11597. Reverse orientation, non-overlapping and no intergenic region. 1KB downstream of SS1G_11596. SS1G_11596 is differentially expressed while SS1G_11597 is not.
Ssc_BI_UF.868.C1___1rc_nr1nt0	New gene between SS1G_07105 and SS1G_07106. Greater than 1kb from both.
Ssc_BI_UF.746.C1___1_nr0nt1	New gene or misannotated SS1G_12567. EST is ~100bp overlapping with 3' end of this gene. But SS1G_12567 is not differentially expressed in microarray.
Ssc_BI_UF.983.C1___1_nr0nt	New gene between SS1G_05337 and SS1G_05338. 4kb downstream of SS1G_05337 and 2kb upstream of SS1G_05338

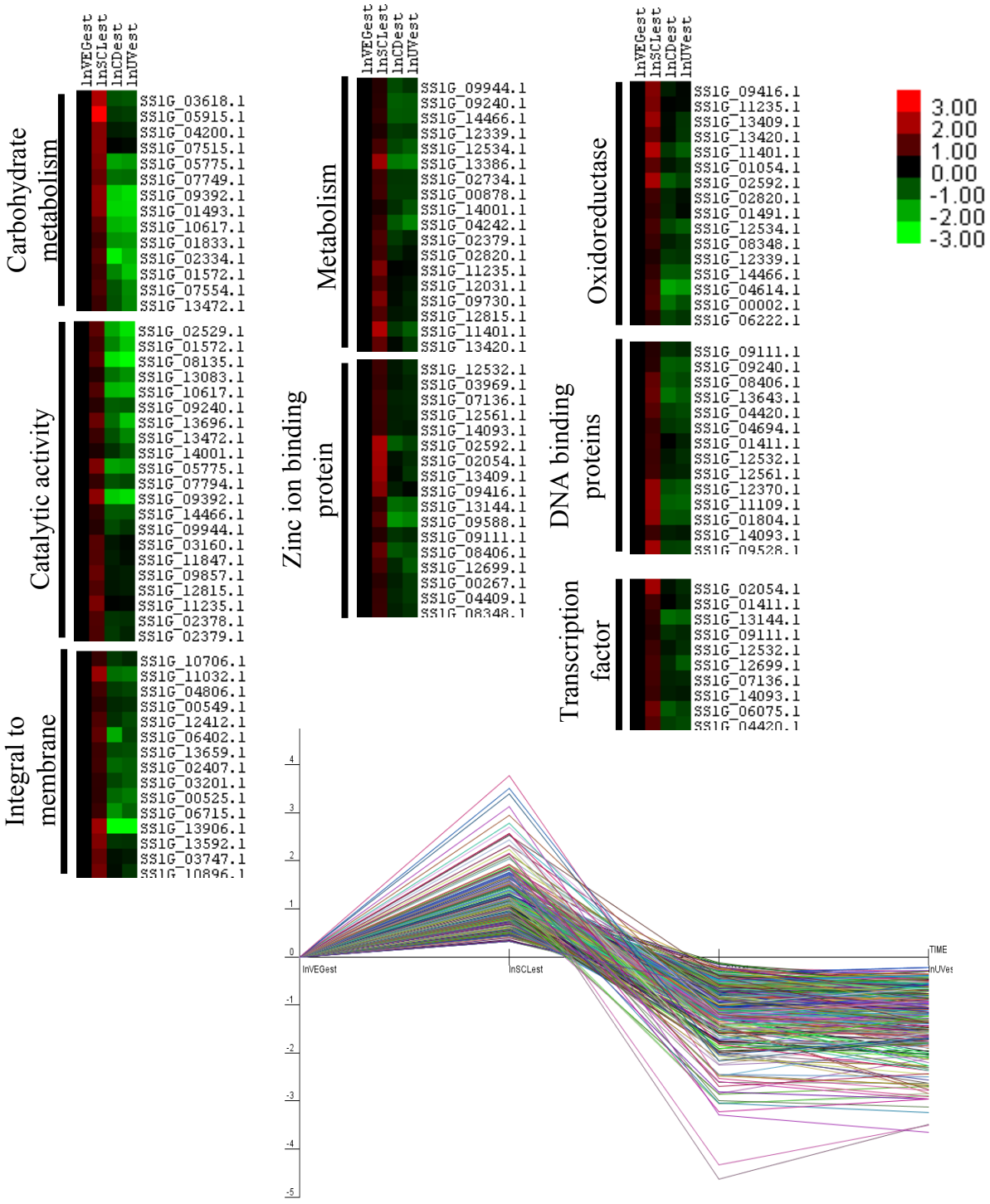


Figure 4-1 Heat maps generated for each functional clusters specifically upregulated during sclerotial initiation and the expression pattern representing these groups. Relative intensities are expressed as natural log values in Y-axis. X-axis represents four developmental stages: vegetative growth (InVEGest), sclerotial initiation (InSCLest), etiolated stipes in constant dark (InCDest) and etiolated stipes treated with UV (InUVest)

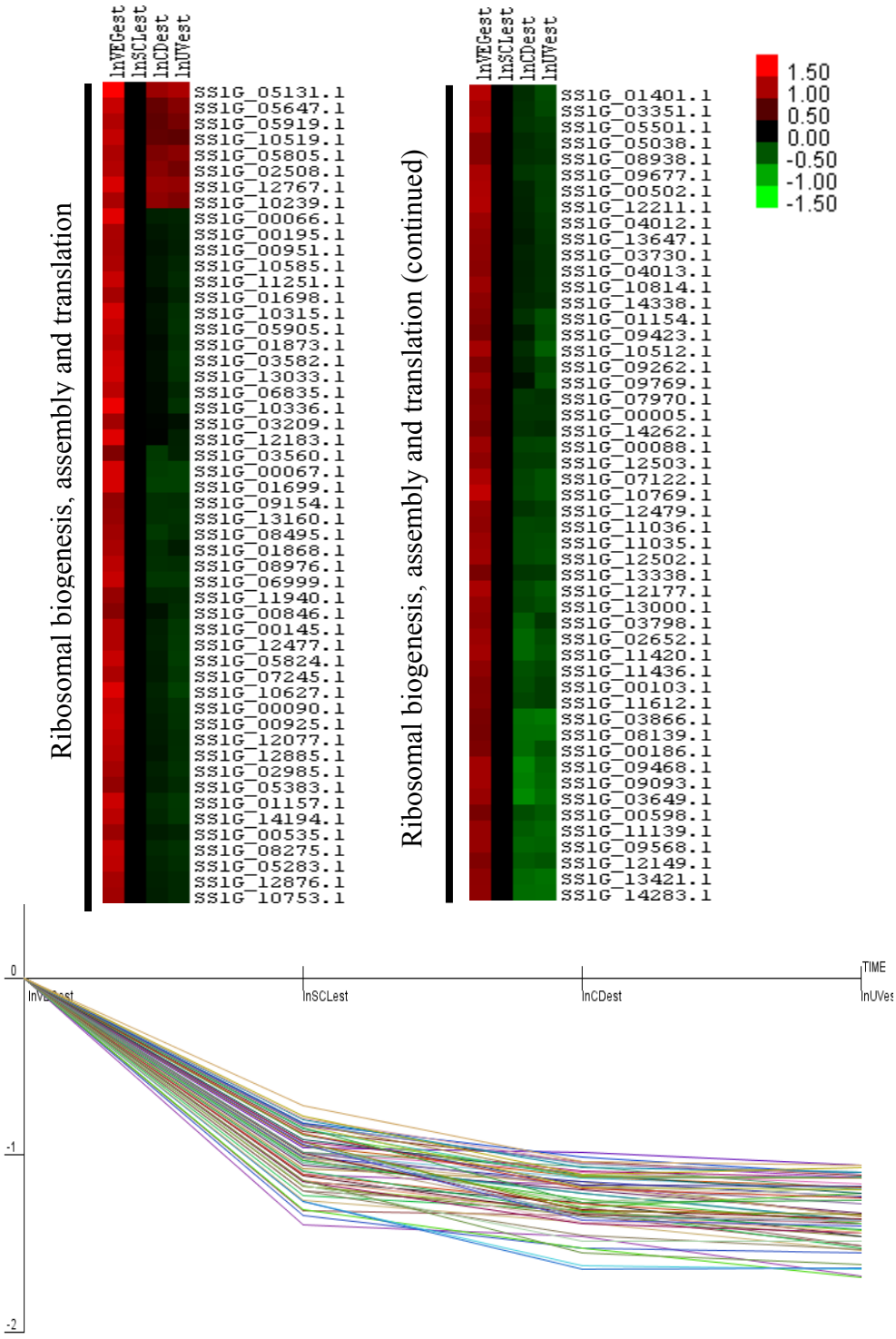


Figure 4-2 Heat maps generated for ribosomal proteins specifically downregulated during sclerotial initiation and the expression pattern representing these genes. Relative intensities are expressed as natural values in Y-axis. X-axis represents four developmental stages: vegetative growth (InVEGest), sclerotial initiation (InSCLest), etiolated stipes in constant dark (InCDest) and etiolated stipes treated with UV (InUVest)

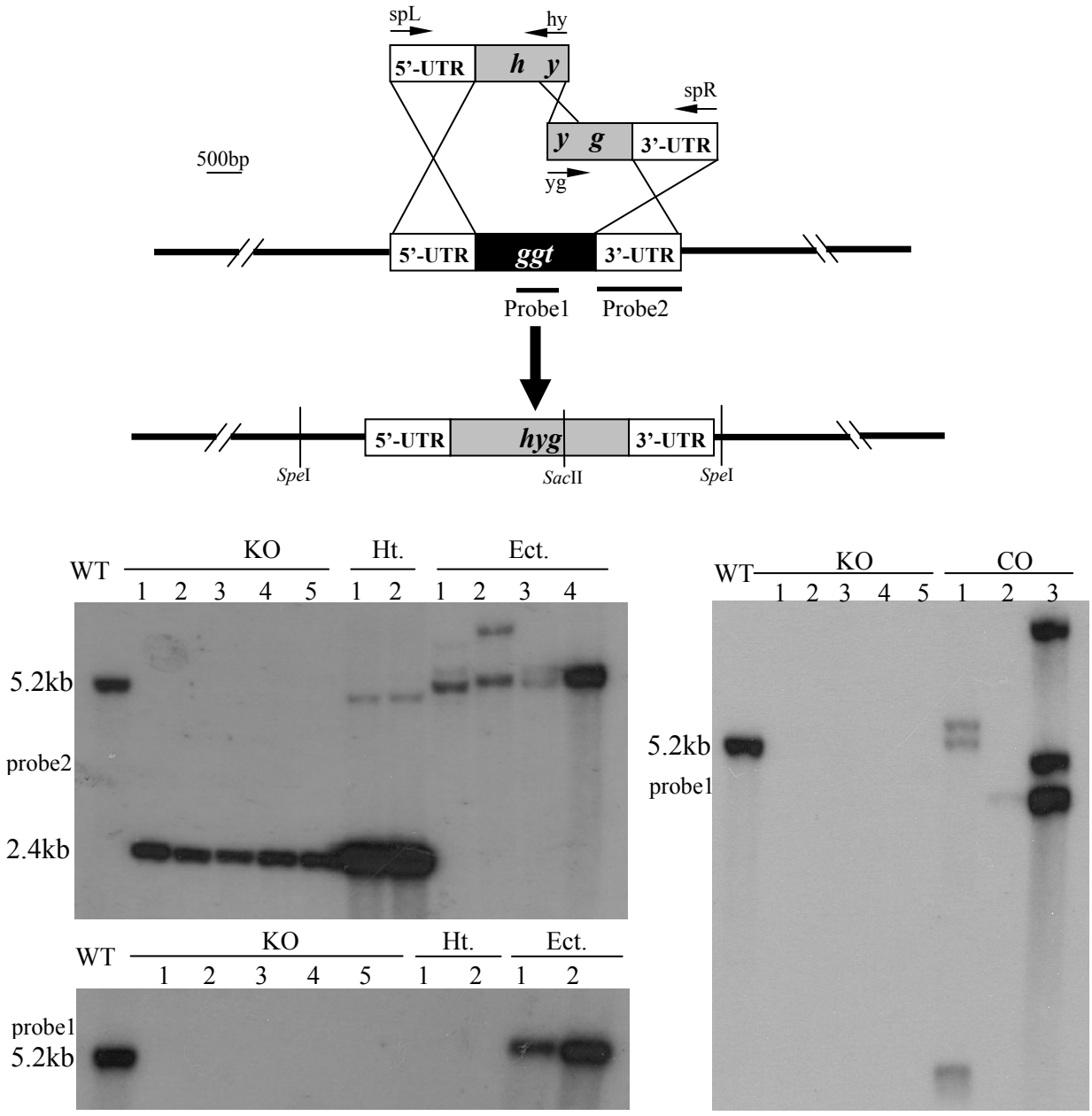


Figure 4-3 The 'split-marker' strategy used for making *ggt* deletion mutants and Southern hybridization for screening homokaryotic *ggt* mutants (KO), heterokaryotic mutants (Ht), ectopic mutants (Ect.) and complemented strains (CO).The wild type *ggt* locus localizes to a 5.2kb band while a homologous integration of the *hph* cassette gives rise to a 2.4kb band when double digested with *Spe*I and *Sac*I and hybridized with Probe2.

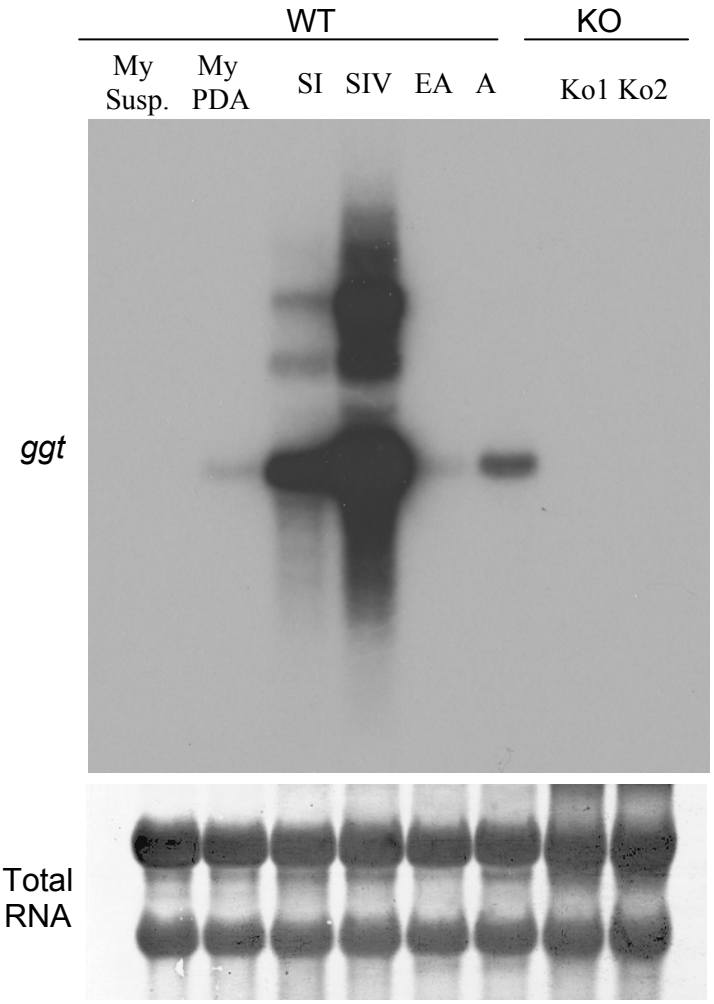


Figure 4-4 Northern hybridization analysis of *ggt* transcript accumulation in different wild type developmental stages (My. Susp.: mycelial suspension culture; My. PDA: mycelia harvested from PDA plates; SI: stage I sclerotia; SIV: stage IV sclerotia; EA: etiolated stipes; A: mature apothecia); Ko1 and Ko2: Stage IV sclerotia from 2 independent *ggt* knock-out mutants.

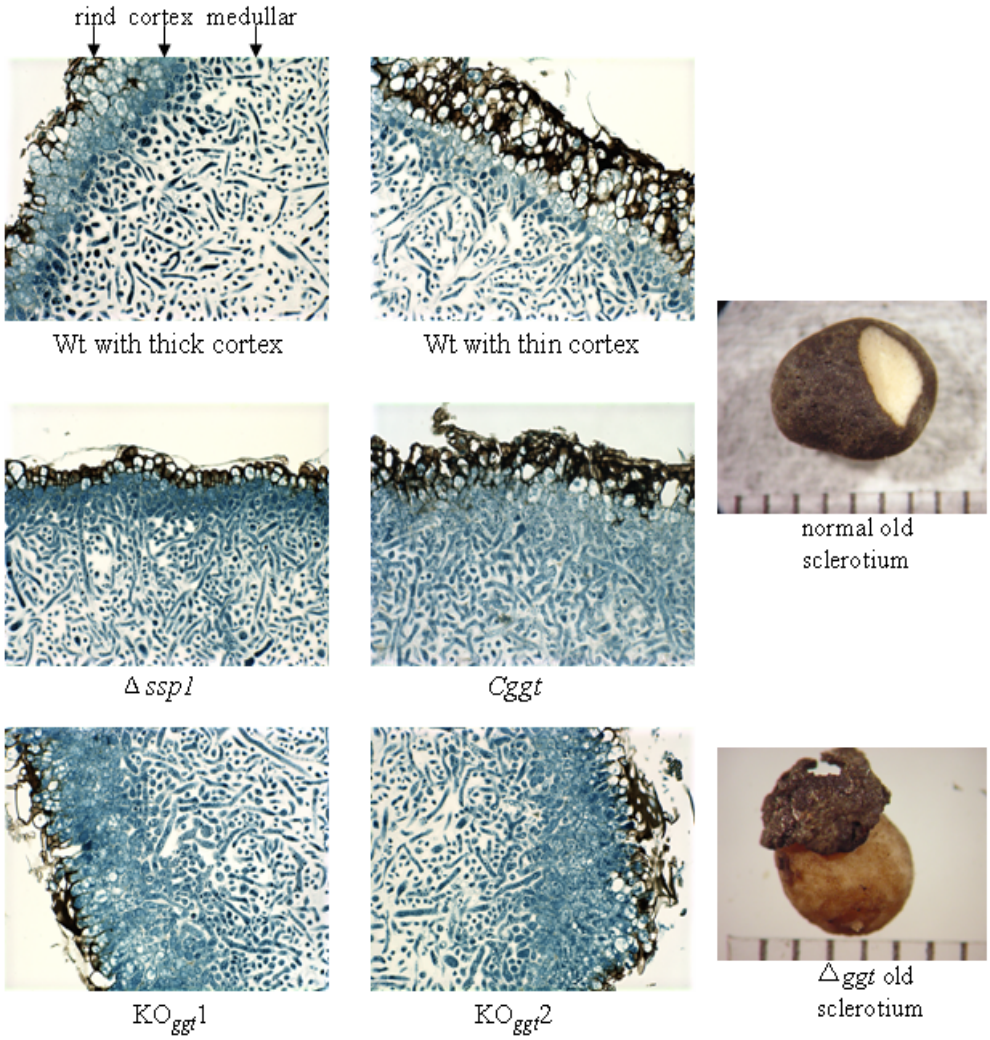


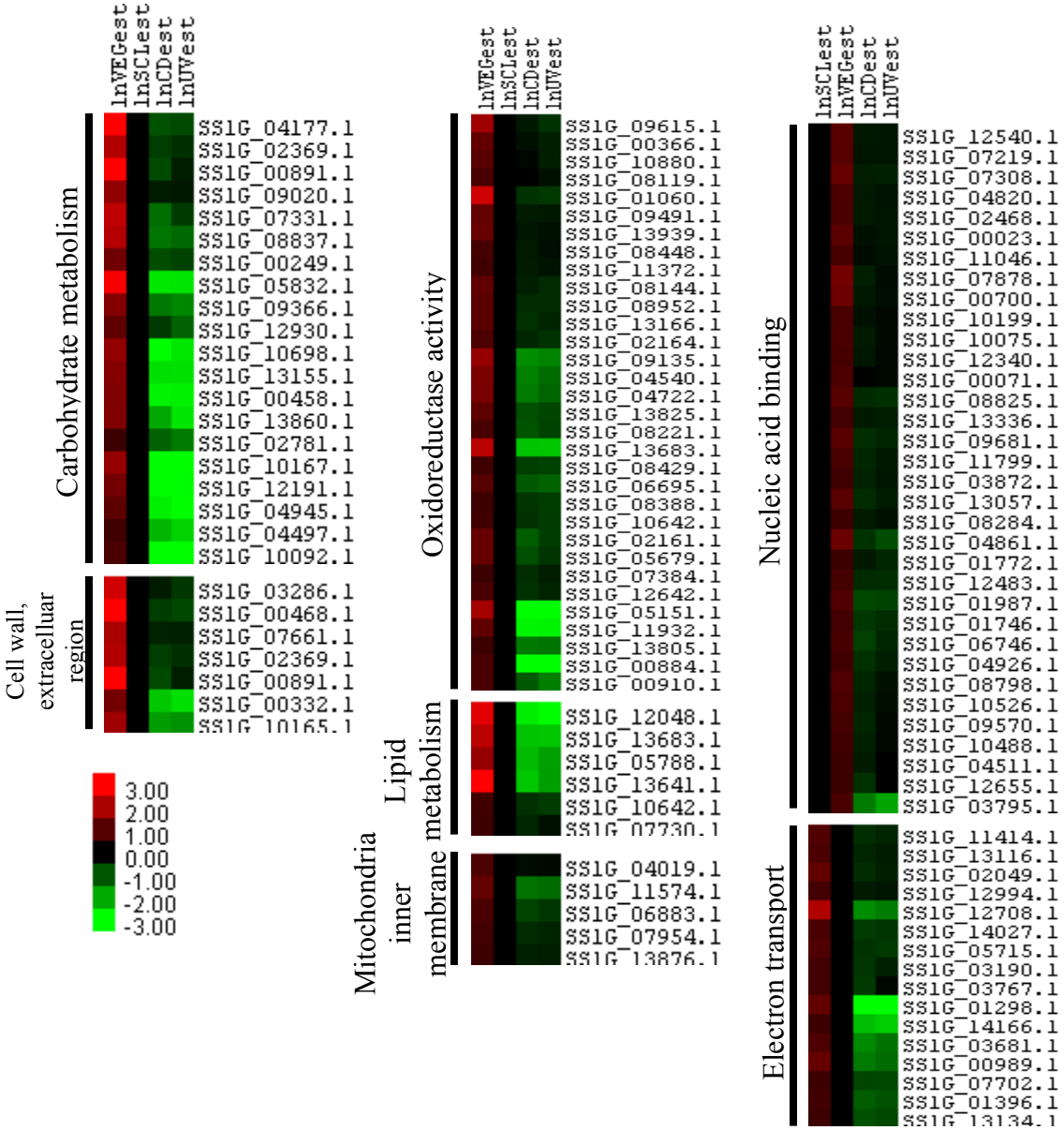
Figure 4-5. Microscopic features (left two columns) and macroscopic features of mature sclerotia (right column) harvested from WT and Δ*ggt* stains. Amido Black section staining displays the rind, cortex and medulla (as indicated by arrows) of mature sclerotia from wild type, ggt knock-out mutants (Δ*ggt*), a Δggt complemented stain and Δssp1. The right column displays the normal fully mature sclerotium and a Δ*ggt* fully mature sclerotium with an easily peeled rind.

CHAPTER 5
CONCLUSIONS

During the course of my dissertation research, genes regulated during sclerotial development in *S. sclerotiorum* were investigated through individual function, gene characterizations and global microarray transcriptome analyses. Ss_Ssp1 is a sclerotial development specific protein described previously. Our study indicated that the transcript accumulation of Ss_Ssp1 is tightly regulated by sclerotial development while the protein accumulates both in sclerotia and apothecia. Ss_Ssp1 in sclerotia is relocated from sclerotia to apothecia during germination. Orthologous sequences were also discovered in other sclerotial-forming Sclerotiniaceae species and even in sclerotia-forming *Aspergillus* species. A homologue of the *Ss_ssp1* sequence (*ssp2*) in the genome of *S. sclerotiorum* was also identified. *ssp2* is highly transcribed in apothecia but not in sclerotia. Observations of the pattern of *Ss_ssp1* transcript accumulation in various sclerotial mutants, natural isolates and other sclerotia-forming species suggests that this gene is a good biomarker to identify early stages of sclerotial development. An ~1kb sequence upstream of the *Ss_ssp1* coding sequence was sufficient to drive tissue-specific expression of sGFP in sclerotia. Beyond understanding fundamental aspects of sclerotial development, the *Ss_ssp1* promoter sequence may have biotechnology utility for expressing and obtaining high quantity, localized, heterologous proteins in sclerotia. To further illuminate the function of this fungal storage protein in sclerotial development and apothecia development, homokaryotic *Ss_ssp1* deletion mutants were generated and the phenotype of the mutant was investigated. This hygromycin selected Δ*Ss_ssp1* strain formed 50% less mature sclerotia than wild type when growing on hygromycin-containing medium. The impaired sclerotial maturation under antibiotic selection seems to be the result of an interplay between *Ss_ssp1* deletion and the effect of hygromycin because the same phenotype was not observed

with the *ΔSs_ssp1* mutant grown on PDA plates without hygromycin or with a strain expressing hygromycin resistance from a randomly integrated *hph* cassette growing on hygromycin-containing medium. No noticeable effects of *Ss_ssp1* deletion on carpogenic germination or apothecial development other than a delay in germination timing were observed. However, I could not conclude that Ss_Ssp1 has no function in sclerotial or apothecial development. The upregulation of *ssp2* transcripts and protein accumulation as well as the higher accumulation of a 16kDa major protein in the Δ*Ss_ssp1* mutant sclerotia compared to the corresponding wild type controls suggests a possible functional compensation for loss of Ss_Ssp1 in *S. sclerotiorum*. Transcriptome profiling during sclerotial initiation was also investigated via long-oligomer microarray analysis. 14.8% of the total genes in the *S. sclerotiorum* genome were determined to be differentially expressed more than two fold during sclerotial initiation. Microarray analysis not only can provide a comparative and comprehensive view of gene regulation during sclerotial development but also can be used to identify new genes. Several putative new genes have been identified from an examination of differentially expressed orphan ESTs from the microarray analysis. A gene that was highly upregulated during sclerotial initiation was the gamma-glutamyl transpeptidase-encoding gene (*ggt*). This gene was chosen to for functional characterization. Lost-of-function mutant of *ggt* exhibit a thicker sclerotial cortex and an easily peeled-off rind layer on fully mature sclerotia. These sclerotia fail to germinate into apothecia as an apparent result of poor environmental protection or loss of integrity due to a discontinuous sclerotial rind. The genes specifically regulated during sclerotial initiation but not comparatively expressed in apothecia development or in vegetative growth are clustered into different functional groups. In the future, these genes will be candidates for investigating their involvement in sclerotial development.

APPENDIX A
GENES DOWNREGULATED IN SCLEROTIAL INITIALS



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BIOGRAPHICAL SKETCH

Moyi Li was born September 9, 1978, in Wuhan, People's Republic of China, to Li Li and Fengying Guo. She grew up in this big city on the YangZi River and the 'outdoor activity' she liked the most before the age of 25 was to search traditional Chinese cuisine in the alleys of Wuhan or hang out in the most prosperous streets of the city with her friends or her mother. But her favorite recreations are still novels, movies, Karaoke and sleeping. Moyi attended Huazhong Agricultural University in September of 1996 where she received a Bachelor of Science degree in microbiology in July 2000. In the Fall of the same year, she was recommended by her undergraduate university to start her graduate school in the School of Life Science and Technology, Huazhong University of Science and Technology. The master's research project focused on elicitation of taxol biosynthesis in *Taxus chinensis* suspension culture by oligosaccharide and polysaccharides secreted by *Aspergillus niger* and she received a Master of Science degree in July of 2003. Moyi was fortunately awarded an Alumni Fellowship from the plant pathology department, University of Florida before the end of her master's studies and then she left China for USA to start her Ph.D program in August 2003. She decided to join Dr. Jeffrey A. Rollins' lab very soon after meeting with him due to his interesting research projects in fungal development and molecular biology. Moyi completed her doctoral studies related to sclerotial development in *S. sclerotiorum* in May 2008 and was awarded a Doctor of Philosophy degree. After graduation, she would like to continue in the area of development and molecular biology as a research scientist.