Sclerotinia sclerotiorum pathogenicity factors: Regulation and interaction with the host

A Thesis Submitted to the College of
Graduate Studies and Research
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in the Department of Food and Bioproduct Sciences
University of Saskatchewan

By
Zafer Dallal Bashi
Saskatoon, Saskatchewan, Canada
April 2011

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Food and Bioproduct Sciences

University of Saskatchewan

Saskatoon, Saskatchewan, S7N 5A8
Canada

ABSTRACT

Sclerotinia sclerotiorum infects over 400 plant species. The pathogen employs several mechanisms to overcome plant defenses including the production of oxalic acid which suppresses the hypersensitive response.It also utilizes hydrolytic enzymes to degrade cell structure and necrosis-inducing molecules to cause cell death. The work described in this thesis: 1) identified and elucidated the role of select proteins and enzymes in *S. sclerotiorum* pathogenesis, 2) determined how their respective genes were regulated throughout the different stages of the infection, and 3) examined their interaction with plant defenses.

The plant surface is covered by a waxy layer referred to as the cuticle. A gene encoding a cutinase was identified in the *S. sclerotiorum* genome. *SsCutA* was expressed after mycelia were placed in contact with the cuticle or other solid surfaces. Expression was limited to the very early stages of the infection suggesting that the enzyme only plays a role in cuticle penetration.

Beneath the cuticle, host integrity is maintained by layers of polysaccharides in the plant cell wall. *S. sclerotiorum* produces a suite of pectin degrading enzymes, and a gene encoding a novel endopolygalacturonase (PG), *SsPg2,* is described. While *SsPg2* was not expressed during infection of *B. napus*, the expression pattern of *SsPg1*indicated that it was involved in infection initiation and lesion expansion. *SsPg1*expression was regulated by a complex network involving feedback inhibition by galacturonic acid, ambient pH, and carbon availability. Two *S. sclerotiorum* PGs were demonstrated to induce necrosis in the host. This activity was inhibited by the *B. napus* polygalacturonase inhibitor proteins BnPGIP1 and BnPGIP2; however, expression of a single BnPGIP in transgenic plants was insufficient to provide practical resistance.

This study also identified two *S. sclerotiorum* Necrosis and Ethylene-inducing Peptides (NEPs) that caused necrotic lesions to form in host plants.

SsNep2 was expressed at a higher level than *SsNep1* during infection of *B. napus* and in both the necrotic zone and the leading margin, indicating that it is involved in lesion expansion and the later necrotic stage.

The signaling mechanisms that are involved in regulating the expression of these pathogenicity-related genes were examined, these included cAMP, Ca^{+} flux, calcineurin and mitogen activated protein kinases (MAPKs). Disruption of the *Smk3*, the orthologue of the *Slt* cell wall integrity MAPK gene from *Saccharomyces cerevisiae*, led to loss of virulence on intact *B. napus* leaves, but not on leaves stripped of cuticle. This phenotype appeared to be associated with alterred mycelial polarity, branching and hydrophobicity and inability to form infection cushions.

In summary, this study examined the integration and coordination of multiple *S. sclerotiorium* pathogencity factors during the infection and their interaction with the host.

ACKNOWLEDGMENTS

I thank my supervisors for the exceptional mentorship they provided. They have enlightened me in many different ways, but I will always remember Dr. Dwayne Hegedus for teaching me how to be a critical thinker, Dr. George Khachatourians for teaching me how to be comprehensive and Dr. Roger Rimmer for teaching me how to be analytical.

I dedicate this thesis to my parents. Their contribution is substantial, but I thank most my father Fayez Dallal Bashi for his inspiration and passion for knowledge and education and my mother Suha Almasri for teaching me to work hard to achieve my goals. I also thank my brother Saeed Dallal Bashi for his support at all times.

I thank my advisory committee members for their valuable discussions and guidance in my research. I thank the Saskatchewan Canola Development Commission for providing the financial support that allowed me to conduct this important work.

I much appreciate the role friends played as an unlimited source of support. I thank everyone for the great times and memories they brought to my life and motivating me to work hard.

Table of Contents

List of Abbreviations 11
CHAPTER ONE 13
GENERAL INTRODUCTION 13
1.1 From *B. napus* to canola 13
1.2 *Sclerotinia sclerotiorum* 15
1.2.1 Biology 15
1.2.2 Sclerotia formation and survival 16
1.2.3 Botanical epidemiology 19
1.2.4 Host penetration and invasion 20
1.2.5 Tissue degradation 22
1.2.6 Role of oxalic acid 25
1.2.7 Disease management strategies 25
1.3 Plant-pathogen interaction 27
1.3.1 Pathogen detection and host inducible defences 27
1.3.2 Interaction of *S. sclerotiorum* with its host(s) 29
1.4 Project Summary 32
CHAPTER TWO 34
INTERACTION BETWEEN *S. SCLEROTIORUM* POLYGALACTURONASES AND *B. NAPUS* POLYGALACTURONASE INHIBITOR PROTEINS 34
2.1 Abstract 34
2.2 Introduction 34
2.3 Results 38
2.3.1 Identification and characterization of *SsPg2* 38
2.3.2 Expression of *S. sclerotiorum* PGs in *Pichia pastoris* 41
2.3.3 Production of *B. napus* PGIPs in *P. pastoris* and concentration of BnPGIP1 . 45
2.3.4 *In vitro* interaction between SsPG6 and BnPGIP1 47
2.3.5 *In planta* interaction between *S. sclerotiorum* PGs and *B. napus* PGIPs 48
2.3.6 Effect of *B. napus* PGIPs on resistance to *S. sclerotiorum* 53
2.4 Discussion: 54
2.4.1 *S. sclerotiorum* PGs have both pectinolytic and necrotizing activity 54
2.4.2 Heterologous expression of *S. sclerotiorum* PGs and *B. napus* PGIPs 55
2.4.3 *In vitro* and *in planta* interaction between *S. sclerotiorum* PGs and *B. napus* PGIPs 57
2.5 Materials and methods 58
2.5.1 Assembly of *SsPg2* 58
2.5.2 Construction of the *SsPg2*-pPICZα cassette and transformation of *P. pastoris* 59
2.5.3 Expression of *S. sclerotiorum* PGs and *B. napus* PGIPs in *P. pastoris* 59
2.5.4 Polygalacturonase assays 60
2.5.5 Purification and concentration of BnPGIP1 61

2.5.6 Effect of light on *S. sclerotiorum* PGs necrotizing activity 62
2.5.7 *In planta* interaction between *S. sclerotiorum* PGs and *B. napus* PGIPs..... 63
2.5.8 Infection of *A. thaliana* with *S. sclerotiorum*..... 63
CHAPTER THREE: 64
CONCERTED REGULATION OF *S. SCLEROTIORUM CUTINASE A* AND *POLYGALACTURONASE 1* TO OVERCOME PLANT PHYSICAL BARRIERS 64
3.1 Abstract: 64
3.2 Introduction 64
3.3 Results 69
3.3.1 The cuticle as a barrier to infection 69
3.3.2 Identification of *S. sclerotiorum* cutinase genes 70
3.3.3 Expression of *SsCutA, SsPg1* and *SsPg2* during the infection..... 71
3.3.4 Regulation of *SsCutA* by contact with surfaces 73
3.3.5 Regulation of *SsPg1* expression by pH 74
3.3.6 Regulation of *SsPg1* expression by galacturonic acid 74
3.3.7 Regulation of *SsPg1* and *SsCutA* expression by carbon availability 76
3.3.8 The role of calcium and cAMP signalling in *SsCutA* and *SsPg1* regulation ... 77
3.4 Discussion 81
3.4.1 Concerted expression of *SsPg1* and *SsCutA* during infection initiation and lesion expansion: 81
3.4.2 Regulation of *SsCutA* and *SsPg1* 83
3.4.3 Intercellular signals regulating *SsCutA* and *SsPg1* expression 85
3.5 Materials and Methods 86
3.5.1 Fungal cultivation 86
3.5.2 Identification of *S. sclerotiorum* cutinase genes 87
3.5.3 Plant bioassays 87
3.5.4 Expression of *SsCutA* in response to surface contact..... 87
3.5.5 Regulation of hydrolytic enzymes in response to carbon source 88
3.5.6 Pharmacological studies 88
3.5.7 RNA isolation and northern blot analysis 89
CHAPTER FOUR: 90
EXPRESSION AND REGULATION OF *S. SCLEROTIORUM* NECROSIS AND ETHYLENE-INDUCING PEPTIDES 90
4.1 Abstract 90
4.2 Introduction 90
4.3 Results 94
4.3.1 Identification of *SsNep1* and *SsNep2* 94
4.3.2 *In planta* expression of *S. sclerotiorum* NEPs 97
4.3.3 Expression of *SsNep1* and *SsNep2* during the infection 98
4.3.4 Expression of *SsNep2* on different surfaces 100
4.3.5 Role of calcium signalling and cAMP in *SsNep2* regulation 101
4.4 Discussion 106
4.4.1 NEPs in phytopathogens 106
4.4.2 Expression of NEP genes during infection 106
4.4.3 Regulation of NEP genes in response to contact surface 107

4.4.4 Signaling mechanisms regulating NEP gene expression 108
4.4.5 Summary 109
4.5 Materials and methods 109
4.5.1 Fungal cultivation 109
4.5.2 Isolation and analysis of *SsNep1* and *SsNep2* 110
4.5.3 *In planta* production of *S. sclerotirum* NEPs via the Potato Virus X (PVX) system 111
4.5.4 Expression of *SsNep2* during the infection 111
4.5.5 Expression of *SsNep2* in response to surface contact 111
4.5.6 Pharmacological studies 111
4.5.7 RNA isolation, northern blot and reverse transcription analysis 111
CHAPTER FIVE 113
ROLE OF THE *S. SCLEROTIORUM* MAPK SMK3 IN PATHOGENICITY 113
5.1 Abstract 113
5.2 Introduction 113
5.3 Results 119
5.3.1 Changes in MAPK phosphorylation in response to contact with different surfaces 119
5.3.2 Characterization of *S. sclerotiorum* pathogenicity and cell wall integrity MAPKs 120
5.3.3 Generation of *Smk3* disruption mutant 123
5.3.4. Growth habits and sclerotia formation in *Smk3υ* 124
5.3.5 Hydrophobicity of *Smk3υ* mycelia 126
5.3.6 Cell wall integrity and heat tolerance in *Smk3υ*. 127
5.3.7 Virulence of *Smk3υ* on *B. napus* 128
5.4 Discussion 130
5.4.1 Identification of pathogenicity-related MAPK 130
5.4.2 Role of *Smk3* in aerial mycelia formation 131
5.4.3 Role of SMK3 in mycelia branching and sclerotia formation 132
5.4.3 Role of SMK3 in response to high temperatures 133
5.4.4 Role of SMK3 in proper cell wall formation 133
5.4.5 Role of SMK3 in virulence 134
5.5 Materials and methods 134
5.5.1 Effect of contact surface and carbon availability on MAPK phosphorylation and gene expression 134
5.5.2 Microscopy 135
5.5.3 Incubation in cell wall degrading enzyme cocktail 135
5.5.4 Hydrophobicity measurement 136
5.5.5 Effect of temperature on growth 136
5.5.6 Pathogenicity assay 136
CHAPTER SIX 137
GENERAL DISCUSSION 137
REFERENCES 145
APPENDIX 172

ISOLATION OF FUNGAL HOMOKARYOTIC LINES FROM HETEROKARYOTIC TRANSFORMANTS BY SONIC DISRUPTION OF MYCELIA 172

List of Figures:

Chapter One

Figure 1.1 Stem rot symptoms on *B. napus* ……..13
Figure 1.2 *S. sclerotiorum* infection on *B. napus*……..19

Chapter Two

Figure 2.1 Sequence and phylogenetic analysis of fungal polygalacturonases. 40
Figure 2.2 Assembly of *SsPg*2 exons.....42
Figure 2.3 Production of *S. sclerotiorum* PGs in *P. pastoris.*.....43
Figure 2.4 Necrosis caused by infiltration of *N. benthamiana* leaves with SsPG3 and SsPg6.....49
Figure 2.5 Effect of cuticle on *S. sclerotiorum* PGs necrotizing activity 50
Figure 2.6 *In planta* inhibition of SsPG3 and SsPG6 by BnPGIP1 and BnPGIP2.....52
Figure 2.7 Susceptibility of *A. thaliana* lines expressing *BnPGIP1* or *BnPGIP2* to *S. sclerotiorum* 53

Chapter Three

Figure 3.1 Role of cuticle and carbon availability in *S. sclerotiorum* infection development. 69
Figure 3.2 Alignment of potential *cutinase* genes from *S. sclerotiorum.* 70
Figure 3.3 Expression of *SsPg1* and *2* under different contact and nutritional conditions. 71
Figure 3.4 Expression of *SsCutA* and *SsPg1* during infection of *B. napus* leaves.....72
Figure 3.5 Expression of *SsCutA* in response to contact with different surfaces.....73
Figure 3.6 Expression of *SsPg1* in response to pectin and pH.....74
Figure 3.7 Regulation of *SsPg1* expression by pectin and galacturonic acid. 76
Figure 3.8 Expression of *SsPg1* in response to carbon availability. 77
Figure 3.9 Effect of calcium and cAMP signalling and calcineurin inhibitors on *S. sclerotiorum* virulence, growth and *SsPg1* and *SsCutA* expression.....79
Figure 3.10 Effect of exogenous cAMP application on *S. sclerotiorum* infectivity and expression of *SsCutA* and *SsPg1*……...81

Chapter Four

Figure 4.1 Sequence and phylogenetic analysis of *S. sclerotiorum* NEPs.....96
Figure 4.2 The Potato Virus X-based binary vector pgR107 used for *in planta* expression of *S. sclerotiorum* NEPs.....97
Figure 4.3 Expression of *S. sclerotiorum* NEPs during infection of *B. napus* leaves....99
Figure 4.4 Expression of *SsNep2* in response to contact with solid surfaces. 100
Figure 4.5 Effect of signalling inhibitors on *S. sclerotiorum* infectivity and growth. .103
Figure 4.6 Effect of signalling inhibitors on *SsNep2* expression during infection of *B. napus* leaves. 104

Figure 4.7 Effect of exogenous cAMP application on *S. sclerotiorum* infectivity and expression of *SsNep2*. 105

Chapter Five

Figure 5.1 Alteration in phosphorylated *S. sclerotiorum* MAPKs in response to contact with solid surfaces.....120
Figure 5.2 Alignment of Slt2-like MAPK orthologues from filamentous fungi.....122
Figure 5.3 Expression of *Smk1* and *Smk3* in response to glucose and contact with various leaf surfaces.....123
Figure 5.4 Effect of *Smk3* mutation on *S. sclerotiorum* mycelial branching. 124
Figure 5.5 Effect of *Smk3* mutation on *S.sclerotiorum* aerial mycelia formation…....125
Figure 5.6 Effect of *Smk3* mutation on *S. sclerotiorum* mycelial hydrophobicity…...126
Figure 5.7 Effect of *Smk3* mutation on *S. sclerotiorum* susceptibility to cell wall degrading enzymes……....127
Figure 5.8 Effect of *Smk3* mutation on *S. sclerotiorum* virulence……...129
Figure 5.9 Effect of *Smk3* mutation on *S. sclerotiorum* infection cushion formation..130

Chapter Six

Figure 6.1 Model for expression regulation of pathogenicity factors during infection.142

List of Abbreviations

AC	Acetone
AS	Ammonium sulfate
Avr	Avirulence
BABA	β-aminobutyric acid
cAMP	Cyclic adenosine monophosphate
DAMPs	Danger associated molecular patterns
DEPC	Diethylpyrocarbonate
DNSA	Dinitrosalicylic acid
DTT	Dithiothreitol
ETI	Effector triggered immunity
ET	Ethylene
EDTA	Ethylene Diamine Tetraacetic Acid
EGTA	Ethylene Glycol Tetraacetic Acid
ERK2	Extracellular signal-regulated protein kinase
GA	Galacturonic acid
OH^-	Hydroxyl radical
H_2O_2	Hydrogen peroxide
HR	Hypersensitive response
ISR	Induced systemic resistance
LTP	Lipid transfer protein
JA	Jasmonic acid
MS	Minimal salt medium
MS-Glu	Minimal salt medium with 1% glucose
MAPK	Mitogen activated protein kinase
NO	Nitric oxide
ORF	Open reading frame
PR	Pathogenesis-related
PAMPs	Pathogen associated molecular patterns

PTI	PAMP triggered immunity
PR	Pathogenesis related
PME	Pectin methyl esterase
PRI	Porcine RNase inhibitor
PDA	Potato dextrose agar
PVX	Potato virus x
PCR	Polymerase chain reaction
PKA	Protein Kinase A
QTL	Quantitative trait loci
R	Resistance protein
QTL	Reactive Oxygen Intermediates
SA	Salicylic acid
ROI	Superoxide
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SAR	Systemic acquired resistance
TBST	Tris Buffered Saline with Tween
TCA	Trichloric acid
4CSA	4 chlorosalicylic acid

CHAPTER ONE

GENERAL INTRODUCTION

1.1 From B. napus *to canola*

Canada exports annually over $3 billion of canola seed, oil and meal. 60,000 people work in canola production and over 2,800 in the processing industries. In total, the canola industry contributes over $13 billion to the Canadian economy (Canola Council of Canada) making canola not only a major crop, but also an important economic element for Canada. Canola is a domesticated form of *Brassica napus* or *B. rapa* in which the erucic acid level is below 2% in the oil and the glucosinolates level is lower than 30 moles per gram in the meal (Ackman, 1990). *Brassica* species are believed to have evolved from three ancestors under a scheme known as the triangle of U. The three original genomes derive from *B. rapa* (n=10, AA), *B. nigra* (n=8, BB) and *B. oleracea* (n=9, CC). *B. oleracea* and *B. rapa* are ancestors of *B. napus* (n=19, AACC), *B. rapa* and *B. nigra* are ancestors of *B. juncea* (n=18, AABB) and *B. oleracea* and *B. nigra* are ancestors of *B. carinata* (n=17, BBCC). This theory was first published in 1935 by Woo Jang-choon who created hybrids between the diploid and tetraploid species and examined chromosome pairing (U, 1935). Modern molecular biology tools for DNA analysis have confirmed these original findings. Besides *B. napus* and *B. rapa* (canola), other *Brassica* species, including *B. carinata* and *B. nigra*, are also being developed as sources of edible oil or for industrial applications.

The first record of rapeseed cultivation dates back to 5000 B.C. in China (Yan, 1990). In India, rapeseed was cited in the literature in 1500 B.C. where the main use of the plant was for the production of lamp oil. In Europe, *Brassica* species have been cultivated since the 13th century and were used to

provide lubricant for steam engines during World War II. By the end of the war, demand for canola oil dropped leading to the search for alternative markets. The main challenges for the use of oilseed rape for the production of edible oil were the green color due to chlorophyll, the pungent taste from glucosinolates and the high erucic acid levels. In the 1970s, Drs. Keith Downey and Baldur Stefansson produced the first line of lower erucic acid *B. napus* through conventional plant breeding. This was made possible by a technique that allowed the division of the seed so that one-half could be evaluated using gas-liquid chromatography and the other half germinated to propagate the line. This allowed for the selection of desirable traits in growing plants and led to the development of 18 different varieties of canola (Phillips and Khachatourians, 2001).

Canola is a host for many pests including the flea beetles (*Phyllotreta sp.*), the diamondback moth (*Plutella xylostella*) and the bertha armyworm (*Mamestra configurata*). Viruses also attack canola, including the western yellow virus which causes loss of production. Most relevant to this study are phytopathogenic fungi, such as *Leptosphaeria maculans* which is responsible for blackleg disease, *Alternaria brassicae* which causes alternaria black spot and *Rhizoctonia solani* which causes root rot. One of the most prevalent diseases of canola in western Canada is stem rot (Dmytriw and Lange, 2005, McLaren et al., 2006, Pearse et al., 2006) which is caused by *S. sclerotiorum*. The economic impact of this disease is significant as it causes 5-100% yield losses on an annual basis. An outbreak of the disease in western Canada in 1982 caused a loss of $15 million dollars (Bailey, 2003). *S. sclerotiorum* is also a global problem as significant damage has been reported in Germany (Kruger, 1975b). In central and eastern parts of Finland the disease prevents the production of oilseed rape (Jamalainen, 1954). Damage can be measured in both quality and yield of the crop. Premature ripening of seed pods causes shattering during harvest (Kruger, 1973) reducing yield (Morral et al., 1977), while reduced seed size translates into reduced oil content and quality (Aggarwal et al., 1997). Sclerotia may also contaminate the seed reducing crop

quality and prohibiting export (Hoes and Huang, 1976, Kruger et al., 1981). An indirect cost associated with the disease is expenditure on disease control. The aim of my study is to provide a better understanding of *S. sclerotiorum* biology, specifically the mechanisms underlying infection and interaction with its host.

Figure 1.1 Stem rot symptoms on *B. napus*

1.2 Sclerotinia sclerotiorum

1.2.1 Biology

S. sclerotiorum is the causative agent of stem rot of canola in western Canada (Nelson, 1998). It is also responsible for a range of other diseases, including cottony soft rot on lettuce, white mould on soybean and watery soft rot on cabbage and cauliflower. The pathogen has long been recognized for the diseases it causes, but the earliest formal description was that of Liebert in 1837 who gave it the name *Peziza sclerotiorum.* Later, Fuckel described in detail members of the genus *Sclerotinia,* but continued to use the name *P. sclerotiorum.* In 1924, Wakefield suggested that the name was inconsistent with the Botanical Nomenclature and the fungus was renamed *S. sclerotiorum.* As de Bary was the first to use this name in 1884, the proper name became *S. sclerotiorum* (Lib.) de Bary. Both *S. sclerotiorum* and *Botrytis cinerea* cause lettuce drop. The distinction between the two did not occur until the early 1900s

when Smith demonstrated that *S. sclerotiorum* was the dominant agent causing the disease (reviewed by Sharan and Mehta, 2007).

S. sclerotiorum is a non-specific pathogen and infects a wide range of plants. Its necrotizing nature allows it to attack different hosts and to apply similar mechanisms to overcome plant defences. This wide host range also makes crop rotation difficult as planting options become limited. Scientists continue to discover hosts for this pathogen which include 408 species from 278 genera in 75 families (Boland and Hall, 1994). Major crops including the seeds of canola, sunflower, soybean, chickpea, lentil and many vegetable crops are among the hosts listed. Most hosts are dicotyledonous herbaceous plants, but a few monocotyledons were also found to be susceptible to the fungus (Saito and Tkachenko, 2003). Identifying the host range of the pathogen allows a better understanding of resistance mechanisms in non-host plants.

The general symptoms of *S. sclerotiorum* infection vary depending on the host. On *B. napus*, stem infection appears as grey, watery lesions developing at the axis of the leaf petiole and the stem where senescent petals collect. The infection spreads down into the stem and up to the leaf. The infected stem becomes bleached and the outer tissues shred, leading to death of the stem and premature ripening of upper tissue. Few or no seeds develop if the infection occurs at flowering. Inside the stem, a cottony, white mold surrounds hard, black structures known as sclerotia, which are able to survive in the soil for an extended time to re-initiate the infection (Adams and Ayers, 1979).

1.2.2 Sclerotia formation and survival

Sclerotia provide a long term survival mechanism for *S. sclerotiorum* and can remain dormant for up to eight years in the soil (Willetts and Wong, 1980; Adams and Ayers, 1979). Sclerotia are formed from dense mycelia that are embedded in a matrix of carbohydrates, primarily β-glucans, and proteins (LeTourneau, 1979). Multiple layers reinforced by melanin surround the viable center and provide a shell that protects the viable core from harsh environmental conditions, such as UV exposure or biotic stresses including soil

microbes and animal ingestion (Henson et al., 1999). Sclerotia are produced at the end of the growth cycle in a three stage process. The first step is initiation, where mycelia aggregate to form dense structures known as sclerotia initials. The initials thicken and increase in size to the point where internal mycelia consolidate; this step is known as development. The final step is maturation in which melanin accumulates in the peripheral cells and the rind darkens (Willetts, 1997). On the cellular level, the walls of cells in the medulla, the inner core of the sclerotia, thicken and become covered with a fibrillar layer composed of β-glucan and protein (Saito, 1974). The rind is usually three cell layers thick and covers the fibrillar matrix that contains the vital core (Colotelo, 1974).

In vitro, sclerotia formation follows a defined and consistent pattern. Upon inoculation of solid medium, mycelia grow in a thin layer on the surface until they reach the outer edge of the plate. Mycelia then thicken to form mycelial aggregates which release exudates. As the aggregate grows, the exudate droplet increases in size, but later disappears coincident with maturation and darkening of the sclerotia. The process of sclerotia formation is completed within one week. Microscopic studies of sclerotia formation showed that anastomosis of long mycelia leads to the formation of the initials which in turn fuse with other initials to form a sclerotium. The medullary cells continue to grow and intercellular spaces fill with the exudate. Rind formation is initiated from cells beneath the surface mycelial network (Colotelo, 1974).

A variety of parameters affect sclerotia development, the most important being a shortage of nutrients. *In vitro* studies demonstrated that an acidic pH is also required for sclerotia formation (Rollins and Dickman, 2001). Mutants that were unable to produce oxalic acid and lower medium pH lost their ability to produce sclerotia; however, lowering the medium pH artificially did not recover sclerotia forming ability suggesting that oxalic acid may play other roles in sclerotia formation (Rollins and Dickman, 2001).

The extracellular influences on sclerotia formation are translated through a complex signalling network. Cyclic adenosine monophosphate (cAMP) was

found to determine the point of transition between mycelial growth and sclerotia formation and supplementation of growth medium with cAMP inhibited sclerotia formation (Rollins and Dickman, 1998). cAMP was also found to inhibit the expression of a gene encoding a MAPK known as *Smk1*. *Smk1* was induced under acidic pH conditions and was proposed to regulate the response to medium pH, while cAMP regulates the response to carbon availability (Chen et al., 2004). Furthermore, protein kinase A (*PKA*) was demonstrated to be involved in sclerotia development since an increase in transcripts corresponded with sclerotia formation and mutants that were impaired in sclerotia formation did not accumulate *PKA* transcripts (Harel et al., 2005).

Sclerotia must resist harsh environmental conditions as they are the primary survival structure. Although large numbers of sclerotia are produced during disease epidemics the soil population of sclerotia remains stable suggesting that the survival rate is rather low (Abawi and Grogan, 1979; Grogan, 1979), though some survive for extended periods of time, up to 8 years, in the soil (Quentin, 2004). Many factors affect sclerotia survival, but the most important are interaction with soil biotic and abiotic factors. Sclerotia survive for longer periods of time in dry soil (Coley-Smith and Cooke, 1971; Imolehin and Grogan, 1980). The ability to prevent water loss is due to resistance of individual cells to desiccation and not the sclerotium rind (Trevethick and Cooke, 1973). High temperatures, such as 37ºC, for extended periods of time reduce sclerotia survival (Adams, 1975) and wet soil combined with high temperatures severely reduces viability (Cook et al., 1975; Willetts and Wong, 1980). Sclerotia buried at a depth of 20 cm for over 18 months survived, while surface sclerotia only survived for 6 months (Sharma and Sharma, 1986). The soil coat protects *S. sclerotiorum* from dramatic change in environmental conditions including heat and moisture. The host from which the sclerotia were derived also factors into their survivorship, as sclerotia were engulfed with soy bean tissue were able to survive for longer periods of time than those that were not (Merriman et al., 1979). Soil microorganisms also

impact sclerotia viability. *Sporidesmium sclerotivorum* (Adams and Ayers, 1979), *Coniothyrium minitans,* and *Trichoderma spp.*have been identified as *S. sclerotiorum* antagonists (Coley-Smith and Cooke, 1971).

1.2.3 Botanical epidemiology

Successive wetting/drying events and low temperature are necessary for sclerotia conditioning prior to germination (Bardin and Huang, 2001). When environmental conditions are favourable, such as temperature 15 ºC and soil water content -0.03 to -0.07 MPa (Hao et al., 2003), sclerotia germinate to produce mycelia (myceliogenic) or apothecia (carpogenic) (Huang and Kozub, 1989; Morrall, 1977; Clarkson et al., 2004). The apothecium is considered the fruiting body and consists of a stipe carrying a receptacle. Rows of asci occur as cylindrical sac-like structures on the receptacle, each containing eight ascospores. Under low nutritional conditions, such as in moist sand, sclerotia germinate carpogenically. When nutrients are available, sclerotia germinates myceliogenically. Apothecia initials are generated in the medulla as a brown knot of interwoven mycelia and eventually break the surface of the sclerotium. The initial then develops into a stipe that under certain light conditions differentiates at one end to form a disk. The disc consists of an ectal excipulum, a medullary excipulum, the hymenium, and the sub-hymenium (Kosasih and Willetts, 1975). Stipes were found to exhibit a dominance effect, as removal of the main stipe from a sclerotium led to the generation of more stipes (Kapoor, 1994; Saito, 1997).When the soil include organic nutrients or the mycelia went through chilling cycle of 3ºC for four weeks, sclerotia germinate to form mycelia that can grow for 2-3 mm (Huang, 1991). Myceliogenic germination also inhibits apothecia formation (Saito, 1977). Crops such as sunflower are infected through mycelia traveling in the soil.

Canola is mostly infected through ascospores which are highly effective in spreading the disease. Changes in atmospheric pressure cause the apothecia to releases puffs of ascospores. Under favourable conditions, a single apothecium may release up to 2.32 million ascospores over a period of one week (Schwartz

and Steadman, 1987). Dependent upon humidity, wind and temperature the apothecium can release ascospores at a rate that can reach 1600 spores per hour for up to 10 days (Clarkson et al., 2003). While the majority of the ascospores infect plants in the same field, some can travel for several kilometres spreading the infection to neighbouring fields (Wegulo et al., 2000). *S. sclerotiorum* also produces microconidia, but these do not germinate and their role is not clear (Kohn, 1979).

1.2.4 Host penetration and invasion

Upon reaching the host, ascospores attach themselves through a sticky layer of mucilage that also allows them to survive ultraviolet light and dry conditions for about 2 weeks (Clarkson et al., 2003). Similar to sclerotia, ascospores survive for longer periods of time under dry conditions and moderate heat (Grogan and Abawi, 1975). At 20 ºC, ascospores develop a germ tube within 6 hours (Abawi and Grogan, 1975). Though they are able to germinate on the leaf surface, ascospores are unable to directly infect vegetative tissues (Lumsden, 1979). On *B. napus*, dead petals provide a source of water and nutrients to generate sufficient mycelial biomass to initiate infection. One of the earliest steps in the infection process is cuticle penetration (Abawi et al., 1975; Lumsden and Dow, 1973). Upon contact with the host, mycelia cells divide to form finger–like appendages that develop into multi-celled, dome-shaped structures referred to as infection cushions (Abawi et al., 1975).

Examination of the infection on rapeseed via scanning electron microscopy revealed that on young petals, ascospores attach, germinate and then penetrate the host and from short germ tubes leading to collapse of epidermal cells (Jaumaux, 1995). No histological studies have examined *S. sclerotiorum* infection on rapeseed vegetative tissue and our understanding of the process is based on the infection of bean (Lumsden and Dow, 1973). After cuticle penetration, *S. sclerotiorum* develops inflated, stain resistant mycelia vesicles between the cuticle and epidermal cells that encourage infection mycelia formation. From these vesicles, mycelia grow between the cuticle and the

epidermal cell layer and downwards between cells of the cortex. The sub-cuticular mycelia aggregate and form a fan-shaped infection front beneath the cuticle. After 12-24 hours, radial mycelia divide into clusters of 18 to 20 mycelia and invade more host tissue. The infection mycelia are present at the advancing margin of the lesion and are responsible for breaking down host defences and colonization of tissue. Factors released from the infection mycelia are probably responsible for symptoms in the advancing margin, including degradation of cell wall pectin (Lumsden and Dow, 1973), cell death and accumulation of fluids (Hancock, 1972).

Figure 1.2 *S. sclerotiorum* infection on *B. napus* .

Senescent petal lodged between the leaf and stem axis that is infected with *S. sclerotiorum*. The mycelia have spread into the leaf and infection established.

Behind the leading margin, secondary mycelia complete the infection cycle by degrading the components of dying cells and scavenging host-derived nutrients. The secondary mycelia are smaller in diameter than infection mycelia, but are still capable of penetrating host cell walls (Lumsden and Dow, 1973). Secondary mycelia also branch extensively and occupy the intercellular spaces in the cortex where they break down the crystalline structure of the host cell wall (Calonge et al., 1969). Upon complete utilization of host tissue, infection mycelia emerge from the stomata or erupt through the cuticle (Lumsden and Dow, 1973). Sclerotia then form on the host surface or under decaying plant parts in the soil. Such a highly evolved interaction must be

orchestrated through coordination of complex biochemical mechanisms governed by factors from the host and the pathogen.

1.2.5 Tissue degradation

A few studies have suggested that cuticle penetration by *S. sclerotiorum* occurs through enzymatic processes, but as yet there is no direct evidence of such. Attempts to distinguish cuticle penetration due to enzymatic activity from that of mechanical pressure failed to detect alteration in cuticle structure due to biochemical changes (Purdy et al., 1958). Later studies suggested that enzymatic action was associated with cuticle penetration. Ultracytochemical techniques showed lipolytic enzyme activity beneath the points of leaf contact with mycelia (Tariq and Jeffries, 1987; Prior and Owens, 1964)

On the other hand, cell wall penetration has been clearly demonstrated to occur through the action of pectinolytic and cellulolytic enzymes (Held, 1955; Morrall et al., 1972; Riou et al., 1991). The earliest studies demonstrated that the pectic content of diseased tissue decreased which suggested that the pathogen secretes pectinolytic enzymes (Hancock, 1966). In parallel, other studies revealed that histochemical and structural changes occur in host middle lamellae where pectin resides during the infection (Calonge et al., 1969). Later studies reported that pectinolytic enzymes were produced during infection as well as in *S. sclerotiorum* cultures *in vitro* (Lumsden, 1976). The first peak in pectinolytic enzyme activity was attributed to an endopolygalacturonase based on the random hydrolysis of pectin polymer. During the infection, a peak of pectinolytic activity was observed after 24 hours, which then declined thereafter. *In vitro*, supplementation of the culture media with glucose suppressed the production of pectinolytic enzymes. It was suggested that hydrolysis products in infected tissue suppressed pectinolytic enzyme production similar to glucose *in vitro*. The advancing margin of the lesion was found to have the highest level of pectinolytic activity, while the necrotic zone had little (Lumsden, 1976).

Pectin is a complex polymer and several enzymes are required to hydrolyze it completely. Pectin is a polymer of galacturonic acid with branches of more complex polysaccharides including homogalacturonan, rhamnogalacturonan 1 and rhamnogalacturonan 2, as well as xylogalacturonan (Harholt, 2010). Pectin methyl esterase (PME) is a key enzyme for the demethylation of pectin. It is released by the fungus during infection and is distinguishable from plant PME by its activity at lower pH; the pH optimum of pathogen PME is 5, while that of host PME is 8 (Barkai-Golan, 1974; Morrall et al., 1972). A suite of enzymes degrade pectin in the middle lamella to facilitate the intercellular invasion of host tissue (Lumsden and Dow, 1973). Demethylated pectin is the preferred substrate for endo- and exo-polygalacturonases (PGs) which further hydrolyze pectate. This provides both a source of carbon and creates space for the mycelia to ramify through the plant tissue.

In addition to pectin degrading enzymes, necrotizing pathogens such as *Sclerotinia* species are expected to release enzymes capable of degrading cellulose. *Sclerotinia minor* secretes a battery of cellulases and hemi-cellulases (Barkai-Golan, 1974; Bauer et al., 1977). Cellulase C1 hydrolyses insoluble cellulose, while Cx hydrolyzes soluble cellulose. β,1-3 glucosidase hydrolyses cellobiose to produce glucose (Lumsden, 1969). During infection, large amounts of cellulase are found in extracts from infected leaves (Barkai-Golan, 1974) which declines in older lesions (Lumsden, 1969). Cellulases alter cellulose structure in the plant cell wall (Calonge et al., 1969) which is necessary for expansion of the infection.

Xylan is an important component of the plant cell wall. It is a complex polysaccharide composed mainly of xylose (Knob et al., 2009). Until recently, no studies have examined the ability of *S. sclerotiorum* to hydrolyze xylan. A recent study identified a putative β-xylosidase, an enzyme that hydrolyses xylan, and demonstrated that disruption of the gene can severely reduce *S. sclerotiorum* virulence on *B. napus* (Yajima et al., 2009).

In addition to cell wall degrading enzymes, other hydrolytic enzymes are produced by *S. sclerotiorum*. Phosphatidase β, an enzyme that hydrolyses

phosphatide components of cell membrane, is produced in abundant amounts early in bean infections (Lumsden, 1970). Proteolytic enzymes, likely involved in both cell wall and protoplasm degradation, were also found to be expressed during infection (Newton, 1972).

Earlier studies of enzymatic activity in *S. sclerotiorum* were based on detection of functions such as pectic or lipolytic. Genomic and proteomic studies confirmed earlier findings through identification of genes responsible for such functions. Examination of *S. sclerotiorum* expressed sequence tags from the infection zone identified genes encoding several enzymes likely to be involved in cell wall degradation (Li et al., 2004a). These included endo-β-1,4-glucanase, β-1,3-glucanase, endoglucanase 1, neutral endopolygalacturonases SSPG1 and SSPG6, acidic endopolygalacturonase SSPG5, 1,4-β-D-glucan cellobiohydrolase B, and two exopolygalacturonases (Li et al., 2004a). Another study characterized five different polygalacturonases from *S. sclerotiorum.* Carbon source, pH and contact with a solid surface were found to regulate the expression of *S. sclerotiorum* PG genes (Li et al., 2004b). Proteomic analysis of the *S. sclerotiorum* secretome further confirmed the genomics results. The majority of the proteins detected during infection were enzymes involved in maceration of host tissue; these included five polygalacturonases, two pectin methyl esterases, one α-arabinofuranosidase (an enzyme involved in cellulose hydrolysis), one cellobiohydrolase and three proteases (Yajima and Kav, 2006). These findings further support the involvement of tissue macerating enzymes in *S. sclerotiorum* infection.

The arsenal of hydrolytic enzymes produced by the pathogen during infection not only ensures the destruction of the host, but that valuable nutrients will be available for perpetuating the infection. Mycelia ramifying beyond the expanding margin were reported to be responsible for the release pectin degrading enzymes and degradation of host cell walls making carbon available (Lumsden and Dow, 1973). Both cellulose and hemicellulose were degraded to some extent early during in the infection, but more extensively in the later stages (Hancock, 1967, Lumsden, 1969). Nitrogen and other nutrients become

available through the activity of enzymes such as phosphatidase and proteases (Newton, 1972).

1.2.6 Role of oxalic acid

Oxalic acid is released by a wide range of fungi and is suspected to play multiple roles in pathogenicity and fungal competition (Dutton and Evans, 1996). The release of oxalic acid during *S. sclerotiorum* infection was first described by de Bary in 1886 (reviewed in Ainsworth, 1981). Infected carrot tissue was highly acidic and possessed oxalate in the form of a calcium salt. Oxalic acid recovered from the infected tissue was shown to cause disease symptoms upon infiltration into the plant (Marciano et al., 1983; Riou et al., 1991). Oxalate oxidase converts oxalic acid into CO_2 and H_2O_2. Expression of the wheat oxalate oxidase gene in *B. napus* improved *S. sclerotiorum* resistance by 88.4-90.2%. (Dong et al., 2008).Accumulation of oxalic acid at the infection margin decreased the ambient pH to 4-5 which both induced the transcription of genes encoding cell wall degrading enzymes and enhanced their activity (Marciano et al., 1983). Low pH may also protect PGs from interaction with inhibitors produced by the host (Favaron et al., 2004). Furthermore, chelation of Ca^{+2} in the middle lamella destabilizes pectin structure and facilitated the action of PGs (Kurian and Stelzig, 1979). Oxalic acid also interfered with Ca^{+2} signalling which is required for induction of the plant defence response (Bateman and Beer, 1965) and suppressed the oxidative burst (Cessna et al., 2000), a major host defence mechanism. It was also found to suppress the activity of certain defence enzymes, such as polyphenol oxidases (Marciano et al., 1983). The low pH caused by oxalic acid is believed to be an indirect cause of cell death and mutants unable to produce oxalic acid were non-pathogenic (Godoy et la, 1990).

1.2.7 Disease management strategies

Many approaches are currently in use for control of stem rot, including crop rotation, tillage control and biological control. The use of fungicide is costly and undesirable due to environmental and health concerns. Crop rotation

is sometimes impractical since the disease can survive in the soil for many years, while stubble removal is laborious and adds to the cost of production. One of the main challenges in breeding lines resistant to *S. sclerotiorum* is the involvement of multiple genes making introgression of the trait difficult. Resistance to *S. sclerotiorum* in *B. napus* was shown to be partially dominant (Huang et al., 2000). One of nine genetic markers associated with glucosinolates content was also associated with resistance to *S. sclerotiorum* (Zhao and Meng, 2003b). Several loci associated with partial resistance to *S. sclerotiorum* were identified in *B. napus* populations (Zhao and Meng, 2003a). Quantitative trait loci (QTL) for resistance of sunflower to *S. sclerotiorum* have also been identified. Three QTL for lesion length and two QTL for head rot were identified (Ronicke et al., 2005). Such information assists breeding efforts in the development of varieties with improved disease tolerance.

The challenges associated with these approaches have directed efforts toward enhancement of plant defences through biotechnology. Oxalate oxidase catalyzes the oxidation of oxalic acid by oxygen to CO_2 and transformation of plants with the corresponding gene improved resistance in many crops (Donaldson et al., 2001; Hu et al., 2003; Kesarwani et al., 2000; Livingstone et al., 2005). Cultivars of *B. juncea* resistant to *S. sclerotiorum* accumulated phenolic compounds at the infection site and exhibited lower fungal pectinolytic activity than susceptible cultivars (Rai et al., 1979). Other genetic engineering strategies have focused on the over-expression of genes involved in plant defences. Introduction of an endochitinase gene under the control of a constitutive promoter into *B. napus* improved resistance to *S. sclerotiorum* (Grison et al., 1996). A successful approach to engineer *B. napus* with resistance to *S. sclerotiorum* was to create an apetalous line to limit the amount of senescent material accumulating in the leaf-stem axis (Liu et al., 1990). Most recently, Yajima et al., (2009) generated a single-chain antibody against SsPG1. *B. napus* transformed with a gene encoding the anti-SsPG1 antibody were more tolerant to *S. sclerotiorum* (Yajima et al., 2010).

1.3 Plant-pathogen interaction

1.3.1 Pathogen detection and host inducible defences

Plants are constantly under attack by a wide range of pathogens. Due to their immobility, they have developed basal and inducible defence mechanisms to protect themselves (Lamb and Dixon, 1997). One form of basal defence is the production of compounds that improve disease tolerance, including hydrocinnamic acid esters, lignins, flavonoids, glucosinolates, salicylic acid and other molecules. In another collaborative project, I demonstrated that a mutation causing sinapine and lignin reduction in *Arabidopsis thaliana* increased susceptibility to *S. sclerotiorum* (Huang et al, 2009). Other secondary metabolites, such as tannins and saponins, also have anti-microbial properties (Osbourn, 2003).

Plants have highly sensitive and selective systems to detect and respond to pathogens which must be overcome to cause disease. During the initial stages of the infection, cuticle hydrolysis by the pathogen releases cutin monomers that are detected by the host. Such metabolites are referred to as danger associated molecular patterns (DAMPs) and are detected by host pattern recognition receptors (PRRs). These receptors may also detect molecules associated directly with the pathogen, such as fungal chitin or bacterial lipopolysaccharides; these are known as pathogen associated molecular patterns (PAMPs) (Boller and Fellx, 2009). A pertinent indication that such interactions induce plant defences comes from the observation that application of hydrolysed chitosan to carrots induced resistance to *S. sclerotiorum* (Molloy et al., 2004); this phenomenon is known as PAMP triggered immunity (PTI). Pathogens secrete a variety of proteins, including hydrolytic enzymes (cutinases and polygalacturonases) and a suite of small proteins (Dodds et al., 2009), that promote pathogen infectivity. Collectively, these proteins are referred to as effectors. Recognition of effectors by host intracellular receptors leads to effector triggered immunity (ETI). Over time, selection has given rise to a diverse set of effectors in pathogens and an equally diverse set of receptors in

the corresponding host. Both PTI and ETI lead to activation of the plant defence system. ETI is generally a stronger inducer and leads to the hypersensitive response and localized cell death, while PTI is highly effective for non-adapted pathogens and leads to non-host resistance.

Two types of inducible resistance may be activated upon recognition of a pathogen effector, systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR was first described in tobacco, when healthy leaves from a plant infected with Tobacco Mosaic Virus showed enhanced resistance (Ross, 1962). SAR was also found to be induced by a wide set of pathogens and improved resistance to a non-inducing organism, for example, pre-inoculation of *Phaseolus vulgaris* with *Colletotrichum lindemuthianum* induced resistance against *C. lindemuthianum* and *Uromyces appendiculatus* (Dann and Deverall, 1995). This defence mechanism was found to be very broad in spectrum and provided improved resistance to viruses, bacteria, oomycetes and fungi (Durrant and Dong, 2004), though it is not always effective across pathogen types as *A. thaliana* treated with benzothiadiazole to induce SAR were still susceptible to the necrotizing pathogen *B. cinerea* (Friedrich et al., 1996). Application of oxalic acid to the leaf of oilseed rape induced significant local resistance to *S. sclerotiorum* in the tissue surrounding the application site and a high level of resistance in the leaf close to the petiole (Toal and Jones, 1999).

SAR occurs in three stages; initiation, establishment and manifestation. The initiation stage is triggered by the interaction between the plant and a microbe and leads to induction of the hypersensitive response (HR) signified by rapid cell death in the region surrounding the infection to prevent pathogen spread (Ryals et al., 1996). This reaction causes accumulation of salicylic acid (SA) at the infection site which can travel through the phloem to the rest of the plant to induce defences in distal tissues (Smith-Becker et al., 1998). The time required to establish SAR varies depending on the host. In cucumber, SAR was detected within seven hours after *P. syringae* inoculation (Smith et al., 1991), while *Peronospora tabacina* spores required 2-3 weeks to establish SAR (Cohen et al., 1987). The establishment stage is measured by the perception of

the signal in non-infected leaves and induction of a micro-HR (Alvarez et al., 1998). More definite indications that SAR has been established are the induction of genes encoding pathogenesis-related (PR) proteins in the distant leaves, similar to those induced at the infection site, and the accumulation of SA. Manifestation occurs when the host produces PR proteins rapidly upon the infection with a second virulent pathogen. Other types of SAR responses include increased lignification and cross-linking of cell wall proteins at the site of the secondary infection (Richter and Ronald, 2000). The effect of SA was tested in parallel with 4-chlorosalicylic acid (4CSA), an active salicylic acid analogue, on resistance of kiwifruit to *S. sclerotiorum*. Both molecules were found to reduce lesion size, though 4CSA had a more pronounced effect, suggesting that they induce defence responses that reduced the progress of *S. sclerotiorum* (Reglinski et al., 1997). Inoculation of tomato or cauliflower with *Trichoderma spp.* induced SAR which in turn reduced symptoms after subsequent *S. sclerotiorum* inoculation (Sharma and Sain, 2004).

Other types of resistance such as, ISR and β-aminobutyric acid (BABA)-induced resistance are distinct from SAR, since they do not induce PR gene expression and are SA-independent. Induced systemic resistance is regulated through jasmonic acid (JA) and ethylene (ET), but not SA (Pieterse et al., 1998). Application of elicitors such as chitosan, oxalic acid and salicylic acid significantly inhibited *S. sclerotiorum* growth in the host (Hilal et al., 2006) The JA related MAPkinase MPK4 is induced during infection of *B. napus* resistant varieties and not susceptible ones. Over expression of the *BnMpk4* in *B. napus* improved resistance to *S. sclerotiorum* and was correlated with reduced accumulation of reactive oxygen intermediates (ROI) (Wang et al., 2008). Drenching of soil with BABA improved resistance of artichoke plants to *S. sclerotiorum* (Chilosi and Magro, 2010).

1.3.2 Interaction of *S. sclerotiorum* with its host(s)

The interaction between *S. sclerotiorum* and its hosts is very complex and gene expression studies have shown that many host genes are involved. In the

partially resistant *B. napus* variety RV289, 686 genes were differentially expressed in response to infection with *S. sclerotiorum*, while 1547 were differentially expressed in the susceptible variety Stellar (Zhao et al., 2007). In the resistant variety, twice as many genes were up-regulated 24-48 hours post-inoculation than during the first 24 hours. In contrast, over 24 times the number of genes were up-regulated in the susceptible variety 24-48 hours post infection (Zhao et al., 2007). This suggested that genes up-regulated early in the resistant variety may have been sufficient to stop progress of the infection. The genes expressed in the resistant variety encoded PR proteins, oxidative burst-related proteins, protein kinases, molecule transporters, those involved in cell maintenance and resistance to abiotic stress, as well as several proteins with undefined functions (Zhao et al., 2007). In another study, two other varieties of *B. napus*, the partially resistant variety ZhongYou 821 and the susceptible variety Westar, were found to respond differently to *S. sclerotiorum.* In the resistant variety, defence gene expression was detected much earlier than in the susceptible variety (Zhao et al., 2009). The group of early-expressed genes included defence genes encoding chitinases, glucanases, osmotins and lectins, as well transcription factors, such as members of the WRKY family. The alteration in gene expression patterns also indicated that synthesis of JA, ethylene and auxin increased and gibberellin was actively degraded. The expression of genes associated with production of ROI and secondary metabolite also increased (Zhao et al., 2009).

Upon recognition of a pathogen, plants accumulate ROI, including superoxide (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2). These molecules are highly oxidative and have been demonstrated to disrupt cellular membranes and metabolism as well as causing oxidative changes in the DNA (Halliwell and Gutteridge, 1998). Another key molecule in the oxidative burst is nitric oxide (NO); this molecule is readily produced by plants and plays a major role in the interaction with pathogens NO triggers a signal transduction pathway that involves a protein kinase, Ca^{+2} mobilization and protein modification. These signalling mechanisms subsequently induce cell death and

activate systemic defence genes (Leshem et al., 1996, Delledonne et al., 1998). The accumulation of ROI leads to localized cell death at the infection site, as noted above this is referred to as the HR, a term introduced by Stakman (1915). The oxidative burst and HR are effective mechanisms to control biotrophic pathogens (Lamb and Dixon, 1997; Lu and Higgins, 1999), but may contribute to necrotroph virulence as these pathogens require dead tissue to grow.

Fungi are able to limit the detrimental effects of ROI by deploying enzymes such as catalases and superoxide dismutases. These enzymes participate in converting O_2^- to H_2O_2 and prevent oxidation of cellular components (Fridovich, 1997). Three types of SODs have been identified in fungi including, a cytosolic Cu, Zn-SOD, a mitochondrial Mn-SOD and an extracellular Cu, Zn-SOD (Fridovich, 1997; Halliwell and Gutteridge, 1998). In an expressed sequence tag study, *S. sclerotiorum* was found to express three different types of Cu, Zn-SOD genes (Li et al., 2004a). Catalase is responsible for the breakdown of hydrogen peroxide to oxygen and water (Emri et al., 1997) and *S. sclerotiorum* was found to express a gene encoding a peroxisomal catalase (Li et al., 2004). Internal peroxide is removed by the glutathione-pathway, a pathway that is known for its role in drug detoxification. Glutathione S-transferases are multifunctional detoxifying enzymes and catalyze the conjugation of xenobiotics to glutathione (Dowd et al., 1997). Genes encoding two glutathione S-transferases and one glutathione-disulfide reductase were expressed by *S. sclerotiorum* during infection of *B. napus* (Li et al., 2004a)

B. cinerea spores were able to germinate in 180 mM of hydrogen peroxide and mycelia were found to be even more resistant (Gil-ad and Mayer, 1999). Surprisingly, fungal catalases were not expressed during infection of tomato by *B. cinerea*, while plant catalase was expressed at a high level. This suggested that this pathogen might have an alternative mechanism to tolerate hydrogen peroxide (Van der Vlugt-Bergmans et al., 1997a). Peroxidases may also be involved in tolerance to ROI as they reduce hydrogen peroxide to water. A wide set of peroxidases have been found in fungi and demonstrated to be highly

active during interaction with the host (Nakayama and Amachi, 1999; Eshdat et al., 1997). *S. sclerotiorum* is known to be able to survive the oxidative burst (Govrin and Levine, 2000) and may be using it to induce apoptosis and create necrotic tissue.

The data generated from the global gene expression studies is in agreement with earlier biochemical and histological studies. It is also a morecomprehensive tool to discover genes that may play a role in host-pathogen interactions. The interaction between polygalacturonases and polygalacturonases inhibitor proteins (PGIPs) is an area that has been studied extensively (De Lorenzo et al., 2001; Hegedus et al., 2008) and a more in-depth review is provided in Chapter 1.

1.4 Project Summary

S. sclerotiorum has been studied for over 100 years; despite this, no definite resistance mechanism to the pathogen has been identified. Researchers continue to examine the *S. sclerotiorum* life cycle to identify points where effective disease management strategies can be applied. The development of molecular tools has allowed better understanding of the pathogen and created new opportunities for research on plant-pathogen interactions and signalling pathways. In this study, I examined the molecular interaction between *S. sclerotiorum* and *B. napus* on several levels. The first level examined cuticle penetration where a *S. sclerotiorum* gene encoding a cuticle-degrading enzyme was identified and its regulation studied. The second level looked at penetration of the cell wall and proliferation of the pathogen in the host. In this regard, the regulation of fungal PGs and the interplay with host PGIPs was examined. The third level examined factors involved in tissue necrosis which was found to involve multiple necrosis-inducing proteins and environmental factors, such as light. Host factors affecting necrosis development, specifically PGIPs, were also studied. Finally, this study attempts to unravel the signalling mechanisms that allow the pathogen to regulate the expression of these pathogenicity

factors. The signalling mechanisms were found to involve calcium, cAMP and at least one MAPK working in concert to coordinate the infection process.

CHAPTER TWO

INTERACTION BETWEEN *S. SCLEROTIORUM* POLYGALACTURONASES AND *B. NAPUS* POLYGALACTURONASE INHIBITOR PROTEINS

2.1 Abstract

S. sclerotiorum releases a battery of polygalacturonases (PGs) during infection which the host plant may cope with through production of polygalacturonase inhibitor proteins (PGIPs). I first scanned the *S. sclerotiorum* genome and identified a gene encoding a novel PG (SsPG2) that was most similar to *B. cinerea* BcPG2; however, *SsPG2* was not expressed during infection of *B. napus*. To study the interaction between *S. sclerotiorum* PGs and *B. napus* PGIPs, I attempted to express five *S. sclerotiorum* PGs and four *B. napus* PGIPs in *Pichia pastoris*. Of these, SsPG3, SsPG6 and BnPGIP1 were successfully produced in the yeast system. *In planta* SsPG3 and SsPG6 both induced light-dependent necrosis when infiltrated into leaves. *In vitro*, BnPGIP1 inhibited SsPG6 enzymatic activity and necrosis caused by the *S. sclerotiorum* PGs was inhibited in *A. thaliana* lines expressing either *BnPGIP1* or *BnPGIP2*. While the onset of symptoms was initially delayed in the line expressing *BnPGIP2*, no longer term effect on *S. sclerotiorum* virulence was observed.

2.2 Introduction

The plant cell wall is composed of cellulose-xyloglucan (50% w/w), pectin (30% w/w) and protein (1-5% w/w) and is considered to be a major barrier to infection. The cellulose-xyloglucan network serves as the skeleton of the plant cell wall and is encased in pectin and protein which provide structural stability (Carpita and Gibeaut, 1993). Pectin is a polymer of galacturonic acid (GA) composed of up to 200 (1→4) α-D-galacturonic acid units (Lau et al., 1985), which are periodically interrupted by rhamnosyl residues (Jarvis, 1984). The

rhamnosyl units may be either rhamnogalacturonan I or rhamnogalacturonan II. Rhamnogalacturonan I units are composed of repeats of (1→2) α-L-rhamnosyl-(1→4) α-D-galacturonic acid. Rhamnogalacturonan II units are less abundant and yield up to 10 different types of sugars upon hydrolysis, including several rare monosaccharides (Darvill et al., 1978). To penetrate this layer, pathogens secrete several types of pectin-degrading enzymes, most notably pectin methylesterases, pectin lyases and polygalacturonases (PG) that weaken the plant cell wall and facilitate spread of the pathogen.

Four *S. sclerotiorum* PG encoding genes have been characterized, herein referred to as *SsPg1*, *SsPg3*, *SsPg5* and *SsPg6* according to the nomenclature used by Li et al. (2004a); these were also described by Kasza et al. (2004). During infection, expression of *SsPg1* preceded that of *SsPg3*, *SsPg5* and *SsPg6* suggesting that SsPG1 may be important for infection establishment (Cotton et al., 2002; Li et al., 2004a; Sella et al., 2005).

Pectin degradation by exo- and endo- PGs causes the release of shorter polymers of GA. These oligogalacturonides have been demonstrated to induce plant defences and part of the host defence mechanism involves the release of proteins and metabolites that inhibit the activity of PGs (De Lorenzo et al., 2001). Pectinolytic enzymes were first shown to be inhibited by secondary metabolites, such as phenolic compounds (Patil et al., 1967), but this was later demonstrated not to be the case for all PGs as the presence of *p*-coumaric acid and ferulic acid from tomato did not inhibit *B. cinerea* PG (Glazener et al., 1982). The first report of PG inhibition by cell wall proteins was by Albersheim and Anderson (1971) and the first gene was cloned 21 years later from *Phaseolus vulgaris* (Toubart et al., 1992).

PGIPs are members of the leucine-rich-repeat protein family which includes the receptor-like proteins encoded by resistance (R) genes. The genes are often organized into multigene families clustered within specific chromosomal regions (Cervone et al., 1990; Leckie et al., 1999). In general, PGIPs are about 300-315 amino acids long with several glycosylation sites. PGIPs are involved in defence against pathogens and in regulating the selective

destruction and regeneration of the plant cell wall during normal plant developmental processes. PGIPs were found in newly-developing tissue or tissue transitioning from mature to elongating areas (Salvi et al., 1990). PGIP levels are also higher in mature fruits than immature ones (Yao et al., 1999). In regard to disease resistance, application of oligogalacturonides or fungal glucan to bean cell suspensions elicited the expression of PGIP genes (Zuppini et al., 2005). *B. napus* PGIP genes were also expressed in response to wounding, different defence hormones and *S. sclerotiorum* infection (Hegedus et al., 2008).

Mechanistically, PGIPs inhibit PGs in a way that is similar to porcine RNase inhibitor (PRI). PGIPs consist of β-turn structural units that organize into parallel β-sheets that impart a horse shoe-like shape on the protein (Kobe et al., 1993). *B. napus* PGIPs have 10 slightly varying repeats of a 24 amino acid leucine-rich domain with the consensus LxxLxLxxNxLt/GxIPxxLxxLxxL. The leucine-rich repeat forms the hydrophobic core, while the side chains of the amino acids flanking the leucine residues are exposed to the solvent and interact with other proteins (Li et al., 2003; Hegedus et al., 2008). Change of even a single amino acid can alter PGIP specificity. A single mutation in sister PGIPs led to a wider range of inhibition or loss of inhibitory ability (Leckie et al., 1999). Two models for PG-PGIP interaction have been described; the first is based on irreversible binding and the second on reversible binding. These models were validated using the data from PG-PGIP interaction studies (Fish and Madihally, 2008).

PGIPs have evolved to be highly specific and selective for their targets. For example, bean PGIP is more effective in inhibiting PGs from the pathogen *C. lindemuthianum* than the closely related non-pathogen *C. lagenarium* (Lafitte et al., 1984). Generally, PGIPs that are induced in response to wounding or infection specifically inhibit fungal PGs and are not effective against plant PGs (Cervone et al., 1990). PGIPs may have specific environmental or biochemical requirements to function properly since PGIPs were demonstrated to function *in planta* failed to do so upon isolation and testing *in vitro* (Albersheim et al.,

1971). Such studies have also suggested that multiple PGIPs interacting with different targets on the PG may be required for inhibition. Genomic studies revealed that many plants have the capacity to produce several PGIP isoforms, *A. thaliana* has two PGIP genes, while *P. vulgaris* has four PGIP genes that are induced in response to *S. sclerotiorum* infection (Ferrari et al., 2003). Multiple PGIP genes were also identified in cotton and grapevine (James and Dubery, 2001; Aguero et al., 2005). The largest PGIP gene family reported to date exists in *B. napus* (Hegedus et al., 2008). Initially, two PGIP genes were described that were induced by wounding and *S. sclerotiorum* infection (Li et al., 2003). Subsequently, 16 *B. napus* PGIP genes were identified representing a large and unexpected expansion of this gene family (Hegedus et al., 2008).

The release of GA during the maceration of the plant tissue causes the release of oligogalacturonides (Ridley et al., 2001). Short oligogalacturonides, 2-20 monomeric, are able to induce virulence gene expression in the pathogen, but also elicit defence responses in the host. Zuppini et al. (2005) demonstrated that host plant intracellular Ca^{+2} levels increased when exposed to both *S. sclerotiorum* PGs and oligogalacturonides; however, the kinetics of Ca^{+2} accumulation and dissipation differed. This suggests that *S. sclerotiorum* PGs are directly inducing defence responses independent of the release of oligogalacturonides.

S. sclerotiorum PGs also induce an apoptosis-like cell death reaction independent of oligogalacturonides through a mitochondria-mediated mechanism as evidenced by the release of cytochrome C and activation of caspase-like proteases. Interestingly PGIPs not only inhibit *S. sclerotiorum* PG pectinolytic activity, but also their ability to induce calcium signalling and apoptosis (Zuppini et al., 2005). The induction of Ca^{+2} signalling was also accompanied by the release of ROI that are known to be associated with the hypersensitive response and therefore tissue necrosis (Tiwari et al., 2002). There is as yet no clear understanding of how PGIPs inhibit PG necrotizing activity. One model suggests that this occurs through formation of a complex with PGs that prevent their recognition by a putative receptor. Other models

suggest that PGIPs might be part of a cell surface receptor complex and could block access to the PG (James and Dubery, 2001).

Gene expression studies demonstrated that individual *B. napus Pgips* and *S. sclerotiorum Pgs* are expressed at different times during the infection (Li et al., 2003; Hegedus et al., 2008). To determine which specific *B. napus* PGIPs may be involved in inhibiting specific *S. sclerotiorum* PGs it was imperative that they be in pure form. PGs have very similar biochemical characteristics and are difficult to separate from one another; the same applies to the separation of PGIPs. The most direct approach to obtain individual PGs or PGIPs is through heterologous expression in a yeast or bacterial system. This study attempted to isolate and express four distinctly different *B. napus* PGIPs and the five *S. sclerotiorum* PGs to examine their interaction *in vitro*. Furthermore, PGIP genes were expressed in plants and *in vivo* studies were conducted.

2.3 Results

2.3.1 Identification and characterization of *SsPg2*

A BLAST search of the *S. sclerotiorum* genome using *B. cinerea* PG2 revealed a previously unknown *S. sclerotiorum* PG gene that I denoted *SsPg2*. According to the 2005 genome annotation, *SsPg2* was predicted to possess three exons interrupted by two introns. Subsequently, the *SsPg2* gene was re-annotated and an intron was identified in what was originally described as the first exon. SsPG2 was deduced to be a 375 amino acid protein with a signal peptide of 19 amino acids. The molecular weight of the mature peptide was calculated to be 38 kDa. SsPG2 was 84.8% identical to BcPG2, while among the *S. sclerotiorum* PGs it was most similar to SsPG1 with 59% identity. The PG active site (CSGGHGLSI/VGS) was found starting at the 112th amino acid of the mature protein (Figure 2.1A). In accordance with the phylogenetic analysis of Li et al. (2004b), SsPG2 clustered in the same clade with *S. sclerotiorum* PGs when compared to other fungal polygalacturonases (Figure 2.1B). PGs can also be classified according to their isoelectric point and *S.*

sclerotiorum PGs are either neutral or acidic. Mature SsPG2 had an isoelectric point of 8.02 and was therefore considered to be a neutral PG.

```
MVNLSVLATF LASSALVASA PSPAPEDLDK RADCTFTSAA TAIAQKKGCS TITLNNVAVP ANTTLDLTGL TTGTKVIFQG
TTTPGYYEWE GPLVSISGTD IVVTGAAGNK LDGGGARWWD KLGSNVTPGN GKVKPKFFAA HKLLGSSSIT GLNFLNAPVQ
CISVGQSVGL SLININIDNS AGDTNQLAHN TDAFDINLSQ NIYISGAIVK NQDDCVAVNS GTNITFTGGN CSGGHGLSIG
SVGGRSGTGA NDVKDVRFLS STVSKSSNGA RIKTVSGKTG TVSGITYQDI TLVGITGYGI VIEQDYENGS PTGTPTSGVP
ITGVTMNNVH GTVTGGQNTY ILCANCSGWT WNKVAITGGT VKKTCKGIPT GASC
```

A

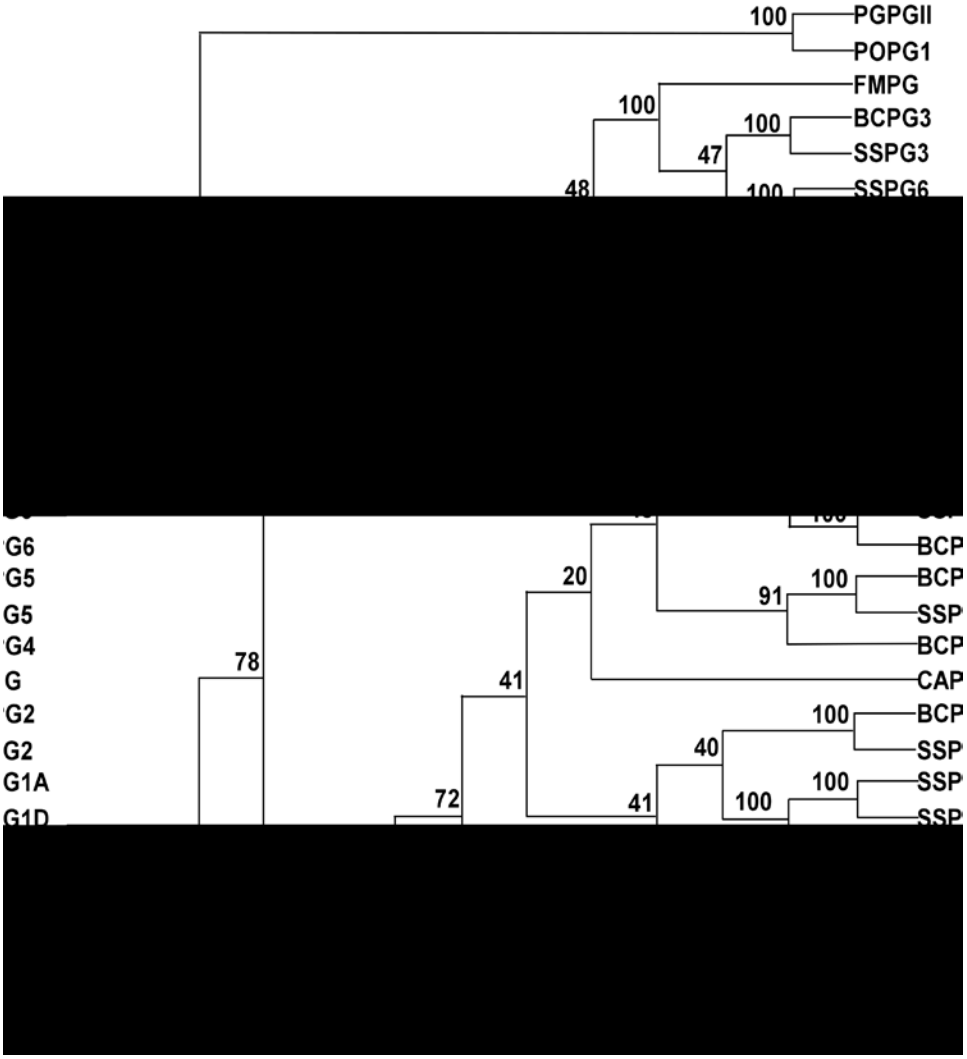


Figure 2.1 Sequence and phylogenetic analysis of fungal polygalacturonases.

A) Amino acid sequence of SsPG2 showing polygalacturonase domain on gray background. **B)** Phylogenetic tree based on analysis of the following endopolygalacturonases: AFPGA, *Aspergillus flavus* (P41749); AFPGB, *A. flavus*, (P41750); ANPGB, *Aspergillus niger* (Y18805); ANPGA, *A. niger* (Y18804); ANPGI, *A. niger* (S17980); AOPGB, *Aspergillus oryzae* (AB007769); APPGA, *Aspergillus parasiticus* (P49575); ATPGII, *Aspergillus tubingensis* (X58894); BCPG3, *B. cinerea* (U68717); BCPG1, *B. cinerea* (U68715); BCPG5, *B. cinerea* (U68721); BCPG4, *B. cinerea* (U68719); BCPG2, *B. cinerea* (U68716); BCPG6, *B. cinerea* (U68722); CLPG1, *C. lindemuthianum* (X89370); CAPG, *Cochliobolus carbonum* (P26215); CPPG, *Cryphonectria parasitica* (U49710) FMPG, *Fusarium moniliforme* (Q07181); FOPG, *Fusarium oxysporum* (AF078156); PGPGII, *Penicillium griseoroseum* (AF195113); PJPG, *Penicillium janthinellum* (D79980); POPG1, *Penicillium olsonii* (AJ243521); SSPG3, *S. sclerotiorum* (AY312510); SSPG6, *S. sclerotiorum* (AF501308); SSPG1a, *S. sclerotiorum* (L12023); SSPG1b, *S. sclerotiorum* (S62742); SSPG1d, *S. sclerotiorum* (AF501307); SSPG5, *S. sclerotiorum* (AY496277). A total of 100 bootstrap trees were generated and the number supporting each branch is indicated.

2.3.2 Expression of *S. sclerotiorum* PGs in *Pichia pastoris*

PGs are difficult to purify from *S. sclerotiorum* cultures due to the release of fungal polysaccharides that increase medium viscosity and hamper purification. A different approach was needed to provide sufficient *S. sclerotiorum* PGs for biochemical analyses. The *SsPg2* exons were amplified individually and the entire open reading frame assembled using PCR (Figure 2.2). *SsPg2* was cloned into the high copy number plasmid pBKS and subsequently sub-cloned into pPICZα which expresses the gene of interest under the control of the alcohol oxidase promoter. The plasmid was introduced into the methylotrophic yeast, *P. pastoris*, to produce the enzyme.

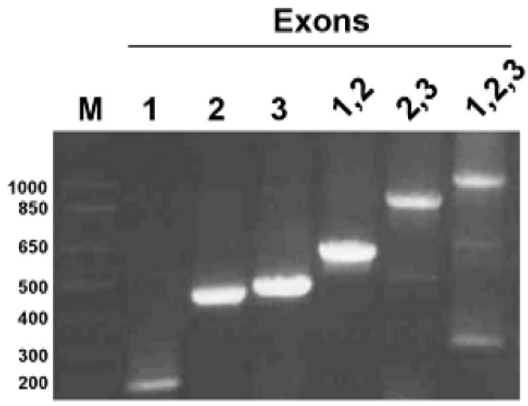


Figure 2.2 Assembly of *SsPg*2 exons.
Agarose gel showing amplification and assembly of *SsPg2* exons. The size of DNA molecular weight markers (lane M) is shown in the left hand margin.

SsPg1, *SsPg3*, *SsPg5* and *SsPg6* had been previously cloned into pPICZα and the constructs transformed into *P. pastoris*. The vector is integrated into the chromosome, therefore, transformants exhibit widely varying levels of expression dependent on the genomic context of the insert. I identified transformants that grew on a high concentration of the selective antibiotic (800 µg/ml Zeocin) which were then tested for protein expression. SsPG3 and SsPG6 were produced in large amounts that were easily detectable on SDS-PAGE gels stained with Coomassie Blue (Figure 2.3A). The vector also added a hexahistidine tail to the enzymes which allowed the use of an anti-hexahistidine antibody for western blot analysis. Using this approach, SsPG2 could be detected in the culture supernatant at five days post-induction (Figure 2.3B), but the concentration was too low to be detected on SDS-PAGE gels. Very low levels of SsPG1 and SsPG5 were detectable in the neat culture supernatant by western blot analysis. The supernatant of *P. pastoris* cells transformed with empty pPICZα was used as control and no proteins were detected on SDS-PAGE gels.

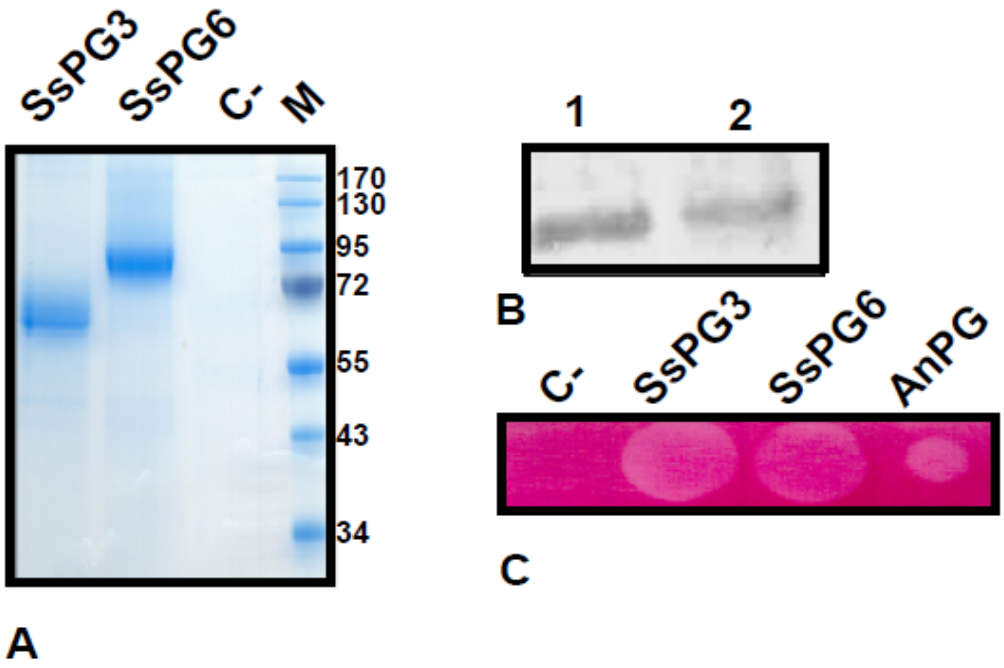


Figure 2.3 Production of *S. sclerotiorum* PGs in *P. pastoris.*
A) SDS-PAGE gel stained with Coomassie Blue showing the production of *S. sclerotiorum* SsPG3 and SsPG6 in *P. pastoris*. No protein bands were observed in the lane from *P. pastoris* transformed with empty pPICZα plasmid (C-). **B)** Western blot showing accumulation of SsPG2 in the medium of two transformed *P. pastoris* lines at five days post induction. The blot was probed with anti-hexahistidine antibody. **C)** Pectin-agar stained with ruthenium red to show activity of SsPG3, SsPG6 and *A. niger* PG as indicated by a zone of clearing. No activity was observed when culture supernatant from *P. pastoris* transformed with empty pPICZα plasmid (C-) was applied.

SsPg1 was expressed at high levels during the infection and was proposed to play a major role in lesion development (Li et al., 2003), therefore, attempts were made to concentrate SsPG1 from the *P. pastoris* culture supernatant. Proteins in the neat supernatant were concentrated using membrane concentration (10,000 KDa pore size). Western blot analysis with an anti-hexahistidine antibody demonstrated that SsPG1 was present in the sample, though at very low levels. The membrane concentration process also accumulated other components besides SsPG1 which produced a highly viscous and dark concentrate that interfered with the separation of the sample on SDS-PAGE gels. Several attempts were made to further purify SsPG1 using nickel

affinity beads that would bind the hexahistidine tag, but these were unsuccessful. Furthermore, imidazole which is used to elute proteins from the nickel beads was found to be toxic to the plant, inducing symptoms that were very similar to necrosis (data not shown) and therefore this method of purification was inappropriate for the intended purposes of the study.

To test whether the enzymes were active, assays using pectin-agar plates and dinitrosalicylic acid (DNSA) were used. The pectin-agar plate assay is a simple semi-quantitative assay for PG activity where Ruthenium Red is used to stain pectin and a zone of clearing indicates that the pectin has been degraded. Using this method, SsPG3 and SsPG6 in neat culture supernatants were demonstrated to be active (Figure 2.3 C). No zone of clearing was found in the culture supernatant from *P. pastoris* transformed with pPICZα.

Enzyme activity was measured quantitatively using the DNSA assay. Increasing the amount of culture supernetant in the reaction was reflected with an increase in pectinolytic activity (Figure 2.4).

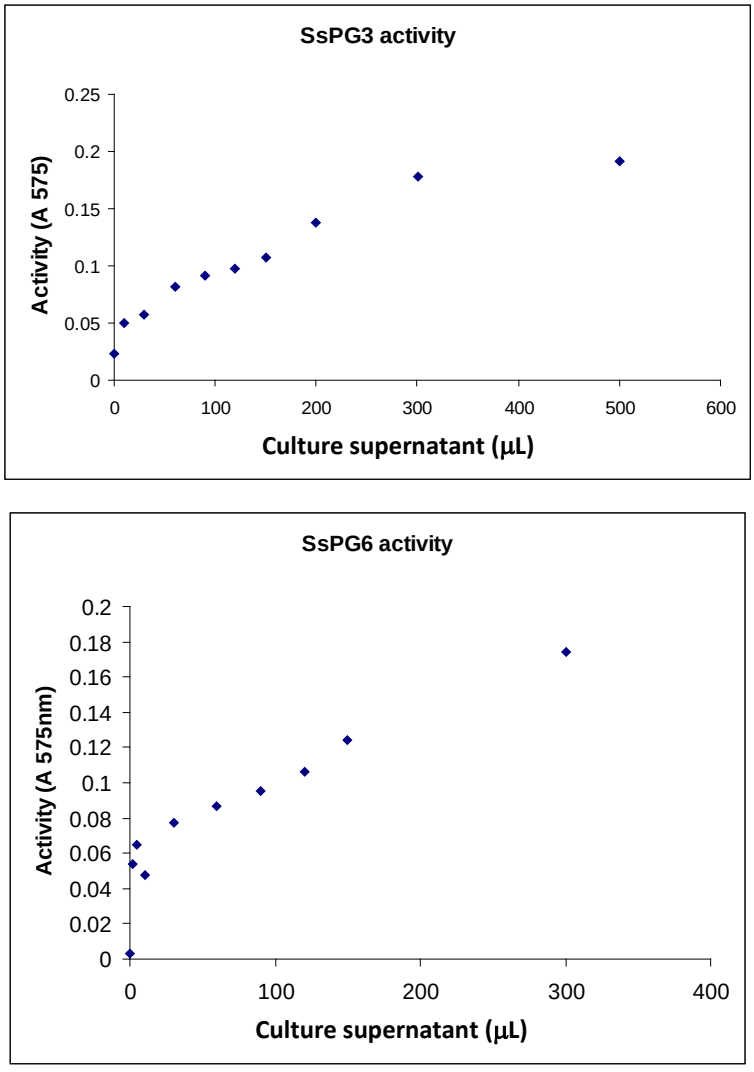


Figure 2.4 Dinitrosalicylic acid assay of SsPG3 and SsPG6 produced in *P. pastoris*.
Spectrophotometric assay measuring pectinolytic activity in the culture supernatants of *P. pastoris* lines producing *S. sclerotiorum* SSPG3 and SSPG6.

2.3.3 Production of *B. napus* PGIPs in *P. pastoris* and concentration of BnPGIP1

Previously, *BnPGIP*1, *BnPGIP2*, *BnPGIP7* and *BnPGIP16* were cloned into pPICZα and the constructs transformed into *P. pastoris*. As above, individual transformants were identified that grew on a high concentration of Zeocin. BnPGIP1, BnPGIP7 and BnPGIP16 were detected by western blot analysis using an anti-hexahistidine antibody in the neat culture supernatants

over a period of six days following induction. The expressed PGIPs could not be seen on SDS-PAGE gels after staining with Coomassie Blue, indicating that protein levels were quiet low. BnPGIP2 was not detected at any time point by either method over a period of six days. The expression of BnPGIP1 was higher and more consistent than that of the other *B. napus* PGIPs and was therefore used for further purification (Figure 2.5A).

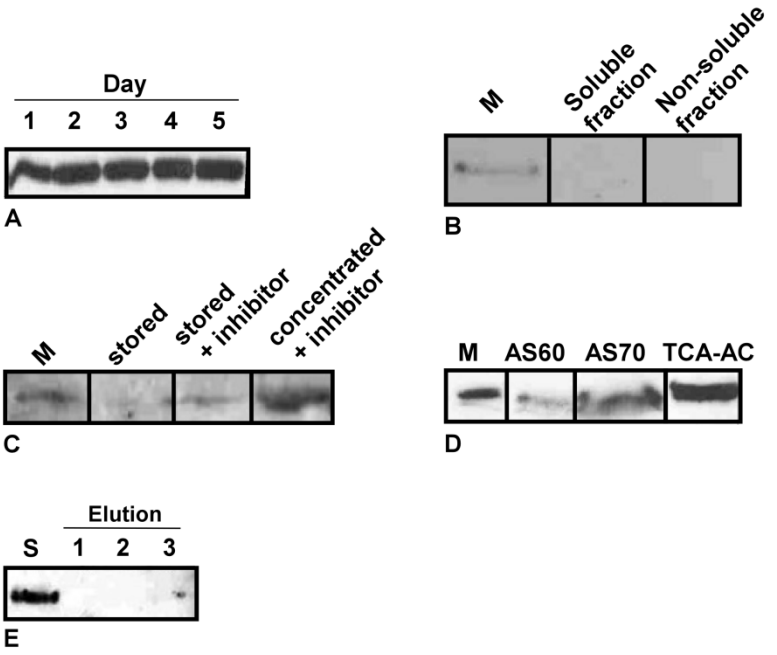


Figure 2.5 Production and purification of *B. napus* PGIP1 in *P. pastoris.* All panels show western blot analysis with anti-hexahistidine antibody. 40 μL of sample was loaded in each lane. **A)** Time course study showing production of BnPGIP1. **B)** Detection of BnPGIP1 in the culture medium (M) and soluble and non-soluble cellular fractions. **C)** Comparison of the BnPGIP1 levels in samples concentrated using a membrane in the presence of protease inhibitor cocktail to samples stored at 4ºC with and without the protease inhibitor or immediately frozen (M). **D)** Different chemical methods for concentrating BnPGIP1 from the the culture medium (M). AS60 and AS70 are the precipitate at 60% and 70% w/v ammonium sulfate saturation. TCA-AC refers to BnPGIP1concentrated using trichloric acid and acetone. **E)** Purification of BnPGIP1 from concentrated culture supernatant (S). BnPGIP1 suspension was combined with nickel resin beads that were then washed with 50 mM, 200 mM and 4 M of imidazole (lanes 1, 2, 3).

Since the concentration of BnPGIP1 in the neat culture supernatant was insufficient to conduct biochemical studies, several methods were tested to

concentrate the protein. Acetone was unable to precipitate the protein, whereas a mixture of trichloroacetic acid and acetone was successful (Figure 2.5D). As these chemicals rely on denaturation of the protein for precipitation, refolding would be required prior to any biochemical testing. Ammonium sulphate precipitation, which is a milder method, was also examined. At 60% ammonium sulphate saturation, BnPGIP1 mainly remained in the supernatant, while at 70% saturation most of the medium BnPGIP1 was precipitated. The precipitate was viscous and did not allow good separation on a SDS-PAGE gel. Contaminants also interfered with the spectrophotometer reading at the wavelength used in the DNSA assay and therefore *in vitro* assays were not possible with this material. An alternative approach that avoided denaturing the protein was to concentrate it using centrifugal membrane filtration. Using this method, BnPGIP1 was concentrated several fold. This is a lengthy procedure, and even though the centrifugation was conducted at 4ºC, some degradation occurred. *P. pastoris* has been demonstrated to secrete small amounts of proteases into the supernatant which may be responsible for the protein degradation (Shi et al., 2003). Including a cocktail of protease inhibitors prevented degradation of the protein during storage at 4ºC and was therefore used during the concentration process (Figure 2.5C). Despite optimal concentration conditions, the amount of BnPGIP1 was still insufficient to be detected on SDS-PAGE gel. Attempts were also made to purify the concentrated BnPGIP1 protein using nickel beads (Figure 2.5E). Several attempts to purify the BnPGIP1 fusion protein using this strategy failed, while purification of SsPG3 and SsPG6 was successful. Overall the best available BnPGIP1 was from the sample concentrated using centrifugal membrane filtration and subsequent interaction studies were performed using this material.

2.3.4 *In vitro* interaction between SsPG6 and BnPGIP1

Due to the limited amount of BnPGIP1 available, *in vitro* inhibition studies were conducted only with SsPG6. Concentrated BnPGIP1 culture supernatant consistently inhibited the activity of SsPG6 (Figure 2.4), though the kinetics of

the interaction could not be determined since the components were not in pure form.

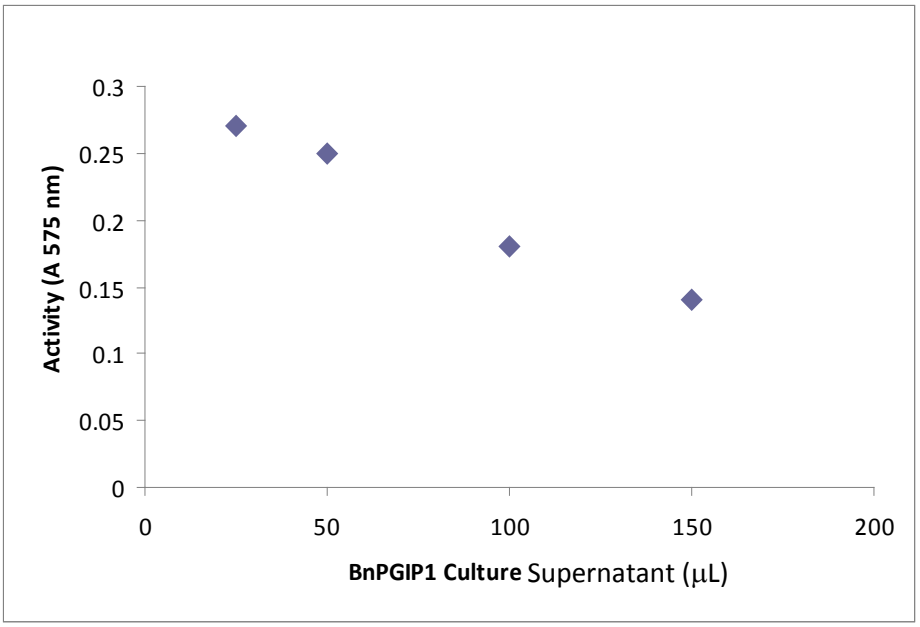


Figure 2.6 *In vitro* inhibition of SsPG6 by BnPGIP1.
Spectrophotometric assay measuring pectinolytic activity of culture supernatant from a *P. pastoris* line producing SsPG6 in the presence of concentrated culture supernatant of a *P. pastoris* line producing BnPGIP1.

2.3.5 *In planta* interaction between *S. sclerotiorum* PGs and *B. napus* PGIPs

The *in vitro* interaction studies demonstrated that BnPGIP1 was able to inhibit SsPG6, though difficulties in obtaining sufficient amounts of *B.napus* PGIPs made this approach impractical. Other studies showed that PGIPs that functioned *in planta* failed to do so *in vitro* indicating that specific environmental conditions from the infection area may also be required for proper interaction (Albersheim et al., 1971). In addition, host-specific glycosylation was deemed to be important for PGIP activity (Lim et al., 2009). In light of these circumstances and potential variables, I elected to test the interactions *in planta*.

The ability to induce necrosis is a key role played by PGs during the infection as this provides the pathogen with access to nutrients. Neat culture supernatants from *P. pastoris* lines producing SsPG3 or SsPG6 caused necrosis

within four days of being infiltrated into *Nicotiana benthamiana* leaves (Figure 2.5). Interestingly, the closely-related *B. cinerea* enzymes, BcPG3 and BcPG6 were unable to induce necrosis in this plant species (Kars et al., 2005).

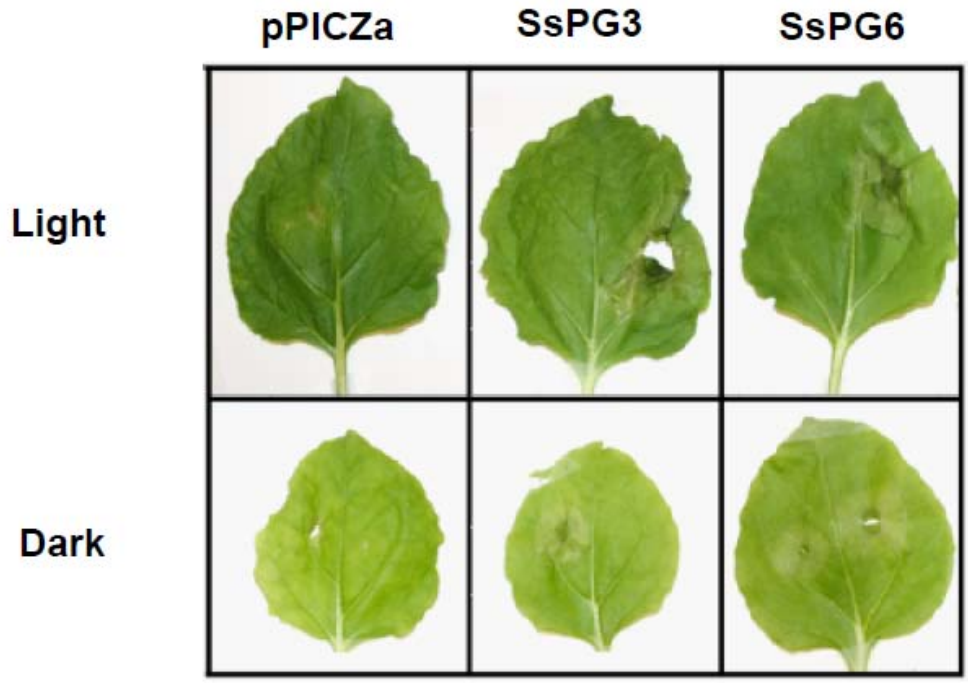


Figure 2.4 Necrosis caused by infiltration of *N. benthamiana* leaves with SsPG3 and SsPg6.
Leaves from one month old plants infiltrated with culture supernatants of a *P. pastoris* line transformed with pPICZα or lines producing SsPG3 or SsPG6. Plants were either grown in the light prior to infiltration and left in the light thereafter or pre-conditioned in the dark, infiltrated and placed in the dark for four days.

The necrosis caused by *S. sclerotiorum* PGs could be due to tissue maceration or the induction of the hypersensitive response (HR). ROI produced by the chloroplast in a light dependent manner are thought to be involved in this process. In other studies, when leaves that had been preconditioned in the dark were infiltrated with toxins that induced the HR in the light, no necrotic symptoms were observed (Qutob et al., 2006). While it was well-established that PGs cause necrosis, it was not known if this was light-dependent and therefore due to HR induction. In this study, leaves that were preconditioned and infiltrated with *S. sclerotiorum* PGs in the dark exhibited markedly reduced symptoms. Although the infiltration zone was chlorotic, necrosis or tissue wrinkling was not observed (Figure 2.5) suggesting that the necrosis is only partially due to HR.

To better understand the role of the cuticle in protecting the leaf, SsPG6 was applied to leaves of *A. thaliana* with either intact or abraded cuticle. In parallel supernatant from *P. pastoris* transformed with pPICZα was tested. SsPG6 was able to induce necrosis only on the leaves with abraded cuticle while the intact cuticle provided full protection for the leaf. Application of *P. pastoris* pPICZα culture supernatant showed no symptoms (Figure 2.6).

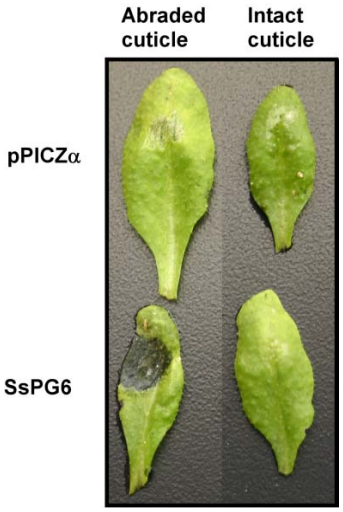


Figure 2.5 Effect of cuticle on *S. sclerotiorum* PGs necrotizing activity
Aliquots of supernatant from *P. pastoris* transformed with empty pPICZα or pPICZα -*SsPg6* were placed on *A. thaliana* intact leaves or leaves with an abraded cuticle and incubated for three days in the light.

Previously, homozygous, single insert *A. thaliana* lines expressing *BnPGIP1* or *BnPGIP2* were generated in the laboratory. I used these to test the ability of *B. napus* PGIPs to inhibit *S. sclerotiorum* PGs *in planta*. Infiltration of *A. thaliana* leaves with the neat supernatant from *P. pastoris* transformed with only the pPICZα, i.e. the negative control, was found to produce necrotic lesions. To overcome this, SsPG3 and SsPG6 were purified by nickel affinity chromatography. Although purified *S. sclerotiorum* PGs were obtained, the imidazole used to elute the protein from the nickel columns was also found to cause necrosis in *A. thaliana* leaves. To remove these components from the negative control, the culture supernatant were subjected to dialysis. Since PGs are most active under acidic conditions, sodium acetate buffer (pH 5.5) was used initially for dialysis; however, the buffer alone caused necrotic lesions. Fortunately, dialysis against ddH_2O removed the necrosis-inducing component(s) from the control supernatant while retaining necrotizing activity in SsPG3 and SsPG6 samples (Figure 2.7). *A. thaliana* lines expressing either *BnPGIP1* or *BnPGIP2* showed reduced symptoms when infiltrated with SsPG3 or SsPG6 relative to the wild-type line. Six days post-infiltration, lines expressing *BnPGIP1* exhibited only limited chlorosis and no necrotic lesions. Furthermore, lines expressing *BnPGIP2* were even more tolerant and exhibited minimal or no chlorotic or necrotic symptoms (Figure 2.7).

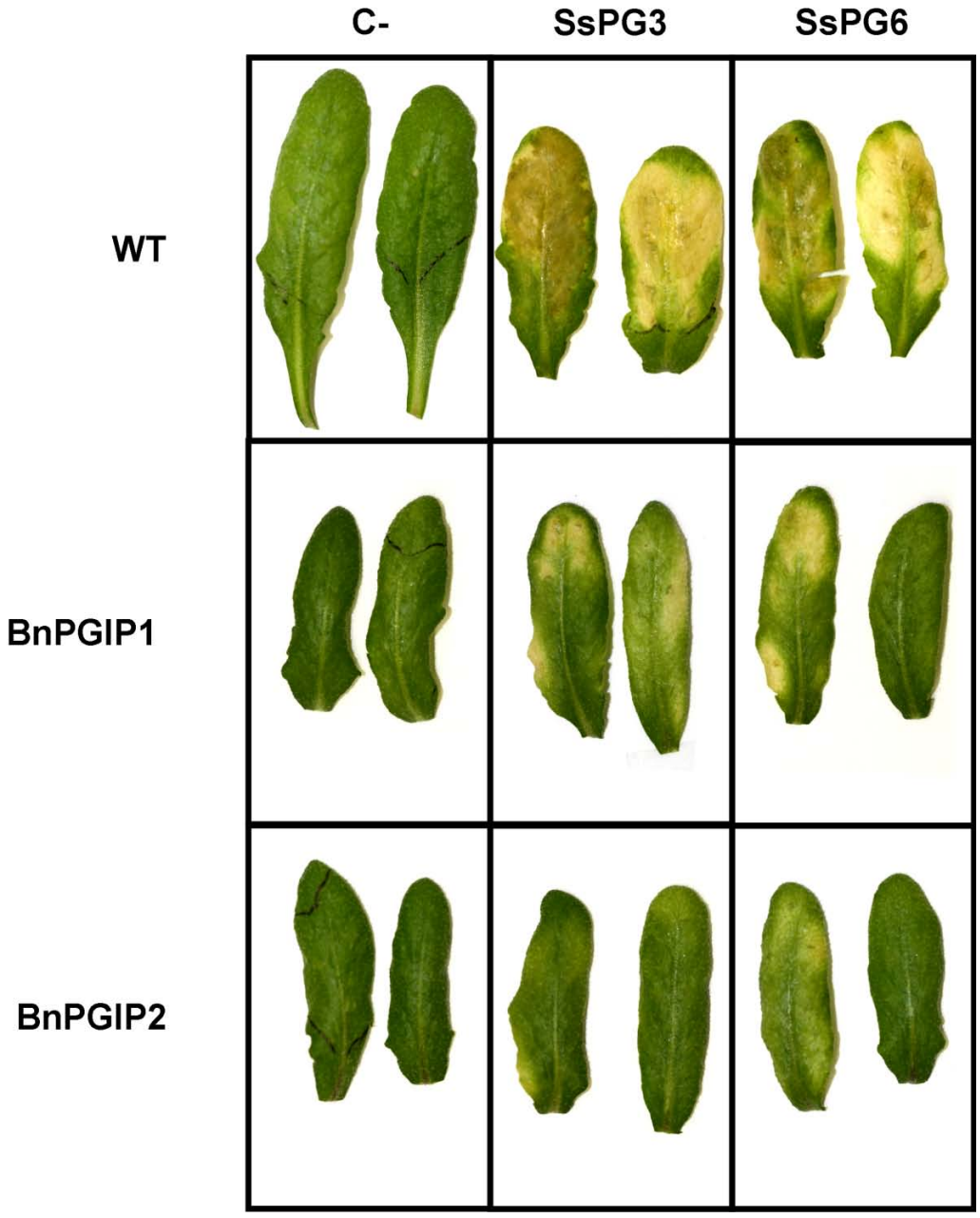


Figure 2.6 ***In planta*** **inhibition of SsPG3 and SsPG6 by BnPGIP1 and BnPGIP2**

Necrosis caused by infiltration of SsPG3 or SsPG6 on leaves of *A. thaliana* lines expressing *BnPgip1* or *BnPgip2*. Enzymes were produced in *P. pastoris* and dialyzed against water. Culture supernatant from *P. pastoris* transformed with pPICZα was used as a control. Five leaves were tested.

2.3.6 Effect of *B. napus* PGIPs on resistance to *S. sclerotiorum*

The *A. thaliana* lines expressing *BnPGIP1* or *BnPGIP2* were also tested for susceptibility to *S. sclerotiorum*. The lesion phenotype was the same in lines producing BnPGIP1 as in wild type. Conversely, lines producing BnPGIP2 exhibited a reduced chlorotic zone surrounding the necrotic region two days post-inoculation. Subsequently, the zone of infection expanded rapidly and no visible differences were noted between the lines. This indicates that BnPGIP2 was capable of delaying the onset of symptoms, but was unable to provide adequate resistance to the pathogen (Figure 2.8).

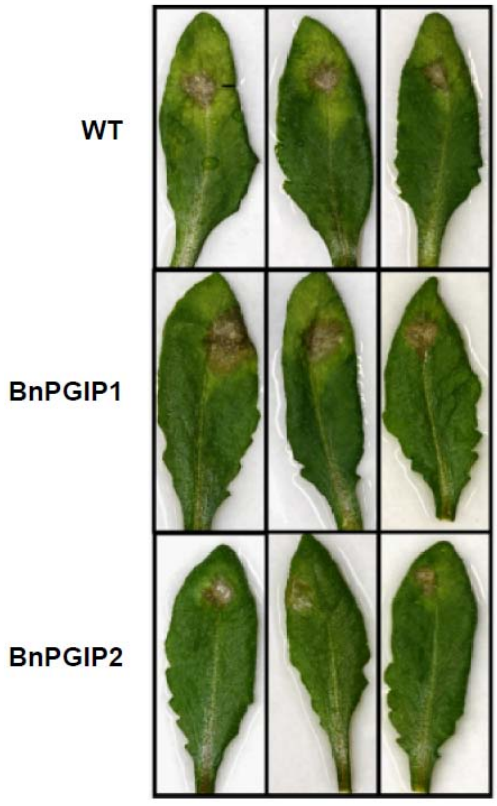


Figure 2.7 Susceptibility of *A. thaliana* lines expressing *BnPGIP1* or *BnPGIP2* to *S. sclerotiorum*
A. thaliana plants expressing *BnPGIP1* or *BnPGIP2* were inoculated with *S. sclerotiorum* and incubated in a Petri dish on Whatman paper. Leaves were photographed two days post-inoculation. Over-expression of *BnPGIP2* reduced the chlorotic zone surrounding the inoculated area. Five replicas were tested

2.4 Discussion:

2.4.1 *S. sclerotiorum* PGs have both pectinolytic and necrotizing activity

S. sclerotiorum hosts are predominantly dicots that possess large deposits of pectin in their cell wall middle lamellae and surrounding the vascular bundles. The pathogen must penetrate these layers to establish an infection. Many studies have reported PGs in a broad range of plant pathogens (Annis and Goodwin., 1997; Staples and Mayer, 1995; Wubben et al., 2000). PGs were found to possess different levels of pectinolytic and necrotizing activity based on the host species (Kars et al., 2005). This may relate to differences in the type and lengths of oligogalacturonides fragments generated. A previous study on *S. sclerotiorum* identified four endo- and two exo-PGs (Li et al., 2004a). In my study, a fifth endo-PG, SsPG2, was found to be encoded by the *S. sclerotiorum* genome. *S. sclerotiorum* and *B. cinerea* have very similar complements of PG genes, with the exception of BcPG4 which is only found in *B. cinerea.* SsPG1 and SsPG2 cluster together in a phylogenetic clade with BcPG1 and BcPG2 and the latter two enzymes have similar biochemical and functional properties. BcPG1 and BcPG2 are most active on non-methylated pectin, produce similar oligogalacturonides and cause comparable levels of necrosis (Kars et al., 2005). Disruption of *BcPg1* caused a 25% reduction in lesion expansion, while disruption of *BcPg2* reduced lesion expansion by 50% (Kars et al., 2005) demonstrating that they are partially redundant. Unlike *BcPg2*, *SsPg2* was not expressed during infection on *B. napus* in a highly virulent strain of *S. sclerotiorum* and therefore SsPG1 may be able to compensate for the lack of SsPG2 activity.

SsPG3 and SsPG6 were shown to cause necrosis when infiltrated into either *A. thaliana* or *N. benthamiana* leaves and in another study, *SsPg3* and *SsPg6* were shown to be expressed in the necrotic lesion (Li et al., 2004b). However, the host range of PGs may be restricted as BcPG1 and BcPG2 were highly necrotizing when infiltrated into *A. thaliana* or *Solanum lycopersicum*

leaves; while, BcPG3 and BcPG6 were unable to cause necrosis on tomato (Kars et al., 2005). BcPG genes were also regulated differently dependent upon the host (ten Have et al., 2000), though similar studies have yet to be conducted with *S. sclerotiorum*.

While BcPG3 and BcPG6 were capable of hydrolyzing pectin to GA (Kars et al., 2005), necrosis did not occur in response to GA monomers or short unbranched polymers (Zuppini et al., 2005; Kars et al., 2005). Further evidence that pectinolytic and necrotizing activity are independent stems from the fact that BcPG1 and BcPG2 are more necrotizing than other *B. cinerea* PGs, yet BcPG1 and BcPG2 have different levels of pectin processivity (Kars et al., 2005). The mechanism(s) through which PGs cause necrosis is not fully understood. Light was required for *S. sclerotiorum* PGs to cause full necrosis upon infiltration into the host. This is similar to the requirement for light and active metabolism for necrosis induction by necrosis and ethylene inducing proteins (NEPs) (Qutob et al., 2006) or AVR/R protein interaction (Chivasa et al., 2005). Light is essential for the production of ROI from the chloroplasts. At low concentrations ROI activate plant HR and subsequently induce cell death. PGs may cause cell death through manipulating the host defence mechanism.

2.4.2 Heterologous expression of *S. sclerotiorum* PGs and *B. napus* PGIPs

S. sclerotiorum and other necrotrophs may have evolved a wide range of PGs to accommodate variations in pectin structure and plant defences including PGIPs. I attempted to express both *S. sclerotiorum* PGs and *B. napus* PGIPs in a heterologous system to obtain sufficient protein for biochemical characterization and interaction studies. Many heterologous protein expression systems have been described including bacteria, yeasts, filamentous fungi, insect cells, mammalian cells, transgenic animals and transgenic plants. Yeast was chosen due to its relative ease of culture and genetic manipulation, as well as the ability to properly process proteins containing disulphide bonds or requiring glycosylation. The *P. pastoris* system is also advantageous as it

secretes the protein into the expression medium facilitating collection and purification.

High levels of active SsPG3 and SsPG6 were produced in *P. pastoris*, while the levels of SsPG1, SsPG2, and SsPG5 were insufficient for subsequent analysis. Similarly, levels of BcPG3 and BcPG6 were 100 to 200-fold higher than BcPG1 and BcPG2 in *P. pastoris* (Kars et al., 2005). *P. pastoris* is one of the more robust and successful protein expression systems; however, better promoter and host strain combinations are being developed to increase protein production efficiency (Cereghino and Cregg, 2006).

B. napus was reported to possess 16 different PGIP genes (Hegedus et al., 2008) of which four genes from distinct PGIP lineages were expressed in *P. pastoris*. I found that *P. pastoris* was unsuitable for the production of high levels of *B. napus* PGIPs. Similar challenges were reported for other PGIPs where in many cases the protein was not detected despite the presence of high levels of gene transcript (De Lorenzo, personal communication). In other cases, high levels of the protein were obtained, but no activity was observed. Furthermore, the bean PvPGIP2 and the soybean GmPGIP1 were inactive when expressed in *Escherichia coli* (De Lorenzo et al., 2001) suggesting that other expression systems are required.

Problems were also encountered when attempts were made to purify BnPGIP1, as the protein failed to bind to nickel affinity beads. It is plausible that the carboxy terminus where the hexahistidine-tag resides was not exposed on the protein surface and therefore inaccessible. Indeed, the amino and carboxy termini of PGIPs form a “knot” consisting of three alpha-helices, so it was not unexpected that the non-denatured BnPGIP1 did not bind to the beads. An anti-hexahistidine antibody recognized the recombinant protein in western blot analysis after separation of denaturing SDS-PAGE gels indicating that the tag was present. Plants have been reported to be a better system for the production of heterologous PGIPs (Berger et al., 2000; Desiderio et al., 1997;

Leckie et al., 1999; Powell et al., 2000) and this strategy was successfully applied to the production of *B. napus* PGIPs in *A. thaliana*.

2.4.3 *In vitro* and *in planta* interaction between *S. sclerotiorum* PGs and *B. napus* PGIPs

In vitro BnPGIP1 was found to consistently inhibit the activity of the *S. sclerotiorum* PG, SsPG6. Expression of *BnPGIP1* and *BnPGIP2* in transgenic plants was found to reduce the necrotizing activity of SsPG3 and SsPG6. BnPGIP2 was a more effective inhibitor since plants expressing *BnPGIP2* showed no signs of necrosis and chlorosis at and around the zone of infiltration, while plants expressing *BnPGIP1* exhibited only reduced symptoms. In agreement, plants expressing *BnPGIP2* had delayed onset of symptoms, a reduced chlorotic zone being the most noticeable difference, after *S. sclerotiorum* inoculation, whereas no differences were seen in plants expressing *BnPGIP1*. Some plants have evolved large families of PGIPs, presumably to counter the multitude of PGs produced by their associated pathogens (Hegedus et al., 2008). Due to the diversity of PGs produced by different pathogens, a single PGIP is not likely to fully inhibit all PG activity and thus correct combinations will be required to provide any degree of practical resistance. For example, expression of a *Vitus vinifera* PGIP in *N. benthamiana* only reduced the symptoms of *B. cinerea* infection (Joubert et al., 2006). *A. thaliana* possesses only two PGIP genes. Over-expression of either *A. thaliana* PGIP gene improved resistance to *B. cinerea* (Ferrari et al., 2003), suggesting that resistance can also be quantitative and clearly dependent on the levels of the target PG.

Expression of *B. napus* PGIP genes in *A. thaliana* had a minimal effect on *S. sclerotiorum* lesion expansion. It was difficult to quantify disease resistance as all cultivars of *A. thaliana* are highly susceptible to this pathogen. Five replicas of the experiment were examined and no visual differences in lesion size was observed. Furthermore, *S. sclerotiorum* ascospores do not infect the leaf directly and mycelia must be used as an inoculum which is difficult to

quantify. The delay in the appearance of the chlorotic zone was found to be consistent in plants expressing *BnPGIP2*, but this did not adequately protect the plant from eventual destruction. Since *S. sclerotiorum* possesses a large family of phytotoxins, including multiple PGs, oxalic acid and NEPS (Chapter 4), more than one mechanism may be required to counter the barrage of pathogenicity factors.

2.5 Materials and methods

2.5.1 Assembly of *SsPg2*

To exclude the introns and assemble the complete *SsPg2* open reading frame (ORF), exons were amplified using primers that had additional sequences at their termini that overlapped with the neighbouring exon. The first step was to amplify each of the three exons separately and in a second PCR reaction assemble two of the exons. A third exon was added through another PCR reaction to the two assembled exons and the ORF amplified. Exon 1 was amplified using the primers SsPG2-EcoRI-exon1-F (5'-GCGAATTCATGGCTCCAGAAGATTTGGACAAGCG-3') and SsPG2-exon1-R (5'-CCCTCCCATTCATAGTAGCCAAACGTAGTAGTTCCTTGGAAGATAAC-3'). Exon 2 was amplified using the primers SsPG2-exon2-F (5'-GTTATCTTCCAAGGAACT ACTACGTTTGGCTAC TATGAATGGGAGG-3') and SsPG2-exon2-R(5'-GTTTCCGCC AGTGAAAGTGATGTTAGTACCGG AGTTAACGGCAACAC-3'). Exon 3 was amplified using the primers SsPG2-exon3-F (5'-GTGTTGCCGTTAACTCCGGTACTAA CATCAC TTTCACTGGCGGAAAC-3') and SsPG2-exon3R (5'-CGTCTAGAGAACAAG AGGCTCCTGTCGGGATAC-3'). The primers also added *Eco*RI and *Xba*I (underlined) to the amplification product for cloning purposes. All PCR reactions were performed with an annealing temp of 58ºC and an extension temp of 72ºC for a period of 1 min per kb. All reactions were

allowed to incubate for 10 min at 72ºC at the end of the reaction to maximize the generation of full length products. The final ORF was cloned into the *EcoR*I and *Xba*I sites of pBKS and introduced to *E. coli* using electroporation. Plasmids were recovered from *E. coli* cells and the insert sequenced to confirm correct assembly.

2.5.2 Construction of the *SsPg2*-pPICZα cassette and transformation of *P. pastoris*

pPICZα (Invitrogen) is an expression vector that is used to produce proteins in *P. pastoris*. The plasmid includes the alcohol oxidase promoter (AOX) that activates gene expression when 0.05% methanol is supplied in the medium. The vector also adds a hexahistidine tail to the protein of interest which allows the use of anti-hexahistidine antibody for protein detection and purification using metal affinity systems. *SsPg2* was cut out of pBKS plasmid using *EcoR*I and *Xba*I and ligated to the same sites in pPICZα using T4 DNA ligase (Invitrogen). In order to introduce the plasmid into *P. pastoris* cells it was necessary to have high concentration of pure linear plasmid and freshly prepared host cells. The vector was obtained using an alkaline plasmid extraction method as described in (Sambrook and Williams, 2001).

Host cell preparation and transformation was carried according to the user manual (Invitrogen). The transformation was confirmed using colony PCR and SsPG2 expression was detected using western blot analysis as described in section 2.5.3.

2.5.3 Expression of *S. sclerotiorum* PGs and *B. napus* PGIPs in *P. pastoris*

BnPGIP1, BnPGIP4, BnPGIP7 and BnPGIP16 were attempted to be expressed. On the other hand all five *S. sclerotiorum* PGs were attempted to be expressed those being SsPG1, SsPG2, SsPG3, SsPG5 and SsPG6. Expression in *P. pastoris* was conducted as in the user manual provided by Invitrogen. Briefly, cells were grown overnight in 25 mL buffered complex glycerol

medium at 28 ºC at to reach OD_{600} of 2-6. Cells were harvested by centrifugation and resuspended in a 100 mL of buffered complex methanol medium. Methanol concentration was maintained at daily 0.5%. Sample of the supernatant was collected daily and cells were removed using centrifugation at 14,000 rpm for 1 min. All samples were immediately frozen in liquid nitrogen and stored at-20 ºC. Samples were tested for the protein of interest by western blot analysis using an anti-hexahistidine anti-body as they carried a six histidine tag that was introduced by the plasmid.

Briefly 10% SDS separation gel was prepared using 3.2 mL of water 1.6 mL of 50% glycerol, 2.5 mL of 1.5 M Tris pH 8.8 and 2.5 mL of 40% acrylamide. After polymerization the gel was topped with a 5% SDS loading gel. The gel was prepared using 2.2 mL of water, 800 µL of 50% glycerol, 1.25 mL of 0.5 M Tris pH 6.9, 625 µL of 40% acrylamide and 50 µL of 10% SDS. Samples were separated at 150 volts for around 2 h and subsequently proteins were transferred to a nitrocellulose membrane at 100 volts for 1.5 h. Membrane was blocked using 5% milk in TBST buffer over night. Primary antibody (Invitrogen) was used at 1/5000 TBST and incubated for 1.5 h and secondary antibody was used at 1/5000 in TBST as well for 1 h. ECL (General electric) kit was used to activate the fluorescent label on the secondary antibody and the film was exposed for 5 min.

2.5.4 Polygalacturonase assays

Pectin-agar plates were prepared with 1% agar (Difco) as a solidifying agent and 0.3% pectin (Sigma-Aldrich) in 0.1 M sodium acetate buffer (pH 5.2). Aliquots (20 µL) of the culture supernatant were placed on the plate and allowed to incubate at 20ºC for one hour. Plates were stained with 0.05% Ruthenium red for 30 min and rinsed gently under running water for 30 sec. A zone of clearing was indicatative of pectin degradation.

The DNSA assay was based on the procedure of Miller (1959) with modifications to stabilize the reaction color. DNSA is an aromatic compound

that reacts with reducing sugars to form 3-amino-5-nitrosalicylic acid which absorbs light at 540 nm. A 0.4 mL aliquot of 0.5% pectin was mixed with enzyme and the final volume was brought up to 500 µL using 0.1M sodium acetate. Samples were incubated at 30ºC for 5 hours. DNSA reagent (44 mM DNSA, 4 mM sodium sulfite, 375 mM NaOH) was then added and the samples were boiled for 10 min to stop the reaction. Sample volume was brought up to 2 mL using ddH_2O and the absorbance measured at 575 nm using a spectrophotometer and water as a blank (Bio-Rad).

2.5.5 Purification and concentration of BnPGIP1

A *P. pastoris* line producing BnPGIP1 was induced with methanol for six days. To determine where the bulk of the protein was located, the culture supernatant was collected after centrifugation at 14,000 rpm in a microcentrifuge for 1 min. In addition, the cell pellet was subjected to 3 rounds of sonication for 10 sec each using a Misonix S-4000 sonicator (Qsonica) with a frequency of 20 kHz set at 50 W. Samples were placed on ice for 10 sec between pulses. The suspension was centrifuged for 5 min at 14,000 rpm in a microcentrifuge and the soluble (supernatant) and insoluble (pellet) fractions recovered.

Proteins were concentrated using Centricon tubes with membranes pore size of 10,000 KDa (Millipore). Samples were loaded in the upper chamber of the tube and centrifuged for 40 min at 5,000 rpm at 4°C. Upon completion, the concentrate was $1/10^{th}$ of the original sample volume. The viscous concentrate was pipetted to microcentrifuge tubes and frozen in liquid nitrogen. All samples were stored at -80°C. Membrane concentrated samples were also dialyzed to remove all medium components and contaminants. Dialysis cassettes (Pierce) with a pore size of 10,000 kDa were used. The cassette was allowed to hydrate, the water removed and 3 mL of sample injected using a syringe. Samples were dialyzed against 1 L of ionized sterile water at 4°C with continuous stirring.

Water was changed three times over 48 hours. Samples were then collected and immediately frozen in liquid nitrogen and stored at -80 °C.

Protein concentration was also attempted using various chemical methods. Ammonium sulphate was added gradually with continued stirring at 4°C in a flask. No salt was allowed to accumulate at the bottom of the flask. At 60% saturation and the precipitate was separated by centrifugation at 14,000 rpm for 5 min. A second fraction was collected at 70% saturation and the pellets dissolved in 50 mM Tris-HCl buffer (pH 7.5). Proteins were detected using western blot analysis as described above. Protein was also precipitated using TCA-acetone solution (1 volume of TCA was mixed with 2 volumes of acetone). The sample was mix with TCA-acetone at 1:3 ratio and was allowed to precipitate at -20°C over night. Samples were then centrifuged at 14,000 rpm and pellet dissolved in 50 mM Tris-HCl buffer (pH 7.5).

Attempts were made to purify SsPG3, SsPG6 and BnPGIP1 using nickel resin. For every 1 mL of sample, 100 µL of resin was used. Resin was prepared according to the user manual (Novagen). Briefly, 50 µL of resin was separated from100 µL preservation buffer by centrifugation. The resin was charged by multiple washes with supplied buffers. The sample was then mixed with resin at a ratio of 4:1 and allowed to incubate at room temperature for 5 min and centrifuged at 3,000 rpm. The resin was washed four times with binding and wash buffer. Finally, the resin was washed with increasing concentrations of imidazole, 50 mM, 200 mM and 4 M and the elutate immediately frozen in liquid nitrogen and stored at -80°C.

2.5.6 Effect of light on *S. sclerotiorum* PGs necrotizing activity

Four-week-old *N. benthamiana* plants were grown in the greenhouse and pre-conditioned in the dark for one hour prior to protein infiltration. Culture supernatants from *P. pastoris* lines transformed with the empty vector or cassettes expressing *SsPg3* and *SsPg6* were infiltrated using 0.5 mL syringe by gently applying pressure to the bottom side of the leaf. Infiltration was

conducted in the dark under red light, the infiltration zone (ca. 1-2 cm) marked and plants incubated in the dark for four days at 20°C. In parallel, plants were infiltrated and incubated in the greenhouse.

2.5.7 *In planta* interaction between *S. sclerotiorum* PGs and *B. napus* PGIPs

A. thaliana leaves grown under normal greenhouse conditions are generally too small to be infiltrated without causing significant damage. Large leaves were obtained by growing the plants for 3-4 weeks at 18ºC in a light:dark period of 16:8 hours/day. Aliquots of *P. pastoris* culture supernatant were infiltrated into the leaves using a 2 mL syringe and ensured to have spread into at least 80% of the leaf area. Leaves were incubated in a Petri plate on wet Whatman #5 paper to maintain humidity under 12:12 hours/day light:dark conditions and symptoms noted over the next 5 days.

2.5.8 Infection of *A. thaliana* with *S. sclerotiorum*

S. sclerotiorum strain 1980 was grown on potato dextrose agar at 20ºC. Thirty plugs, 3 mm diameter, were excised from the advancing margin of the colony and used to inoculate 200 mL of minimal salt-glucose (MS-Glu) medium (10 g/L glucose, 2 g/L NH_4NO_3, 1 g/L KH2 PO4, 0.1 g/L $MgSO_4.7H_2O$, 0.5 g/L yeast extract, 3 g/L DL-malic acid, 1 g/L NaOH). The culture was incubated at 20ºC in a 1 L flask with shaking (80 rpm) for 3 days. Mycelia were harvested by centrifugation and resuspended in the same volume of MS-Glu. This was homogenized using a Waring blender by applying ten pulses for 2 sec each. Aliquots (20 µL) of the medium were placed onto detached 21-day-old leaves of wild type lines and transgenic lines expressing *BnPGIP1* or *BnPGIP2*. Leaves were placed in a Petri plate on wet Whatman paper in a sealed chamber to maintain humidity.

CHAPTER THREE:

CONCERTED REGULATION OF *S. SCLEROTIORUM CUTINASE A* AND *POLYGALACTURONASE 1* TO OVERCOME PLANT PHYSICAL BARRIERS

3.1 Abstract:

S. sclerotiorum releases hydrolytic enzymes that sequentially degrade structural components of the plant cuticle, middle lamellae, primary and secondary cell walls. The cuticle was determined to be a barrier to *S. sclerotiorum* infection as leaves stripped of cuticle were more effectively colonized. The factors affecting the regulation of genes encoding SsPG1 and a newly identified cutinase (SsCUTA) were examined. *In vitro, SsCutA* transcripts were detected within 1 hpi of leaves and expression was primarily governed by contact of mycelia with solid or semi-solid surfaces. Expression of *SsPg1* was mainly restricted to the expanding margin of the lesion. The balance between pectin and galacturonic acid (GA) was found to regulate the expression of *SsPg1* where GA repressed induction by pectin. Carbon starvation was also found to induce *SsPg1* expression. Disruption of calcium signalling reduced *SsCutA* and *SsPg1* expression and decreased *S. sclerotiorum* virulence, whereas elevated cAMP levels reduced virulence, but did not affect expression of either gene.

3.2 Introduction

In the previous chapter, *S. sclertiorum* polygalacturonses (PGs) were found to cause necrosis only when applied to damaged leaves or infiltrated into the leaf tissue. This finding indicates that this aspect of *S. sclerotiorum* PG activity is dependent on physical or other enzymatic mechanisms to reach host epidermal cells. This chapter examines the mechanisms used by *S. sclerotiorum*

to breach the plant cuticle, the first barrier to infection, as well as the interplay between cutinases and PGs in plant-pathogen interactions.

The plant cuticle is comprised of several chemically and mechanically distinct layers (Jetter et al., 2000). The outermost layer is composed of intracuticular wax or cutin, a polymer of 16 and 18 carbon hydroxy fatty acids linked by ester bonds covered by a film of soluble lipids referred to as epicuticular wax (Baker, 1982). Many pathogens degrade cuticular lipids by releasing cutinase which hydrolyzes the ester bond linking the fatty acids (Baker and Bateman, 1978). In *F. solani*, cutinase activity was required for pathogenicity on potatoes and increased virulence was correlated with increased cutinase activity (Morid et al., 2009). Interestingly, some pathogens that lack cutinase, such as *Mycosphaerella* species, can only infect wounded plants and insertion of a cutinase gene conferred the ability to infect intact papaya fruits (Dickman et al., 1989). The necrotrophic pathogen, *B. cinerea,* expresses genes encoding both cutinase (Vlugt-Bergmans et al., 1997b) and lipase early in the infection (Reis et al., 2005); however, pathogenicity was not compromised in mutants lacking these enzymes (van Kan et al., 1997; Reis et al., 2005). *S. sclerotiorum* also secretes lipases beneath mycelia in contact with the host cuticle (Tariq and Jeffries, 1987), but little is known about the complement of cuticle-penetrating enzymes or how they arose.

The majority of phytopathogenic fungi have multiple genes encoding cutinase-like enzymes. A recent study proposed that a gene encoding a cutin-degrading enzyme evolved after the origin of land plants in a microbe living closely with plants. Eukaryotic plant pathogens acquired cutinase genes by lateral gene transfer from prokaryote pathogens (Belbahri et al., 2008). *Phytophthora infestans* has two cutinase A (*CutA*) genes, while *P. sojae* and *P. ramorum* have 16 and 4 paralogs, respectively. Bioinformatics analysis found orthologues of these genes in Actinobacteria suggesting that *Phytophthora* acquired the *CutA* gene from this prokaryote. The multiple *CutA* paralogs existing in close proximity in the genome of *Phytophthora* species likely arose

from recent gene duplication (Belbahri et al., 2008). Similar clusters of genes encoding PGs were found in *Phytophthora cinnamomi* and they were suggested to have evolved in a similar fashion (Gotesson et al., 2002). This illustrates the evolutionary pressure the development of the cuticle had on pathogens. As with pectin, epicuticular wax is chemically complex and multiple enzymes are required to fully hydrolyze it. While the biochemical specificity of cutinases from phytopathogens has not been examined, different isoforms may have greatly different activities. As an example from another pathosystem, *Mycobacterium tuberculosis* acquires lipids from the degradation of host cell membranes which it uses as carbon source during the infection (Schue et al., 2010). The bacterium has seven genes encoding cutinase-like enzymes, one of which hydrolyzes medium-chain carboxylic esters and monoacylglycerols, while another is a phospholipase (Schue et al., 2010).

The cutin monomers derived from cutinase activity are signalling molecules for the pathogen and regulates processes that take place early in the infection. Cutin induces appressorium formation in *Magnaporthe grisea* (Francis et al., 1996) and removing cuticular waxes reduced germination of *Blumeria raminis* on barley (Zabka et al., 2008). *F. solani* has three highly similar, but differentially regulated cutinase genes. *FsCut1* is induced by cutin monomers, while *FsCut2* and *FsCut3* are constitutively expressed, but can be induced further by starvation. The expression of *FsCut1* is restricted by a repressor that binds to the *FsCut1* promoter, but not to that of *FsCut2* or *FsCut3.* In the presence of cutin monomers, the repressor is released allowing induction of *FsCut1* expression (Li et al., 2002).

Cuticle permeability also plays a major role in determining the outcome of host plant-pathogen interactions. *A. thaliana* mutants with increased cuticle permeability were more resistant to *S. sclerotiorum* infection (Bessire et al., 2007). The leaves of the mutant line were found to be more perceptive of elicitors since they accumulated antifungal compounds in response to the application of sterile culture broths in which the pathogen has been grown,

while wild type leaves did not (Bessire et al., 2007). Therefore, the lack of cuticle permeability may be disadvantageous to the plant if this delays perception of pathogen elicitors and early induction of defences. Cutin hydrolysis leads to the release of cutin monomers, which are described earlier as danger-associated molecular patterns (DAMPs) that induce plant defences (Reina-pinto and Yephremoy, 2009). Expression of fungal cutinase in *A. thaliana* led to complete resistance to *B. cinerea* infection, while an inactive enzyme did not which indicated that perception of cutin monomers and not the enzyme itself is responsible for inducing defence responses. The same set of defence genes were expressed in both the transgenic plants expressing cutinase and those infected with *B. cinerea* (Chassot et al., 2008).

The second barrier to infection is the plant cell wall which is composed of cellulose-xyloglucan (50% w/w), pectin (30%) and protein (1-5%). The cellulose-xyloglucan network serves as the skeleton of the plant cell wall and is encased in pectin and protein which provide structural stability (reviewed by Carpita and Gibeaut, 1993). Pectin is a polymer of galacturonic acid (GA), up to 200 (1→4) α-D-galacturonic acid units (Lau et al., 1985), which is periodically interrupted by rhamnosyl residues (Jarvis, 1984). The rhamnosyl units may be either rhamnogalacturonan I or rhamnogalacturonan II. Rhamnogalacturonan I is composed of repeats of (1→2) α-L-rhamnosyl-(1→4) α-D-galacturonic acid. Rhamnogalacturonan II is less abundant and yields up to 10 different types of sugars upon hydrolysis including several rare monosaccharides (Darvill et al., 1978). Plant pathogens release several types of pectin degrading enzymes, most notably pectin methylesterases, pectin lyases and PGs that weaken the plant cell wall and facilitate the spread of the pathogen.

The production of *S. sclerotiorum* PG is induced by pectin, while glucose represses PG synthesis (Riou et al., 1992; Fraissinet-Tachet and Fevre, 1996; Li et al., 2004a). Four *S. sclerotiorum* PG genes have been characterized, herein referred to as *SsPg1*, *SsPg3*, *SsPg5* and *SsPg6* according to the nomenclature

used by Li et al. (2004b), but also described by Fraissinet-Tachet et al (1995) and Kasza et al (2004). *In planta*, expression of *SsPg1* preceded that of *SsPg3*, *SsPg5* and *SsPg6* suggesting that SSPG1 may be important for infection establishment (Cotton et al., 2002; Li et al., 2004b; Sella et al., 2005). The expression of *SsPg3*, *SsPg5* and a gene encoding an exo-PG, *SsxPg1*, was induced by pectin and galacturonic acid (GA), but repressed by glucose (Li et al., 2004b). Early studies that examined the regulation of *SsPg1* expression reported that the gene was not expressed in the presence of glucose. This observation, together with the identification of a CREA repressor-binding site in the promoter, suggested that *SsPg1* expression was subject to carbon catabolite repression (Cotton et al., 1996). The regulation of *SsPg1*, however, is not clear as subsequent reports indicated that *SsPg1* expression was unaffected by glucose or GA (Cotton et al., 2003), while others found it to be partially repressed by glucose (Li et al., 2004b).

Additional factors are also involved in the regulation of *S. sclerotiorum* PG gene expression. PGs are released in parallel with oxalic acid and these function collaboratively during the infection. Oxalic acid reduces ambient pH, and *SsPg1* was shown to be expressed in the pH range of 3.0-3.8. pH-dependent expression is negatively regulated by the transcription factor PAC and disruption of the *S. sclerotiorum Pac* gene caused *SsPg1* to be expressed at a higher pH range from 4.6-6.6 (Rollins, 2003). PAC is also a positive regulator of *SsPg6* expression, which is normally expressed at neutral pH (Tae kim et al., 2007).

This chapter describes a *S. sclerotiorum* cutinase gene (*SsCutA*) and identifies the factors that regulate the expression of *SsCutA* and *SsPg1* during the infection. The intracellular signalling mechanisms involved in the expression of these genes are also examined.

3.3 Results

3.3.1 The cuticle as a barrier to infection

The role of the cuticle in *S. sclerotiorum*-host plant interactions was examined by comparing the progress of the infection on intact *B. napus* leaves to leaves that had been stripped of surface wax (Figure 3.1). On either intact or stripped leaves no symptoms were observed at 1 hour post-inoculation (hpi). By 12 hpi, infection foci were observed beneath the inoculum on the stripped leaf, but not on the intact leaf. At 24 hpi, the inoculum area was completely necrotic on stripped leaves, while only small necrotic foci were observed on intact leaves. *Sclerotia* initials were observed on the stripped leaves at 32 hpi, indicating that the infection was in its final stages. The appearance of sclerotia suggests that the leaf tissue has been fully digested and available nutrients have been depleted. Only at 48 hpi were the symptoms on intact leaves comparable to those seen at 32 hpi on stripped leaves, indicating that the progress of infection was delayed by the cuticle.

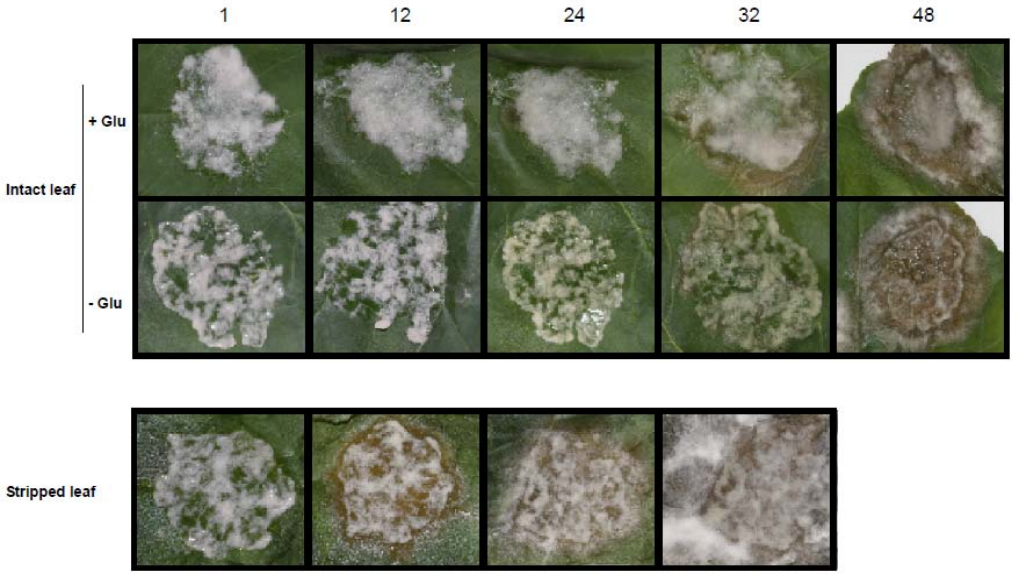


Figure 3.1 Role of cuticle and carbon availability in *S. sclerotiorum* infection development.
Time course(hours) showing the progress of *S. sclerotiorum* infection on *B. napus* leaves stripped of cuticular wax and intact leaves in the presence or absence of glucose. Leaves were inoclulated with mycelia placed in a 5 cm diameter circle.

3.3.2 Identification of *S. sclerotiorum* cutinase genes

A search of the *S. sclerotiorum* genome using BLAST with the *B. cinerea cutinase A* (*BcCUTA*) ORF identified four genes encoding cutinase-like enzymes. The enzymes were encoded by SS1G_07661.1, SS1G_12709.1, SS1G_13386.1 and SS1G_12907.1, and exhibited 81 %, 63 %, 58 % and 52 % identity, respectively, to BcCUTA (Figure 3.2). SS1G_07661.1 contained an open reading frame that encoded a 203 amino acid protein, compared to 202 amino acids for BcCUTA, and was designated SsCUTA (Figure 3.2). The most probable signal peptide cleavage site was predicted to be between amino acids 22 and 23 and the predicted molecular weight of the mature enzyme was around 20.14 KDa.

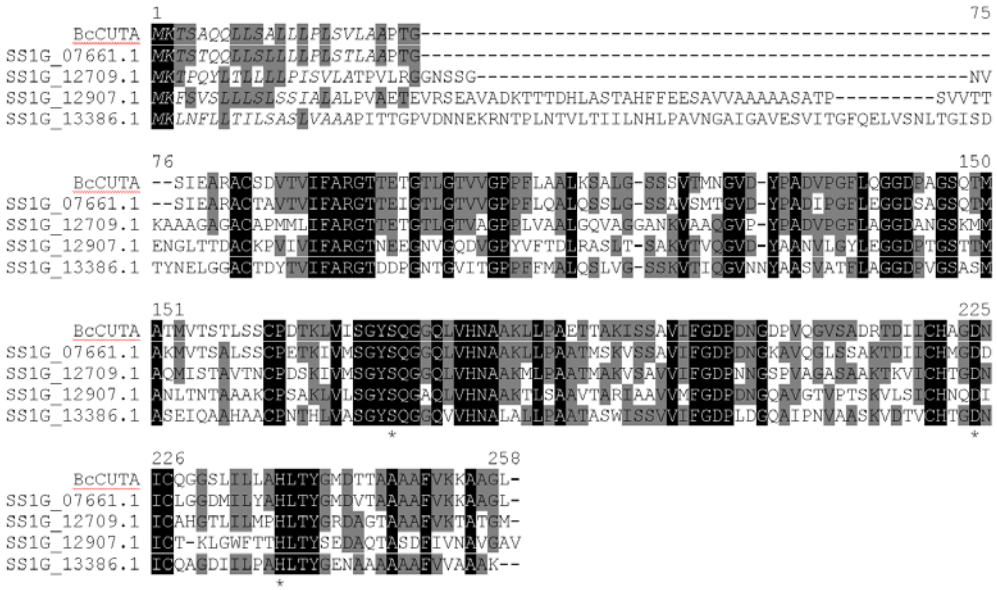


Figure 3.2 Alignment of potential *cutinase* genes from *S. sclerotiorum*. Alignment of *B. cinerea* CUTA with proteins encoded by four homologues genes from *S. sclerotiorum*. Signal peptides (italics), identical residues (white on black background), conserved residues (black on grey) and amino acids forming the esterase catalytic triad (*) are shown.

3.3.3 Expression of *SsCutA*, *SsPg1* and *SsPg2* during the infection

SsPg2 was found not to be expressed during infection in the highly aggressive strain of *S. sclerotiorum* 1980. Alterations of other environmental parameters such as the provision of pectin or galacturonic acid or contact with a solid surface did not induce gene expression. The same samples were found to express *SsPg1* at a high level and therefore further studies focused on *SsPg1* (Figure 3.3).

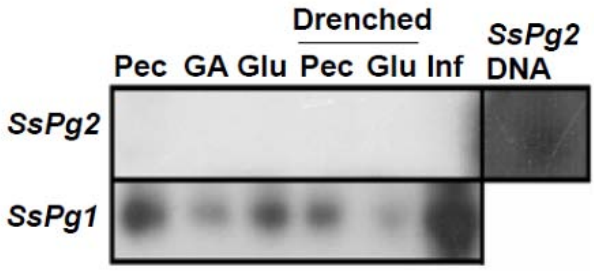


Figure 3.3 Expression of *SsPg1* and *2* under different contact and nutritional conditions.
S. sclerotiorum mycelia were grown in minimal salt medium with the carbon sources indicated above the blot and then spread on Parafilm or *B. napus* leaves surface. Furthermore Ms-glucose (Glu) or Ms-pectin (Pec) were provided (Drenched) to examine the effect of contact surface and carbon source in combination. Northern blots were performed using *SsPg2* DNA as a probe control and the same membrane was stripped and probed again with *SsPg1*.

To better understand the interplay between cutinase and PG, the expression of *SsCutA* and *SsPg1* was examined during the first 48 hpi. *SsCutA* was induced within 1 hpi and its expression increased steadily until 24 hpi. By 48 hpi, the leaf with the inoculum was fully necrotic and expression of *SsCutA* declined dramatically (Figure 3.4A). *SsPg1* exhibited a different pattern of expression. It was expressed slightly at 1 hpi and transcript levels were approximately the same at 6 hpi. By 24 hpi, transcript levels increased dramatically and then declined by 48 hpi as the initial inoculation site became fully necrotic.

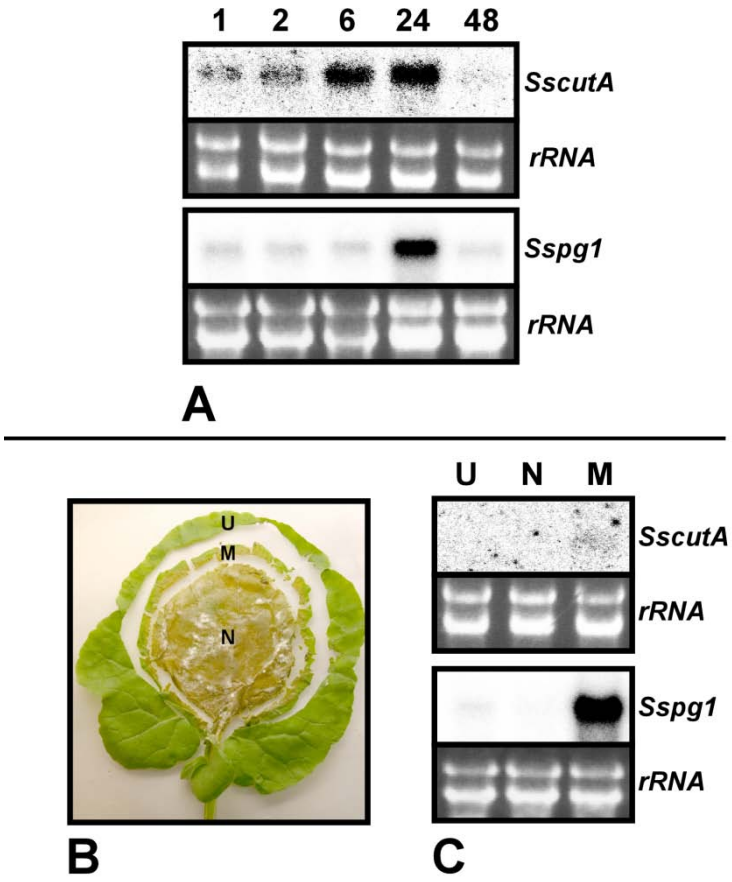


Figure 3.4 Expression of *SsCutA* and *SsPg1* during infection of *B. napus* leaves.
A) Northern blot analysis showing expression in infected tissue at 1, 2, 6, 24, 48 hpi. **B)** Dissection of the leaf into three distinct zones: uninfected (U), margin (M) and necrotic (N). **C)** Northern blots showing *SsCutA* and *SsPg1* expression in each zone. Ribosomal RNA loaded in each lane is shown in lower panels and experiment was repeated three times.

At 48 hpi, the expanding lesion was subdivided into specific zones, namely the necrotic tissue, the lesion margin and asymptomatic tissue. *SsCutA* expression was detected only at the lesion margin, though at very low levels, suggesting that the primary role of SsCUTA is to breach the cuticle during the initial stages of the infection. When the same zones were examined, high levels

of *SsPg1* transcripts were detected at the margin of the expanding lesion, but not in the necrotic or asymptomatic regions (Figure 3.4C).

3.3.4 Regulation of *SsCutA* by contact with surfaces

SsCutA expression was detected within 1 hpi. Whether this was a response to elicitors on the leaf surface or to interaction with solid or semi-solid surfaces was examined. *SsCutA* transcripts were not detected when mycelia were transferred to liquid MS medium or MS medium supplemented with glucose or finely granulated leaf wax (Figure 3.5). Conversely, *SsCutA* expression was sharply induced within 18 h after mycelia were transferred to solid or semi-solid surfaces, namely intact leaves, leaves stripped of wax, leaf wax on glass, PDA, Parafilm™ or glass. In the presence of glucose, *SsCutA* expression was fairly uniform regardless of the surface. In the absence of glucose, surfaces that contained plant components were more conducive to *SsCutA* expression, though this may be due to the availability of a carbon source.

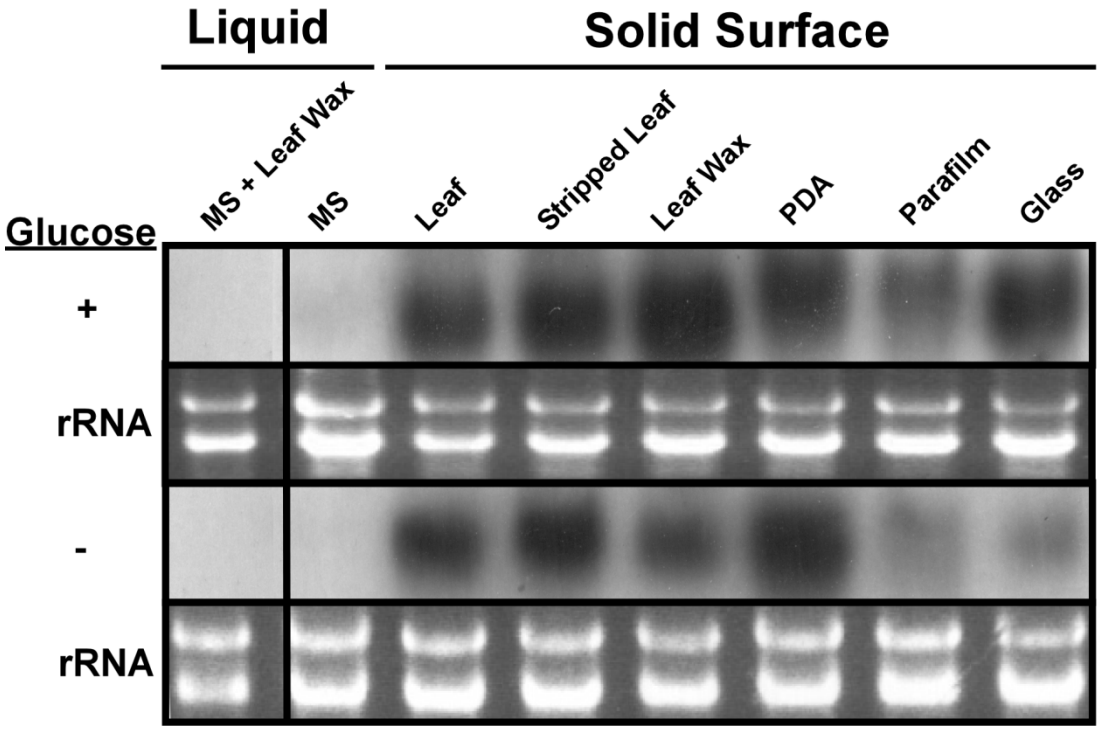


Figure 3.5 Expression of *SsCutA* in response to contact with different surfaces. Northern blot analysis showing the expression *S. sclerotiorum SsCutA* in mycelia after transfer to minimal salts (MS) liquid medium supplemented with wax granules or solid surfaces in the presence or absence of glucose or leaf wax. rRNA loaded in each lane is shown in the bottom panel.

3.3.5 Regulation of *SsPg1* expression by pH

During infection, the pH within the infected region is reduced by the release of oxalic acid and *SsPg1* expression was reported to be optimal at low pH (Rollins, 2003). In MS medium supplemented with pectin, *SsPg1* expression was detected at substantially higher levels at pH 3.0 than at pH 7.0 (Figure 3.6). Interestingly, two peaks of *SsPg1* expression were observed; an initial peak on the first day and a second peak at approximately the fourth day.

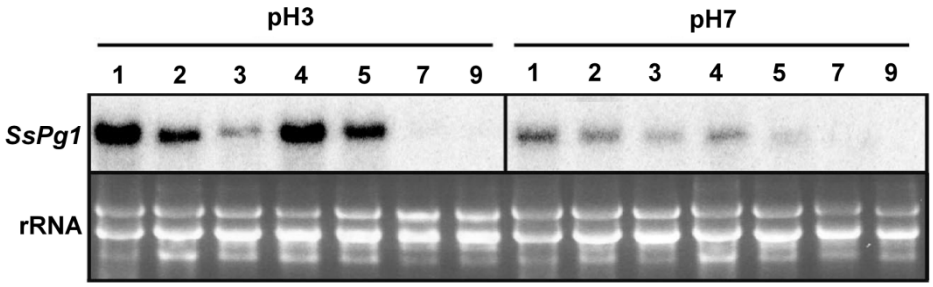


Figure 3.6 Expression of *SsPg1* in response to pectin and pH.
Northern blot analysis showing a time course (days) analysis of *SsPg1* expression in liquid minimal salts medium supplemented with 1% pectin. The culture supernatant was buffered at pH 3 or 7. rRNA loaded in each lane is shown below the blot.

3.3.6 Regulation of *SsPg1* expression by galacturonic acid

Previous studies demonstrated that pectin and GA stimulated *S. sclerotiorum* PG production and induced the expression of several PG genes (Cotton et al., 2003; Li et al., 2004b). In the latter study, mycelial plugs were used as the inoculum which already had high initial levels of *SsPg1* transcripts since they were excised from PDA, therefore, the effect of pectin and GA could not be properly determined (Li et al., 2004b). In the current study, mycelia propagated in MS-Glu liquid medium, in which *SsPg1* is not induced, were used as a source of inoculum to avoid this problem. *SsPg1* expression was monitored in MS and MS-pectin media buffered at pH 3.0 and supplemented with a range of GA concentrations (Figure 3.7). When MS medium was

supplemented with the lower GA concentrations tested (0.01-0.05%), *SsPg1* was expressed at similar levels over the entire nine day period. Conversely, higher concentrations of GA (0.1 -1.0%) resulted in a gradual reduction of *SsPg1* expression from that observed on the first day. At GA concentrations of 0.5 and 1%, *SsPg1* transcripts at day five or thereafter were very low. GA was also found to suppress *SsPg1* expression in the presence of pectin which had in earlier studies been reported to be an inducer. Supplementation of the MS-pectin medium with increasing concentrations of GA (0.01%-1%) resulted in a corresponding decrease in *SsPg1* expression over time. Furthermore, higher concentrations (1.0 %) of GA abolished the expression of *SsPg1*, even that seen on the first day at lower GA concentrations. Two peaks of expression were again observed in MS-pectin medium in the absence of GA.

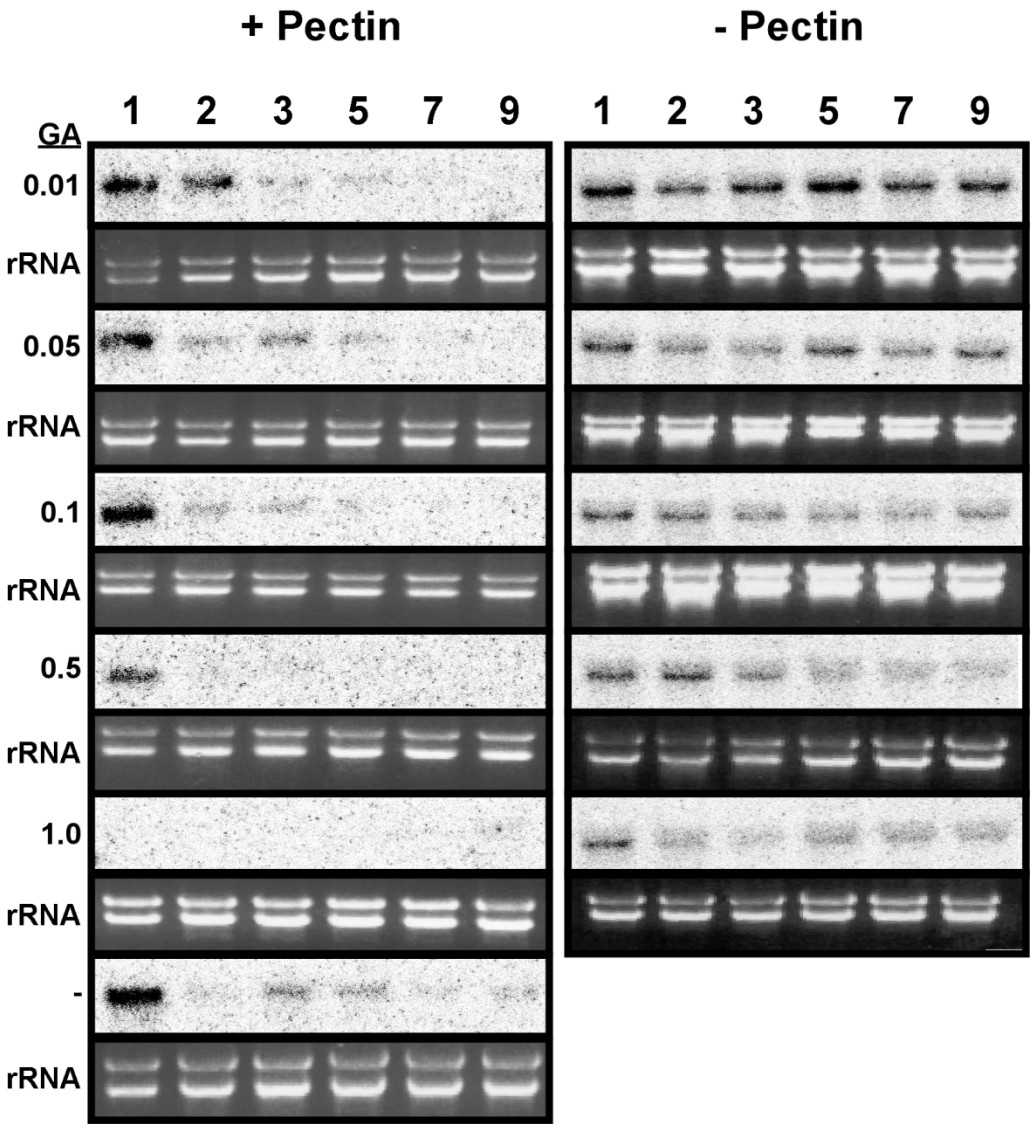


Figure 3.7 Regulation of *SsPg1* expression by pectin and galacturonic acid. Northern blot showing the expression of *SsPg1* in liquid MS medium supplemented with galacturonic acid (GA) and/or pectin over the course of nine days. Values indicated in the left margin are the concentrations of GA (% w/v). rRNA loaded in each lane is shown below the blot.

3.3.7 Regulation of *SsPg1* and *SsCutA* expression by carbon availability

In a natural setting, *S. sclerotiorum* uses dead petals as an initial source of carbon to establish sufficient mycelial biomass to initiate the infection. When mycelia were applied to the leaf surface in MS medium, they were slower to establish an infection than mycelia in MS supplemented with glucose. In the

presence of glucose, necrosis was observed at 12 hpi, while in the absence of glucose necrosis was observed only at 32 hpi (Figure 3.1).

The expression of *SsPg1* in response to organic carbon starvation was examined. In MS liquid medium alone, *SsPg1* expression gradually increased and peaked on the fourth day (Figure 3.8). When glucose was provided as a carbon source, *SsPg1* expression was delayed and did not peak until the seventh day, indicating that *SsPg1* expression is responsive to carbon limitation. *SsCutA* expression was not detected in either the MS or MS-Glu liquid medium, indicating that *SsCutA* is not responsive to carbon starvation or glucose supplementation in liquid culture.

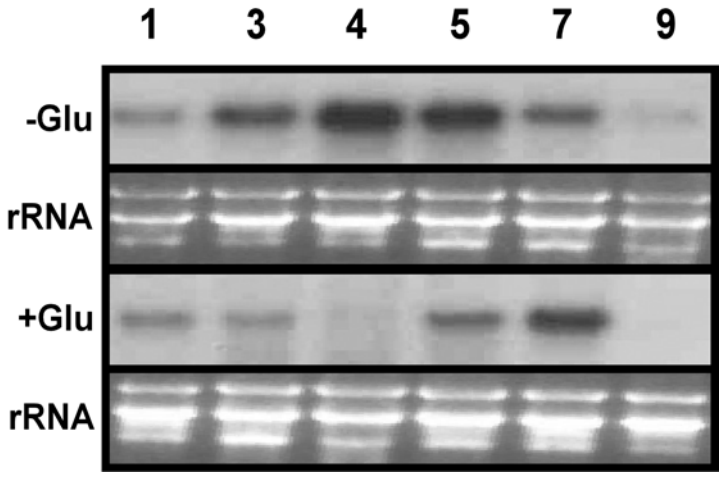


Figure 3.8 Expression of *SsPg1* in response to carbon availability.
Northern blot analysis showing the expression of *SsPg1* in liquid MS medium or MS medium supplemented with 1% glucose over the course of nine days as indicated above the panels. rRNA loaded in each lane is shown below the blot.

3.3.8 The role of calcium and cAMP signalling in *SsCutA* and *SsPg1* regulation

Extracellular factors, such as contact with surfaces and carbon availability, were found to control the expression of *SsPg1*and *SsCutA.* Here, a pharmacological study was conducted to examine the role of calcium and cAMP-mediated intracellular signalling in the overall infection process and specifically in regulating *SsCutA* and *SsPg*1 expression.

Lanthanum chloride, a plasma membrane calcium channel blocker, reduced *S. sclerotiorum* virulence and expression of both *SsCutA* and *SsPg1*, though the effect on *SsPg1* was more pronounced. EGTA, a chelator of external calcium, did not affect virulence or gene expression, suggesting that mobilization of external calcium is not required for infection. Compounds 48/80 and U73122 both inhibit phospholipase C activity, but also promote the release of calcium from intracellular stores (Mogami et al., 1997). Treatment with 48/80 slightly reduced virulence; however, both reduced *SsPg1* and *SsCutA* expression. At the concentrations tested, none of these compounds inhibited mycelial growth indicating that any reduction in gene expression was not due to cytotoxicity (Figure 3.9). Calcineurin, a calcium and calmodulin-dependent Ser/Thr protein phosphatase, is also involved in fungal pathogenesis (Fox and Heitman, 2002). The calcineurin inhibitor, cyclosporin A, reduced virulence and expression of *SsPg1* and *SsCutA*, though mycelial growth was also reduced.

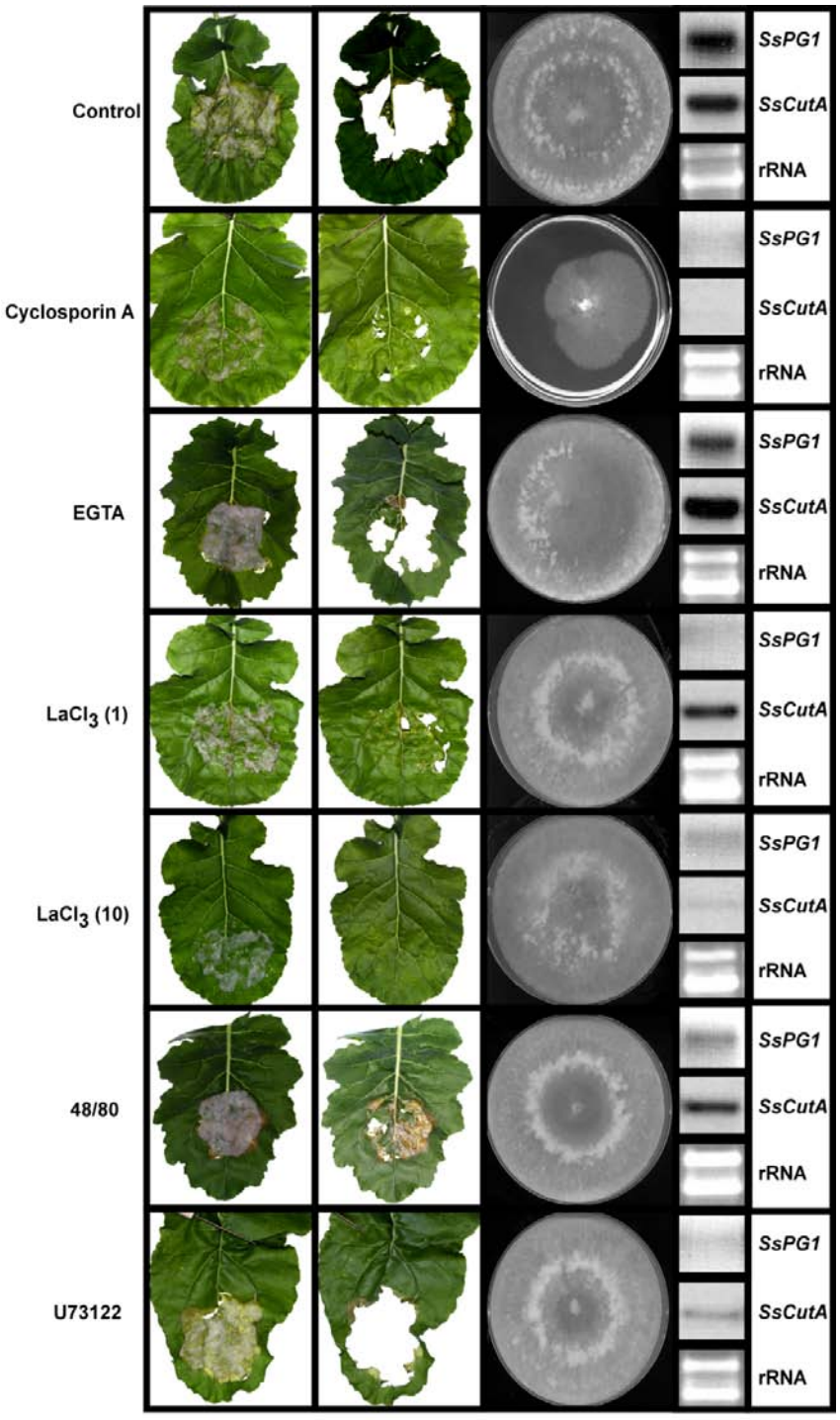


Figure 3.9 Effect of calcium and cAMP signalling and calcineurin inhibitors on *S. sclerotiorum* virulence, growth and *SsPg1* and *SsCutA* expression.

The effect of compounds interfering with cAMP levels and calcium signaling on infection of *B. napus* leaves 16 h after inoculation with either untreated *S. sclerotiorum* mycelia or mycelia treated with cyclosporin A (4.9 nM), EGTA (10 mM), lanthanum chloride ($LaCL_3$; 1 and 10 mM), compound 48/80 (5 μM) or compound U73122 (10 mM). The leaf on the right has had the inoculum removed to reveal the lesion beneath. The effect of the various compounds on mycelial growth on MS-Glu agar after five days is shown next to the leaves. The panels to the right show northern blot analysis of *SsPg1* and *ScCutA* expression during infection of the leaves with rRNA below the blot.

cAMP signalling plays a role in many aspects of fungal biology including virulence (D'Souza and Heitman, 2001). Caffeine has been used to increase cAMP accumulation in fungi through inhibition of phosphodiesterases (Berridge, 1997); however, it also increases internal calcium levels by activating ryanodine receptors on the endoplasmic reticulum leading to the release of calcium stores (Berridge, 1997). Caffeine sharply reduced the level of *SsPg1*expression and to a lesser extent the expression of *ScCutA* (data not shown). To directly examine the role of cAMP, *S. sclerotiorum* lesion formation as well as *SsPg1* and *SsCutA* expression were monitored after treatment of mycelia with exogenous cAMP. At 0.1 mM and 1 mM concentrations, cAMP did not affect lesion development, while lesion formation was reduced at 10 mM cAMP (Figure 3.10). Interestingly, *SsPg1* and *SsCutA* expression were not dramatically affected at any of the cAMP concentrations tested.

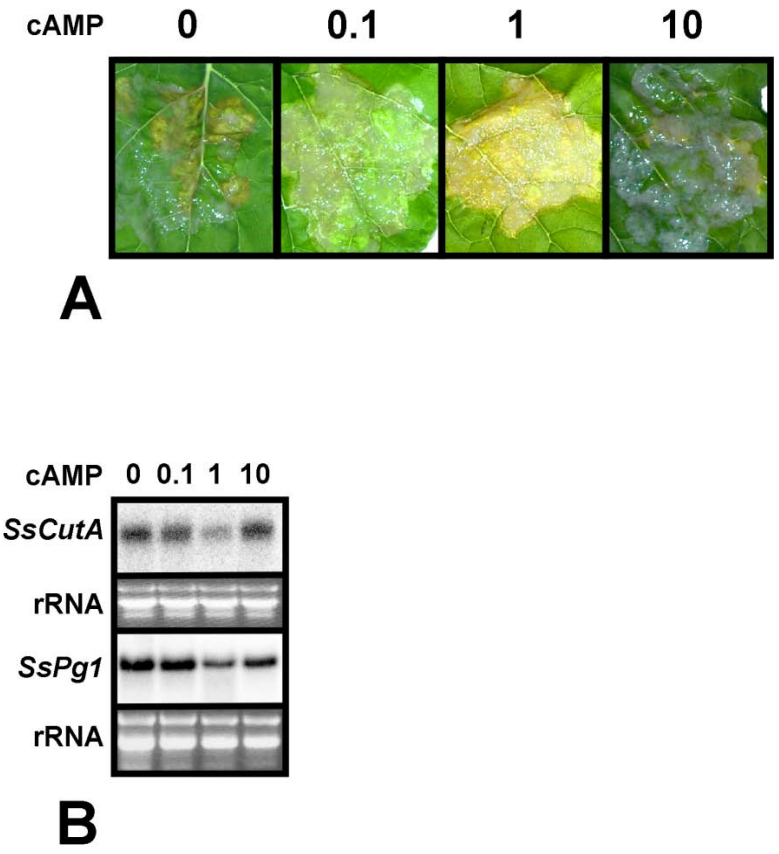


Figure 3.10 Effect of exogenous cAMP application on *S. sclerotiorum* infectivity and expression of *SsCutA* and *SsPg1*
A) Lesions forming on *B. napus* leaves when mycelia from *S. sclerotiorum* strain 1980 were treated with increasing levels of cAMP (mM) prior to inoculation. **B)** Northern blot analysis showing *SsCutA* and *SsPg1* expression 24 h after inoculation of *B. napus* leaves with mycelia treated with cAMP. The bottom panel shows the rRNA loaded in each lane

3.4 Discussion

3.4.1 Concerted expression of *SsPg1* and *SsCutA* during infection initiation and lesion expansion:

To penetrate the leaf cuticle and the plant cell wall, *S. sclerotiorum* must release degradative enzymes at the appropriate stage of the infection. This study examined the expression of *SsCutA* and *SsPg1* at the time when the cuticle is being breached and subsequently as the lesion develops and expands.

Leaves that had been stripped of cuticle were more susceptible to *S. sclerotiorum* infection. The plant cuticle contains a complex mixture of long

chain saturated hydrocarbons, primary and secondary alcohols, esters and ketones (Purdy and Truter, 1963). Lipase activity was detected beneath *S. sclerotiorum* infection cushions in contact with the leaf surface (Tariq and Jeffries, 1987). This activity was proposed to be involved in cuticle degradation. Indeed, when an anti-lipase antibody was applied to the leaf surface it prevented the infection of tomato leaves by *B. cinerea* (Commenil et al., 1998); however, mutation of *BcCutA* alone (Van Kan et al., 1997) or in combination with *BcLip1* (Reis et al., 2005) had little or no impact on *B. cinerea* pathogenicity. In this study, I identified four genes encoding putative cutinases in the *S. sclerotiorum* genome, of which *SsCutA* was most similar to *BcCutA*.

SsCutA transcripts were detected within one hour after contact with the leaf surface and continued to increase up to 24 hpi. Similarly, *BcCutA* was expressed at the early stages of the infection; a *BcCutA* promoter-GUS fusion was active within 6 h of conidial germination on gerbera flowers (van Kan et al., 1997). This suggests that cutinase is involved in hydrolyzing the cuticle during the very early stages of the infection. Unlike the strong early induction of *SsCutA*, *SsPg1* expression was induced only slightly within 1 h after contact with *B. napus* leaves, but its expression increased sharply at 24 hpi. At this point, necrotic foci were detected beneath the inoculum indicating that the cuticle had been breached and the infection was at a stage where cell death was occurring. The rapid induction of *SsCutA* soon after inoculation and the coincidental appearance of necrotic foci with induction of *SsPg1* expression, indicate that SsCUTA and SsPG1 work in concert to initiate and progress the infection.

On *B. napus* leaves, the highest levels of *SsPg1* transcripts were detected at 24 hpi; however, both the host plant and tissue may affect when the gene is expressed. On carrot roots (Kasza et al., 2004) and sunflower cotyledons (Cotton et al., 2002), *SsPg1* transcripts, referred to as *pg1-3* or *pg2*, were detected at 16 hpi and continued to increase until 36 hpi. During infection of

soybean leaves, *SsPg1* (*SsPgb*) expression was detected within 8 hpi before peaking at 16 hpi (Sella et al., 2005).

In this study, *SsPg1* was expressed mainly at the margin of the necrotic zone, suggesting that it is involved in lesion expansion. In other studies, *SsPg1* was expressed sooner than *SsPg3*, *SsPg5* or *SsPg6* (Cotton et al., 2002; Li et al., 2004b; Sella et al., 2005) indicating that other *S. sclerotiorum* PGs may be involved in the maceration of tissues within the necrotic zone. This notion is supported by studies which demonstrated that *BcPg1,* the *B. cinerea* orthologue of *SsPg1*, was expressed at the margin of the infection zone in apple fruit, but to a lesser degree in the center of the lesion. Furthermore, *BcPg1* mutants generated a primary infection, but were less able to expand the lesion (ten Have et al., 1998).

3.4.2 Regulation of *SsCutA* and *SsPg1*

The first opportunity for a plant pathogen to detect the presence of a suitable host is by physical interaction with its surface. *SsCutA* expression was detected soon after inoculation of the leaf with mycelia or after contact with other solid surfaces. Contact with a solid surface was required for the release of cutinase from *Erysiphe graminis* f. sp. *hordei* spores (Pascholati et al., 1992). Cutinase is also a part of the hydrolytic enzyme milieu within the spore extracellular matrix (Pascholati et al., 1993). Surface contact was also shown to be important for spore germination and appressorium formation in other fungal pathogens (Kim et al., 1998; Chaky et al., 2001; Doehlemann et al., 2006). Expression of *SsCutA* was also higher in the presence of readily useable carbon source which may mimic the natural situation where this is provided by senescent petals. Leaf wax granules in liquid medium did not induce *SsCutA*; however, its orthologue *BcCutA* was induced in response to the cutin monomer 16-hydroxyhexadecanoic acid (Van der Vlugt-Bergmans et al., 1997b). The lack of induction of *SsCutA* by wax in liquid medium may be due to the inability to solubilise wax components provided in the form of finely granulated

particles. Furthermore, since *SsCutA* is not expressed constitutively, it may not be available to degrade the wax granules to cutin monomers that could induce expression. Down-regulation of *SsCutA* expression after cuticle penetration may be due to the lack of cutin monomers between the epidermal cells beneath the cuticle. It is also possible that certain molecules released from the cell wall, such as galacturonic acid, signal the end of cuticle breach and subsequently repress *SsCutA* expression. Cutinases, or more precisely the products they generate, also regulate other aspects necessary for a successful infection. Mutation of the *M. grisea cutinase2* (*MgCUT2*) gene severely reduced virulence. In addition to being involved directly in cuticle penetration, MgCUT2 was also required for the release of cutin monomers that induce appressorium formation. In fact, supplementation of the *MgCut2* mutant with monomeric cutins fully restored the pathogenicity defects (Skamnioti and Gurr, 2007).

Expression of *SsPg1* is regulated by multiple biochemical parameters. *SsPg1* was shown to be induced slightly in response to contact with the leaf or other surfaces (Li et al., 2004b). In this study, expression was sharply induced as necrotic foci began to appear, but declined thereafter coincident with maceration of the infected tissue. Within the infection zone, expression of *SsPg1* was only detected in the lesion margin where deposits of intact pectin exist and simple sugars are lacking. *SsPg1* expression is induced by carbon starvation which would allow the pathogen to hydrolyze the polymers and access these carbon reserves. Previous studies suggested that starvation is one component of the signal required for the induction of pathogenicity determinants and growth on the host (Coleman et al., 1997), for example, the *avr9* gene from *Cladosporium fulvum* is induced in response to nitrogen starvation (van den Ackerveken et al., 1994).

Pectin degradation releases rhamnogalacturonan I and rhamnogalacturonan II which upon further hydrolysis release GA and several rare monosaccharides (Darvill et al., 1978). As such, monosaccharides, including GA, are readily

available in the center of the lesion where *SsPg1* transcripts were not detected and indeed, *SsPg1* expression is repressed by GA. This was also observed with *BcPg1*, where the induction of expression by pectin was transient and *BcPg1* transcript levels decreased upon transfer of mycelia from glucose to GA (Wubben et al., 1999). In MS-pectin medium two peaks of *SsPg1* expression were observed; the initial peak may be due to induction by pectin or pectin components followed by a decline due to the release of GA. The second peak may be due to consumption of the GA and induction of expression by carbon starvation or remaining pectin components, though this remains to be tested. Regulation of *SsPg1* by carbon starvation and increasing GA concentrations supports its proposed role in lesion expansion. Previously, GA was shown to induce the expression of two other *S. sclerotiorum* PG genes, *SsPg3* and *SsPg6*, suggesting they are involved in tissue maceration during the later stages of the infection (Li et al., 2003).

3.4.3 Intercellular signals regulating *SsCutA* and *SsPg1* expression

SsCutA expression is responsive to physical interaction with a solid surface. *SsPg1* is also partially regulated in this manner, but its expression is also affected by availability of carbon, GA concentration, pH and the stage of the infection indicating that multiple signalling pathways may be involved. Intracellular cAMP levels regulate many aspects of *S. sclerotiorum* biology (Rollins and Dickman, 1998; Chen et al., 2004; Chen and Dickman, 2005). Reducing cAMP levels through mutation of the adenylate cyclase gene, *sac1*, caused pleiotropic effects including the formation of abnormal sclerotia (Rollins, 2003), the inability to form infection cushions and reduced virulence (Jurick and Rollins, 2007). In this study, application of exogenous cAMP reduced virulence, but did not affect *SsPg1* or *SsCutA* expression.

Calcium signalling is also involved in regulating aspects of the infection process. In *C. gloeosporioides*, spore germination and appressoria formation in response to contact with a solid surface were calcium-calmodulin dependent

(Kim et al., 1998). Treatment of mycelia with the general calcium channel blocker, lanthanum chloride, reduced virulence and expression of *SsPg1* and *SsCutA* suggesting that calcium signalling is involved in regulating the expression of these genes. EGTA did not affect virulence or gene expression indicating that mobilization of external calcium is not required for infection.

Calcineurin was also found to be involved in the regulation of *SsCutA*, *SsPg1* and virulence. Calcineurin encodes a Ser/Thr phosphatase involved in attenuating MAPK cascades in a calcium-dependent manner. Expression of an anti-sense calcineurin gene reduced *S. sclerotiorum* virulence (Harel et al., 2006). In this study, treatment of *S. sclerotiorum* mycelia with the calcineurin inhibitor, cyclosporin A, reduced virulence as well as *SsCutA* and *SsPg1* expression which is in agreement with that reported for *B. cinerea BcPg1* (Viaud et al., 2003; Schumacher et al., 2008). This suggests that MAPK signal transduction pathways may be involved in the regulation of *SsCutA* and *SsPg1*, and possibly other aspects of pathogenicity.

In conclusion, *S. sclerotiorum* employs multiple, overlapping mechanisms to coordinate the expression of key hydrolytic enzymes. Studies using Ca^{+}, MAPK and cAMP pathways inhibitors have contributed to the identification of the signalling pathways that may be involved. Disruption of genes encoding components of these signalling pathways will better our understanding of how the pathogen initiates and transitions through the various stages of the infection. The availability of the *S. sclerotiorum* genome sequence and efficient homologous recombination systems has provided the tools to systematically undertake such endeavours.

3.5 Materials and Methods

3.5.1 Fungal cultivation

S. sclerotiorum strain 1980 (ATCC18683) was cultivated as described in section 2.5.8

3.5.2 Identification of *S. sclerotiorum* cutinase genes

The amino acid sequence of the *B. cinerea* Cutinase A (GenBank Accession Z69264) was used in a BLAST search of the *S. sclerotiorum* protein database (http://www.broadinstitute.org/annotation/genome/sclerotinia_sclerotiorum/MultiHome.html) to identify genes encoding similar proteins. Signal peptides were identified using SignalP (www.expasy.org).

3.5.3 Plant bioassays

To examine the role of the cuticle as a barrier to infection, leaf wax was removed by dipping *B. napus* cv. Westar leaves in chloroform for 30 sec and the chloroform was allowed to evaporate. Mycelia grown in MS-Glu medium were washed with MS or MS-Glu medium and then placed on the upper surface of either intact or stripped leaves and incubated on wet Whatman paper #5 for up to 24 h in a sealed chamber.

To study the expression of *SsCutA* and *SsPg1* in different infection zones, 1 g of mycelia (wet weight) was spread over a 5 cm diameter area of *B. napus* leaf surface and incubated in a humidified chamber for 48 h. Leaves from 45-day-old plants were used. Samples were collected from three parts of the infection zone including unaffected tissues beyond the lesion, a 0.5 cm zone at the edge of the lesion and from the necrotic tissue. In time course studies, mycelia were collected from the lesion at 1 to 48 hpi as indicated. Plant material was removed and the samples frozen immediately in liquid nitrogen.

3.5.4 Expression of *SsCutA* in response to surface contact

Mycelia were grown in MS-Glu liquid medium as described above, washed with MS medium and then re-suspended in MS or MS-Glu medium. Mycelia were spread over the surface of either intact *B. napus* leaves, leaves stripped of surface waxes, Parafilm™, potato dextrose agar (PDA) plates or glass Petri dishes with or without a coating of leaf wax. Leaf wax was isolated by dipping *B. napus* leaves in chloroform for 30 sec and evaporating the chloroform under

a stream of liquid nitrogen. Wax was applied by dissolving the crystals in a small volume of chloroform, spreading it to form a thin film on a glass Petri dish and allowing the chloroform to evaporate. Samples were incubated on the various surfaces in a humidified chamber at 20°C for 18 h after which the mycelia were harvested, frozen in liquid nitrogen and RNA extracted as described in section 3.5.7.

3.5.5 Regulation of hydrolytic enzymes in response to carbon source

Mycelia were grown in MS-Glu liquid medium as described above, washed with MS medium and 20 g (wet weight) transferred to 200 mL of MS-Glu, MS-galacturonic acid (1 %), MS-pectin (1 %) or a combination of pectin and galacturonic acid as indicated. MS medium was supplemented with finely granulated leaf wax crystals (1 %) prepared by homogenization using a Virtis mechanical homogenizer.

To examine the effect of GA concentration on *SsPg1* expression, MS medium was supplemented with GA (0.01, 0.05, 0.1, 0.5 and 1 %) alone or with pectin (1 %). The pH of the media was maintained at 3.0 using 0.1 M citric acid-phosphate buffer. Aliquots were collected each day, mycelia harvested by centrifugation at 10,000 rpm for 5 min, frozen in liquid nitrogen and stored at -80°C.

3.5.6 Pharmacological studies

Mycelia were grown in MS-Glu liquid medium as described above, washed with MS medium, and then resuspended in MS medium with compounds known to affect calcium signalling and cAMP levels. The mycelia were incubated with the tested compounds for 18 h at 20°C in the dark prior to inoculation. Compound U73122 was dissolved in 95 % ethanol and used at final concentration of 10 mM. EGTA was used at 10 mM, compound 48/80 at 5 μM and lanthanum chloride at 1 and 10 mM and all were dissolved in water. Cyclosporin A was dissolved in dimethylsulfoxide and used at a final

concentration of 4.9 nM. Mycelia were transferred to *B. napus* leaves and maintained under humid conditions for 16 h at which time mycelia were collected and used for gene expression analysis. The effect of these compounds on mycelial growth was tested by placing mycelia, treated in the same manner as that applied to the *B. napus* leaves, on MS-Glu agar and incubating at 20°C. To test the effect of cAMP, mycelia were incubated for 18 h in the dark in solutions of cAMP (0, 0.1, 1 and 10 mM) dissolved in water.

3.5.7 RNA isolation and northern blot analysis

To isolate total fungal RNA, 150 mg of mycelia (wet weight) was ground in liquid nitrogen and dispensed into 1.5 mL microcentrifuge tubes containing 600 μL extraction buffer [0.1 M NaCl, 2 % SDS, 50 mM Tris–HCl (pH 9.0), 10 mM EDTA] and 600 μL phenol/chloroform/isoamyl alcohol (25:24:1). The samples were mixed for 30 sec using a vortex and centrifuged for 10 min at 15,000 g. The aqueous phase was extracted again with an equal volume of chloroform, transferred to a new microcentrifuge tube and the RNA precipitated using lithium chloride (2 M final concentration) for 16 h at 4°C. The pellet was washed once with 2 M lithium chloride and once with 75 % ethanol. The RNA was dried in air for 10 min and dissolved in 35 μL ddH_2O treated with diethyl pyrocarbonate. Northern blot analysis was performed as described in Li et al. (2004b). The *SsCutA* probe consisted of a 300 bp region amplified from exon 1 using the following primers; SsCutAF (5'-CTCAACCCAACAACTCTTGTC-3') and SsCutAR (5'- ATAGTCTGACTACCAGCAGA-3'). The *SsPg1* probe consisted of a 600 bp fragment excised using *Bam*HI from an *SsPg1* cDNA cloned into pGEM-T Easy.

CHAPTER FOUR:

EXPRESSION AND REGULATION OF *S. SCLEROTIORUM* NECROSIS AND ETHYLENE-INDUCING PEPTIDES

4.1 Abstract

Successful host colonization by necrotrophic plant pathogens requires the induction of plant cell death to provide the nutrients needed for infection establishment and progression. Two genes encoding necrosis and ethylene-inducing peptides (NEPs) from *S. sclerotiorum* were cloned, which were named *SsNep1* and *SsNep2*. The peptides encoded by these genes induced necrosis when expressed transiently in tobacco leaves. *SsNep1* was expressed at a very low level relative to *SsNep2* during infection. The expression of *SsNep2* was induced by contact with solid surfaces and occurred in both the necrotic zone and at the leading margin of the infection. *SsNep2* expression was dependent on calcium and cAMP signalling, as compounds affecting these pathways reduced or abolished *SsNep2* expression coincident with a partial or total loss of virulence.

4.2 Introduction

In the Chapter 1, SsPG3 and SsPG6 were both found to induce necrosis in the host. Although, BnPGIP1 and BnPGIP2 were able to inhibit their necrotizing activity, this was insufficient to reduce the virulence of the pathogen. This observation suggested that other necrosis-inducing molecules are released by *S. sclerotiorum* during the infection. The following study identified two additional proteins that work in concert with PGs to induce necrosis and examined how the expression of their genes is regulated.

Plant cell death is a key factor for the success of necrotrophic fungal pathogens. It can occur through an organized and controlled sequence of events leading to the destruction of the host cells, a process known as apoptosis (Kerr

et al., 1972), or more rapidly without involving such molecular events, this is commonly referred to as necrosis. Morphologically, apoptosis is accompanied by cell shrinkage, nuclear condensation, DNA fragmentation and lysis of the cells to form apoptotic bodies (Adrian and Martin, 2001). Apoptosis is mediated by the activation of a family of cysteine proteases known as caspases (Wolf and Green, 1999). Triggering of the caspase cascade can occur internally through the release of cytochrome C from the mitochondria or externally through the activation of death receptors (Green and Reed, 1998; Adrain and Martin, 2001). Studies on animal cells found that the type of cell death was dependent on the amount of stress the cells were exposed to. At low stress levels, cells initiated apoptosis, while at high stress levels they died through necrosis (Lennon et al., 1991). This theory was tested on plant cells by exposing carrots to various levels of heat stress. At temperatures up to 55ºC, the protoplast detached from the cell wall and cell death occurred. Above 75ºC, cells died without going through the protoplast detachment step. The detachment of the protoplast from the cell wall is a distinct feature of apoptosis (McCabe et al., 1997). Cell death is not completely understood in plants and plant pathogens release molecules that induce different forms of cell death. Photoactive metabolites have been demonstrated to be released during plant-pathogen interactions and cause host cell death. *S. sclerotiorum* produces a suite of secondary metabolites, such as sclerin which promotes root formation and increases dry weight (Satomura and sato, 1964) and sclerotinin-A, sclerotinin - B and sclerone which regulate plant growth. Phytotoxic metabolites were also found to be produced by *S. sclerotiorum* (Pedras and Ahiahonu, 2004). *B. cinerea* was found to produce botrydial, botrydienal and 8, 9-epibotrydial which promote development of the infection (Colmenares et al., 2002). The activity of these toxins was light dependent, but they were not host-specific.

Necrotrophs also manipulate plant defences, such as the oxidative burst, to cause cell death. The release of reactive oxygen intermediates (ROI) is an effective defence mechanism against biotrophic fungi. Killing the cells

surrounding the infection zone can limit the progress of infection when the pathogen is adapted to absorbing nutrients from living host cells, i.e. biotrophs. Conversely, necrotrophs have developed mechanisms to survive the high concentrations of ROI in the host. Furthermore, ROI also cause host cell death leading to the formation of necrotic tissue which provides the nutrients required by these types of pathogens. Indeed, some necrotrophs, such as *B. cinerea*, release ROI (Tenhaken et al., 1995). ROI have different functions dependent upon their concentrations during the infection. At low levels, ROI were found to induce disease response genes in the host. When concentrations increased to between 6 and 8 mM, ROI cause hypersensitive cell death or apoptosis (Tenhaken et al., 1995), whereas even higher concentrations caused necrosis (Apostol et al., 1989). The release of ROI by the pathogen may trigger the hypersensitive response (HR), including necrosis and thus the pathogen may be manipulating the host defence system for its own advantage.

A new family of host specific toxins that are being investigated in different organisms are the NEPs (Pemberton and Salmond, 2004). The first NEP, NEP1, was isolated from *F. oxysporum* culture filtrates as a 24 kDa protein that caused necrosis and induced ethylene production in the coca plant, *Erythroxylum coca* (Bailey et al., 1995, Bae et al., 2006). In the past few years, NEP1-like proteins (NLPs) have been identified in taxonomically diverse organisms including bacteria, fungi and oomycetes. They have also been found in non-pathogenic organisms, such as *Vibrio pommerensis* (Jores et al., 2003), and were expressed in the hemibiotrophic fungus *Moniliophthora perniciosa* during the biotrophic stage (Garcia et al., 2007). Transformation of *C. coccodes* with *F. oxysporum Nep1* increased virulence and the spectrum of hosts that could be infected (Amsellem et al., 2002). NLP elevate internal Ca^{+2} levels, activate MAPKs, and promote the production of ROI, ethylene and pathogenesis-related proteins (Jennings et al., 2001; Veit et al., 2001; Fellbrich et al., 2002; Keates et al., 2003; Qutob et al., 2006). In *A. thaliana*, global gene expression patterns in response to *Phytophthora parasitica* NLP were similar to that observed in

response to bacterial flagellin (Qutob et al., 2006). The mechanism by which NLPs bring about these phenomena is unknown, but they were proposed to associate with the outer surface of the plasma membrane to trigger apoptosis (Qutob et al., 2006; Schouten et al., 2007). Schouten et al (2007) provided direct evidence that NEPs also alter the cell membrane, a phenomenon not related to apoptosis. Furthermore, when caspase inhibitors were applied, cell death was delayed but not prevented, indicating that NEPs may also cause cell death via non-apoptotic mechanisms. In this case, 40-60% of the cells were swollen and did not exhibit signs of DNA fragmentation. This suggested that NEPs not only induce apoptosis, but also cause necrotic cell death. The same study found that NEPs induce massive accumulation of ROI in the chloroplast and therefore induce cell death through at least two distinct pathways.

Common to all NLPs is a conserved GHRHDWE domain and an amino-terminal register of conserved cysteine residues. Based on the number of cysteine residues, NLPs can be classified as either Type I or Type II which have two or four cysteine residues, respectively. Both types of NLPs are produced by fungi and bacteria; however, only Type I NLPs have been reported in oomycetes (Gijzen and Nurnberger, 2006). The conserved GHRHDWE domain was not solely responsible for activity (Schouten et al., 2007). Mutation of the conserved cysteine residues or other alterations that affect the tertiary structure abolished the ability to induce cell death in protoplasts or to generate necrotic lesions (Fellbrich et al., 2002; Qutob et al., 2006).

The majority of studies conducted on NLPs have focused on their interaction with the host. Little is known about factors governing the regulation of NLP gene expression. In *Erwinia carotovora*, *Nep* was expressed only when the bacteria were grown on solid medium (Mattinen et al., 2004). Here I report the characterization of two NLP genes from *S. sclerotiorum*, referred to as *SsNep1* and *SsNep2*. The peptides encoded by these genes caused necrosis and *SsNep2* was expressed soon after contact of mycelia with the leaf or other solid surfaces. I also investigated the role of cAMP and Ca^{+2} in the regulation of

SsNep2 expression. This study further illustrates the significance of having multiple effectors to initiate host apoptosis and/or tissue necrosis for successful infection by necrotic pathogens.

4.3 Results

4.3.1 Identification of *SsNep1* and *SsNep2*

To identify *S. sclerotiorum* NEP (*SsNep*) genes, a BLASTX search of the *S. sclerotiorum* genome sequence (http://www.broadinstitute.org/annotation/genome/sclerotinia_sclerotiorum/MultiHome.html) with *F. oxysporum Nep1* (AF036580) was conducted. Two *Nep1* homologues were identified with expectation values of 0 (SS1G_03080.1) and 1.06 e-^{31} (SS1G_11912.1) which were named *SsNep1* and *SsNep2*, respectively. The *SsNep1* gene contains two introns of 53 and 58 bp with the resultant 738 bp open reading frame encoding a 246 amino acid protein with a predicted molecular weight of 26.7 kDa. The *SsNep2* gene contains two introns of 61 and 58 bp with a 735 bp open reading frame that encodes a 245 amino acid protein predicted to have a molecular weight of 26.0 kDa.

Comparison of NLPs from several fungal pathogens revealed a diverse phylogenetic arrangement (Figure 4.1B). SsNEP1 and SsNEP2 were most closely related to *B. elliptica* Nep1 (ABB43266; 82% identity) and Nep2 (CAJ31369; 79% identity), respectively. SsNEP1 was also related to an *A. oryzae* NLP (XP_001824353), while SsNEP2 was related to an *A. fumigatus* NLP (EAL86241). The oomycete NLPs from *Phytophthora* and *Pythium* species formed a clade that was distinct from those including SsNEP1 and SsNEP2, though the former was more similar to the oomycete NLP sequences. SsNEP1 and SsNEP2 are highly divergent and exhibit only 35% identity at the amino acid level. The conserved GHRHDWE sequence is located between positions 103-110 in SsNEP1 and 109-116 in SsNEP2. Both appear to be secreted as they were predicted to possess signal peptides. Conserved cysteine residues define the type or class of NLP and both SsNEPs are Type I with two

conserved cysteine residues at positions 47 and 73 in the mature protein (Figure 4.1A). SsNEP1 contains a third cysteine residue, while SsNEP2 contains three additional cysteines. The third cysteine in SsNEP1 and two of the three additional cysteines in SsNEP2 are conserved among the orthologs in *Botrytis* species, but not so in other NLP.

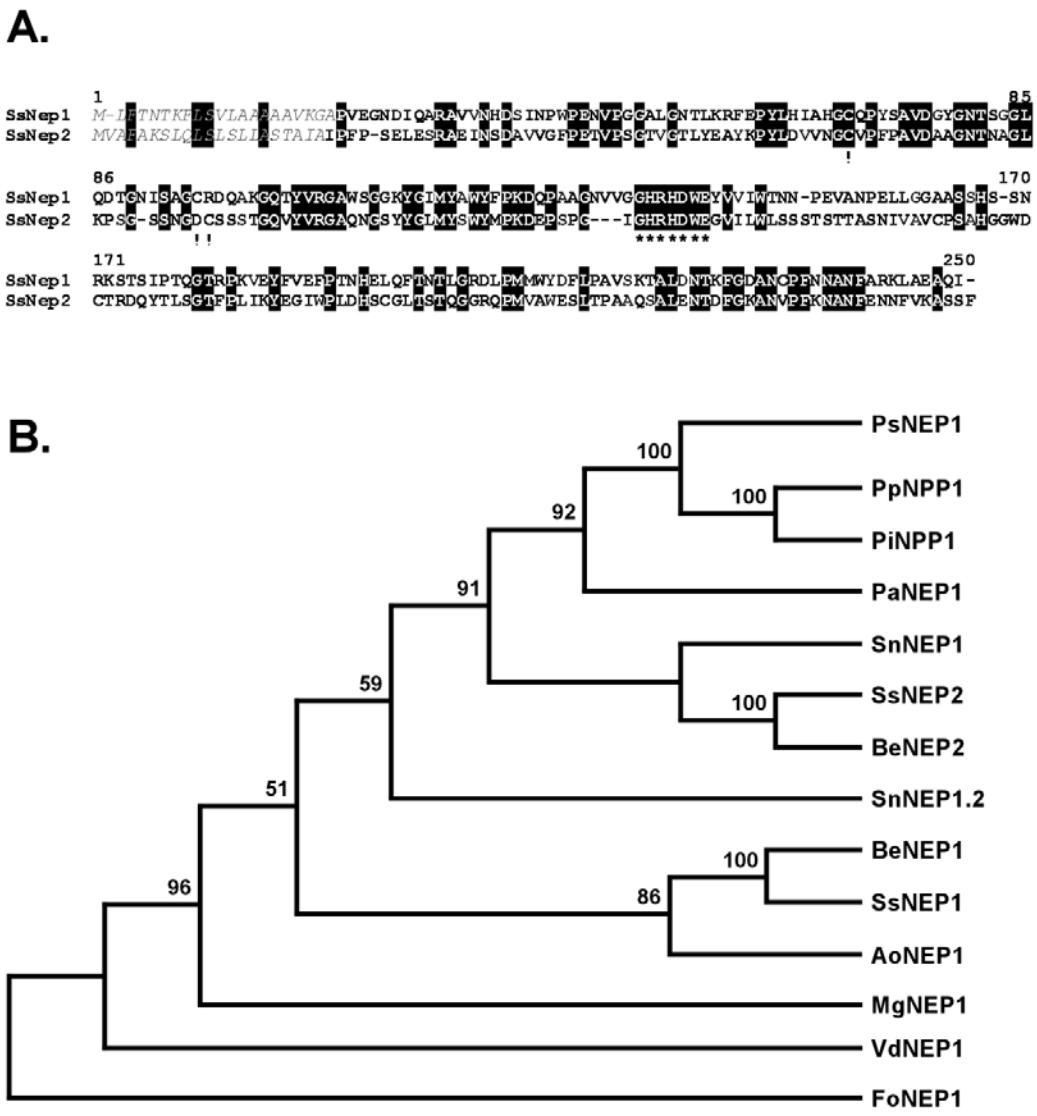


Figure 4.1 Sequence and phylogenetic analysis of *S. sclerotiorum* NEPs.
A) Alignment of SsNEP1 and SsNEP2 showing identical residues (white on black background), signal peptides (italics), conserved cysteine residues (!) indicative of Type I NEP1-like proteins (NLPs) and the GHRHDWE motif (*). **B)** Phylogenetic relationship between NLPs from various fungi and oomycetes. The proteins used in the analysis are coded with the first two letters representing the organism followed by the GENBANK accession number. Af, *A. fumigatus*; As, *A. nidulans*; Ao, *A. oryzae*; Be, *Botrytis elliptica*; Fo, *F. oxysporum*; Mg, *M. grisea*; Pa, *Pythium aphanidermatum* NEP1, Pi, *Phytophthora infestans*; Pp, *Phytophthora parasitica*; Ps, *Phytophthora sojae*; Sn, *Stagonospora nodorum*; Ss, *S. sclerotiorum*; Vd, *Verticillium dahliae*. Type I and Type II NLPs were classified according to Gijzen and Nürnberger (2006). Confidence values for each node are based on 100 bootstrap analyses.

4.3.2 *In planta* expression of *S. sclerotiorum* NEPs

Recombinant PVX vectors allowing for expression of *SsNep1* and *SsNep2* under the direction of the CaMV promoter were introduced into *N. benthamiana* leaves. In each case, endogenous SsNEP fungal signal peptides were included in the heterologous expression vectors and therefore the proteins were expected to be secreted into the apoplast. Leaves infiltrated with constructs expressing either *SsNep1* or *SsNep2* developed lesions within 10-12 days post inoculation (dpi). The lesion started near the margin of the inoculation site and then proceeded to expand to the surrounding tissue by 14-16 dpi (Figure 4.2). No differences were observed in the rate of lesion development or the severity of symptoms between the *SsNep1*and *SsNep2* constructs.

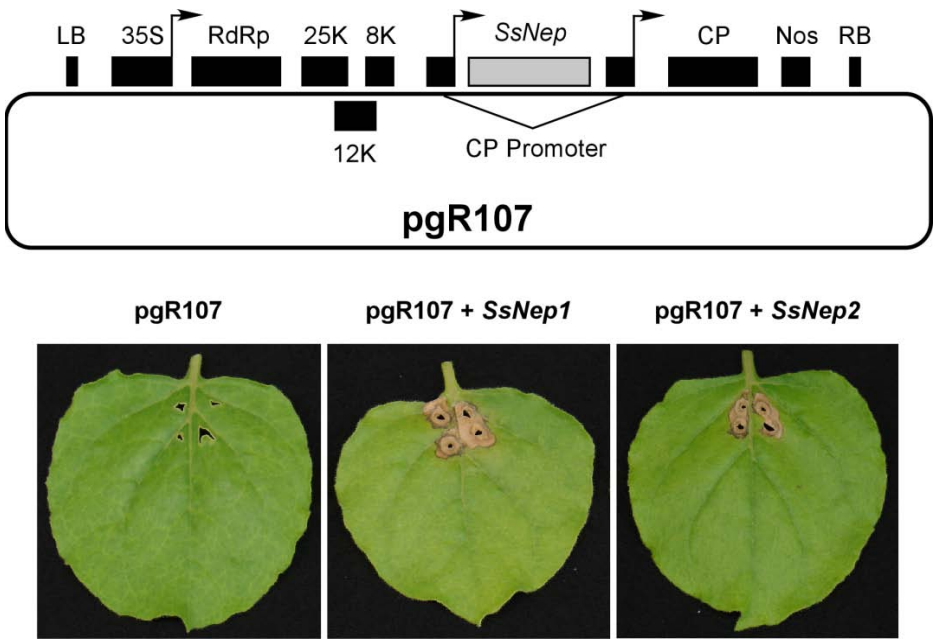


Figure 4.2 The Potato Virus X-based binary vector pgR107 used for *in planta* expression of *S. sclerotiorum* NEPs.
Elements shown are: LB, left border; 35S, cauliflower mosaic virus 35S promoter; RdRp, viral RNA-dependent RNA polymerase; M1–M3, viral movement proteins; CP, coat protein, Nos, *Agrobacterium tumefaciens* nopaline synthetase transcriptional terminator; RB, right border. Necrotic symptoms observed on *N. benthamiana* leaves 2 weeks after inoculation with PVX virus, PVX + *SsNep1* or PVX + *SsNep2*

4.3.3 Expression of *SsNep1* and *SsNep2* during the infection

The expression of *SsNep1* and *SsNep2* was examined during the course of the infection. Necrotic symptoms were visible within 6 hours post-inoculation (hpi) of *B. napus* leaves with *S. sclerotiorum*. By 24 hpi, most of the area beneath the inocula had become necrotic and by 48 hpi the leaf tissue was completely macerated. *SsNep1* transcripts were detected at very low levels from 6 to 24 hpi (Figure 4.3), but were undetectable at 48 hpi. *SsNep2* transcripts were also detected within 6 hpi and peaked at 24 hpi. The expression of *SsNep1* was always much less than that of *SsNep2*, with the former often being barely detectable using either northern blot analysis or RT-PCR.

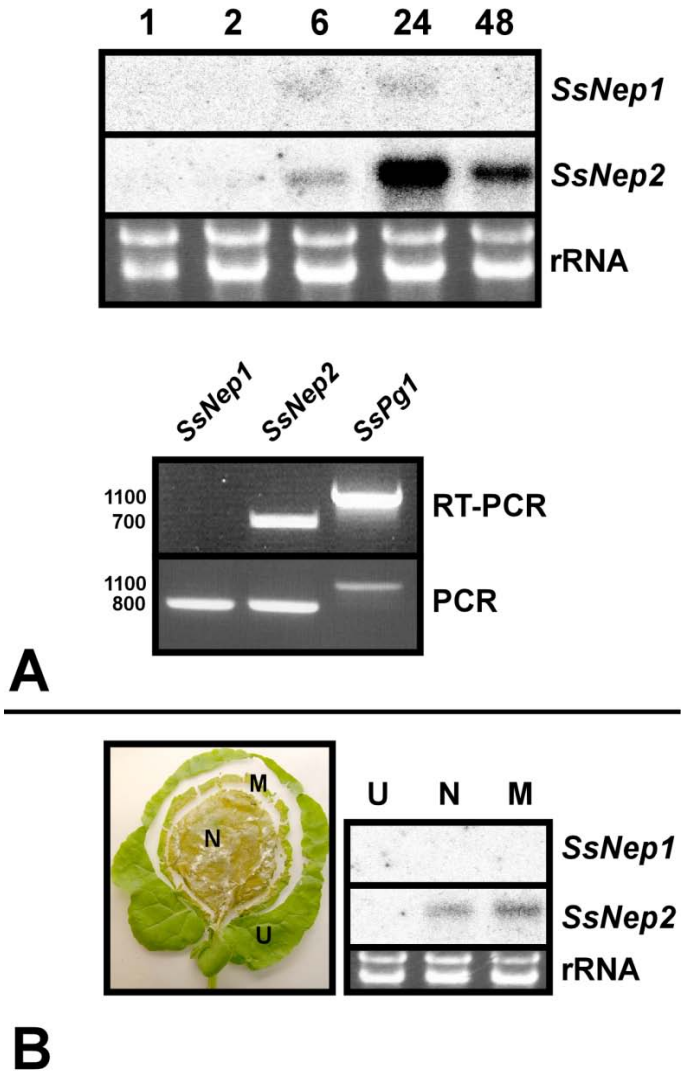


Figure 4.3 Expression of *S. sclerotiorum* NEPs during infection of *B. napus* leaves.
A) Northern blot analysis showing expression in mycelia at various times (hours) after inoculation. The rRNA loaded in each lane is also shown. Lower panel shows *SsNep1*, *SsNep2* and *SsPg1* reverse transcriptase-polymerase chain reaction (RT-PCR) products amplified from mycelia 24 h after inoculation. Amplification of the corresponding loci using genomic DNA was conducted as a positive PCR control. **B)** Division of the lesion into uninfected (U), margin (M) and necrotic (N) zones and northern blot analysis showing expression in each zone.

The expression of the *SsNep2* genes was also examined in various infection zones, namely necrotic tissue, the lesion margin and uninfected or

asymptomatic tissue (Figure 4.3). *SsNep1* transcripts were not detected by northern blot analysis in any of the zones tested, whereas *SsNep2* was expressed in necrotic region and the expanding margin of the lesion.

4.3.4 Expression of *SsNep2* on different surfaces

A previous study found that the *Erwinia carotovora Nep* gene was expressed only when the bacterium was grown on solid medium (Mattinen et al., 2004). Therefore, the effect of different contact surfaces on *SsNep2* expression, the more highly expressed of the two *S. sclerotiorum Nep* genes, was examined. *SsNep2* transcripts were not detected in liquid media including MS (starvation), MS-Glu or MS supplemented with finely granulated leaf wax (Figure 4.4). Conversely, when mycelia were placed on solid or semi-solid surfaces (intact leaves, leaves stripped of wax, leaf wax on glass or glass) *SsNep2* expression was sharply induced within 24 h. Interestingly, *SsNep2* was not expressed in response to contact with the hydrophobic material Parafilm™ in either the presence or absence of glucose.

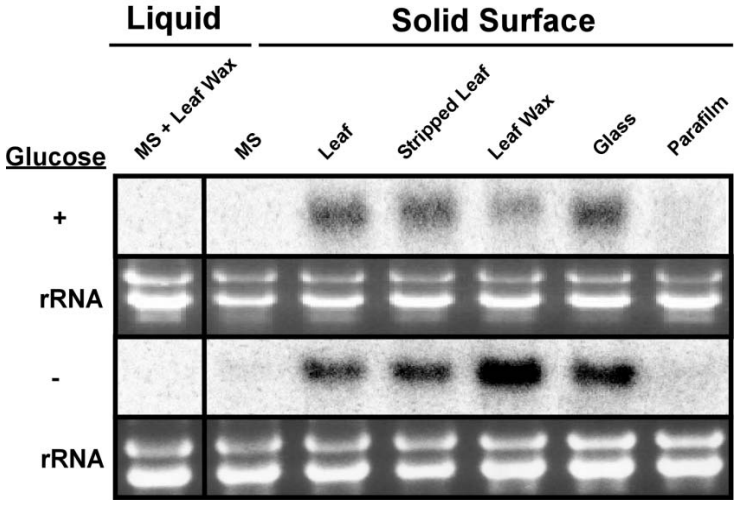
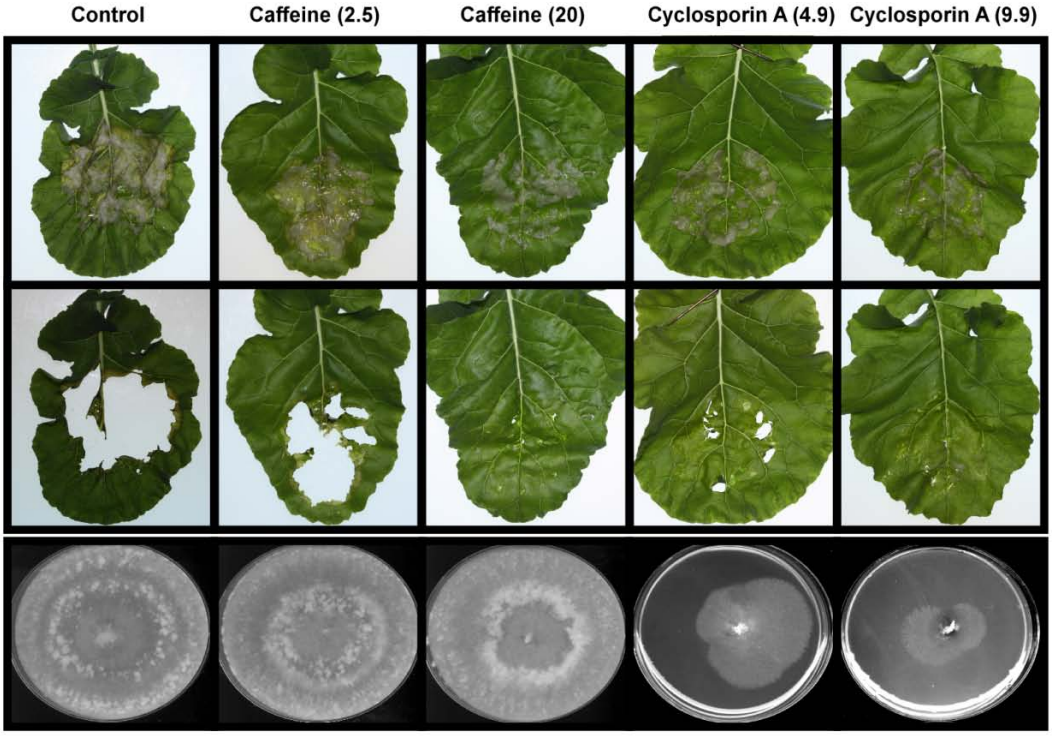


Figure 4.4 Expression of *SsNep2* in response to contact with solid surfaces. Northern blot analysis showing the expression of *SsNep2* in mycelia in liquid media in the presence or absence of leaf wax or after transfer to solid surfaces in the presence or absence of glucose. The bottom panel shows the rRNA loaded in each lane.

4.3.5 Role of calcium signalling and cAMP in *SsNep2* regulation

To study the role of calcium in *SsNep2* expression, the effect of several compounds that affect calcium signalling were tested. Caffeine increases cytosolic calcium levels by activating ryanodine receptors on the endoplasmic reticulum leading to the release of internal calcium stores (Berridge, 1997). Treatment of mycelia with 20 mM caffeine resulted in near complete inability to penetrate *B. napus* leaf cuticle, while 2.5 mM caffeine did not affect virulence (Figure 4.5). Neither treatment affected growth MS-Glu agar.


Control
Caffeine (2.5)
Caffeine (20)
Cyclosporin A (4.9)
Cyclosporin A (9.9)

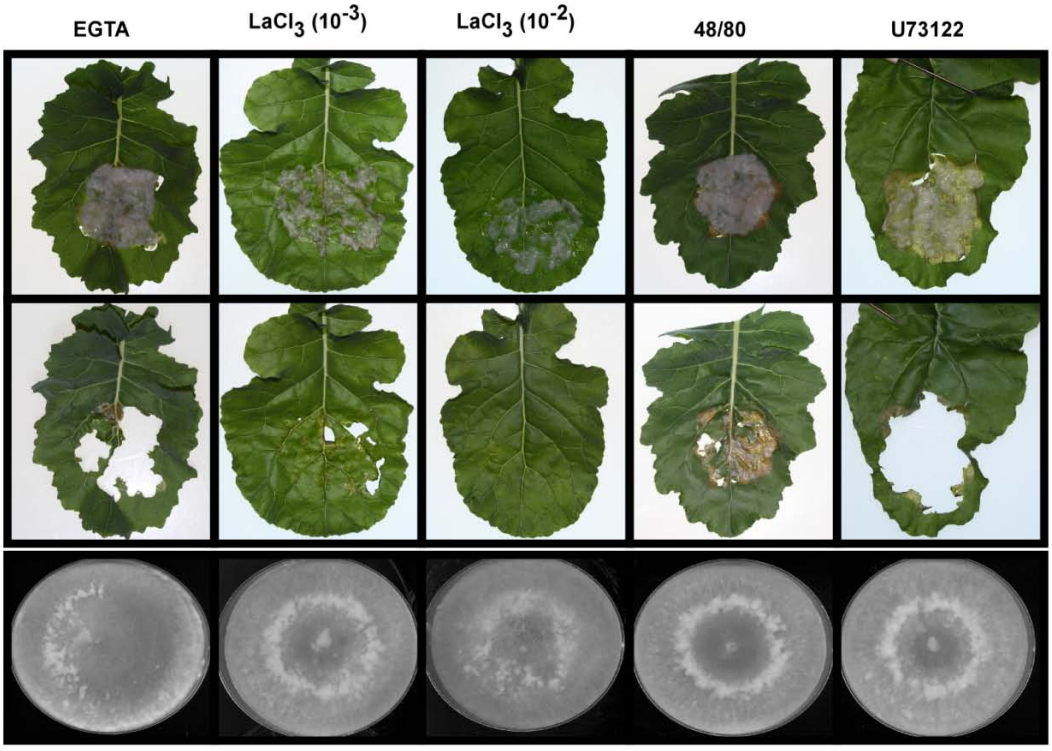

EGTA
LaCl3 (10-3)
LaCl3 (10-2)
48/80
U73122

Figure 4.5 Effect of signalling inhibitors on *S. sclerotiorum* infectivity and growth. The top panels show leaves 16 h after inoculation (upper frame), as well as the lesions beneath (lower frame), for mycelia that were untreated (control) or treated with caffeine (2.5 and 20 mM), cyclosporin A (CspA; 4.9 and 9.9 nM), [ethylenebis (oxonitrilo)]tetra acetic acid (EGTA) (10 mM), lanthanum chloride (LaCl3; 1 and 10 mM), compound 48/80 (5 mM) or compound U73122 (10 mM). The bottom panels show the effect of these compounds on mycelial growth. A sample of the mycelial preparation used to inoculate the leaves was also placed in the centre of a minimal salts–glucose (MS-Glu) plate and radial growth was assessed after 5 days.

Both concentrations of caffeine decreased *SsNep2* expression after 24 h on the leaf surface (Figure 4.6). Lanthanum chloride, a plasma membrane calcium channel blocker, greatly reduced *S. sclerotiorum* virulence and expression of *SsNep2*. EGTA, which chelates extracellular calcium ions, had no effect suggesting that mobilization of internal calcium stores is required for infection and *SsNep2* expression. Compounds 48/80 and U73122 inhibit phospholipase C activity, but were found to promote the release of calcium from intracellular stores (Mogami et al., 1997). Treatment with 48/80 slightly reduced virulence; however, both reduced *SsNep2* expression. None of the compounds tested above were found to inhibit mycelial growth at the concentrations used (Figure 4.5). Calcineurin, a calcium and calmodulin-dependent Ser/Thr protein phosphatase, has been shown to play a central role in fungal pathogenesis (Fox and Heitman, 2002). The calcineurin inhibitor cyclosporin A, reduced virulence and strongly reduced the expression of *SsNep2*, though mycelial growth was also reduced. The northern blot analysis was conducted with similar amounts of total RNA from each sample; however, to determine the extent to which the treatments affected housekeeping gene expression, the same blots were probed with the *SsActin* gene. The intensity of the hybridization signal in the control lane for both *SsNep2* and *SsActin* were similar. In all cases where *SsNep2* expression was reduced, the level of actin expression was similar to the control or was affected to a lesser degree than *SsNep2* indicating that the compounds had a greater effect on *SsNep2* expression than *SsActin*.

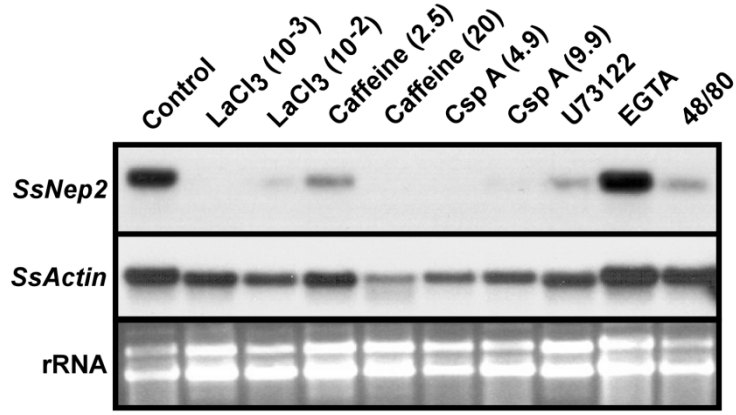


Figure 4.6 Effect of signalling inhibitors on *SsNep2* expression during infection of *B. napus* leaves.
Northern blot analysis showing the expression of *SsNep2* and *SsActin* genes in mycelia treated with compounds affecting cyclic adenosine monophosphate (cAMP) levels (caffeine) and calcium signaling and placed on *B. napus* leaves for 16 h. The bottom panel shows the rRNA loaded in each lane.

Caffeine also elevates internal cAMP levels by inhibiting cAMP-phosphodiesterase activity which converts cAMP to a non-cyclic form (Berridge, 1997). Several studies have reported that cAMP signalling plays a role in many aspects of fungal biology including virulence and development (D'Souza and Heitman, 2001). To test the effect of cAMP on colonization of *B. napus* by *S. sclerotiorum* lesion formation and *SsNep2* expression after application of exogenous cAMP were monitored (Figure 4.7). At 0.1 mM and 1 mM concentrations, cAMP had no effect on disease development or *SsNep2* expression; however, at 10 mM lesion formation was severely reduced. *SsNep2* expression also decreased; however, the expression of *SsActin* also declined with increasing cAMP levels indicating that the loss of virulence may be due to factors in addition to reduced *SsNep2* expression. The *S. sclerotiorum* adenylate cyclase deletion mutant, s*ac1*, which is unable to generate cAMP was also non-pathogenic (Jurick and Rollins, 2007). This mutant grew slowly and while mycelial rRNA levels were similar to the wild-type strain I was unable to detect *SsNep2* or *SsActin* indicating that the mutation has broad pleiotropic effects. Furthermore, application of exogenous cAMP to the *sac1* mutant failed to restore pathogenicity or *SsNep2* expression.

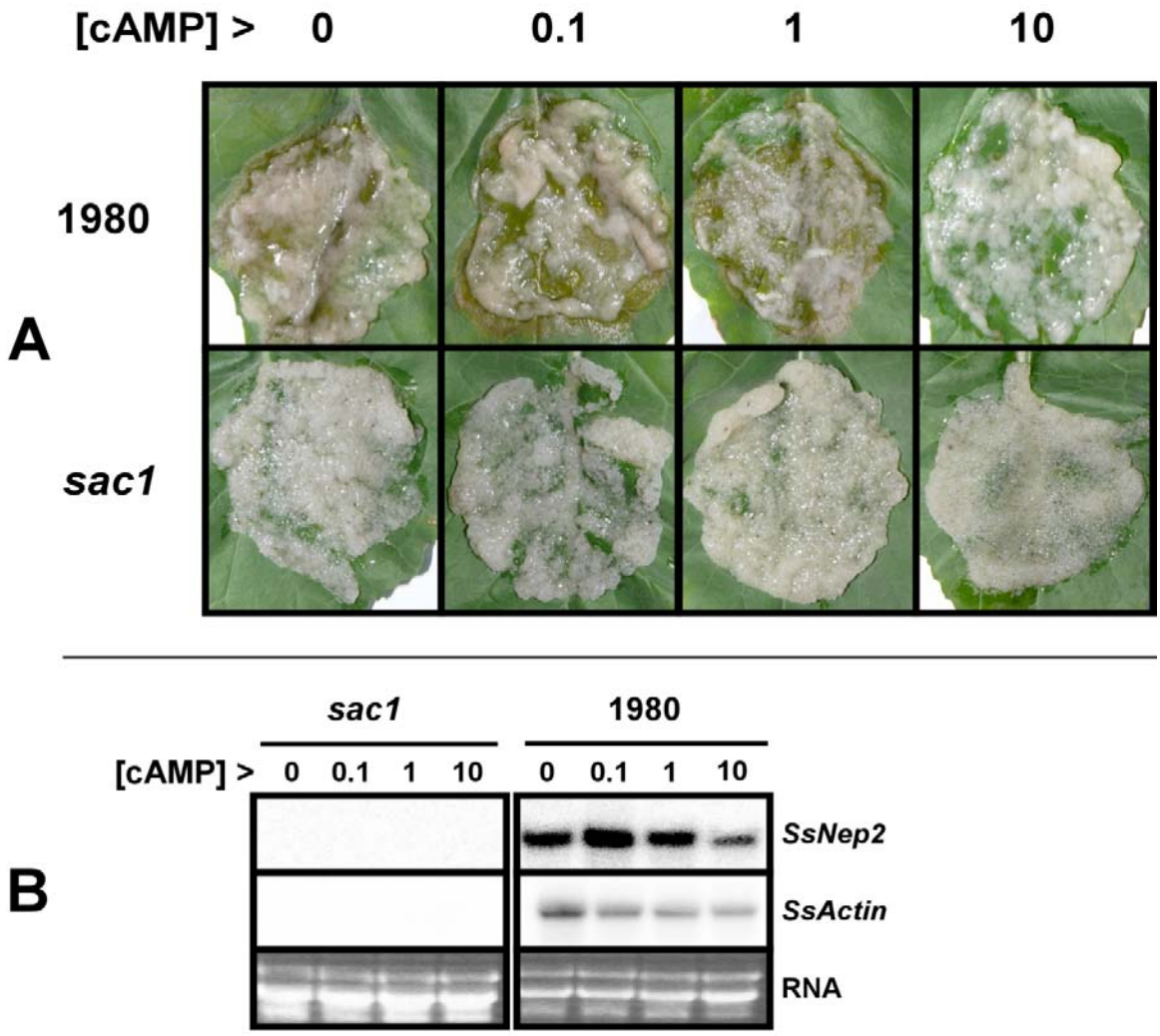


Figure 4.7 Effect of exogenous cAMP application on *S. sclerotiorum* infectivity and expression of *SsNep2*.
A) Lesions forming on *B. napus* leaves when mycelia from *S. sclerotiorum* strain 1980 and the *sac1* adenylate cyclase mutant were treated with increasing levels of cAMP (mM) prior to inoculation. **B)** Northern blot analysis showing *SsNep2* expression 24 h after inoculation of *B. napus* leaves with mycelia treated with cAMP. The bottom panel shows the rRNA loaded in each lane

4.4 Discussion

4.4.1 NEPs in phytopathogens

The redundancy in function between PGs and NEPs reflects the complex nature of plant pathogen interactions. As plants continued to develop defence mechanisms against fungal toxins, the pathogen produced new toxins that can overcome plant defences. Inhibitors have been identified for PGs, but receptors or other host proteins that interact with NEPs have not been found yet. Previous studies have documented the necrotic effect of NEPs from different organisms, but no studies have investigated how expression of their corresponding genes is regulated.

Induction of host cell death is crucial for *S. sclerotiorum* pathogenicity (Dickman et al., 2001) and its genome was found to contain two *NLP* genes. The *Phytophthora sojae* and *Phytophthora ramorum* genomes contain 50-60 NLP genes and pseudogenes, while the *M. grisea* and *Gibberella zeae* genomes have only four (Gijzen and Nurnberger, 2006). The *S. sclerotiorum* NEP proteins were most similar to those from *B. elliptica* (Staats et al., 2007), the genus closest to *Sclerotinia*. Though the *S. sclerotiorum* NEPs are highly divergent, each possessed features highly conserved among NLP and were effective in causing necrosis when introduced into *N. benthamiana* leaves using the PVX expression system.

4.4.2 Expression of NEP genes during infection

In *S. sclerotiorum* 1980, *SsNep2* was expressed at a much higher level than *SsNep1* during infection of *B. napus* leaves, which was confirmed by both northern blot and RT-PCR analysis. Differential expression of genes encoding pathogenicity factors has been shown elsewhere, for example, in *F. solani* f. sp. *pisi* the pectate lyase gene *pelD* was expressed only in infected pea tissue, whereas *pelA* expression was undetectable (Guo et al., 1996; Rogers et al., 2000). I also noted that *SsNep2* was expressed soon after mycelial inoculation and in both the necrotic region and at the expanding margin of the lesion. In the

hemi-biotrophic pathogen *P. sojae*, the highest level of *Nep* expression coincided with the transition from the biotrophic to the necrotrophic stage (Qutob et al., 2002). There is an accumulating body of evidence indicating that NLPs contribute to virulence. Transgenic *C. coccodes* expressing *Nep1* from *F. oxysporum* were more virulent on the original host and were able to infect tobacco and tomato lines that were resistance to wild type *C. coccodes* (Amsellem et al., 2002). Similarly, expression of the *E. carotovora Nip* gene, which encodes a NLP, in a low virulence strain resulted in larger lesions and increased necrosis of host tissues (Mattinen et al., 2004).

4.4.3 Regulation of NEP genes in response to contact surface

The expression of *SsNep2* was also found to be regulated by the physical properties of the contact surface. *SsNep2* transcripts were not detected in MS-based liquid cultures, but its expression was induced by mycelial contact with a solid or semi-solid surface. Many studies have examined the effect of physical properties and chemical signals associated with the leaf surface on pathogen development and differentiation of infectious structures. Appressoria formation by *Colletotrichum* species was related to leaf topography (Kolattukudy et al., 1995), whereas in *B. graminis* f. sp. *hordei*, these were formed in response to barley leaf wax (Tsuba et al., 2002). The induction of *SsNep2* expression in response to contact with a solid surface is in accordance with the observation that it is expressed in the very early stages (detectable within 6 h by northern blot analysis) of the infection. Interestingly, NLPs may affect the structure of host cuticle as application of *F. oxysporum* NEP1 to leaves of several plant species resulted in a thinning and/or sloughing of the cuticle (Keates et al., 2003). This may be due to the direct interaction of NEP1 with lipophilic compounds in the cuticle or to chloroplast disruption and reprofiling of gene expression that occurs soon after NEP1 application (Keates et al., 2003). Regardless of the underlying mechanism, the rapid induction of NLP gene expression by contact with the leaf surface and the ability to erode cuticle

would allow them to work in concert with cutinases and plant cell wall degrading enzymes to breach this primary plant defence.

4.4.4 Signaling mechanisms regulating NEP gene expression

In *Colletotrichum*, several genes are induced in response to contact with solid surfaces, including *Colletotrichum* Hard Surface Induced Protein 1 (*CHIP1*) which encodes a ubiquitin-conjugating enzyme, *CHIP2* which encodes a transcription factor and *CHIP3* which was predicted to encode a membrane-localized protein. The expression of these genes under these circumstances is mediated by calcium and calmodulin signaling pathways (Liu and Kolattukudy., 1998; Kim et al., 1998). I showed that compounds affecting intracellular calcium levels (caffeine, compounds 48/80 and U73122) also affect *SsNep2* expression, though the latter two compounds had little if any affect on virulence at the concentrations tested. Conversely, down regulation of *SsNep2* expression after treatment with the general calcium channel blocker lanthanum chloride coincided with a reduction in virulence. Although such compounds are known to have pleiotropic effects, the expression of the housekeeping gene *SsActin* was generally maintained. The importance of calcium-dependent signalling on *SsNep2* expression was also indicated by inhibition of calcineurin, a calcium and calmodulin-dependent Ser/Thr protein phosphatase, by cyclosporin A which resulted in reduction in virulence and expression of *SsNep2*. Harel and colleagues (2006) showed that inhibition of calcineurin impaired *sclerotia* development without affecting the secretion of oxalic acid, which is another important virulence factor.

cAMP was also found to be involved in the regulation of *SsNep2* expression and *S. sclerotiorum* pathogenicity; however, this seems to be related to fine control of cAMP levels. The *S. sclerotiorum sac1* mutant, in which the adenylate cyclase gene is deleted, was cAMP deficient and exhibited reduced *sclerotia* development (Jurick and Rollins, 2007); however, my studies showed that it was also non-pathogenic and failed to express *SsNep2*. I also observed

that elevation of cAMP levels by application of exogenous cAMP had similar effects. In much the same way, increasing cAMP levels also inhibited *sclerotia* development in *S. sclerotiorum* (Rollins and Dickman, 1998). While some of the developmental abnormalities associated with the adenylate cyclase mutant were corrected by the application of exogenous cAMP (Jurick and Rollins, 2007), this was not the case for pathogenicity or *SsNep2* expression. Surprisingly, expression of *SsActin* was not observed in the *sac1* mutant under any of these conditions suggesting that the mutation has far-reaching pleiotropic effects.

4.4.5 Summary

In summary, I showed that the *S. sclerotiorum* NEPs were able to cause necrosis when expressed *in planta*, but their genes were differentially expressed during the infection. The initial expression of at least *SsNep2* was dependent upon interaction with solid surfaces and calcium and cAMP signaling are likely involved. While the lack of *SsNep2* expression often coincided with reduced virulence or loss of pathogenicity, some pharmacological treatments reduced *SsNep2* expression without any apparent affect on virulence. Interestingly, expressed sequence tags generated from cDNA libraries made from infected *B. napus* (Li et al., 2004a) or infection cushions (Sexton et al., 2006) failed to identify genes encoding either NEP. As such, further studies, such as testing NEP mutants, will be required to determine their overall contribution to pathogenesis.

The above chapter has been published (Dallal Bashi et al, 2010).

4.5 Materials and methods

4.5.1 Fungal cultivation

S. sclerotiorum strain 1980 (ATCC18683) was propagated as per section 2.5.8. The adenylate cyclase deletion mutant, *sac1*, was obtained from J. Rollins (Jurick and Rollins, 2007).

4.5.2 Isolation and analysis of *SsNep1* and *SsNep2*

RNA was extracted from *B. napus* leaves infected with *S. sclerotiorum* as described below. cDNA was synthesized using an oligo dt20 primer and Superscript 2 reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Using the cDNA as a template, the *SsNep1* and *SsNep2* open reading frames were amplified using SsNep1-F (5'-ATCAGGATCCATGCTTTTCACCAACACA-3') and SsNep1-R (5'-ATAAGAATGCGGCCGCTTAGATTTGTGCCTCTGCGAG-3') or SsNep2-F (5'-TACTGAATTCGTCGACGGATCCATGGTTGCCTTTGCCAAATCTC-3') andSsNep2-R (5'-ACAACTCGAGGCGGCCGCCTAGAAACTACTAGCCTTCAC-3'), respectively. Due to low levels of *SsNep1* transcripts, the product from the first PCR reaction (50 cycles) was purified and re-amplified to generate sufficient material for cloning. *SsPg1* was amplified using SsPg1-F (5'-CTCGAGAAGAGAGCTCCAGCACCAGCACCA-3') and SsPg1-R (5'-GGAGGAAGAGACGGTACCGTGAACG-3').

Signal peptides were identified using SignalP. Intron-exon boundaries were inferred using NNSPLICE 0.9 (www.fruitfly.org/seq_tools/splice.html). Dendograms arising from phylogenetic analysis were constructed according to the neighbour-joining method and confidence values for the branches determined using bootstrap analysis where 100 trees were generated from randomly resampled data generated by CLUSTAL W.16 as provided in PHYLIP version 3.5C (distributed by J. Felsenstein at http://www.med.nyu.edu/rcr/rcr/phylip/main.html). The CLUSTALW alignment was performed using the Blosum scoring matrix with the following gap penalties; opening gap (10), end gap (10), extending gap (0.05) and separation gap (0.05).

4.5.3 *In planta* production of *S. sclerotirum* NEPs via the Potato Virus X (PVX) system

The amplified *SsNep1* fragment was digested with *Bam*HI and *Not*I and cloned into the GATEWAY entry vector pTK172 (a modified GATEWAY entry 1A vector with Zeocin resistance, gift from K. Rozwadowski). To convert pGR107 (Chapman et al., 1992) into a GATEWAY destination vector, the GATEWAY cassette A was cloned into the *Sma*I site of pGR107 as a blunt end fragment. This vector was named pGR107-GWY. The *SsNep*1 open reading frame was transferred from pTK172 to pGR107-GWY by recombination as per by the GATEWAY protocol (Invitrogen). The *SsNep2* open reading frame was amplified and cloned into pGEM-T Easy (Promega). The insert was excised from the pGEM-T Easy vector by digestion with *Sal*I and *Xho*I and cloned into the *Sal*I site of the PVX vector pGR107 (Chapman et al., 1992). The PVX constructs were transferred to *Agrobacterium tumefaciens* Gv3101 and grown in Luria broth agar. Leaves were inoculated by piercing the base of the leaf with a bacteria-laden toothpick near the main vein of four to six week-old *N. benthamiana* plants.

4.5.4 Expression of *SsNep*2 during the infection

Conducted as per section 3.5.6.

4.5.5 Expression of *SsNep2* in response to surface contact

Conducted as per section 3.5.4

4.5.6 Pharmacological studies

Conducted as per section 3.5.6

4.5.7 RNA isolation, northern blot and reverse transcription analysis

To isolate total fungal RNA, 150 mg of mycelia (wet weight) was ground in liquid N_2 and dispensed into a 1.5 mL microcentrifuge tube containing 600 µL of extraction buffer [0.1 M NaCl, 2 % SDS, 50 mM Tris–HCl (pH 9.0), 10 mM EDTA] and 600 µL phenol/chloroform/isoamyl alcohol (25:24:1). Samples

were mixed for 30 s using a vortex and centrifuged for 10 min at 15000 g. The aqueous phase was extracted again with an equal volume of chloroform, transferred to a new microcentrifuge tube and the RNA precipitated using lithium chloride (2 M final concentration) overnight at 4°C. The pellet was washed once with 2 M lithium chloride and once with 75 % ethanol. The RNA was air dried for 10 min and dissolved in 35 μL DEPC-treated ddH_20. Northern blot analysis was done as described in Li et al. (2004b). *SsNep* cDNA was amplified using the same primers mentioned above, labelled with ^{32}P-CTP and used as a probe. A segment of the *SsActin* gene (GenBank accession No. XP_001589969) was amplified using the SsActinF (5'-CAGCGTTCTACGTCTCTATC-3') and SsActinR (5'-CGAACATCAACATCACAC-3') primers.

For RT-PCR analysis, mRNA was converted to cDNA using the Superscript First Strand Synthesis System (Invitrogen) followed by PCR using the primer pairs described above. The PCR reaction mixture contained 2.5 μL 10 X buffer, 2 μL dNTPs (5 mM), 2 μL forward primer (2.5mM), 2 μL reverse primer (2.5mM), 0.5 μL rTaq polymerase I, 2 μL of a 1/20th dilution of the cDNA and 14 μL dH_20. A 2 μL aliquot of genomic DNA (40 ng/μL) was used as a control. The PCR conditions used were as follows: initial denaturation at 94°C for 5 min followed by 27 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final extension of 72°C for 7 min.

CHAPTER FIVE

ROLE OF THE *S. SCLEROTIORUM* MAPK SMK3 IN PATHOGENICITY

5.1 Abstract

MAPKs play a central role in transferring signals and regulating gene expression in response to extracellular stimuli. Disruption of the *S. sclerotiorum Smk3* gene severely reduced virulence on intact leaves, but not on leaves stripped of cuticle. This was attributed to loss of the ability to form infection cushions. The mutation also caused hyper-branching, loss of the ability to produce sclerotia, increased aerial mycelia and altered mycelial hydrophobicity. Mutants also had slower radial expansion rates on solid media and were more tolerant to high temperatures. Loss of SMK3 appears to have impaired the ability of *S. sclerotiorum* to sense its surrounding environment leading to misregulation of a variety of functions.

5.2 Introduction

The previous studies found that many genes, including those encoding cutinase, polygalacturonases (PGs) and necrosis and ethylene inducing peptides (NEPs) were expressed at different times during the infection or responded differently to physical, environmental and biochemical cues. These enzymes and proteins work in concert during the infection and therefore the expression of the genes encoding them must be highly coordinated. Many signalling mechanisms have been identified in eukaryotes, including calcium flux, potassium flux, iron flux, hormonal pathways, cAMP levels and mitogene activated protein kinase (MAPK) cascades. To better understand how the expression of these genes is governed, this study focused on MAPKs that regulate the response to exterior environmental stimuli and known to be involved in pathogenesis in other phytopathogenic fungi.

An important step in pathogenesis is adhesion to the host tissue. Subsequently, the plant cell wall is breached through the formation of specialized structures, such as appressoria or infection cushions, followed by the release of hydrolytic enzymes. Proper fungal cell wall formation is important as high turgor pressures are developed during penetration. Complex networks of proteins help to shape the structure of the fungal cell wall and allow it to function in adhesion and penetration. Changes to fungal cell wall architecture have been demonstrated to be regulated through MAPKpathways. Earlier chapters in this thesis focused on understanding the regulation of genes during infection, including penetration of host physical barriers. This chapter examines the signalling mechanism that links cell wall formation and surface sensing with hydrolytic enzyme gene expression. A MAPK pathway involved in cell wall integrity was identified as a candidate for this study since it was reported to be involved in both cell wall formation and gene expression in other systems. Cell wall integrity MAPK pathways have been studied in several filamentous fungi, including *M. grisea* (Xu and Hamer 1996), *C. lagenarium* (Takano et al., 2000), *F. oxysporum* (Di Pietro et al., 2001), *C. heterostrophus* (Lev et al., 1999), and *Ustilago maydis* (Mayorga and Gold, 1999). MAPK pathways are conserved in eukaryotes and transfer signals from the external environment to the transcription control apparatus. All MAPK pathways share a common organization. Protein kinases and GTPases interact with sensory proteins at the plasma membrane where they sense external conditions and translate that message through phosphorylation of a MAP kinase kinase kinase (MAPKKK). Subsequently, the MAPKKK phosphorylates a MAPKK which finally phosphorylates the MAPK. A distinct feature of MAPKs that distguishes them from other kinases is that they are phosphorylated at conserved threonine and tyrosine residues. The MAPK transfers the signal to a transcription factor or other molecules that either activate or repress gene expression. Several models through which specific signals are transduced have been proposed.

The first model is based on the sequestration of pathway-specific components away from another pathway, allowing the components of the activated pathway to come into close proximity. Three different mechanisms of sequestration have been described. The first mechanism for sequestration is through cell-specific protein expression that restricts certain proteins to specific cell types (Volmat et al., 2001). This hypothesis may be valid for organisms with multiple types of tissues, but is less likely to be the case in filamentous fungi. An example of such mechanism is from *Caenorhabditis elegans* where activation of the same MAPK pathway led to activation of different transcription factors dependent on where the pathway was being activated. This led to the differentiation of cells into different tissues (Tan et al., 1998). A second mechanism for sequestration is through compartmentalization of specific elements of a pathway within a cell. In one scenario, the specific proteins were restricted to the nucleus, while others were in the cytoplasm. This prevented cross talk and ensured the activation of specific pathways. This mechanism is used to govern the response of neuron distal axon terminals to nerve growth factor (Watson et al., 2001). A third mechanism for sequestration is through temporal restriction of expression of proteins that can cross talk. This mechanism is employed during the cell cycle where the expression of a cyclin-dependent kinase is timed to specific points in the cell cycle (Murray, 2004). The fourth mechanism for sequestration is through scaffolding. Over eighteen scaffold proteins have been identified in mammalian MAPK pathways. Scaffolds are believed to accelerate signalling through binding components of the pathway bringing them into proximity. The scaffold proteins were suggested to have higher affinity for activated forms of specific signalling proteins ensuring that only the correct pathway is activated. The scaffold may also assist in the targeting of certain proteins for degradation specifically when bound to the scaffold (reviewed by Morrison and Davis, 2003).

A second model for MAPK signalling is through combinatorial signalling (Madhani and Fink, 1997). This applies to situations where eliciting more than

one pathway is required for activation of a response. This mechanism prevents unnecessary activation of the pathway due to perception of only one elicitor. For example, the hormone N-WASP induces polymerization of actin by the Arp2/3 complex. Transduction of the N-WASP signal requires activation of both Cdc42 (cell division control protein) and PIP2 (phosphatidylinositol 4,5 biophosphate) (Prehoda et al., 2000; Rohatgi et al., 2000). Similar mechanisms may be employed to activate gene expression when a fungal pathogen is interacting with an incompatible host. Two independent parameters may have to be optimal to activate gene transcription.

The third model is through cross pathway inhibition where one pathway is stimulated leading to activation of pathway components, including components shared with other pathways; while components of the second pathway are degraded or inhibited. Formation of alternate protein scaffolds may be responsible for inhibiting components of the second pathway (Madhani and Fink, 1998).

The fourth model is through signalling kinetics, which is believed to be more sensitive and quantitative (Murphy et al., 2002). The response can be different depending on the duration and amplitude of the input. One proposed mechanism involves an unstable downstream protein that requires constant activation to remain functional and to conduct the signal. For example, expression of the gene encoding the transcription factor c-Foc was induced by many extracellular signals, such as growth factors, through a MAPK pathway and was subsequently activated by a MAPK in a second pathway to ensure that the protein is only active in the presence of a sustained signal (Murphy et al., 2004).

Five main MAPK pathways have been characterized in *S. cerevisiae*; these are the mating pheromone response pathway, the filamentation-invasion pathway, the spore wall assembly pathway, the HOG pathway and the cell integrity pathway (Gustin et al., 1998). These pathways are highly conserved

among yeasts and filamentous fungi, but have evolved to regulate different functions or physiological features.

The mating pheromone response pathway is activated when a mating pheromone is received. The mating pheromone receptor is a seven transmembrane domain G-protein coupled receptor. Recognition of the pheromone causes the heterotrimeric G-protein to be released which in turn activates a MAPK pathway. This pathway is partially responsible for a group of changes necessary for mating including, polarized growth towards the mating partner, cell cycle arrest in the G1 phase and production of adhesion and fusion proteins. A well-studied MAPK in this pathway is Fus3 (Bardwell, 2004).

The filamentation-invasion pathway regulates in the transition from yeast-like to filamentous growth as noted by the formation of extended cells and colonies with rough edges. Filamentous growth allows invasion of solid medium to promote nutrient acquisition. One of the inducers of filamentous growth is nitrogen starvation. A well studied MAPK in this pathway is the Kss1 (Davenport et al., 1999). The orthologue of Kss1 in *S. sclerotiorum*, *Smk1*, was found to play a central role in coordinating signals from pH, cAMP and oxalic acid in the formation of sclerotia. Mutation of *Smk1* led to a deficiency in sclerotia formation (Chen et al., 2004).

The HOG pathway is responsible for regulating the response to osmotic pressure. This is critical for survival of yeast under stresses related to increasing external osmolarity. The pathway has two upstream branches. The first branch starts with the putative membrane protein Sho1p, while the other branch contains a three component signalling protein complex composed of Sln1p, Ypd1p, and Ssk1p. When osmolarity increases, the signalling pathway is activated leading to the expression of genes involved in the accumulation of intracellular glycerol that provides osmotic balance with the external environment (Meikle et al., 1988).

The spore wall assembly pathway regulates sporulation in response to nutrient stresses. Nitrogen and carbon starvation cause diploid yeast cells to

form spores that are resistant to biotic and abiotic stresses. Under these conditions, the cells proceed through meiosis 1 and 2 followed by assembly of walls that surround each of the four nuclei to form haploid spores (Kupiac et al., 1997).

Most related to the regulation of *S. sclerotiorum* pathogenicity factors in response to environmental stimuli is the cell wall integrity pathway. In *Candida albicans*, mutation in this pathway causes increased sensitivity to cell wall degrading enzymes (Navarro-Garcia et al., 1995). Orthologues of the *S. cerevisiae* MAPK involved in this pathway, SLT2, have been identified in many filamentous fungi, including plant and animal pathogens. Mutants frequently have osmotic-remediable lytic phenotypes caused by impaired cell wall biogenesis (De nobel, 2000; Levin, 2005). In *C. albicans*, *MKC1* has been shown to be essential in sensing contact with surfaces and transition to invasive growth (Kumamoto, 2005). The *Mkc1* mutant was unable to form filamentous invasive mycelia and was non-pathogenic. In addition, the mutant had increased sensitivity to cell wall degrading enzymes suggesting that cell wall architecture was altered (Navarro-Garcia et al., 1995). Mutation of *Mps1* in *M. grisea* caused a range of changes in physiological functions including reduced aerial growth and conidation. Colonies also exhibited central progressive autolysis that was recoverable with the addition of 1 M sorbitol to the medium, indicating a deficiency in tolerating osmotic pressure. As with *C. albicans*, this mutant was also more susceptible to treatment with cell wall degrading enzymes. Interestingly, the mutant was completely avirulent (Xu et al., 1998). In *F. graminearum*, mutation of *Fsk* caused reduced growth only on solid medium and reduced aerial mycelia formation and conidation. Mutant colonies also had reduced pigmentation and were white rather than pink in colour. Similar to those above, this mutant was more susceptible to cell wall degrading enzymes and high temperatures and was less virulent. Interestingly, the mutant was found to be only male fertile with reduced fusion mycelia events and heterokaryon formation (Hou et al., 2002). In *B. cinerea*, a close relative of *S.*

sclerotiorum, the mutation caused a severe reduction in aerial mycelia formation and conidation similar to the *F. graminearum* mutant. Generally, radial expansion of mutant colonies was slower than the wild type and supplementation with nutrients failed to recover the phenotype. Unlike previously characterized mutants in other filamentous fungi, the *B. cinerea* mutant did not exhibit heat sensitivity or susceptibility to cell wall degrading enzymes. The frequency of conidia germination was unaffected, but germ tubes elongated excessively and were unable to penetrate the host. Mycelia also exhibited reduced epidermal cell penetration and fewer lesions formed on intact leaves, but to a less extent on wounded leaves where the cuticle had been damaged (Rui and Hahn, 2007). These observations clearly demonstrate the diversity in functions controlled by the cell wall integrity MAPK pathway and its importance in pathogenicity.

In this chapter, I identify the *Slt2* homologue in *S. sclerotiorum*, named *Smk3,* and examine the phenotype of a mutant in which the gene was disrupted.

5.3 Results

5.3.1 Changes in MAPK phosphorylation in response to contact with different surfaces

The literature clearly indicates that activation of MAPK pathways is involved in various aspects related to pathogenesis. The majority of MAPK pathways include ERK1/ERK2 (extracellular signal-regulated protein kinase)-type MAPK and antibodies that discriminate between the phosphorylated and non-phosphorylated forms are available commercially. *S. sclerotiorum* mycelia were applied to different surfaces for 10 min to mimic events occurring during the very initial stages of infection establishment. Western blot analysis of total cell protein from these mycelia revealed that phosphorylation of ERK1/ERK2 type proteins increased upon mycelia contact with a solid surface (Figure 5.1).

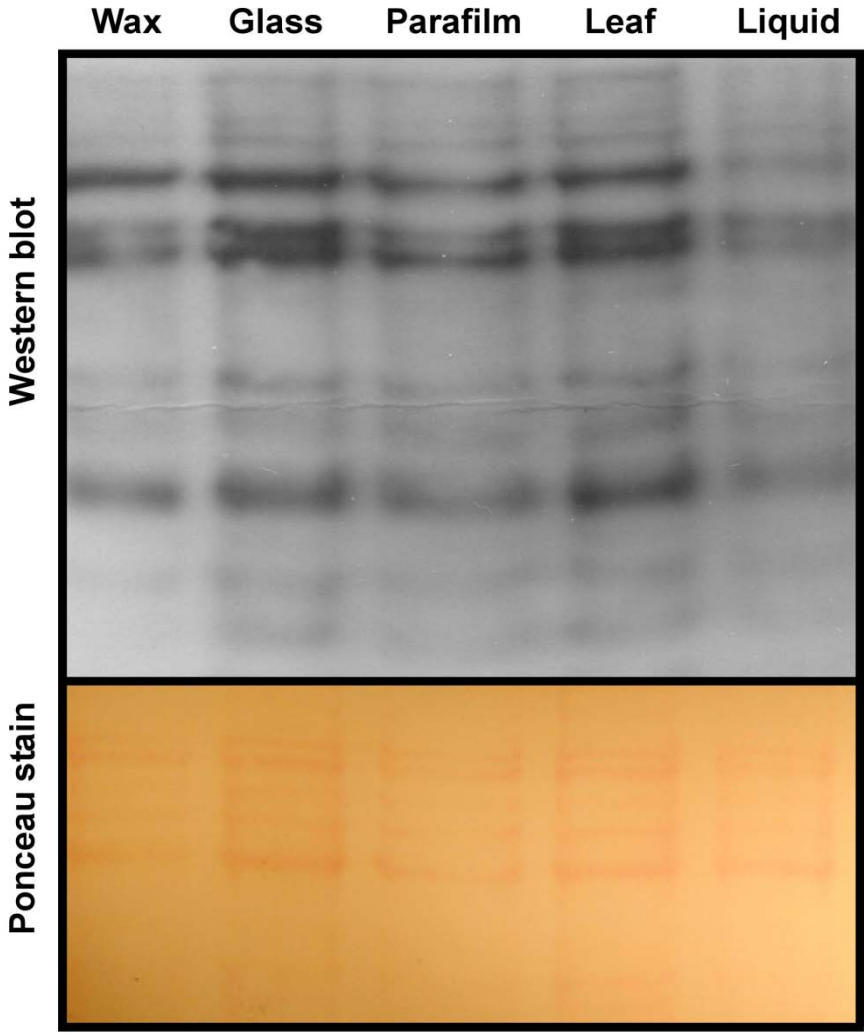


Figure 5.1 Alteration in phosphorylated *S. sclerotiorum* MAPKs in response to contact with solid surfaces.
Mycelia were grown in MS-Glu liquid medium and then placed in contact with a solid surface for 10 min. Western blot was conducted using anti-phosphorylated ERK1/2 antibody. The bottom panel shows the same membrane treated with Ponceau stain to detect protein loaded in each lane.

5.3.2 Characterization of *S. sclerotiorum* pathogenicity and cell wall integrity MAPKs

Since ERK1/ERK2-type MAPK were being phosphorylated, and presumably activated, in response to conditions that mimic the early stages of infection, I characterized two MAPKs that may be involved in pathways

relevant to this stage; the cell wall integrity MAPK and the pathogenicity MAPK.

A BLAST search of the *S. sclerotiorum* genome with the *B. cinerea* cell wall integrity MAPK *Bmp3* identified a similar gene that I referred to as *Sclerotinia MAPK3* (*Smk3*). The *Smk3* gene was predicted to have 4 introns and 5 exons resulting in an open reading frame encoding a protein of 413 amino acids that was predicted to be 47 kDa. SMK3 was most similar to *B. cinerea* (99.8% identity) and least to *U. maydis* (43% identity). SMK3 has a conserved TEY domain (Figure 5.2) in which the threonine and tyrosine are phosphorylated leading to activation of the kinase.

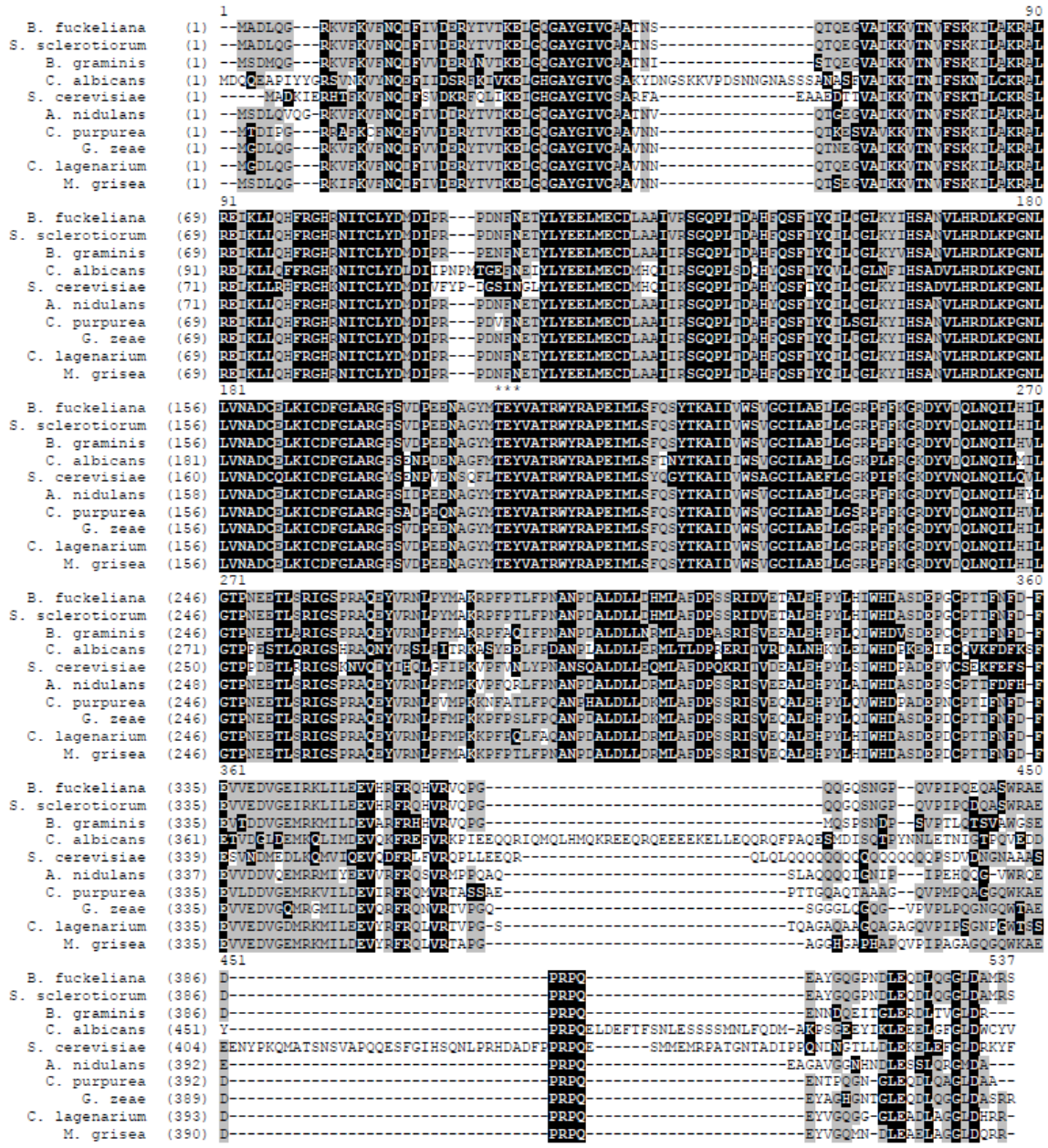


Figure 5.2 Alignment of Slt2-like MAPK orthologues from filamentous fungi. Alignment of *S. sclerotiorum* SMK3 with SLT2 orthologues from *B. cinerea* BMP3 (DQ986330.1), *S. sclerotiorum* SMK3, *B. graminis* MPK2 (AF301166), *C. albicans* MKC1 (P43068), *S. cerevisiae* SLT2 (X59262), *A. nidulans* MPKA (U59214), *C. purpurea* CPMK2 (AJ320496), *G. zeae* MGV1 (AF492766), *C. lagenarium* MAF1 (AY064246), *M. grisea* MPS1 (AF020316 http://www.ncbi.nlm.nih.gov/nuccore/77022312). The figure shows identical residues (white on black background) and residues conserved in five out of ten sequences (black on gray). ERK1/2 type phosphorylation site is also indicated (*).

Northern blot analysis was used to examine changes in the expression of *Smk3*, as well as the pathogenicity MAPK *Smk1* in response to contact with different surfaces in the presence or absence of glucose. Glucose was added for two reasons; firstly, to provide an exogenous utilizable carbon source that would naturally be provided to the mycelia from decaying petals and secondly, since it was shown to increase the expression of *SsCutA* in mycelia that are in contact with the leaf or other solid surfaces. *Smk1* transcript levels were unchanged in mycelia growing in liquid culture or after transfer to intact leaves or leaves stripped of surface wax (Figure 5.3). The presence of glucose reduced the expression of *Smk1* under these conditions, indicating that it may be subject to catabolite repression. Conversely, *Smk3* expression was induced on intact leaves in the presence of glucose, which should simulate the conditions present during infection. Furthermore, expression was unaffected on leaves stripped of wax indicating that the natural conditions or context is important for up-regulation of Smk3 expression.

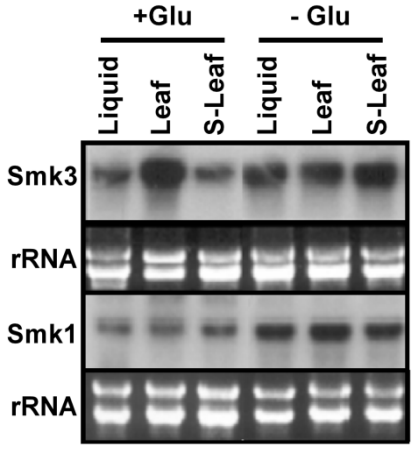


Figure 5.3 Expression of *Smk1* and *Smk3* in response to glucose and contact with various leaf surfaces.
Northern blot analysis showing gene expression in liquid minimal salts medium or after contact with intact *B. napus* leaves (24 h) and leaves stripped of cuticular wax in the presence or absence of glucose. The lower panels show the rRNA loaded in each lane.

5.3.3 Generation of *Smk3* disruption mutant

Smk3 expression was induced during the early stages of the infection and orthologues of *Smk3* were shown to be involved in aspects of pathogenicity in other fungal

phytopathogens. To determine the role of SMK3 in *S. sclerotiorum* pathogenicity, a *Smk3* disruption mutant (*Smk3υ*) was generated. This work also led to the development of an efficient new method for generating homokaryons from heterokaryotic transformants of filamentous fungi. This is most useful for fungi that do not produce haploid spores. Details of the protocol were published separately and can be found in Appendix A.

5.3.4. Growth habits and sclerotia formation in *Smk3υ*

Two independent *Smk3υ* mutants were examined. *Smk3υ* colonies grew slower and the rate of radial expansion was only 63-82 % that of the wild-type. Microscopic examination of mycelia grown in liquid culture revealed that the *Smk3υ* had much higher rate of branching (Figure 5.4). In liquid culture, *S. sclerotiorum* mycelia grew as spherical aggregates. At the edge of these aggregates, wild type mycelia radiate or project outward, whereas mutant mycelia branched more frequently and closer to the growing tip which increased aggregate density.

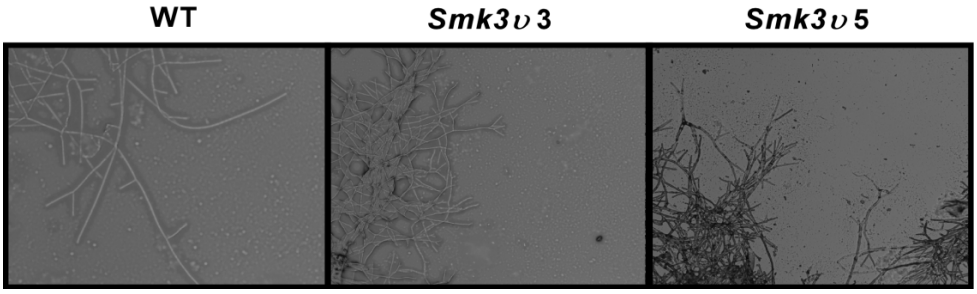


Figure 5.4 Effect of *Smk3* mutation on *S. sclerotiorum* mycelial branching.
Light microscopic images of wild type (WT) and *Smk3* disruption mutants (*Smk3υ*) growing in minimal salt-glucose liquid medium. Mycelial branching is more frequent in the mutant colonies than in the wild type.

Microscopic examination of a vertical section of a colony growing on solid medium revealed a dense mat of mycelia in the mutant colonies (Figure 5.5). Wild-type mycelia grew parallel to the surface, while mutant mycelia tended to grow erect, with the branched mycelia elevating off the surface of the plate. This would explain the reduction in the colony expansion rate of the mutants

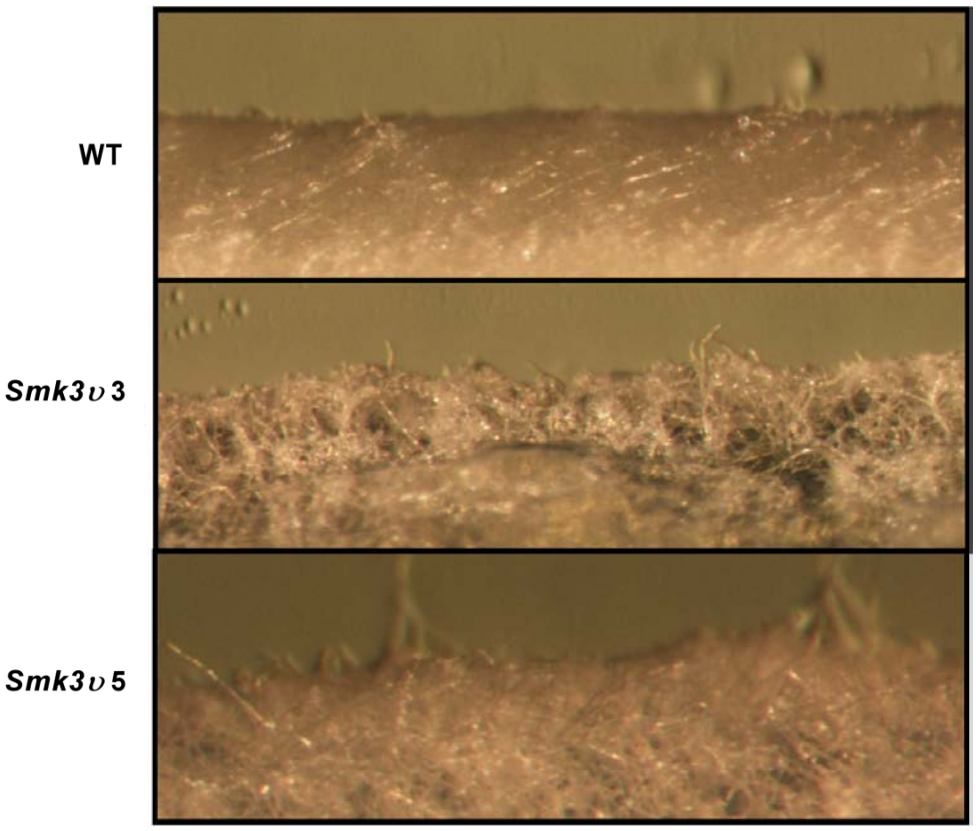


Figure 5.5 Effect of *Smk3* mutation on *S.sclerotiorum* aerial mycelia formation
Light microscopic images of wild type (WT) and *Smk3* disruption mutants (*Smk3υ*) growing on potato dextrose agar culture. Examination of vertical sections from the colonies showed increase in aerial mycelia formation in the mutants.

5.3.5 Hydrophobicity of *Smk3υ* mycelia

The *Smk3υ* colonies had increased aerial mycelial biomass. Aerial mycelia formation is promoted by increased surface tension and hydrophobicity. Hydrophobicity was determined using a technique that measures water droplet contact angle on the mycelial surface. Wild type mycelia were more hydrophobic at the edge of the colony than in the centre with water droplet contact angles of 91º and 64º, respectively (Figure 5.6). The *Smk3υ* mutants had the opposite pattern as the center was more hydrophobic than the edge with water droplet contact angles of 33-71º and 95-96º, respectively. The increased hydrophobicity towards the center of the mutant colonies correlates with the higher density of aerial mycelia formation in this region.

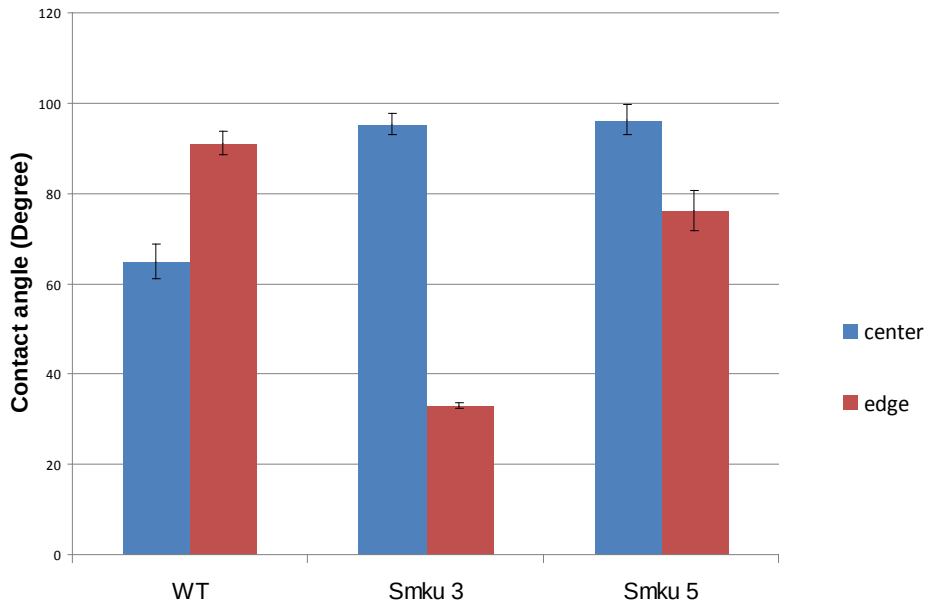


Figure 5.6 Effect of *Smk3* mutation on *S. sclerotiorum* mycelial hydrophobicity. Water droplet contact angle on *S. sclerotiorum* wild type and *Smk3υ* mycelia surface in the center and the edge of the colony. Contact angle was determined immediately after placement of the sample droplet. Error bars indicate standard deviation of 3 replicates.

5.3.6 Cell wall integrity and heat tolerance in *Smk3υ*.

Previous studies demonstrated that the *Smk3* orthologue in other fungi played a role in construction of the cell wall as indicated by the decreased resistance to high temperatures and cell wall degrading enzymes in mutants. To compare the cell wall integrity of wild type and *Smk3υ* colonies, mycelia were fragmented and incubated in lyase, a solution of cell wall degrading enzymes from *Trichoderma harzianum*. After 1.5 h the mutant colonies formed protoplasts, while the wild type mycelia remained intact (Figure 5.7). This suggests that the cell wall in the mutant is either thinner, lacking a critical component or is organized in such a way that enzymes may access its component polymers more easily.

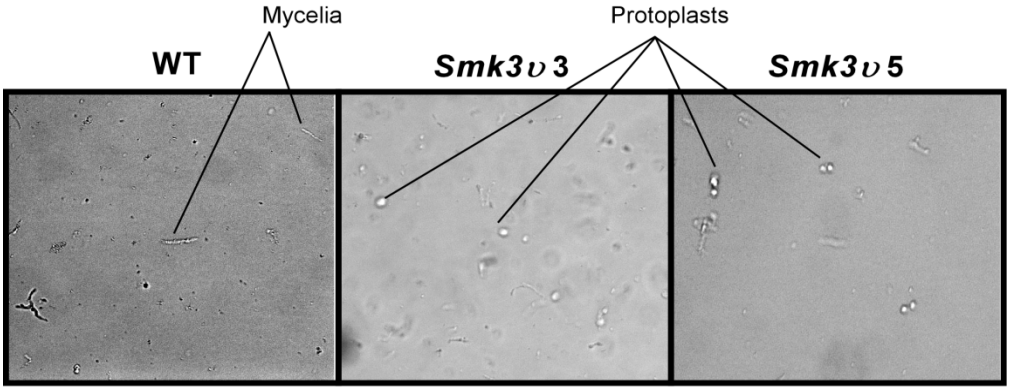


Figure 5.7 Effect of *Smk3* mutation on *S. sclerotiorum* susceptibility to cell wall degrading enzymes.
Light microscope image of wild type (WT) and *Smk3υ* mycelia after incubation with lyase for 1.5 h. Protoplasts were formed in the two mutant colonies, but not in the WT.

Cell wall integrity has also been examined as a function of tolerance to high temperatures (Hou et al., 2002). This study and the related studies noted above, examined only the effect of high temperature on cell wall integrity as measured by collapse of the mycelia. Therefore, they concluded that the mutants had alterations in cell wall structure that caused them to be less resistant to higher temperatures. These studies did not take into consideration the effect of temperature on growth. Growth of the *Smk3υ* colonies was compared to wild type at different temperatures. Neither the mutant nor wild type was able to grow at temperatures above 37°C. When ratio of colony expansion at 20°C and 28°C was compared, the mutant colonies were found to be more tolerant of the higher temperature. At 28°C, the wild type colony grew at 22% of its rate at 20°C, whereas the

Smk3υ colonies grew at 47-51% of their rates at 20°C. This indicates that SMK3 may be involved in regulating growth and cell wall formation in response to temperature.

5.3.7 Virulence of *Smk3υ* on *B. napus*

As mentioned above, loss of the cell wall integrity MAPK was shown to reduce the virulence of phytopathogenic fungi and *Smk3υ* mutants exhibited reduced virulence on intact *B. napus* leaves (Figure 5.8). Within 24 hpi wild type mycelia established a necrotic zone covering the entire area beneath the inoculum, while the mutant colonies only formed infection initials, small necrotic spots, around the leaf veins.

Further experiments were conducted to determine the reason underlying the impaired virulence of the *Smk3υ* mutants. Oxalic acid is a key element in the infection and is involved in chelating cell wall calcium to allow access by hydrolytic enzymes. The release of oxalic acid also reduces the ambient pH which is required for expression and activity of cell wall hydrolytic enzymes. The pH of wild type and *Smk3υ* liquid cultures was measured after 3 days of incubation in MS-Glu medium and no difference was noted (pH 3).

The cuticle is the first physical barrier to infection. The virulence of wild type and *Smk3υ* mutants was examined on intact leaves or leaves stripped of cuticle. Surprisingly, the *Smk3υ* mutants were as or more virulent than the wild type on stripped leaves showing complete necrosis beneath the inoculum within 24 hpi (Figure 5.8). This suggested that the mutation impaired cuticle penetration, but not the ability to infect and consume the underlying tissue.

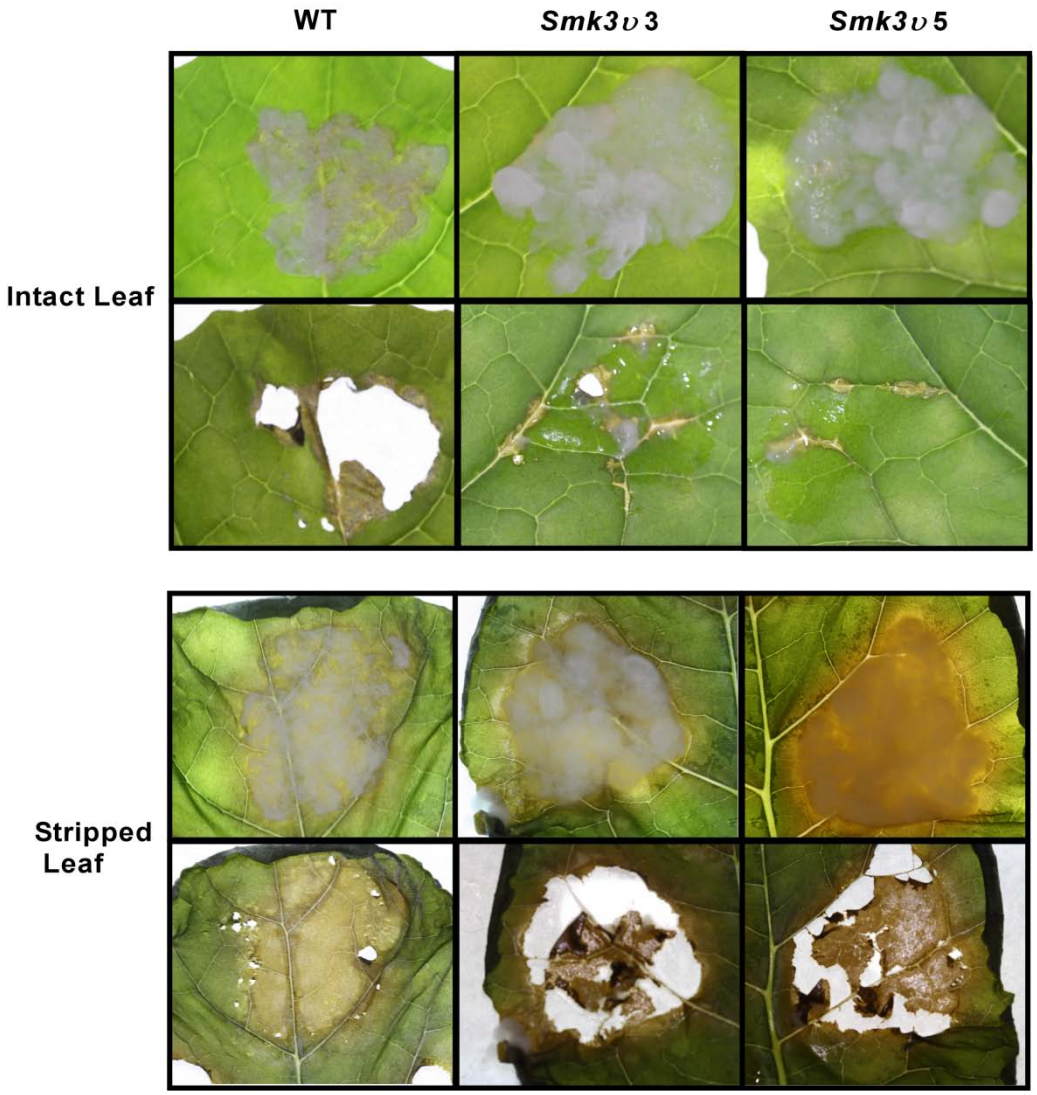


Figure 5.8 Effect of *Smk3* mutation on *S. sclerotiorum* virulence.
Intact *B. napus* leaves or leaves stripped of surface wax 24 hpi with wild type *S. sclerotiorum* or *Smk3υ* mutants. The upper panels show leaves with the mycelial inoculum, while in the lower panels mycelia has been removed to reveal the affected tissue beneath.

Droplets of MS-Glu medium containing mycelia were placed on the leaf surface and examined after 26 h using a dissecting microscope. Wild type mycelia had radiated out of the droplet and formed infection cushions that are responsible for cuticle penetration (Figure 5.9). Indeed, a large necrotic area had already begun to form around the infection cushions by this time. Conversely, the mycelia of both mutants grew parallel to the leaf

surface. They failed to orient toward the leaf to form infection cushions and instead continued to radiate horizontally.

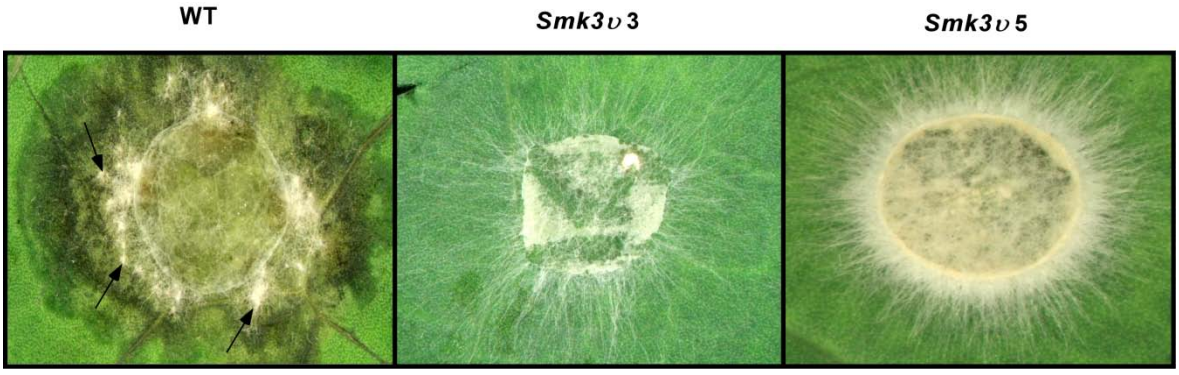


Figure 5.9 Effect of *Smk3* mutation on *S. sclerotiorum* infection cushion formation. *B. napus* leaves 26 hours post inoculation with mycelial suspensions of wild type (WT) *S. sclerotiorum* or *Smk3υ* mutants. Arrows indicate infection cushions.

5.4 Discussion

5.4.1 Identification of pathogenicity-related MAPK

Two types of MAPKs have been demonstrated to be important for plant pathogenesis by filamentous phytopathogenic fungi; the pathogenicity MAPK and the cell wall integrity MAPK. In *S. sclerotiorum*, the pathogenicity MAPK, *Smk1*, was found to be involved in mediating the cross-talk between cAMP and pH to coordinate *sclerotia* formation (Chen et al., 2004). While the virulence of *Smk1* mutants was not examined, disruption of orthologues in other phytopathogenic fungi severely reduced virulence and in some cases rendered them non-pathogenic (Jin-Rong et al., 1998; Hou et al., 2002). My studies demonstrated that the expression of the gene encoding *SsCutA* which is involved in cuticle penetration was dependent on contact with a solid surface, while the expression of *SsPg1* was restricted to certain infection zones, such as the expanding margin of the lesion. Regulation of both genes requires that the fungus be able to sense its environmental context and therefore the cell wall integrity MAPK pathway may be involved.

In this study, the *S. sclerotiorum Slt2* orthologue, *Smk3*, was identified. *Slt2*-like MAPKs are involved in the signalling pathway that is activated in response to extracellular

factors that cause cell wall perturbation. MAPKs are highly conserved in fungi and yeasts and are functionally interchangeable between different organisms (Fujioka et al., 2007, Mey et al., 2006). *Smk3* was also up-regulated during infection which further supports the notion that SMK3 may play a role in *S. sclerotiorum*-host interactions.

5.4.2 Role of *Smk3* in aerial mycelia formation

The most common approach to characterize MAPKs is to create a loss-of-function mutation which causes direct and measurable effects. Disruption of *Smk3* caused visually detectable alterations in colony morphology. *Smk3υ* colonies were less pigmented and more elevated than the wild type, which upon microscopic examination was found to be due to a higher density of erect aerial mycelia. Unlike *S. sclerotiorum,* mutations in *Slt2* orthologues in *B. cinerea* (Rui and Hahn, 2007), *F. graminearum* (Hou et al., 2002), *M. grisea* (Xu et al., 1998) and *A. nidulans* (Bussink and Osmani, 1999) reduced aerial mycelia formation, while complete loss was observed in *Mycosphaerella graminicola* (Mehrabi et al., 2006). Similar to *Smk3υ*, a *S. sclerotiorum* adenylate cyclase mutant formed dense aerial mycelia (Jurick and Rollins, 2007). The similarity of phenotype between the *Smk3υ* and adenylate cyclase mutants suggests that SMK3 may also coordinate physiological functions through cross talk with cAMP.

The degree of aerial mycelia formation is a function of small (~ 100 amino acid), surface-active, secreted proteins with eight conserved cysteine residues known as hydrophobins (Wosten and Willey, 2000; Wessels, 1997). Once secreted, hydrophobins self assemble at the hydrophilic-hydrophobic interface into a 10 nm thick amphipathic protein film. This film increases water tension allowing formation of aerial mycelia. The increase in aerial mycelia in *Smkυ* mutants could be due to SMK3 interaction with transcription factors and up-regulation of hydrophobin gene expression. *Smkυ* mutant colonies were found to be more hydrophobic at the center of the colony than at the edge, while the wild type strain was more hydrophobic at the edge than the center. Fungi exhibit apical dominance which allows the colony to differentiate between the edge and the center of the colony. Lack of proper apical dominance may also be the reason for extensive branching in the *Smkυ* mutant. The shift in the hydrophobic zone towards the edge of the

colony in the *Smkυ* mutant may be a secondary effect of the lack of apical dominance and extensive branching. Hyper-branching in the *Smkυ* mutants was consistent with an earlier finding in *C. purpurea* which exhibited a similar phenotype upon mutation of its *Slt2* orthologue. Branching in fungi is also regulated through cAMP as low concentrations increase branching (Scott and Solomon, 1975; Rosenberg and Pall, 1979) and disruption of the adenylate cyclase gene decreased the branching angle in *S. sclerotiorum* (Jurick and Rollins, 2007). The involvement of SMK3 and cAMP in aerial mycelia formation and branching is further evidence that a cross talk might occur between the two signalling mechanisms.

5.4.3 Role of SMK3 in mycelia branching and sclerotia formation

The *Smk3υ* mutants were unable to form sclerotia or sclerotia initials. Proper mycelial branching is known to be necessary for sclerotia formation in the close relative *Sclerotinia rolfsii.* The mycelia of *S. rolfsii* were classified as either leading mycelia with 40 nuclei or lateral mycelia with 10 nuclei. Lateral mycelia with shorter intrudes (240 μ) were proposed to be responsible for sclerotia formation (Goujon et al., 1970). Apical dominance is regulated through a hormone-like substance and prevents lateral branching and therefore sclerotia formation (Burnett, 1968). In *S. sclerotiorum*, the expanding margin may have an apical dominance mechanism that represses sclerotia formation in the center of the colony. When starved, the expanding margin forms sclerotia and only later do other parts of the colony follow and produce sclerotia as well. In all cases, sclerotia formation is strongly related to proper branching which is altered in the *Smk3υ* mutant.

Smk3υ mutant colonies expanded on solid media at a slower rate than wild type colonies. Disruption of *Slt2* orthologues in *F. graminearum* (Hou et al., 2002), *C. albicans* (Navarro-Garcia, 1995) and *B. cinerea* (Rui and Hahn, 2007) caused reduced expansion on solid media similar to *S. sclerotiorum*. In *M. graminicola*, no alterations in growth phenotype were observed until the fifth day on which cells showed polarized growth with swollen cells forming by the eleventh day (Mehrabi et al., 2006). Unlike other fungi, the mutation had no effect on colony expansion rate in *M. grisea* (Jin-Rong et al., 1998) or *C. lagenarium* (Kojima et al., 2002). Reduced colony expansion rate has been suggested to be

due to lack of osmotic tolerance. Addition of an osmotic stabilizer to cultures of a *B. cinerea Slt2* orthologue disruption mutant recovered 80% of the mycelial growth and 100% of the germling elongation rate (Rui and Hahn, 2007). It is also possible that the reduced colony expansion rate in the *Smk3υ* mutant was due to hyper branching which leads to dense rather than expansive growth.

5.4.3 Role of SMK3 in response to high temperatures

Mutations in *Slt2* orthologues have been demonstrated to cause sensitivity to high temperatures. Cells of *F. graminearum* (Hou et al., 2002), *S. pombe* (Toda et al., 1996) and *C. albicans* (Navarro-Garcia et al., 1996) *Slt2* mutants burst upon exposure to high temperatures. Surprisingly, the colony expansion rate was higher in the *Smk3υ* mutant than the wild type when incubated at the more restrictive temperature of 28ºC compared to 20ºC. No studies have been conducted on the role of MAPKs in the response to heat in filamentous fungi. In tomato, MAPK phosphorylation led to activation of a heat stress transcription factor indicating that the MAPKs play a role in transducing the heat stress signal (Link et al., 2002). SMK3 may be responsible for reducing growth rate at high temperatures to avoid lethal events, such as cell burst, which would explain why the wild type strain grew slower than the mutant. It is also possible that cell wall structure or components have been altered in an advantageous way that causes the growth of the *Smk3υ* mutant to be less affected at higher temperatures.

5.4.4 Role of SMK3 in proper cell wall formation

The *Smk3υ* mutant was found to be more susceptible to cell wall degrading enzymes than the wild type. Similar results were found in *Slt2* mutants in *M. grisea* (Navarro-Garcia et al., 1998), *F. graminearum* (Hou et al., 2002) and *M. graminicola* (Mehrabi et al., 2006). A *Slt2* orthologue and a second gene were found to play a redundant role in regulating cell wall integrity in *C. lagenarium* as mutation of the *Slt2* orthologue alone did not increase the susceptibility of the cell wall to these enzymes (Kojima et al., 2002). *Smk3υ* mutant cell walls may be less rigid due to lack of certain components or structural changes. This flexibility could have allowed the *Smk3υ* mutant to grow better at high temperatures, but made it more susceptible to cell wall degrading enzymes than the wild type.

5.4.5 Role of SMK3 in virulence

The *Smk3υ* mutant was found to be at least as virulent as the wild type strain on leaves stripped of cuticle, but were much less virulent on intact leaves. Mutations in the *slt2* orthologue in *B. cinerea* also reduced virulence on intact leaves. *B. cinerea* spore germlings elongated excessively due to failure in sensing the leaf surface and were unable to form appressoria to attach themselves to the plant (Rui and Hahn, 2007). A *M. graminicola Slt2* orthologue mutant was able to initiate infection through the stomata, but was unable to expand in the plant tissue (Mehrabi et al., 2006). *C. lagenarium Slt2* orthologue mutant spores lost their ability to form appressoria, but retained a low level of virulence due to the release of cell wall degrading enzymes and mycotoxins (Kojima et al., 2002). A wide range of factors may be affected by mutation of *Slt2* orthologues in filamentous fungi. Since *Smk3υ* mutant mycelia were able to expand in the leaf tissue beyond the cuticle, only cuticle penetration appears to be impaired. This could be due to lack of proper infection cushion formation as observed on the leaf surface which also occurred in the adenylate cyclase mutant colony (Jurick and Rollins, 2007). Failure to form infection cushions may be due to inability to sense the leaf surface and respond accordingly. It could also be due to loss of a physiological function that is necessary for infection cushion development, such as proper branching. Considering the overlap in functions between cAMP and SMK3 signalling, including aerial mycelia formation, branching and sclerotia formation, it is possible that both signalling mechanisms regulate infection cushion formation as well.

5.5 Materials and methods

5.5.1 Effect of contact surface and carbon availability on MAPK phosphorylation and gene expression

To examine MAPK phosphorylation in response to contact surface, *S. sclerotiorum* was cultivated in MS-Glu as described in section 2.5.8. Three-day-old mycelia were harvested by centrifugation at 10,000 rpm and spread on wax, glass parafilm leaf or kept in liquid culture. Samples were allowed to incubate at 20°C for 10 min and were immediately frozen in liquid nitrogen. Total protein was extracted by grinding mycelia in liquid

nitrogen and incubating in extraction buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 2% DTT)at a 1:5 w/v ratio for 2 min. Samples were then centrifuged at 14,000 rpm for 5 min and the supernatant transferred to a separate tube and stored at -20°C. Western blot analysis was conducted using commercially available polyclonal and monoclonal antibodies that can specifically detect phosphorylated ERK1/2 type MAPKs (Cell Signalling). The protocol for western blot analysis is outlined in section 2.5.2
The effect of contact surface and carbon availability on *Smk1* and *Smk3* gene expression was also examined. Mycelia were grown as described in section 3.5.1and spread on wax, glass parafilm leaf or kept in liquid culture. Samples were incubated for 24 h on wet Whatman #5 paper in a sealed chamber to provide the necessary humidity. Mycelia were then collected and immediately frozen in liquid nitrogen. Northern blot analysis was conducted as described in section 3.5.7 using DNA fragments amplified from *Smk1* and *Smk3* as probe templates. The following primers were used to amplify fragments from *S. sclerotiorum* genomic DNA: *Smk1* forward (5'-CTTCTCGCATTTAATCCTG-3') and s *Smk1* reverse (5'-CCTTGGTGAGGTTGTCC-3') and *Smk3* forward (5'-CTTCTCGCATTTAATCCTG-3') and s *Smk3* reverse (5'- CCTTGGTGAGGTTGTCC-3').

5.5.2 Microscopy

A plug from the growing margin of a colony was used to inoculate a potato dextrose agar (PDA) plate incubated at 20°C for 3 days. To examine aerial mycelia formation, vertical sections were made in the colony using a razor and a 1 mM thick slice was laid on a slide. Samples were examined at 20 times magnification using a dissecting scope (Nikon SMZ1500). To determine the branching pattern, mycelia was cultivated in MS-Glu liquid medium as above and then examined under the microscope at 200 times magnification.

5.5.3 Incubation in cell wall degrading enzyme cocktail

Mycelia were grown in 25 mL of potato dextrose broth for 3 days at 20 ºC and washed with sterile water once and once with buffer (0.8 M $MgSo_4.7H_2O$, 0.2 M Sodium citrate.$2H_2O$ pH5.5). Samples were then chopped coarsely and incubated in 10 mg /mL *Trichoderma harzianum* Lysine enzymes (Sigma-Aldrich) in lyase buffer (1 M Sorbitol, 50

mM Sodium citrate, pH 5.8). The mix was incubated at 28 C° with shaking at 100 rpm. Samples were collected and observed under a microscope every 30 minutes.

5.5.4 Hydrophobicity measurement

Samples were prepared as per Chau et al. (2009). Briefly, microscope slides were sterilized using 70% ethanol solution. Slides were transferred to a sterile Petri dish and allowed to dry in a sterile flow chamber. When solidified, PDA was poured on the slide to form 1 mm layer and plates were closed to allow media to solidify. Using tweezers a pin head size of mycelia was transferred to the edge of the slide and samples were incubated at 20 ºC for 2 days. Contact angles of 3 droplets of water were measured at the center of the colony as well as at the edge. Water droplets (10 μL) were placed on the mycelia gently as described in Chau et al., 2009 and image was taken immediately (less than a sec). Images were analyzed using ImageJ (http://rsb.info.nih.gov/ij/) and a plug in for the analysis of a drop shape was used to measure the contact angle. This is also publically available at http://bigwww.epfl.ch/demo/dropanalysis/. The use of this software was originally described in Stalder et al., 2006.

An average of three droplets was calculated and standard deviation is indicated in the chart.

5.5.5 Effect of temperature on growth

Petri dishes including PDA were inoculated using a 3 mm plug from the edge of a growing colony. Samples were incubated at 20 ºC, 28 ºC, 37 ºC and 52 ºC. The experiment was conducted in three replicates. Colony diameters were measured on daily basis and readings value from 28 ºC were divided by readings value at 20 ºC. This calculation accounts for the difference of colony expansion rate between the WT and the mutants and measures only the change in expansion ratio.

5.5.6 Pathogenicity assay

Pathogenicity assay was conducted as per 3.5.3

CHAPTER SIX

GENERAL DISCUSSION

S. sclerotiorum has been of interest to phytopathologists and mycologists for over a hundred years. The importance of the fungus comes from its ability to infect a wide range of plant species and cause financial loss in the hundreds of millions of dollars annually. In addition to the dollar amount, the pathogen causes inefficiencies in human food production that are becoming less tolerable with increasing food demand. *S. sclerotiorum* employs several proteins and other molecules to kill the host. Such advanced and complex infection mechanisms were unexplored until the last thirty years with the increasing capabilities of molecular analysis tools. Most studies in this area focused on the mechanisms of infection during penetration of a specific plant barrier e.g. the cell wall. Such studies provided the foundation of this thesis which used a comprehensive approach to examine the co-regulation and function of multiple pathogenicity proteins concurrently. It examined cuticle and cell wall hydrolysis and the induction of cell death. It also clarified the complex signalling mechanisms that are involved in the governance of differentiation and infection.

This study aimed to identify *B. napus* PGIPs that are able to suppress the activity of the early released *S. sclerotiorum* PGs. The production and purification of an individual *S. sclerotiorum* PG or *B. napus* PGIP can be useful in studying the kinetics of enzyme-inhibitor *in vitro* interaction. It is implicit that this may not be an accurate reflection of *in vivo* interaction. Other research groups experienced difficulty in the production of PGs and PGIPs in current expression systems. Therefore, transient expression becomes a higher throughput approach that can be more effective in the selection of target proteins. Also, in the *in vivo* assay, PG-PGIP interaction occurs *in planta* which are comparable conditions to the natural environment. The above considerations can be utilized to target pathogenicity proteins that are produced early during the infection. Inhibition of infection initiation should allow the host to ramp up defences and limit the development of the disease.

Both BnPGIP1 and BnPGIP2 were able to limit the necrotic effects of SsPG3 and SsPG6. Although BnPGIP2 limited *S. sclerotiorum* PG symptoms, *A. thaliana* over-expressing *BnPgip2* did not show any measurable improvement in *S. sclerotiorum* tolerance. This further suggested that *S. sclerotiorum* during infection overwhelms the host by releasing not only polygalacturonases, but a battery of other enzymes including pectin methyl esterases and pectin lyases. Array analysis of gene expression during infection may be utilized for comprehensive understanding of gene expression at different stages of the infection. This will allow identification of highly expressed proteins early during the infection which can be targeted for engineering resistant plants. Similar studies have been conducted in other microorganisms including *F. oxysporum* (Di Pietro et al., 2003)

S. sclerotiorum, through millions of years of evolution, developed a variety of hydrolytic enzymes. As a result, the host developed a range of inhibitor proteins to combat them. *A. thaliana* was found to have a pectin methylesterase inhibitor (Lionetti et al., 2007) and sugar beet possesses a pectin lyase inhibitor (Bugbee, 1993). The majority of research on inhibitors was focused on PGIPs since they were the first to be identified over 30 years ago. Since over expression of *BnPGIP1* and *BnPGIP2* was insufficient to increase resistance, a more comprehensive approach that includes pectin methylesterase inhibitors and pectin lyase inhibitors may be necessary. The inhibitor selection should not be limited to pectin degrading enzymes, but also can include cellulose hydrolytic enzymes. *Triticum aestivum* encodes for a xylanase inhibitor (Fierens et al., 2007; Elliott et al., 2003) and a xyloglucan endoglucanase inhibiting protein was found in potato (Jones et al., 2006). Such genes can be transferred to plants of interest and tested for improved disease tolerance. A comprehensive approach in combating hydrolytic enzymes may be necessary for successful engineering of disease tolerance.

Due to the complexity and diversity in the inhibitors of cell wall degrading enzymes, it is important to understand the recognition sites and mechanism. The availability of such information will allow the engineering of new inhibitors with multiple recognition sites that can be effective against a broad range of enzymes. It will also permit transforming crops with fewer key genes that can produce the desired effect at a lower cost to the plant.

This thesis was the first to reveal a cutinase expressed by *S. sclerotiorum* during infection. Other enzymes that were found to be encoded in *S. sclerotiorum* genome include cutinases, lipases and esterases that are also expected to play a role in cuticle penetration. *SsCutA* expression was induced within one hour of contact with the leaf surface. *SsCutA* was induced solely in response to contact with a solid surface, unlike *SsPG1* whose regulation was significantly more complex and dependent on the available carbon source and the medium pH. Mechanistically, the simple and rapid induction of *SsCutA* is necessary due to the roles of released cutin monomers in inducing other cutinases. In *F. graminearum*, constitutively active cutinase releases cutin monomers which activate the expression of inducible cutinases (Li et al, 2002). The simplicity in *SsCutA* expression has a further advantage in that it could also allow rapid production of SsCUTA to avoid inducing plant defences. If cutinase activity is detected too early, the host produces a wide range of defence molecules and prevents the pathogen from establishing the infection. *A. thaliana* transformed with the *B. cinerea* orthologue of *SsCutA*, *CUTE*, were resistant to *B. cinerea.* An inactivated mutant form was unable to generate the same response indicating that the activity is essential for the detection of the cutinase (Chassot et al., 2007). Further examination of the defence mechanism found that it was not due to hormone-induced resistance. The expression pattern of marker genes, *PR-1, PR-3, PR-4, PDF1.2*, did not change in the *CUTE* plants. Furthermore, mutation in any of the key genes from the hormonal pathways did not affect CUTE based resistance (Chassot et al., 2007).

Microarray and gene expression studies confirmed that CUTE based resistance was independent of the hormonal pathway and involved up-regulation of genes encoding lipid transfer protein (LTP), peroxidase and protein inhibitor gene families (Chassot et al., 2007). LTP proteins were found to be toxic against fungi (Garcia-Olmedo, 1998) and over-expression of pepper LTP in *A. thaliana* caused complete resistance to *B. cinerea* (Jung et al., 2005). Peroxidases are believed to reinforce cell wall structure through lignin-cross linking (McLusky et al., 1999; Tognolli et al., 2002) and protease inhibitors interfere with hydrolytic enzymes involved in penetration (Dunaevskii et al., 2005). Interestingly, expression of *CUTE* in plants constitutively activates the expression of *AtPgip2* and up regulates the expression of *AtPgip1* (Chassot, 2006). Expression of *CUTE* in plants over-

expressing antisense PGIP did not induce resistance to *B. cinerea.* These results suggest that PGIPs are key for resistance and work in combination with LTP, peroxidase and protease inhibitors (Chassot, 2006). Together these findings further support our earlier conclusion of the necessity to employ a comprehensive approach in developing disease resistance.

SsCutA is rapidly induced, but is also strictly controlled as the expression is repressed at 24 hpi. It is also differentially expressed in the infection zone as no transcript was detected in the center of the necrotic lesion while the gene was slightly expressed at the expanding margin where some cutin is found. These results suggested that although cutinase is induced in response to contact with a solid surface, molecules in the active infection zone may repress the expression of the gene after the cuticle has been penetrated. These findings are critical in developing crops with improved disease resistance as further research should be directed toward improving earlier detection of the pathogen and induction of natural plant defences.

Unlike the relatively simple mechanism that governs the expression of *SsCutA*, *SsPg1* was controlled by multiple parameters including carbon source and pH conditions. This study clarifies the roles of carbon starvation and GA in the regulation of *SsPg1* expression. A feedback regulation mechanism allows strict control of enzyme levels and may prevent the release of excessive amounts of GA which can shift the pH of the medium outside the optimal range. *SsPg1* was expressed exclusively in the leading margin and the absence of a readily available carbon source in the undamaged cell walls may signal the induction of *SsPg1* expression. SsPG1 may then hydrolyse the pectin to release GA which then inhibits *SsPg1* expression in the necrotic zone where host tissues are being macerated by other hydrolytic enzymes. Such feedback regulation of *SsPg1* also explains the difference in *SsPg1* transcript accumulation between the center of the lesion and the progressing margin.

After cuticle and cell wall penetration, the third critical stage in pathogenesis is the point when cell death is induced. As a necrotroph, *S. sclerotiorum* is expected to express cell death-inducing molecules. This study is the first to isolate and prove the activity of two NEPs from *S. sclerotiorum.* NEPs research has been focused on understanding the function of the protein in the host and this study was the first to explore how the expression of their

genes is regulated. *SsNep2* expression was coordinated with that of *SsCutA* and *SsPg1*, and transcripts were detected within 6 hpi. Similarly to *SsCutA*, *SsNep2* was induced in response to contact with a solid surface whether it was hydrophobic or hydrophilic in nature. Since only *SsNep 2* was expressed on *B. napus, S. sclerotiorum* NEP gene expression may be regulated selectively based on the host similarly to genes encoding PGs (ten Have et al., 2001).

To have a better understanding of the mechanisms that orchestrate the expression of *SsCutA*, *SsPg1* and *SsNep2*, signalling pathways including Ca^{+2}, cAMP, calcineurin and MAPKs were examined. Crosstalk between signalling cascades is the mechanism for information transfer and coordination of biological functions in fungi. This integration can cause agonist effects of two different inducers or antagonist effects between an inducer and suppressor. Lanthanum chloride, a calcium channel blocker, reduced the expression of *SsCutA*, *SsPg1* and *SsNep2* and reduced *S. sclerotiorum* virulence but not viability. Treatment with cyclosporin, a calcineurin inhibitor, also reduced expression of all three genes, but not the house-keeping gene actin. Similarly, phospholipase C inhibitors reduced expression of all three pathogencity genes. Interestingly, application of exogenous cAMP reduced virulence and expression of *SsNep2*, but not *SsPg1* or *SsCutA*. The common expression patterns suggest that these genes share a limited number of signalling pathways. cAMP may not have as dominant an effect on the regulation of pathogenicity genes as previously reported as it only influenced the expression of *SsNep2*. A model outlining the integration and coordination of signalling pathways involved in the expression of *S. sclerotiorum* pathogenicity factors is provided in Figure 6.1.

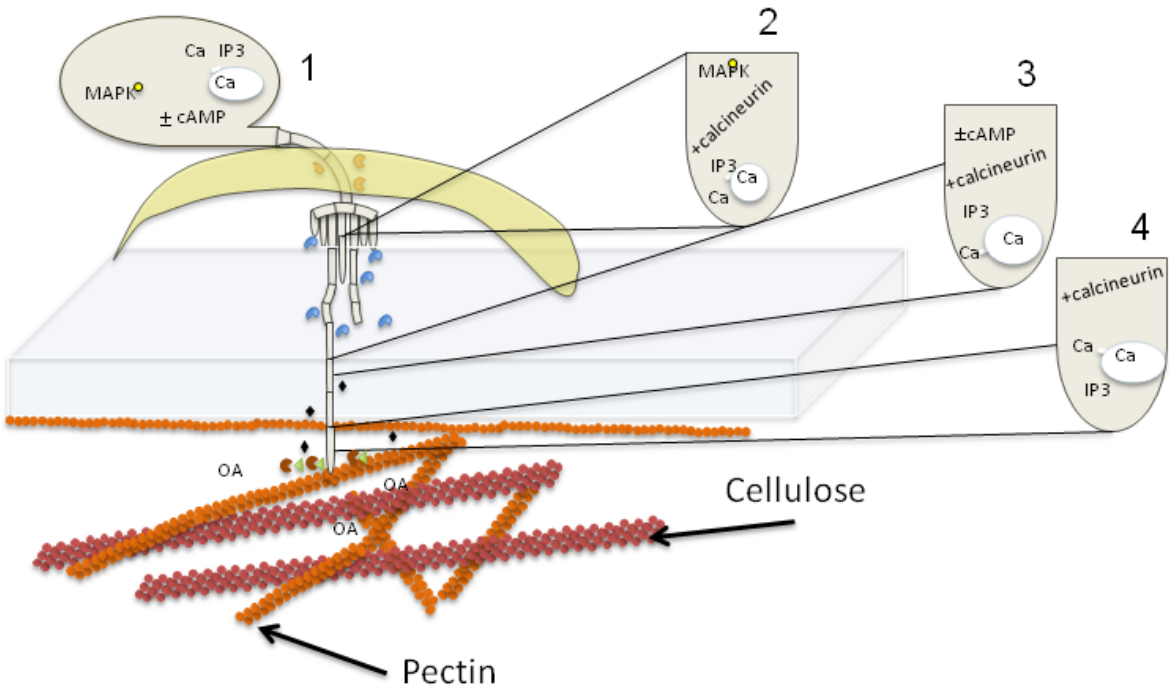


Figure 6.1 Model for expression regulation of pathogenicity factors during infection The model describes four of the major infection stages and the signalling pathways involved in activation of gene expression. 1) After ascospores land on a senescent petal, MAPK signaling pathways are activated in response to contact with a solid surface. Release of inositol triphosphate (IP3) from the membrane opens internal calcium channels. Calcium flux and alteration in cAMP levels occur prior to the release of hydrolytic enzymes. Degradation of the petal tissue provides the necessary nutrients for further development of the germ tube and infection cushion. 2) Upon contact with the cuticle surface MAPK pathways are activated. Calcineurin activity and calcium flux precede the expression of *S. sclerotiorum* cutinase which hydrolyses the cuticle allowing the mycelia to pass through it. 3) Upon reaching the epithelial cells beneath the cuticle, *SsNep2* expression is induced. Three signalling pathways including cAMP, calcineurin and calcium coordinate the expression of *SsNep2*. 4) When mycelia reach the pectin rich layer, calcineurin activity and calcium flux are required for the induction of *SsPg1*expression and synthesis of oxalic acid (OA). Plant induced defences include the production of PGIPs (green triangles) to prevent cell wall pectin hydrolysis by PGs (orange circles).

MAPKs expression profile was altered in *S. sclerotiorum* when it was in contact with a solid surface. MAPKs may be involved in transferring a signal relating to the nature of the contact surface and control gene expression. Studying the role of MAPKs in regulation of gene expression can be achieved using chemicals yet they are not specific enough to block a single kinase (Davies, 2000). Therefore, a genetic approach was used e.g. disruption mutation. A pleiotropic effect of the disruption mutation was expected causing the development of extensive branching, an increase in aerial mycelia and a decreased colony expansion rate.

Smk3 homologues have been tested in a range of phytopathogenic fungi but it is yet not clear what genes they regulate. The inducers of the cell wall integrity MAPK pathway are not fully understood yet and further research is required for identification of upstream proteins and downstream genes. One reason for the lack of clear understanding of the cell wall integrity pathway functions is the diversity of roles it plays in different organisms. Despite the highly conserved MAPKKK–MAPKK–MAPK model between different organisms the functions of each of the pathways differ significantly. Examples of different phenotypes due to mutations in *Smk3* homologues were outlined in chapter 5. In *S. sclerotiorum* only one other MAPKs have been studied, *Smk1* (Chen et al, 2004). Better understanding of the other MAPK pathways will help us understand their role in pathogenesis and other functions. It may also allow the identification of points of cross talk between different signalling pathways.

Furthermore, MAPKs can be targeted using RNAi. Targeting MAPKs in disease resistance is an evolving concept that emerged out of cancer research (Sebolt-Leopold, 2000). In humans, research in this area is yet at the lab scale due to the complexity of the targeted proteins functions. The technology was initially used against viruses but is currently being developed against eukaryotic organisms (Jun-Hai et al., 2010). Such solutions are being developed in phytopathogenic fungi. This strategy, although proving to be effective, is not without its challenges. The process is inefficient and the frequency of a single insert in which the cassette has only replaced the target locus with no integration in other sites is low (Temple et al., 2009). Nonetheless, it is more difficult to develop against necrotrophs as they hydrolyse plant components externally.

The studies in this thesis contributed to an understanding of *S. sclerotiorum* pathogenicity and it was concluded that an integrated approach must be taken to developing disease tolerance. Previously, studying a gene or family of genes independently has generated only partial answers. High throughput technology for gene expression profiling must be utilized to identify the proteins that are most important at the three major stages in infection: cuticle penetration, tissue degredation and cell death. Broad range inhibitors of hydrolytic enzymes must be identified and possibly engineered through better understanding of enzyme-inhibitor interaction.

REFERENCES

Adams, P. B. and Ayers, W. A. (1979) Ecology of *Sclerotinia* species. *Phytopathology*. **69,** 896–899.

Adams, P. B. (1975) Factors affecting survival of *Sclerotinia sclerotiorum* in soil. *Plant Dis Rep*. **59,** 599–603.

Abawi, G. S. and Grogan, R. G. (1979) Epidemiology of diseases caused by *Sclerotinia* species. *Phytopathology*. **69,** 899–904.

Abawi, G. S. and Grogan, R. G. (1975) Source of primary inoculum and effects of temperature and moisture on infection of beans by *Whetzelinia sclerotiorum. Phytopathology*. **65,** 300–309.

Abawi, G. S., Polach, F. J. and Molin, W. T. (1975) Infection of bean by ascospores of *Whetzelinia sclerotiorum. Phytopathology*. **65,** 673–678.

Ackman, R.G. (1990) Canola fatty acids: an ideal mixture for health, nutrition, and food use. In: F. Shahidi (Ed.) Canola and rapeseed, production, chemistry, nutrition and processing technology. Van Nostrand Reinhold, New York, USA. pp. 81-98.

Adrian, C. and Martin, S.J. (2001). The mitochondrial apoptosome: a killer unleashed by the cytochrome seas. *Trends Biochem Sci.* **26,** 390–397.

Aggarwal, R. A. K., Kumar, A. and Thakur, H. L. (1997) Effect of *Sclerotinia* rot on oil quality in low erucic acid cultivars of rapeseed. *Cruciferae Newslett*. **19,** 103–104.

Aguero, CB., Uratsu, SL., Greve, C., Powell, A., Labavitch, JM., Meredith, CP. and Dandejar, AM. (2005) Evaluation of tolerance to Pierce's disease and *Botrytis* in transgenic plants of *Vitus vinifera* L. expressing the pear PGIP gene. *Mol Plant Pathol.* **6,** 43–51.

Ainsworth, C. (1981) Introduction to the History of Plant Pathology. Cambridge University Press, Cambridge, UK.

Albersheim, P. and Anderson, A. (1971) Proteins from plant cell walls inhibit polygalacturonases secreted by plant pathogens. *PNAS.* **68,** 1815–1819.

Alvarez, M.E., Pennell, R.I., Meijer, P.J., Ishikawa, A., Dixon, R.A. and Lamb, C. (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell.* **92,** 773-784.

Amsellem, Z., Cohen, B.A. and Gressel, J. (2002) Engineering hypovirulence in a mycoherbicidal fungus for efficient weed control. *Nature Biotech.* **20,** 1035–1039.

Annis, S. and Goodwin. P. (1997) Recent advances in the molecular genetics of plant cell wall-degrading enzymes produced by plant pathogenic fungi. *Eur J Plant Pathol.* **103,** 1–14.

Apostol, I., Heinstein, P.F. and Low, P.S. (1989) Rapid stimulation of an oxidative burst during elicitation of cultured plant cells : role in defense and signal transduction. *Plant Physiol*. **90,** 109-116.

Bae, H., Moon, S.K., Richard, C.S., Bae, H-J. and Bailey, B. (2006) Necrosis and ethylene inducing peptide from *Fusarium oxysporum* induces a complex cascade of transcripts associated with signal transduction and cell death in *Arabidopsis. Plant Physiol.* **141,** 1056-1067.

Bailey, B.A. (1995) Purification of a protein from culture filtrate of *Fusarium oxysporum* that induces ethylene and necrosis in leaves of *Erythroxylum coca*. *Phytopathology*. **85,** 1250-1255.

Bailey, K. L. (2003) Plant diseases and the Canadian economy. *Can Phytopathol Soc. Ottawa, Canada*. 290.

Baker, E.A. (1982) Chemistry and morphology of plant epicuticular waxes. In: Cutler, DF, Alvin, KL and Price, CE, The Plant Cuticle. *Academic Press*. **15,** 139-165.

Baker, C. J. and Bateman, D. F. (1978) Cutin degradation by plant pathogenic fungi. *Phytopathology*. **69,** 1577-1584.

Bardin, S.D. and Huang, H.C. (2001) Research on biology and control of *Sclerotinia* diseases in Canada. *Can J Plant Pathol*. **23,** 88–98.

Barkai-Golan, R. (1974) Production of cellulose and poly-galacturonase by *Sclerotinia minor.Mycopathol Mycol Appl*. **54,** 297–302.

Bardwell, L. (2004) A walk-through of the yeast mating pheromone response pathway. *Peptides*. **25,** 1465-1476.

Bateman, D. F. and Beer, S. V. (1965) Simultaneous production and synergistic action of oxalic acid and polygalacturonase during pathogenesis by *Sclerotinia rolfsii. Phytopathol.* **55,** 204–211.

Bauer, W. D., Bateman, D. F. and Whalen, C. H. (1977) Purification of an endo-β-l, 4 galactanase produced by *Sclerotinia sclerotiorum:* effects on isolated plant cell-walls and potato tissue. *Phytopathology.* **67,** 862–868.

Belbahri, L., Calmin, G., Mauch, F. and Andersson, J. (2008) Evolution of the cutinase gene family: Evidence for lateral gene transfer of a candidate Phytophthora virulence factor. *Gene.* **408,** 1–8.

Berger, D., Oelofse, D., Arendse, M., Du Plessis, E. and Dubery, I. (2000) Bean polygalacturonase inhibitor protein-1 (PGIP-1) inhibits polygalacturonases from *Stenocarpella maydis*. *Physiol Mol Plant Pathol.* **57,** 5–14.

Berridge, M.J. (1997) Elementary and global aspects of calcium signaling. *J Physiol*. **499,** 291-306.

Bessire, M., Chassot, C., Jacquat, A.C., Humphry, M., Borel, S., Petetot, J.M., Metraux, J.P. and Nawrath, C. (2007) A permeable cuticle in Arabidopsis leads to a strong resistance to *Botrytis cinerea*. *EMBO J.* **26,** 2158-2168.

Boland, G.J. and Hall, R. (1994) Index of plant hosts of *Sclerotinia sclerotiorum*. *Can J Plant Pathol*. **16,** 93–108.

Boller, T. and Felix, G. A (2009) Renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu Rev Plant Biol.* **60**, 379–406.

Bugbee, W. (1993) Pectin lyase inhibitor protein from cell walls of sugar beet. *Phytopathology*. **83,** 63-68.

Burnett, H. (1968) Fundamentals in Mycology. Arnold: London, UK.

Bussink, H. and Osmani, S. (1999) A mitogen-activated protein kinase (MPKA) is involved in polarized growth in the filamentous fungus *Aspergillus nidulans. FEMS Microbiol Lett*. **173,** 117-125.

Calonge, F. D., Fielding, A. H., Byrde, R. J. W. and Akinrefon, D. A. (1969) Changes in ultrastructure following fungal invasion and the possible relevance of extra cellular enzymes *J Exp Bot*. **20,** 350–357.

Carpita, C. and Gibeaut, D. (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *The Plant J*. **3,** 1-30.

Cereghino, J. L. and Cregg, J. M. (2000) Heterologous protein expression in the methylotrophic yeast *Pichia pastoris. FEMS Microbiol Rev*. **24,** 45–66.

Cervone, F., De Lorenzo, G., Pressey, R., Darvill, A. and Albersheim, P. (1990) Can *Phaseolus* PGIP inhibit pectic enzymes from microbes and plants?. *Phytochemistry*. **29,** 447–449.

Cessna, S. G., Sears, V. E., Dickman, M. B. and Low, P. S. (2000) Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum* suppresses the oxidative burst of the host plant. *Plant Cell.* **12,** 2191–2199.

Chaky, J., Anderson, K., Moss, M. and Vaillancourt, L. (2001) Surface hydrophobicity and surface rigidity induce spore germination in *Colletotrichum graminicola*. *Phytopathology*. **91,** 558–564.

Chassot, C., Nawrath, C. and Métraux, J.P. (2008) The cuticle: Not only a barrier for plant defence A novel defence syndrome in plants with cuticular defects. *Plant Signal Behav.* 3, 142–144.

Chassot, C. (2006) Cuticular defects or wounding lead to full immunity to a major plant pathogen. PhD dissertation, University of Fribourg, Switzerland.

Chapman, S., Kavanagh, T.A. and Baulcombe, D.C. (1992) Potato virus X as a vector for gene expression in plants. *Plant J.* **2,** 549-557.

Chau, H., Si, B., Goh, Y. and Vujanovic, V. (2009) A novel method for identifying hydrophobicity on fungal surfaces. *Mycol Res.* **113,** 1046-1052.

Chiosi, G. and Magro, P (2010) Induced resistance by β-aminobutyric acid in artichoke against white mould caused by *Sclerotinia sclerotiorum. J Phytopathol.* **158,** 659-667.

Chen, C., Harel, A., Gorovoits, R., Yarden, O. and Dickman, M.B. (2004) MAPK regulation of sclerotial development in *Sclerotinia sclerotiorum* is linked with pH and cAMP sensing. *MPMI*. **17,** 404-413.

Chen, C. and Dickman, M.B. (2005) cAMP blocks MAPK activation and sclerotial development via Rap-1 in a PKA-independent manner in *Sclerotinia sclerotiorum*. *Mol Microbiol.* **55,** 299-311.

Chivasa, S., Ndimba, B.K., Simon, W.J., Lindsey, K. and Slabas, A.R. (2005). Extracellular ATP functions as an endogenous external metabolite regulating plant cell viability. *Plant Cell.* **17,** 3019–3034.

Clarkson, J. P., Phelps, K., Whipps, J. M., Young, C. S., Smith, J. A. and Watling, M. (2004) Forecasting *Sclerotinia* disease on lettuce: toward developing a prediction model for carpogenic germination of sclerotia. *Phytopathology*. **94,** 268–279.

Clarkson, J. P., Staveley, J., Phelps, K., Young, C. S. and Whipps, J. M. (2003) Ascospore release and survival in *Sclerotinia sclerotiorum. Mycol Res*. **107,** 213–222.

Cohen, Y., Peer, S., Balass, O. and Coffey, M. (1987) A fluorescent technique for studying growth of *Peronospora tabacina* on leaf surfaces. *Phytopathology*. **77,** 201- 204.

Coleman, M., Henricot, B., Jose, A. and Oliver, R. (1997) Starvation-induced genes of the tomato pathogen *Cladosporium fulvum* are also induced during growth *in planta*. *MPMI*. **10,** 1106-1109.

Coley-Smith, J. R. and Cooke, R. C. (1971) Survival and germination of fungal sclerotia. *Annu Rev Phytopathol*. **9**, 65–92.

Colmenares, A. J., Aleu, J., Durán-Patrón, R., Collado, I. G. and Hernández-Galán, R. (2002) The putative role of botrydial and related metabolites in the infection mechanism of *Botrytis cinerea. J Chem Ecol.* **28,** 997–1005.

Colotelo, N. (1974) A scanning electron microscope study of developing sclerotia of *Sclerotinia sclerotiorum. Can J Bot.* **52,** 1127–1130.

Commenil, P., Belingheri, L. and Dehorter, B. (1998) Antilipase antibodies prevent infection of tomato leaves by *Botrytis cinerea*. *Physiol Mol Plant Pathol*. **52,** 1-14.

Cook, G. E., Steadman, J. R. and Boosalis, M. G. (1975) Survival of *Whetzelinia sclerotiorum* and initial infection of dry edible beans in Western Nebraska. *Phytopathology.* **65,** 250–255.

Cotton, P., Rascale, C. and Fevre, M. (2002) Characterization of PG2, an early endoPG produced by *Sclerotinia sclerotiorum*, expressed in yeast. *Microbiol Lett.* **213,** 239-244.

Cotton, P., Fraissinet-Tachet, L. and Fevre, M. (1996) Expression of the *Sclerotinia sclerotiorum* polygalacturonase *pg1* gene: Possible involvement of CREA in glucose catabolite repression. *Curr Genet*. **30,** 240-245.

Cotton, P., Kasza, Z., Bruel, C., Rascle, C. and Fevre, M. (2003) Ambient pH controls the expression of endopolygalacturonase genes in the necrotrophic fungus *Sclerotinia sclerotiorum*. *Microbiol Lett.* **227,** 163-169.

D'Souza, C.A. and Heitman, J. (2001) Conserved cAMP signaling cascades regulate fungal development and virulence. *FEMS Microbiol Rev*. **25,** 349–364.

Dallal Bashi, Z., Hegedus, D., Buchwaldt, L., Rimmer, R. and Borhan, M. (2010) Expression and regulation of Sclerotinia sclerotiorum necrosis and ethylene-inducing peptides (NEPs). *Mol Plant Pathol.* **11,** 43-53.

Davenport, K., Williams, K., Ullmann, B. and Gustin, M. (1999) Activation of the *Saccharomyces cerevisiae* filamentation/invasion pathway by osmotic stress in high-osmolarity glycogen pathway mutants. *Genetics*. **153,** 1091-1103.

Dann, E.K. and Deverall, B.J. (1995) Effectiveness of systemic resistance in bean against foliar and soil-borne pathogens as induced by biological and chemical means. *Plant Pathol*. **44,** 458-466.

Darvill, A., McNeil, M. and Albersheim, P. (1978) Structure of plant cell walls. A new pectic polysaccharide. *Plant Physiol.* **62,** 418-422.

Davies, S., Reddy, H., Caivano, M. and Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J*. **351,** 95–105.

Delledonne, M., Xia, Y., Dixon, R.A. and Lamb, C. (1998) Nitric oxide functions as a signal in plant resistance. *Nature*. **394,** 585–588.

De Lorenzo, G., D'Ovidio1, R. and Cervone F. (2001) The role of polygalacturonase inhibiting proteins (PGIPS) in defense against pathogenic fungi. *Annu Rev Phytopathol*. **39,** 313–335.

De Nobel, H. (2000) Cell wall maintenance in fungi. *Trends Microbiol.* **8,** 344-345.

Desiderio, A., Aracri, B., Leckie, F., Mattei, B. and Salvi, G. (1997) Polygalacturonase-inhibiting proteins (PGIPs) with different specificities are expressed in *Phaseolus vulgaris. MPMI.* **10,** 852–860.

Dickman, M., Podila, G. and Kolattukudy, P. (1989) Insertion of cutinase gene into a wound pathogen enables it to infect intact host. *Nature.* **342,** 446-448.

Dickman, M.B., Park, Y.K., Oltersdorf, T., Li, W., Clemente, T. and French, R. (2001) Abrogation of disease development in plants expressing animal antiapoptotic genes. *PNAS.* **98,** 6957-6962.

Di Pietro, A., Garcia-MacEira, I., Meglecz, E. and Roncero, I. (2001). A MAP kinase of the vascular wilt fungus *Fusarium oxysporum* is essential for root penetration and pathogenesis. *Mol Microbiol.* **39,** 1140-1152.

Dowd, C., Buckley, C. and Sheehan, D. (1997) Glutathione S-transferases from the white-rot fungus, *Phanerochaete chrysosporium. Biochem J.* **324,** 243-248.

Dodds, P. N., Lawrence, G. J., Catanzariti, A. M., Ayliffe, M. A. and Ellis, J. G. (2004) The *Melampsora lini* AvrL567 avirulence genes are expressed in haustoria and their products are recognized inside plant cells. *Plant Cell.* **16**, 755–768.

Doehlemann, G., Berndt, P. and Hahn, M. (2006) Different signalling pathways involving a G- protein, cAMP and a MAP kinase control germination of *Botrytis cinerea* conidia. *Mol Microbiol*. **59,** 821-835.

Donaldson, P. A., Anderson, T., Lane, B. G., Davidson, A. L. and Simmonds, D. H. (2001) Soybean plants expressing an active oligomeric oxalate oxidase from the wheat gf-2-8 (germin) gene are resistant to the oxalate secreting pathogen *Sclerotinia sclerotiorum. Physiol Mol Plant Pathol*. **59,** 297–307.

Dong, X., Ji, R., Guo, X., Foster, S., Chen, H., Dong, C., Liu, Y., Hu, Q. and Liu, S (2008) Expressing a gene encoding wheat oxalate oxidase enhances resistance to *Sclerotinia sclerotiorum* in oilseed rape (*Brassica napus*). *Planta.* **228,** 331-340.

Dunaevskii, Y., Tsybina, T., Belyakova, G., Domash, V., Sharpio, T., Zabreiko, S. and Belozerskii, M. (2005) Proteinase inhibitors as antistress proteins in higher plants. *Appl Biochem Microbiol*. **41,** 344-348.

Durrant, W.E., and Dong, X. (2004) Systemic acquired resistance. *Annu Rev Phytopathol.* **42,** 185-209.

Dutton, M. V. and Evans, C. S. (1996) Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. *Can J Microbiol*. **42,** 881–895.

Elliott, G., McLauchlan, W., Williamson, G. and Kroon, P. (2003) A wheat xylanase inhibitor protein (XIP-I) accumulates in the grain and has homologues in other cereals. *J Cereal Sci.* **37,** 187-194.

Emri, T., Pocsi, I. and Szentirmai, A. (1997) Glutathione metabolism and protection against oxidative stress caused by peroxides in *Penicillium chrysogenum*. *Free Radical Biol Med*. **23,** 809–814.

Eshdat, Y., Doron, H., Zehava, F. and Gozal, B.H. (1997) Plant glutathione peroxidases. *Physiol Plant*. **100,** 234–240.

Favaron, F., Sella, L. and D'Ovidio, R. (2004) Relationships among endopolygalacturonase, oxalate, pH and plant polygalacturonase inhibiting protein (PGIP) in the interaction between *Sclerotinia sclerotiorum* and soybean. *MPMI*. **17,** 1402–1409.

Fellbrich, G., Romanski, A., Varet, A., Blume, B., Brunner, F., Enelhardt, S., Felix, G., Kemmerling, B., Krzymowska, M. and Nurnberger, T. (2002) NPP1, a *phytophthora-associated* trigger of plant defense in parsley and *Arabidopsis*. *Plant J*. **32,** 375-390.

Ferrari, S., Vairo, D., Ausubel, F. M., Cervone, F. and De Lorenzo, G. (2003) Tandemly duplicated Arabidopsis genes that encode polygalacturonaseinhibiting proteins

are regulated coordinately by different signal transduction pathways in response to fungal infection. *Plant Cell.* **15,** 93-106.

Fierens, E., Rombouts, S., Gebruers, K., Goesaert, H., Brijs, S., Beaugrand, J., Volckaert, G., Van, Campenhout, S., Proost, P., Courtin, S. and Delcour, J. (2007) TLXI, a novel type of xylanase inhibitor from wheat (*Triticum aestivum*) belonging to the thaumatin family. *Biochem J.* **403,** 583–591.

Fish, W. W. and Madihally, SV. (2004) Modeling the inhibitor activity and relative binding affinities in enzyme-inhibitor-protein systems: application to developmental regulation in a PG-PGIP system. *Biotechnol Prog.* **20,** 721-727.

Fox, D.S. and Heitman, J. (2002) Good fungi gone bad: The corruption of calcineurin. *BioEssays.* **24,** 894-903.

Fraissinet-Tachet, L., Reymond-Cotton, R. and Fevre, M. (1995) Characterization of a multigene family encoding an endopolygalacturonase in *Sclerotinia sclerotiorum*. *Curr Genet.* **29,** 96-100.

Fraissinet-Tachet, L. and Fevre, M. (1996) Regulation by galacturonic acid of pectinolytic enzyme production by *Sclerotinia sclerotiorum*. *Curr Microbiol*. **33,** 49-53.

Francis, S., Dewey, F.M. and Gurr. S.J. (1996) The role of cutinase in germling development and infection by *Erysiphe graminis* f. sp. *hordei*. *Physiol Mol Plant Pathol.* **49,** 201-211.

Fridovich, I. (1997) Superoxide anion radical [O2], superoxide dismutases, and related matters. *J Biol Chem*. **272,** 18515–18517.

Friedrich, L., Lawton, K., Ruess, W., Masner, P., Specker, N., Gut-Rella, M., Meier, B., Dincher, S., Staub, T. and Uknes, S., Métraux, J.P., Kessmann, H. and Ryals, J. (1996) A benzothiadiazole derivative induces systemic acquired resistance in tobacco. *Plant J.* **10,** 61-70.

Fujioka, T., Mizutani, O., Furukawa, K., Sato, N., Yoshimi, A.,Yamagata, Y., Nakajima, T. and Abe, K. (2007) MpkA-dependent and -independent cell wall integrity signaling in *Aspergillus nidulans*. *Eukaryot Cell.* **6,** 1479-1510.

Garcia-Olmedo, F., Molina, A., Alamillo, J. and Rodriguez-Palenzuela, **P.** (1998) Plant defence peptides. *Biopolymers*. **47,** 479-491.

Garcia, O., Macedo, J., Tiburcio, R., Zaparoli, G., Rincones, J., Bittencourt, L., Ceita, G., Micheli, F., Gesteira, A., Mariano, A., Schiavinato, M., Medrano, F., Meinhardt, L., Pereira, G. and Cascardo, J. (2007) Characterization of necrosis and ethylene-

inducing proteins (NEP) in the basidiomycete *Moniliophthora perniciosa*, the causal agent of witches' broom in *Theobroma cacao*. *Mycol Res*. **3,** 443-455.

Gijzen, M. and Nürnberger, T. (2006) Nep1-like proteins from plant pathogens: Recruitment and diversification of the NPP1 domain across taxa. *Phytochemistry*. **67,** 1800–1807.

Gil-ad, N.L. and Mayer, A.M. (1999) Evidence for rapid breakdown of hydrogen peroxide by *Botrytis cinerea*. *FEMS Microbiol Lett*. **176,** 455–461.

Glazener, J. (1982) Accumulation of phenolic compounds in cells and formation of lignin-like polymers in cell walls of young tomato fruits after inoculation with *Botrytis cinerea. Physiol Mol Plant Pathol*. **20,** 11–25.

Godoy, G., Steadman, J., Dickman, M., and Dam, R. (1990) Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum on Phaseolus vulgaris. Physiol Mol Plant Pathol.* **37,** 179-191.

Gotesson, A., Marshall, J.S., Jones, D.A. and Hardham, A.R. (2002). Characterization and evolutionary analysis of a large polygalacturonase gene family in the oomycete plant pathogen *Phytophthora cinnamomi*. *MPMI.* 15, 907–921.

Goujon, M. (1970) Mecanismes physiologiques de la formation des sclerotes chez Ie *Corticium rofsii* (Sacc.) curzi. *Physiol Veg.* **8,** 349-360.

Govrin, E.M. and Levine, A. (2000) The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Curr Biol.* **10,** 751–757.

Green, D.R. and Reed, J.C. (1998). Mitochondria and apoptosis. *Sci.* 281, 1309–1312.

Grison, R., Grezes-Besset, B., Schneider, M., Lucante, N., Olsen, L., Leguay, J. J. and Toppan, A. (1996) Field tolerance to fungal pathogens of *Brassica napus* constitutively expressing a cimeric chitinase gene. *Nature Biotech*. **14,** 643–646.

Grogan, R. G. and Abawi, G. S. (1975) Influence of water potential on growth and survival of *Whetzelinia sclerotiorum. Phytopathology.* **65,** 122–138.

Grogan, R. G. (1979) *Sclerotinia* species: Summary and comments on needed research. *Phytopathol.* **69,** 908–910.

Guo, W., Gonzalez-Candelas, L. and Kolattukudy, P.E. (1996) Identification of a novel *pelD* gene expressed uniquely *in planta* by *Fusarium solani* f. sp. *pisi* (*Nectria haematococca*, mating type VI) and characterization of its protein product as an endo-pectate lyase. *Arch Biochem Biophys.* **332,** 305-312.

Gustin, M., Albertyn, J., Alexander, M. and Davenport, K. (1998) MAP kinase pathways in the yeast *Saccharomyces cerevisiae. Microbiol Mol Biol Rev*. **62,** 1264-1300.

Halliwell, B. and Gutteridge, J.M.C. (1998) Free Radicals in Biology and Medicine. Clarendon, Oxford.

Hancock, J. G. (1966) Degradation of pectic substances associated with pathogenesis by *Sclerotinia sclerotiorum* in sunflower and tomato stems. *Phytopathology.* **56,** 975–979.

Hancock, J. G. (1967) Hemicellulose degradation in sunflower hypocotyls infected with *Sclerotinia sclerotiorum. Phytopathology.* **57,** 203–206.

Hancock, J. G. (1972) Changes in cell membrane permeability in sunflower hypocotyls infected with *Sclerotinia sclerotiorum. Plant Physiol*. **49,** 358–364.

Hao, J., Subbarao, K. and Duniway (2003) germination of *Sclerotinia minor* and *S. sclerotiorum* sclerotia under various soil moisture and temperature combination. *Epidemiol.* **94,** 443-450.

Harel, A., Gorovits, R. and Yarden, O. (2005) Changes in protein kinase A activity accompany sclerotial development in *Sclerotinia sclerotiorum. Phytopathology.* **95,** 397–404.

Harel, A., Bercovich, S. and Yarden, O. (2006) Calcineurin is required for sclerotial development and pathogenicity of *Sclerotinia sclerotiorum* in an oxalic acid independent manner. *MPMI.* **19,** 682-693.

Harholt, J., Suttangkakul, A. and Scheller, A. (2010) Biosynthesis of pectin. *Plant Physiol.* **153,** 384-395.

Hegedus. D., Li, R., Buchwaldt, L., Parkin, I., Whitwill, S., Coutu, C., Bekkaoui, D. and Rimmer, R. (2008) *Brassica napus* possesses an expanded set of polygalacturonase inhibitor protein genes that are differentially regulated in response to *Sclerotinia sclerotiorum* infection, wounding and defense hormone treatment. *Planta.* **228,** 241–253.

Held, Y. M. (1955) Physiological differences between a normal and a degenerate strain of *Sclerotinia trifoliorum. Phytopathology.* **45,** 39–42.

Henson, J. M., Butler, M. J. and Day, A. W. (1999) The dark side of mycelium; melanins of phytopathogenic fungi. *Annu Rev Phytopathol*. **37,** 447–471.

Hilal, A., Nada, M. and Zaky, W. (2006) Induced resistance against *Sclerotinia sclerotiorum* disease in some umbelliferous medicinal plants as a possible and effective control mean. *Egypt J Phytopathol.* **34,** 85-101.

Hoes, J. A. and Huang, H. C. (1976) Control of *Sclerotinia* basal stem rot of sunflower: a progress report. In: *Proceedings of the Sunflower Forum*. Fargo, ND. p. 18-19.

Hou, Z., Xue, C., Peng, Y., Katan, T., Kistler, H,C. and Jin-Rong, X. (2002) A Mitogen-Activated Protein Kinase gene (MGV1) in *Fusarium graminearum* is required for female fertility, heterokaryon formation, and plant infection. *MPMI*. **15,** 1119-1127.

Hu, X., Bidney, D. L., Yalpani, N., Duvick, J. P., Crasta, O., Folkerts, O. and Lu, G. (2003) Over expression of a gene encoding hydrogen-peroxide generating oxalate oxidase evokes defense responses in sunflower. *Plant Physiol*. **133,** 170–181.

Huang, H. C. and Kozub, G. C. (1989). A simple method for production of apothecia from sclerotia of *Sclerotinia sclerotiorum. Plant Prot Bull*. **31,** 333–345.

Huang, J., Bhinu, V., Li, X., Dallal Bashi, Z., Zhou, R. and Hannoufa, A. (2009) Pleiotropic changes in Arabidopsis f5h and sct mutants revealed by large-scale gene expression and metabolite analysis. *Planta.* **230,** 1057-1069.

Huang, R. C. and Janzen, H. H. (1991) Control of carpogenic germination of sclerotia of *Sclerotinia sclerotiorum* by volatile substance from urea. *Plant Prot.* **33,** 283–289.

Huang, Yong Ju, Chen, J. and Li, Y. C. (2000) Genetic study of *Sclerotinia sclerotiorum* resistance in rapeseed (*Brassica napus* L.) I. Its inheritance and combining ability. *Chin J Oil Crop Sci*. **22,** 1–5.

Imolehin, E. D. and Grogan, R. G. (1980) Factors affecting survival of sclerotia and effect of inoculum density, relative position and distance of sclerotia from the host on infection of lettuce by *Sclerotinia minor. Phytopathology.* **70,** 1162–1167.

Jamalainen, E. A. (1954) Over-wintering of cultivated plants under snow. *F.A.O. Plant Prot Bull* **2,** 12-13.

James, J. T. and Dubery, I. A. (2001) Inhibition of polygalacturonase from *Verticillium dahliae* by a polygalacturonase inhibiting protein from cotton. *Phytochemistry.* **57,** 149–156.

Jamaux, I., Gelie, B. and Lamarque, C. Early stages of infection of rapeseed petals and leaves by *Sclerotinia sclerotiorum* revealed by scanning electron microscopy. *Plant Pathol.* **44,** 22-30.

Jarvis, M. (1984) Structure and properties of pectin gels in plant cell walls. *Plant Cell Environ.* **7,** 153-164.

Jennings, J.C., Apel-Birkhold, P.C., Mock, N.M., Baker, C.J., Anderson, J.D. and Bailey, B.A. (2001) Induction of defense responses in tobacco by the protein Nep1 from *Fusarium oxysporum. Plant Sci*. **161,** 891–899.

Jetter, R., Schaffer, S. and Riederer, M. (2000) Leaf cuticular waxes are arranged in chemically and mechanically distinct layers: evidence from *Prunus laurocerasus* L. *Plant Cell Environ.* **23,** 619-628.

Jin-rong, X., Staiger, C. and Hamer, J. (1998) Inactivation of the mitogen-activated protein kinase Mps1 from the rice blast fungus prevents penetration of host cells but allows activation of plant defense responses. *PNAS.* **95,** 12713-12718.

Jones, M., Ospina-Giraldo, M. and Deahl, K. (2006) Gene Silencing Indicates a Role for Potato Endoglucanase Inhibitor Protein in Germplasm Resistance to Late Blight. *Amer J Potato Res*. **83,** 41-46.

Jores, J., Appel, B. and Lewin, A. (2003) Cloning and molecular characterization of a unique hemolysin gene of *Vibrio pommerensis* sp. nov.: Development of a DNA probe for the detection of the hemolysin gene and its use in identification of related *Vibrio* spp. from the Baltic Sea. *FEMS Microbiol Lett*. **229,** 223–229.

Joubert, D., Kars, I., Wagemakers, L., Bergmann, C., Kemp, G., Vivier, A. and van Kan, J. (2006) A polygalacturonase-inhibiting protein from grapevine reduces the symptoms of the endopolygalacturonse BcPg2 from *Botrytis Cinerea* in *Nicotiana Benthamiana* leaves without any evidene for in vitro interaction. *MPMI*. **20,** 392-402.

Jurick, W. and Rollins, J. (2007) Deletion of the adenylate cyclase (*sac1*) gene affects multiple developmental pathways and pathogenicity in *Sclerotinia sclerotiorum. Fung Genet Biol*. **44,** 521-530.

Jung, H., Kim, K. and Hwang, B. (2005) Identification of pathogen-responsive regions in thepromoter of a pepper lipid transfer protein gene (*CALTPI*) and the enhanced resistance of the CALTPI transgenic *Arabidopsis* against pathogen and environmental stresses. *Planta*. **221,** 361-73.

Jun-Hai, N, Heng, J., Jian-Mei, X., Yang-Dong, G. and Qian, L. (2010) RNAi technology extends its reach: Engineering plant resistance against harmful eukaryotes. *African J Biotech*. **9,** 7573-7582.

Kars, I., Krooshof, G., Wagemakers, L., Joosten, R., Benen, J. and van Kan J. (2005) Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris. Plant J*. **43,** 213-25.

Kasza, Z., Vagvolgyi, C., Fevre, M., and Cotton, P. (2004) Molecular characterization and in planta detection of *Sclerotinia sclerotiorum* endopolygalacturonase genes. *Curr Microbiol*. **48,** 208-213.

Kapoor, K. S. (1994) Stipe regeneration potential of sclerotia of *Sclerotinia sclerotiorum* (Lib.) de Bary. *Plant Dis Res*. **9,** 98–100.

Keates, S.E., Kostman, T.A., Anderson, J.D. and Bailey, B.A. (2003) Altered gene expression in three plant species in response to treatment with Nep1, a fungal protein that causes necrosis. *Plant Physiol*. **132,** 1610–1622.

Kerr, J.F., Wyllie, A.H. and Currie, A.R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British J Cancer*. **26,** 239–257.

Kesarwani, M., Azam, M., Natarajan, K., Mehta, A. and Datta, A. (2000) Oxalate decarboxylase from *Collybia velutipes*. Molecular cloning and its over expression to confer resistance to fungal infection in transgenic tobacco and tomato. *J Biol Chem*. **275,** 7230–7238.

Kim, Y., Li, D. and Kolattukudy, P. (1998) Induction of Ca^{2+}-calmodulin signaling by hard-surface contact primes *Colletotrichum gloeosporioides* conidia to germinate and form appressoria. *J Bacteriol*. **180,** 5144–5150.

Knob, A. and Carmona, E. (2009) Cell-associated acid beta-xylosidase production by *Penicillium sclerotiorum*. *Nature Biotech.* **26,** 60-67.

Kobe, B. and Deisenhofer, J. (1993) Crystal structure of porcine ribonuclease inhibitor, a protein with leucine-rich repeats. *Nature.* **366,** 751–56.

Kohn, L. M. (1979) Delimitation of the economically important plant pathogenic *Sclerotinia* species. *Phytopathology.* **69,** 881–886.

Kolattukudy, P.E., Rogers, L.M., Li, D., Hwang, C.S. and Flaishman., M.A. (1995) Surface signaling in pathogenesis. *PNAS*. **92,** 4080 **-** 4087.

Kojima, K., Kikuchi, T., Takano, Y., Oshiro, E. and Okuno, T. (2002) The mitogen-activated protein kinase gene *maf1* is essential for the early differentiation phase of appressorium formation in *Colletotrichum lagenarium. MPMI.***15,** 1268-1276.

Kosasih, B.D. and Willetts, H.J. (1975) Types of abnormal apothecia produced by *Sclerotinia sclerotiorum*. *Mycologia.* **67,** 89–97.

Kruger, W. (1975) Influence of weather on the attack of rape by *Sclerotinia sclerotiorum* Lib.) de Bary. *Nachrichtenble Devet. Pflanzenschutzd*. (*Braunschweig*) **27,** 1–6.

Kruger, W. (1973) Control measures for *Sclerotinia sclerotiorum* in rape. *Phytopathology*. 77, 125–137.

Kruger, W., Marguard, W. R. and Schosser, E. (1981) Plant disease product quality. II. Influence of stem canker *Sclerotinia sclerotiorum* (Lib.) de Bary on the quality of rapeseed. *Rev Plant Pathol*. **60,** 2949-2950.

Kumamoto, C. (2005) A contact-activated kinase signals Candida albicans invasive growth and biofilm development. *PNAS.* **102,** 5576-81.

Kupiac, M., Byers, B., Esposito, R. and Mitchell, A. (1997) Meiosis and sporulation in *Saccharomyces cerevisiae*. *Mol Gen Genet*. **221,** 889–1036.

Kurian, P. and Stelzig, D. A. (1979) The synergistic role of oxalic acid and endopolygalacturonase in bean leaves infected by *Cristulariella pyramidalis. Phytopathology.* **69,** 1301–1304.

Lamb, C. and Dixon, R.A. (1997) The oxidative burst in plant disease resistance. *Ann Rev Plant Physiol Plant Mol Biol.* **48,** 251–275.

Lau, J., McNeil, M., Darvill, A. and Albersheim, P. (1985) Structure of the backbone of rhamnogalacturonan 1, a pectic polysaccharide in the primary walls of plants. *Carb Res*. **137,** 111-125.

Lafitte, C., Barthe, J., Montillet, J. and Touz´e, A. (1984) Glycoprotein inhibitors of *Colletotrichum lindemuthianum* endopolygalacturonase in near isogenic lines of *Phaseolus vulgaris* resistant and susceptible to anthracnose. *Physiol Plant Pathol.* **25,** 39–53.

Leckie, F., Mattei, B., Capodicasa, C., Hemmings, A. and Nuss, L., (1999) The specificity of polygalacturonase-inhibiting protein (PGIP): a single amino acid substitution in the solvent-exposed strand turn region of the leucine-rich repeats (LRRs) confers a new recognition capability. *EMBO J.* **18,** 2352–2363.

Leshem, Y.Y. (1996) Nitric oxide in biological systems. *Plant Growth Regul*. **18,** 155–159.

LeTourneau, D. (1979) Morphology, cytology and physiology of *Sc1erotinia* species in culture. *Phytopathology.* **69,** 887–890.

Lev, S., Sharon, A., Hadar, R., Ma, H. and Horwitz, B. (1999) A mitogen-activated protein kinase of the corn leaf pathogen *Cochliobolus heterostrophus* is involved in conidiation, appressorium formation, and pathogenicity: diverse roles for mitogen-activated protein kinase homologs in foliar pathogens. *PNAS.* **96,** 13542-13547.

Levin, D. (2005) Cell wall signaling in *Saccharomyces cerevisiae. Microbial Mol Biol Rev.* **69,** 262-291.

Lennon, S.V., Martin, S.J. and Cotter, T.G. (1991). Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli. *Cell Prolif.* **24,** 203–214.

Li, D., Sirakova, T., Rogers, L., Ettinger, W.F., Kolattukudy, P.E. (2002) Regulation of constitutively expressed and induced cutinase genes by different zinc finger transcription factors in *Fusarium solani* f. sp. *pisi* (*Nectria haematococca*). *J Biol Chem*.**8,** 7905-12.

Li, R., Rimmer, R., Buchwaldt, L., Sharpe, A., Seguin-Swartz, G., Coutu, C. and Hegedus, D. (2004a) Interaction of *Sclerotinia sclerotiorum* with a resistant *Brassica napus* cultivar: expressed sequence tag analysis identifies genes associated with fungal pathogenesis. *Fungal Genet Biol.* **41,** 735-753.

Li, R., Rimmer, R., Buchwaldt, L., Sharpe, A., Seguin-Swartz, G. and Hegedus, D. (2004b) Interaction of *Sclerotinia sclerotiorum* with *Brassica napus*: cloning and characterization of endo-and exo-polygalacturonases expressed during saprophytic and parasitic modes. *Fungal Genet Biol.* **41,** 754-765.

Li, R., Rimmer, R., Yu, M., Sharpe, A., Séguin-Swartz, G., Lydiate, D. and Hegedus, D. (2003) Two Brassica napus polygalacturonase inhibitory protein genes are expressed at different levels in response to biotic and abiotic stresses. *Planta*. **217,** 299-308.

Lim, J., Aoki, K., Angel, P., Garrison, D., King, D., Tiemeyer, M., Bergmann, C. and Wells, L. (2009) Mapping glycans onto specific N-linked glycosylation sites of Pyrus communis PGIP redefines the interface for EPG-PGIP interactions. *J Proteome Res*. **8,** 673-680.

Link, V., Sinha, A., Vashista, P., Hofmann, M., Proels, R., Ehness, R. and Roitsch, T. (2002) A heat-activated MAP kinase in tomato: a possible regulator of the heat stress response. *FEBS Lett.* **531,** 179-183.

Lionetti, V., Raiola, A., Camardella, L., Giovane, A., Obel, N., Pauly, M., Favaron, Cervone, F. and Bellincampia, D. (2007) Overexpression of *Pectin Methylesterase Inhibitors* in *Arabidopsis* Restricts Fungal Infection by *Botrytis cinerea. Plant Physiol.* **143,** 1871-1880.

Liu, C. G., Du, D. Z., Zou, C. S. and Huang, Y. J. (1990) Initial studies on tolerance to *Sclerotinia sclerotiorum* (Lib.) de Bary in *Brassica napus* L. *Proceedings of Symposium of China International Rapeseed Science*, 70–71.

Liu, Z.M. and Kolattukudy, P.E. (1998) Identification of a gene product induced by hard-surface contact of *Colletotrichum gloeosporioides* conidia as a ubiquitin-conjugating enzyme by yeast complementation. *J Bacteriol*. **180,** 3592-3597.

Livingstone, D. M., Hampton, J. L., Phipps, P. M. and Grabau, E. A. (2005) Enhancing resistance to *Sclerotinia minor* in peanut by expressing a barley oxalate oxidase gene. *Plant Physiol*. **137,** 1354–1362.

Lu, H. and Higgins, V.J. (1999) The effect of hydrogen peroxide on the viability of tomato cells and of the fungal pathogen *Cladosporium fulvum*. *Physiol Mol Plant Pathol*. **54,** 131–143.

Lumsden, R. D. (1969) *Sclerotinia sclerotiorum* infection of bean and the production of cellulose. *Phytopathology*. **59,** 653–657.

Lumsden, R. D. (1970) Phosphatidase of *Sclerotinia sclerotiorum* produced in culture and in infected bean. *Phytopathology.* **60,** 1106–1110.

Lumsden, R. D. and Dow, R. L. (1973) Histopathology of Sclerotinia sclerotiorum infection of bean. *Phytopathology.* **63,** 708–715.

Lumsden, R. D. (1976) Pectolytic enzymes of *Sclerotinia sclerotiorum* and their localization in infected bean. *Can J Bot*. **54,** 2630–2641.

Lumsden, R. D. (1979) Histology and physiology of pathogenesis in plant disease caused by *Sclerotinia* species. *Phytopathology*. **69,** 890–896.

Madhani, H. and Fink, R. (1997) Combinatorial control required for the specificity of yeast MAPK signaling. *Science*. **275,** 1314-1317.

Marciano, P., Lenna, P. Di and Magro, P. (1983) Oxalic acid, cell wall-degrading enzymes and pH in pathogenesis and their significance in the virulence of two *Sclerotinia sclerotiorum* isolates on sunflower. *Physiol Mol Plant Pathol*. **22,** 339–345.

Mattinen, L., Tshuikina, M., Mae, A. and Pirhonen, M. (2004) Identification and characterization of Nip, necrosis-inducing virulence protein of *Erwinia carotovora subsp. carotovora*. *MPMI.* **17,** 1366–1375.

Mayorga, M. and Gold, S. (1999) A MAP kinase encoded by the *ubc3* gene of *Ustilago maydis* is required for filamentous growth and full virulence. *Mol Microbiol.* **34,** 485-497.

McCabe, P.F., Levine, A., Meijer, P.J., Tapon, N.A. and Pennell, R.I. (1997). A programmed cell death pathway activated in carrot cells cultured at low cell density. *Plant J*. 12, 267–280.

McLaren, D. L., Graham, A. D., Kaminski, D. A., and Lange, R. M. (2006) Canola diseases in Manitoba: Distribution, prevalence and incidence in 2005. *Can Plant Dis Surv*. **86,** 96-97.

McLusky, S., Bennett, M., Beale, M., Lewis, M., Gaskin, P. and Mansfield, J. (1999) Cell wall alterationsand localized accumulation of feruloyl-3 '-methoxytyramine in onion epidermis atsites of attempted penetration by *Botrytis allii* are associated with actin polarisation, peroxidases activity and suppression of flavonoid biosynthesis. *Plant J*. **17,** 523-34.

Mehrabi, R., Van der Lee, T., Waalwijk, C. and Kema, G. (2006) MgSlt2, a Cellular integrity MAP kinase gene of the fungal wheat pathogen *Mycosphaerella graminicola*, is dispensable for penetration but essential for invasive growth. *MPMI.* **19,** 389–398.

Merriman, P. R., Pywell, M., Harrisen, G. and Nancarrow, J. (1979) Survival of sclerotia of *Sclerotinia sclerotiorum* and effects of cultivation practices on disease. *Soil Biol Biochem*. **11,** 567–570.

Meikle, J., Reed, H. and Gadd, M. (1988) Osmotic adjustment and the accumulation of organic solutes in whole cells and protoplasts of *Saccharomyces cerevisiae*. *J Gen Microbiol.* **134,** 3049-3060.

Mey, G., Held, K., Scheffer, J., Tenberge, K. and Tudzynski, P. (2006) CPMK2, an SLT2-homologous mitogen-activated protein (MAP) kinase, is essential for pathogenesis of *Claviceps purpurea* on rye: evidence for a second conserved pathogenesis-related MAP kinase cascade in phytopathogenic fungi. *Mol Microbiol.* **46,** 305-318.

Mogami, H., Lloyd Mills, C. and Gallacher, D.V. (1997) Phospholipase C inhibitor, U73122, releases intracellular $Ca^{2+,}$ potentiates Ins(1,4,5)P3-mediated Ca^{2+} release and directly activates ion channels in mouse pancreatic acinar cells. *Bioch J*. **324,** 645-651.

Molloy, C., Cheah, L. H. and Koolaard, J. P. (2004) Induced resistance against *Sclerotinia sclerotiorum* in carrots treated with enzymatically hydrolyzed chitosan. *Postharvest Biol Techol*. **33,** 61–65.

Morid, B., Zare, R., Rezaee, S., Zamani-zadeh1, H. and Hajmansour, S. (2009) The relationship between cutinases and the pathogenicity/virulence of *Fusarium solani* in potato tubers. *Phytopathol Mediterr.* **48,** 403–410.

Morrall, R. A. A., Duczek, I. J. and Sheard, J. W. (1972) Variation and correlation within and between morphology, pathogenicity and pectolytic enzyme activity in *Sclerotinia* from Saskatchewan. *Can J Bot*. **50,** 767–786.

Morrall, R. A. A. (1977) A preliminary study of the influence of water potential on sclerotium germination in *Sclerotinia sclerotiorum. Can J Bot* **55,** 8–11.

Morrison, D. and Davis, R. (2003) Regulation of map kinase signaling modules by scaffold proteins in mammals. *Annu Rev Cell Devel Biol.* **19,** 91-118

Murphy, L., MacKeigan, J. and Blenis, J. (2004) A network of immediate early gene products propagates subtle differences in mitogen-activated protein kinase signal amplitude and duration. *Mol Cell Biol.* **24,** 144–53.

Murphy, L., Smith, S., Chen, R., Fingar, D. and Blenis, J. (2002) Molecular interpretation of ERK signal duration by immediate early gene products. *Nat Cell Biol.* **4,** 556– 564.

Murray, A. (2004) Recycling the cell cycle: cyclins revisited. *Cell.* **116,** 221–234.

Nakayama, T. and Amachi, T. (1999) Fungal peroxidase: its structure, function, and application. *J Mol Catalysis B Enzym*. **6,** 185–198.

Navarro-garcia, F., Sanchez, M., Pla, J. and Nombela, C. (1995) Functional characterization of the *MKC1* gene of *Candida albicans*, which encodes a mitogen-activated protein kinase homolog related to cell integrity. *Mol Cell Biol.* **15,** 2197-2206.

Nelson, B. (1998) Biology of Sclerotinia. In: Proceedings of the 10th International Sclerotinia workshop, 21 Jan. 1998, Fargo, ND, USA. North Dakota State University Department of Plant Pathology, Fargo, ND, pp. 1–5.

Newton, H. K. (1972) *Sclerotinia sclerotiorum* an incitant of lettuce drop: Sources of inoculum, host resistance and mechanism of pathogenesis. Ph.D. thesis, , University of Murofilm Inc., Ann Arbor, MI, 135 pp.

Osbourn, A. E. (2003) Saponins in cereals. *Phytochemistry*. **62,** 1-4.

Patil, S. and Dimond, A. (1967) Inhibition of *Verticillium* polygalacturonase by oxidation products of polyphenols. *Phytopathology*. **57,** 492–496.

Pascholati, S., Deising, L., Anderson, D. and Nicholson, R. (1993) Cutinase and non specific esterase activities in the conidial mucilage of *Colletotrichum graminicola. Physiol and Mol Plant Pathol*. **42,** 37-51.

Pascholati, S., Yoshioka, H., Kunoh, H. and Nicholson, R. (1992) Preparation of the infection court by *Erysiphe graminis* f. sp. *hordei*: Cutinase is a component of the conidial exudates. *Physiol and Mol Plant Pathol*. **41,** 53-59.

Pearse, P. G., Morrall, R. A. A., Kutcher, H. R., Yasinowski, J. M., Harris, C. L., Gugel, R. K., and Bassendowski, K. A. (2006) Survey of canola diseases in Saskatchewan, 2005. *Can. Plant Dis Surv*. **86,** 94- 95.

Pedras, M.S. and Ahiahonu, P.W. (2004) Phytotoxin production and phytoalexin elicitation by the phytopathogenic fungus *Sclerotinia sclerotiorum*. *J Chem Ecol*. **30,** 2163-2179.

Pemberton, C.L. and Salmond, G.P.C. (2004) The Nep1-like proteins-a growing family of microbial elicitors of plant necrosis. *Mol Plant Pathol*. **5,** 353-359.

Phillips, P.W.B. and Khachatourians, G.G. (2001). The biotechnology revolution in global agriculture: invention, innovation and investment in canola sector. CABI Publishing, Wallingford, Oxon, UK.

Pieterse, C.M.J., Van Wees, S.C., Van Pelt, J.A., Knoester, M., Laan, R., Gerrits, H., weisbeek, P. and Leendert, C (1998) A novel signaling pathway controlling induced systemic resistance in Arabidopsis. *Plant Cell.* **10,** 1571-1580.

Prior, G. D. and Owen, J. H. (1964) Pathological anatomy of *Sclerotinia trifoliorum* on clover and alfalfa. *Phytopathology.* **54,** 784–787.

Purdy, L. H. (1958) Some factors affecting penetration and infection by *Sclerotinia sclerotiorum. Phytopathology.* **48,** 605–609.

Powell, L., van Kan, J., ten Have, A., Visser, J., Greve, C., Bennett, B. and Labavitch, M. (2000) Transgenic expression of pear PGIP in tomato limits. *Annu Rev Phytopathol.* **39,** 313-335.

Prehoda, K., Scott, J., Mullins, R. and Lim, A. (2000) Integration of multiple signals through cooperative regulation of the N-WASP-Arp2/3 complex. *Science.* **290,** 801–806.

Purdy, J. and Truter, E. (1963) Constitution of the surface lipid from the leaves of *Brassica oleracea*: isolation and quantitative fractionation. *Proc Royal Soc London.* **158,** 536-543.

Quentin, U. (2004) *Sclerotinia sclerotiorum*, occurrence and control. *Kartoffelbau.* **8**, 318–319.

Qutob, D., Kemmerling, B., Brunner, F., Kufner, I., Engelhardt, S., Gust, A., Luberacki, B., Seitz, H., Stahl, D., Rauhut, T., Glawischnig, E., Schween, G., Lacombe, B., Watanabe, N., Lam, E., Schlichting, R., Scheel, D., Nau, K., Dodt, G., Hubert, D., Gijzen, M. and Nurenberger, T. (2006) Phytotoxicity and innate immune responses induced by Nep1-like proteins. *Plant Cell.* **18,** 3721-3744.

Qutob, D., Kamoun, S. and Gijzen, M. (2002) Expression of *Phytophthora sojae* necrosis-inducing protein occurs during transition from biotrophy to necrotrophy. *Plant J*. **32,** 361-373.

Rai, J. N., Singh, R. P. and Saxena, V. C. (1979) Phenolics and cell wall degrading enzymes in *Sclerotinia* infection of resistant and susceptible plants of *Brassica juncea. Indian J Mycol Plant Pathol*. **9,** 160–165.

Reglinski, T., Poole, P. R., Whitaker, G. and Hoyte, S. M. (1997) Induced resistance against *Sclerotinia sclerotiorum* in kiwi fruit leaves. *Plant Pathol*. **46,** 716–721.

Reina-Pinto, J. and Yephremov, A. (2009) Surface lipids and plant defenses. *Plant Physiol Biochem*. **47,** 540-549.

Reis, H., Pfiffi, S. and Hahn, M. (2005) Molecular and functional characterization of a secreted lipase from *Botrytis cinerea*. *Mol Plant Pathol*. **6,** 257-267.

Richter, T.E. and Ronald, P.E. (2000) The evolution of disease resistance genes. *Plant Mol Biol*. **42,** 195-205.

Ridley, L., O'Neill, A. and Mohnen, D. (2001) Pectins: Structure, biosynthesis and oligogalcturonide-related signaling. *Phytochemistry*. **57,** 929-967.

Riou, C., Fraissinet-Tachet, L., Freyssinet, G. and Fevre, M. (1992) Purification and characterization of extracellular pectinolytic enzymes produced by *Sclerotinia sclerotiorum. Appl Environ Microbiol*. **58,** 578–583.

Riou, C., Freyssinet, G. and Fevre, M. (1991) Production of cell wall degrading enzymes by the phytopathogenic fungus *Sclerotinia sclerotiorum. Appl Environ Microbiol*. **57,** 1478–1484.

Rogers, L.M., Kim, Y.K., Guo, W., Gonzalez-Candelas, L., Li, D. and Kolattukudy, P.E. (2000) Requirement for either a host- or pectin-induced pectate lyase for infection of *Pisum sativum* by *Nectria haematococca***.** *PNAS.* **97,** 9813-9818.

Rohatgi, R., Ho, H. and Kirschner, M. (2000) Mechanism of N-WASP activation by CDC42 and phosphatidylinositol 4, 5-bisphosphate. *J Cell Biol.* **150,** 1299–1310.

Rollins, J. (2003) The *Sclerotinia sclerotiorum pac1* gene is required for sclerotial development and virulence. *MPMI.* **16,** 785-795.

Rollins, J. A. and Dickman, M. B. (2001) pH signaling in *Sclerotinia sclerotiorum* identification of a *pac C*/ *RIM 1* homolog. *Appl Environ Microbiol*. **67,** 75–81.

Rollins, J. A. and Dickman, M. B. (1998) Increase in endogenous and exogenous cyclic AMP levels inhibits sclerotial development in *Sclerotinia sclerotiorum. Appl Environ Microbiol.* **64,** 2539–2544.

Ronicke, S., Hahn, V., Vogler, A. and Friedt, W. (2005) Quantitative trait loci analysis of resistance to *Sclerotinia sclerotiorum* in sunflower. *Phytopathology*. **95,** 834–839.

Rosenberg, G. and Pall, M. (1979) Properties of two cyclic nucleotide-deficient mutants of *Neurospora crassa*. *J Bacteriol.* **137,** 1140-1144.

Ross, A.F. (1961) Systemic acquired resistance induced by localized virus infections in plants. *Virology.* **14,** 340-358.

Rui, O. and Hahn, M. (2007) The Slt2-type MAP kinase Bmp3 of *Botrytis cinerea* is required for normal saprotrophic growth, conidiation, plant surface sensing and host tissue colonization. *Mol Plant Pathol.* **8,** 173-184.

Ryals, J., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.Y. and Hunt, M. (1996) Systemic acquired resistance. *Plant Cell.* **8,** 1809-1819.

Saito, I. (1977) *Sclerotinia sclerotiorum* (Lib.) de Bary. Studies on the maturation and germination of sclerotia of *Sclerotinia sclerotiorum* (Lib.) de Bary, a causal fungus of bean stem rot. *Report Hokkaido Prefectural Agric. Exp. Stn. No. 26, March, 1977. Hokkaido Central Agricultural Experiment Station,* 069–13, Japan, 106 p.

Saito, I. (1997) Sclerotinia nivalis sp. nov. the pathogen of snow mold of herbaceous dicots innorthern Japan. *Mycoscie*. **38,** 227–236.

Saito, I. (1974) Utilization of β-glucans in germinating sclerotia of *Sclerotinia sclerotiorum* (Lib.) de Bary. *Ann Phytopathol Soc* [Japan] **40,** 372–374.

Salvi, G., Giarrizzo, F., De Lorenzo, G. and Cervone, F. (1990) A polygalacturonaseinhibiting protein in the flowers of *Phaseolus vulgaris* L. *Plant Physiol.* **136,** 513–18.

Sambrook, J. and Russell, D. (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. USA.

Satomura, Y. and Sato, A. (1964) Isolation and physiological activity of sclerin, a metabolite of *Sclerotinia* fungus. *Agri Biol Chem*. **29,** 337-344.

Scott, W. and Solomon, B. (1977) Adenosine 3',5'-cyclic monophosphate and morphology in *Neurospora crassa*: Drug-induced alterations. *J Bacteriol*. **122,** 454-463.

Schouten, A., Van Baarlen, P. and Van Kan, J. (2007) Phytotoxic Nep1 like proteins from the necrotrophic fungus *Botrytis cinerea* associate with membranes and the nucleus of plant cells. *New Phytol.* **177,** 493-505.

Schué, M., Maurin, D., Dhouib, R., Bakala N′Goma, J.C., Delorme, V., Lambeau, G., Carrière, F. and Canaan, S. Two cutinase-like proteins secreted by *Mycobacterium tuberculosis* show very different lipolytic activities reflecting their physiological function. *FASEB J.* **24,** 1893-1903.

Schumacher, J., Viaud, M., Simon, A. and Tudzynski, B. (2008) The G alpha subunit BCG1, the phospholipase C (BcPLC1) and the calcineurin phosphatase co-ordinately regulate gene expression in the grey mould fungus *Botrytis cinerea*. *Mol Microbiol.* **67,** 1027-1050.

Schwartz, H. F., Casciano, D. H., Asenga, J. A. and Wood, D. R. (1987) Field measurement of white mold effects upon dry beans with genetic resistance on upright plant architecture. *Crop Sci*. **27,** 699–702.

Schwartz, H. F. and Steadman, J. R. (1978). Factors affecting sclerotium population of and apothecium production by *Sclerotinia sclerotiorum. Phytopathology.* **68,** 383–388.

Sebolt-Leopold. S. (2000) Development of anticancer drugs targeting the MAP kinase pathway. *J. Oncogene*. **19,** 6594-6599.

Sella, L., Tomassini, A., D'Ovidio, R. and Favaron, F. (2005) Expression of two *Sclerotinia sclerotiorum* endo-*PG* genes correlates with endo-polygalacturonase activity during *Glycine max* colonization. *J Plant Plathol.* **87,** 199-205.

Sexton, A.C., Cozijnsen, A.J., Keniry, A., Jewell, E., Love, C.G., Batley, J., Edwards, D. and Howlett, B.J. (2006) Comparison of transcription of multiple genes at three developmental stages of the plant pathogen *Sclerotinia sclerotiorum. FEMS Microbiol Lett.* **258,** 150–160.

Sharma, P. and Sain, S. K. (2004) Induction of systemic resistance in tomato and cauliflower by *Trichoderma* spp. against stalk rot pathogen *Sclerotinia sclerotiorum* (Lib) de Bary. *J Biol Control.* **18,** 21–27.

Sharma, R. C. and Sharma, S. L. (1986) Survival of *Sclerotinia sclerotiorum* and initial infection of cauliflower in Himachal Pradesh. *Indian J Ecology.* **13,** 367–369.

Shi, X., Karkut, **T., Chamankhah, M., Alting-Mees, M., Hemmingsen**, **S. M. and Hegedus, D. (2003)** Optimal conditions for the expression of a single-chain antibody (scFv) gene in *Pichia pastoris. Protein Express Purif*. **28,** 321-330.

Skamnioti, P. and Gurr, S. (2007) *Magnaporthe grisea* cutinase 2 mediates appressorium differentiation and host penetration and is required for full virulence. *Plant Cell*. **19,** 2674-2689.

Smith-Becker, J., Marois, E., Huguet, E., Midland, S., Sims, J. and Keen, N. (1998) Accumulation of salicylic acid and 4-hydroxybenzoic acid in phloem fluids of cucumber during systemic acquired resistance is preceded by a transient increase in phenylalanine ammonia-lyase activity in petioles and stems1. *Plant Physiol.* **116,** 231-238.

Smith, J.A., Hammerschmidt, R. and Fulbright, D.W. (1991) Rapid induction of systemic resistance in cucumber by *Pseudomonas syringae* pv. *syringae*. *Physiol Mol Plant Pathol*. **38,** 223-235.

Staats, M., Van Baarlen, P., Schouten, A. and Van Kan, J. (2007) Functional analysis of NLP genes from *Botrytis elliptica*. *Mol Plant Pathol*. **8,** 209-214.

Staples, R. and Mayer. A. (1995) Putative virulence factors of *Botrytis cinerea* acting as a wound pathogen. *FEMS Microbiol Lett.* **134,** 1–7.

Tae Kim, Y., Pursky, D. and Rollins, J. (2007) An activating mutation of the *Sclerotinia sclerotiorum pac1* gene increases oxalic acid production at low pH but decreases virulence. *Mol Plant Pathol*. **8,** 611-622.

Takano, Y., Kikuchi, T., Kubo, Y., Hamer, E., Mise, K. and Furusawa, I. (2000) The *Colletotrichum lagenarium* MAP kinase gene CMK1 regulates diverse aspects of fungal pathogenesis. *MPMI.* **13,** 374-383.

Tan, P., Lackner, M. and Kim, S. (1998) MAP kinase signaling specificity mediated by the LIN-1 Ets/LIN-31 WH transcription factor complex during *C. elegans* vulval induction. *Cell.* **93,** 569–80.

Tariq, V. and Jeffries, P. (1987) Cytochemical localization of lipolytic enzyme activity during penetration of host tissues by *Sclerotinia sclerotiorum*. *Physiol Mol Plant Pathol*. **30,** 77-91.

Temple, B., Bernier, L. and Hintz, W. (2009) Characterization of the polygalacturonase gene of the Dutch elm disease pathogen ophiostoma novo-ulmi. *NZ J Sci.* **39,** 29–37

Tenhaken, R., Levine, A., Brisson, L. F., Dixon, R. A. and Lamb, C. (1995) Function of the oxidative burst in hypersensitive disease resistance. *PNAS*. **92,** 4158–4163.

ten Have, A., Mulder, W., Visser, J. and van Kan, J. (1998) The endopolygalacturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea*. *MPMI*. **11,** 1009-1016.

ten Have, A., Breuil, W., Wubben, J., Visser, J. and van Kan, J. (2000) *Botrytis cinerea* endopolygalacturonase genes are differentially expressed in various plant tissues. *Fungal Genet Biol*. **33**, 97-105.

Tiwari, B. S., Belenghi, B. and Levine, A. (2002) Oxidative stress increased respiration and generation of reactive oxygen species, resulting in ATP depletion, opening of mitochondrial permeability transition, and programmed cell death. *Plant Physiol*. **128,** 1271-1281.

Toal, E. S. and Jones, P. W. (1999) Induction of systemic resistance to *Sclerotinia sclerotiorum* by oxalic acid in oilseed rape. *Plant Pathol*. **48,** 759–767.

Toda, T., Dhut, S., Superti-Furga, G., Gotoh, Y., Nishida, E., Sugiura, R. and Kuno, T. (1996) The fission yeast pmk1+ gene encodes a novel mitogen-activated protein kinase homolog which regulates cell integrity and functions coordinately with the protein kinase C pathway. *Mol Cell Biol.* **16,** 6752-6764.

Tognolli, M., Penel, C., Greppin, H. and Simon, P. (2002) Analysis and expression of the class III peroxidaselarge gene family in *Arabidopsis thaliana*. *Gene*. **288,** 129-138.

Toubart, P., Desiderio, A., Salvi, G., Cervone, F., Daroda, L. and De Lorenzo, G. (1992) Cloning and characterization of the gene encoding the endopolygalacturonase-inhibiting protein (PGIP) of *Phaseolus vulgaris* L. *Plant J.* **2,** 367–373.

Trevethick, J. and Cooke, R. C. (1973) Non nutritional factors influencing sclerotia formation in some *Sclerotinia* and *Sclerotium* species. *Trans Br Mycol Soc*. **60,** 559–566.

Tsuba, M., Katagiri, C., Takeuchi, Y., Takada, Y. and Yamaoka, N. (2002) Chemical factors of the leaf surface involved in the morphogenesis of *Blumeria graminis*. *Physiol Mol Plant Pathol*. **60,** 51-57.

U, N. (1935) Genome analysis in *Brassica* with special reference to the experimental formation of *Brassica napus* and peculiar mode of fertilization. *Japan J Bot*. **7,** 389-452.

van den Ackerveken, G., Dunn, R., Cozijnsen, A., Vossen, J., van den Broek, H. and de Wit, P. (1994) Nitrogen limitation induces expression of the avirulence gene avr9 in the tomato pathogen Cladosporium fulvum. *Mol General Genet*. **243,** 277-285.

van Kan, J., van't Klooster, J., Wagemakers, C., Dees, D. and van der Vlugt-Bergmans, C. (1997) Cutinase A of *Botrytis cinerea* is expressed, but not essential, during penetration of gerbera and tomato. *MPMI.* **10,** 30-38.

Van der Vlugt-Bergmans, C.J.B., Wagemakers, C.A.M., Dees, D.C.T. and van Kan, J.A.L. (1997a) Catalase A from *Botrytis cinerea* is not expressed during infection on tomato leaves. *Physiol Mol Plant Path*. **50,** 1–15.

Van der Vlugt-Bergmans, C. J. B., Wagemakers, C. A. M. and van Kan, J. A. L. (1997b) Cloning and Expression of the Cutinase A Gene of *Botrytis cinerea*. *Phytopath Society*. **10,** 21-29.

Veit, S., Worle, J.M., Nurnberger, T., Koch, W. and Seitz, H.U. (2001) A novel protein elicitor (PaNie) from *Pythium aphanidermatum* induces multiple defence responses in carrot, *Arabidopsis*, and tobacco. *Plant Physiol*. **127,** 832–841.

Viaud, M., Brunet-Simon, A., Brygoo, Y., Pradier, J. and Levis, C. (2003) Cyclophilin A and calcineurin functions investigated by gene inactivation, cyclosporin A inhibition and cDNA array approaches in the phytopathogenic fungus *Botrytis cinerea*. *Mol Microbiol.* **50,** 1451-1465.

Volmat, V., Camps, M., Arkinstall, S., Pouyssegur, J. and Lenormand, P. (2001) The nucleus, a site for signal termination by sequestration and inactivation of p42/p44 MAP kinases. *J Cell Sci*. **114,** 3433-43.

Wang, Z., Mao, H., Dong, C., Ji, R., Cai, L., Fu., H and Liu., S. (2008) Overexpression of Brassica napus MPK4 Enhances Resistance to *Sclerotinia sclerotiorum* in Oilseed Rape. *MPMI*. **22,** 235-244.

Watson, F., Heerssen, H., Bhattacharyya, A., Klesse, L., Lin, M. and Segal, R. (2001) Neurotrophins use the Erk5 pathway to mediate a retrograde survival response. *Nat Neurosci.* **4,** 981–88.

Wegulo, S. N., Sun, P., Martinson, C. A. and Yang, X. B. (2000) Spread of *Sclerotinia* stem rot of soybean from area and plant sources of apothecial inoculum. *Can J Plant Sci*. **80,** 389–402.

Wessels, J. (1997) Hydrophobins, proteins that change the nature of the fungal surface. *Adv Microbial Physiol.* **38**, 1-45.

Willetts, H. J. (1997) Morphology, development and evaluation of stromata sclerotia and macroconidia of the *Sclerotiniaceae*. *Mycol Res*. **101,** 939–952.

Willetts, H. J. and Wong, J. A. L. (1980) The biology of *Sclerotinia sclerotiorum, S. trifoliorum* and *S. minor* with emphasis on specific nomenclature. *Bot Rev*. **46,** 101–165.

Wolf, B.B. and Green, D.R. (1999). Suicidal tendencies: apoptotic cell death by caspase family proteinases. *J Biol Chem*. **274,** 20049–20052.

Wosten, H. and Willey, J. (2000) Surface-active proteins enable microbial aerial hyphae to grow into the air. *Microbiology.* **146,** 767–773.

Wubben, J., ten Have, A., van Kan, J. and Visser, J. (2000) Regulation of endopolygalacturonase gene expression in *Botrytis cinerea* by galacturonic acid, ambient pH and carbon catabolite repression. *Curr Genet*. **37,** 152–157.

Wubben, J., Mulder, W., Ten Have, A., Van Kan, J. and Visser, J. (1999) Cloning and partial characterization of Endopolygalacturonase genes from *Botrytis cinerea*. *Appl Environ Microbiol*. **65**, 159-1602.

Xu, J. and Hamer, J. (1996) MAP kinase and cAMP signaling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. *Genes Dev*. **10,** 2696-2706.

Xu, J., Staiger, C. and Hamer, J. (1998) Inactivation of the mitogen-activated protein kinase Mps1 from the rice blast fungus prevents penetration of host cells but allows activation of plant defense responses. *PNAS.* **95,** 12713-12718

Yajima, W. and Kav, N. (2009) The proteome of the phytopathogenic fungus *Sclerotinia sclerotiorum. Proteomics.* **6,** 5995-6007.

Yajima, W., Verma, S., Shah, S. and Rahman, M. (2010) Expression of anti-sclerotinia scFv in transgenic *Brassica napus* enhances tolerance against stem rot. *Nature Biotech.* **27,** 816-21

Yajima, W., Liang, Y. and Kav, N. (2009) Gene disruption of anarabinofuranosidase/beta-xylosidase precursor decreases *Sclerotinia sclerotiorum* virulence on canola tissue. *MPMI,* **22,** 783-789.

Yao, C., Conway, W., Ren, R., Smith, D., Ross, G. and Sams, C. (1999) Gene encoding polygalacturonase inhibitor in apple fruit is developmentally regulated and activated by wounding and fungal infection. *Plant Mol Biol.* **39,** 1231–1241.

Zabka, V., Stangl. M., Bringmann, G., Vogg, G., Riederer, M. and Hildebrandt, U. (2008) Host surface properties affect prepenetration processes in the barley powdery mildew fungus. *New Phytol*. **177,** 251-63.

Zuppini, A., Navazio, L., Sella, L., Castiglioni, C., Favaron, F. and Mariani, P. (2005) An endopolygalacturonase from *Sclerotinia sclerotiorum* induces calcium-mediated signalling and programmed cell death in soybean cells. *MPMI.* **18,** 849-855.

Zhao, J., Wang, J., An, L., Doerge, R.W., Chen, Z.J., Grau, C.R., Meng, J. and Osborn, T.C. (2007) Analysis of gene expression profiles in response to *Sclerotinia sclerotiorum* in *Brassica napus. Planta*. **227,** 13-24.

Zhao, J., Buchwaldt, L., Rimmer, S.R., Sharpe, A., McGregor, L., Bekkaoui, D., Hegedus, D. (2009) Patterns of differential gene expression in *Brassica napus* cultivars infected with *Sclerotinia sclerotiorum. Mol Plant Pathol*. **10,** 635-649.

Zhao, J. and Meng, J. (2003a) Genetic analysis of loci associated with partial resistance to *Sclerotinia sclerotiorum in* rapeseed (*Brassica napus* L.) *Theor Appl Genet*. **106,** 759–764.

Zhao, J. and Meng, J. (2003b) Detection of loci controlling seed glucosinolate content and their association with *Sclerotinia* resistance in *Brassica napus. Plant Breed*. **122,** 19–23.

APPENDIX

ISOLATION OF FUNGAL HOMOKARYOTIC LINES FROM HETEROKARYOTIC TRANSFORMANTS BY SONIC DISRUPTION OF MYCELIA