Characterization and expression of an endopolygalacturonase gene from a lupin anthracnose fungus identified as *Colletotrichum lupini* var. *setosum*

By

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any University for a degree.

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Summary

Endopolygalacturonases (PGs) are the first cell wall degrading enzymes that are produced when pathogenic fungi encounter the host cell wall (Albersheim and Anderson, 1971). The role that these enzymes play in pathogenicity has been investigated for numerous pathogenic fungi. Although the results are not conclusive, there is evidence for some fungi that these enzymes are significant for their pathogenecity. Furthermore, plants contain polygalacturonase inhibiting proteins (PGIPs) in their cell walls, which are able to inhibit PGs (De Lorenzo *et al*, 2001; 2002).

Colletotrichum SHK2148 is a pathogenic fungus causing anthracnose of lupin plants in South Africa. The identity of the fungus has been described as *Colletotrichum tortuosum* (Koch, 1996). However, this was based on morphological evidence only. Thus, the classification of the South African lupin- associated *Colletotrichum* isolates was re-assessed by comparing *Colletotrichum* SHK2148 on a morphological and molecular level to the recently described *Colletotrichum lupini* species (Nirenberg *et al*, 2002) as well as previously described *Colletotrichum acutatum* lupin anthracnose isolates (Talhinas *et al*, 2002). Based on the culture morphology, ITS and β-tubulin sequence data, it was concluded that *Colletotrichum* SHK2148 groups with *C. lupini*, more specifically, *C. lupini* var. *setosum*.

The fungus, renamed *Colletotrichum lupini* SHK2148, was evaluated for its PG activity in pectin media (pH 5) over a 12 day growth period by using an agarose diffusion assay. The specific PG activity reached its highest level after three days, whereafter it decreased. Previous studies performed at the ARC, revealed that the fungus produced PG activity and this crude activity was inhibited by a PGIP produced in apple. A study was launched to isolate and characterise the gene(s) responsible for PG production. PG gene sequences from *Colletotrichum gloeosporioides* f.sp. *malvae* and *Colletotrichum lindemuthianum* were compared and conserved regions were identified from which primers were designed to amplify a fragment of a PG gene from *C. lupini* SHK2148. Inverse PCR was used to resolve the 5' and 3' sequences of the PG gene whereafter a complete copy of the gene was isolated from the genome of the

fungus and characterised. The isolated gene was approximately 1Kb, contained a single intron of 59 bp and was very similar to the PG gene from *C. gloeosporioides* f.sp. *malvae* (*cmpgII*) as well as one of the PG genes (*clpg2*) from *C. lindemuthianum*. Southern blot analyses revealed that the gene was present as a single copy in the genome of the fungus. The *in vitro* expression of the PG gene from *C. lupini* SHK2148, grown in pectin media (pH 5), was investigated via northern blot analyses as well as RT-PCR, which revealed that the gene was expressed in the same time period that the highest PG activity was observed. A full cDNA copy of the PG gene was isolated using mRNA harvested from mycelia that was grown for 4 days on pectin. The cDNA copy confirmed the predicted intron position of the previously isolated genomic PG gene.

Due to the unavailability of a full cDNA copy of the *C. lupini* SHK2148 PG gene at the time when expression studies were initiated, a complete cDNA copy was constructed by swapping an internal cDNA PG fragment with its counterpart in the complete genomic PG gene copy. The resulting cDNA PG copy was used as a template from which PG constructs were prepared for expression in *Pichia pastoris*. Constructs containing the PG gene with its native signal peptide, the PG gene with the α-MF signal peptide factor as well as hybrid constructs where the N terminal part of the mature PG proteins of *Fusarium moniliforme* and *C. lupini* SHK 2148 were exchanged, were transformed into *P. pastoris*. No PG activity was observed with an agarose diffusion assay for any of the *Pichia* clones. SDS-PAGE analyses were used to evaluate total protein isolations from the *P. pastoris* clones. The supernatant and cells of the clones were subjected to western blot analyses using antibodies directed against *Aspergillus niger* PG as well as *F. moniliforme* PG. The only positive hybridisation signal was observed between the *A. niger* antibody and a protein in supernatant extracts of the *P. pastoris* clones. However, the size of the hybridising band was very large. This could be due to glycosylation of the *C. lupini* SHK 2148 PG in *P. pastoris*, although the size increase is unusually large. The results indicated that it is unlikely that the *C. lupini* SHK 2148 PG was expressed in *P. pastoris* transformed with any of these constructs.

Opsomming

Endopolygalakturonase ensieme is van die eerste selwand degraderende ensieme wat plant patogeniese swamme produseer sodra hulle in kontak kom met plantselwande (Albersheim and Anderson, 1971). Die patogeniese rol van dié ensieme is al menigmaal ondersoek en alhoewel dit nog nie absoluut duidelik is nie, is daar wel gevalle waar die ensieme krities was vir die virulensie van sekere swamme (hoofstuk 1). Alhoewel, in plantselwande word poligalakturonase-inhiberende proteïne aangetref wat daartoe instaat is om endopolygalakturonases van swampatogene te inhibeer (De Lorenzo *et al*, 2001; 2002).

Colletotrichum SHK2148 is ‘n patogeniese swam wat antraknose veroorsaak op lupiene plante in Suid Afrika. Die swamme is vroeër geklassifiseer as *Colletotrichum tortuosum* (Koch, 1996), maar die klassifikasie studie was slegs gebasseer op morfologiese kenmerke. Dus is die identiteit van die *Colletotrichum* SHK 2148 swam, wat antraknose van lupiene veroorsaak, geherevalueer deur die swamme op ‘n molekulêre en morfologiese vlak te vergelyk met die onlangs geïdentifiseerde *C. lupini* isolate (Nirenberg *et al*, 2002) as ook vorige geïdentifiseerde *C. acutatum* isolate (Talhinas *et al*, 2002). Volgens die morfologiese, ITS en β-tubulin resultate, lyk dit asof die *Colletotrichum* SHK 2148 isolaat baie naby verwant is aan die *C. lupini* spesie of meer spesifiek een van die spesie se variante, *C. lupini* var. *setosum*.

Die PG aktiwiteit sowel as die proteïen produksie van die *Colletotrichum lupini* SHK 2148 swam, wat vir ‘n 12 dag periode gegroei is in ‘n pektien medium (pH 5), is onderskeidelik bepaal met ‘n agarose diffusie en Bradford toets. Die hoogste PG aktiwiteit was waargeneem nadat die swam 3 dae in die pektien medium gegroei het, die aktiwiteit het daarna afgeneem. Vorige studies wat plaasgevind het by die LNR, het daarop gedui dat die *Colletotrichum* swamme, wat antraknose van lupiene veroorsaak, PG aktiwiteit besit en dat ‘n ongesuiwerde PG ekstrak van *Colletotrichum* se aktiviteit geïnhibeer word deur PGIP wat uit appels geïsoleer is. Dus is ‘n studie onderneem om die geen/gene te identifiseer wat verantwoordelik was vir die PG aktiwiteit. PG gene van *C. gloeosporioides* f.sp. *malvae* (*cmpgII*) en

C. lindemuthianum (*clpg2)* is met mekaar vergelyk om gekonserveerde gebiede te identifiseer vir die ontwerp van voorlopers wat aangewend is om ‘n interne fragment van die PG geen te isoleer van die genoom van *Colletotrichum lupini* SHK 2148. Om die ontbrekende dele van die PG geen te bepaal is ‘n inverse polimerase ketting reaksie (PKR) gebruik. Gevolglik is die volledige PG geen van die genoom van *Colletotrichum lupini* SHK 2148 geamplifiseer en gekarakteriseer. Die geen was ongeveer 1 Kb groot, onderbreek deur ‘n 59 bp intron byna identies aan die *cmpgII* geen van *C. gloeosporioides* f.sp. *malvae* asook die *clpg2* geen *C. lindemuthianum*. Volgens die “Southern” klad, word die geen aangetref as ‘n enkel kopie in die genoom van die swam. Die geen se *in vitro* uitdrukking is bepaal, in pektien medium (pH5), met behulp van ‘n “Northern” klad en omgekeerde transkripsie polimerase ketting reaksie. Die geen word uitgedruk terselfdetyd as wat die hoogste PG aktiwiteit waargeneem is. Die geen word nie uitgedruk wanneer die swam vir 5 of 12 dae in die pektien medium gegroei het nie. ‘n Volledige cDNS (kopie dieoksieribonukleïensuur) kopie van die PG geen is geïsoleer van die boodskapper ribonukleïensuur (mRNS) van die swam wat vir 4 dae in die pektien medium (pH 5) gegroei het. Die cDNS kopie het die posisie van die voorspelde intron in die genomiese PG geen kopie bevestig.

Aangesien nog geen cDNS PG geen kopie beskikbaar was tydens die gis uitdrukkings studies nie, is ‘n cDNS kopie gekonstrueer deur ‘n interne cDNS PG fragment te ruil met die ooreenstemmende fragment in die genomiese PG kopie. Dié gekonstrueerde cDNS PG geen is as templaat gebruik waarvan PG konstrukte saamgestel is vir die uitdrukking studies in *P. pastoris*. Onder andere is ‘n PG geen met sy eie seinpeptied, ‘n PG geen met die α-MF (α-mating factor) seinpeptied sowel as hibried PG gene, waarvan die N-terminale van die volledige PG proteïene van *F. moniliforme* en *Colletotrichum lupini* SHK 2148 met mekaar omgeruil is, uitgedruk in *P. pastoris*. Geen PG aktiwiteit is waargeneem met die agarose difussie toets vir enige van die konstrukte se klone nie. ‘n SDS-PAGE toets is gedoen om die proteïen isolasies van die klone te evalueer. Die supernatant sowel as die selle van die klone is gebruik in ‘n “Western” klad analise met die teenliggame vir die PG gene van *A. niger* PG en *F. moniliforme*. Die enigste positiewe sein wat waargeneem is, was met die teenliggaam van die *A. niger* PG en die supernatant van die klone. Die sein se bandjie

het baie groot vertoon. Glikosilering van die PG proteïen mag moontlik die grootte van die proteïen verhoog, maar waarskynlik nie tot so ‘n groot mate nie. Die resultate toon waarskynlik dat geen van die PG konstrukte in *P. pastoris* uitgedruk is nie.

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List of abbreviations

α-MF	alpha mating factor
AA	amino acids
ADA	agarose diffusion assay
AFLP	amplified fragment length polymerisation
AOX1	alcohol oxidase gene 1
ARC	Agricultural Research Council
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
CWDE	cell wall degrading enzymes
CreA protein	DNA binding protein involved in carbon catabolite repression
DIG	digoxygenin
dATPs	deoxy adenosine triphosphate
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediamine tetraacetic acid
EndoPGs	endopolygalacturonases
EtBr	etidium bromide
EtOH	ethanol
ExoPGs	exopolygalacturonases
FA	formaldehyde
GAP	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
IPTG	isopropyl-β-D-thiogalactopyranoside
ITS	internal transcribed spacer region
kB	kilo base pair
kV	kilo volt
LB	Luria Bertani
LMW	low molecular weight
LRR	leucine-rich repeat
LSLB	low salt LB
MCS	multiple cloning site
$MgCl_2$	magnesium chloride
mM	millimolar
M-MLV	Moloney Murine Leukemia Virus
mRNA	messenger ribonucleic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBT	nitroblue tetrazolium chloride
ng	nanogram
OD	optical density
OGAs	oligogalacturonides
PAGE	polyacrylamide gel electrophoresis

PCR	polymerase chain reaction
PDA	potato dextrose agar
PGA	polygalacturonic acid
PGs	polygalacturonases
PGIPs	polygalacturonase Inhibiting Proteins
RAPD	random amplified polymorphic DNA
RNA	ribonucleic acid
RNAse	ribonuclease
ROS	reactive oxygen species
Rpm	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
Sec.	seconds
SNA	saltwater nutrient agar
SOE PCR	splicing by overlap extension by PCR
SSC	sodium chloride/sodium citrate
TAE	Tris-acetate ethylenediamine tetraacetic acid
TCA	trichloroacetic acid
TE	Tris ethylenediamine tetraactic acid
TNE	Tris-sodium chloride EDTA
TT	transcription termination
U	unit
UV	ultraviolet
µg	microgram
µl	microlitre
µM	micromolar
VCG	vegetative compatibility grouping
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
YPD	Yeast Extract Peptone Dextrose
BMMY	Buffered methanol complex medium
HCl	Hydrochloric acid

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Aim of study

Lupins have been cultivated for at least 2000 years and of the more than 300 existing species only five are cultivated worldwide today. The plants are grown as both forage and grain legume in the USSR, Poland, Germany, South Africa and the Mediterranean, while Australia cultivates lupin primarily as an export product for European and Asian feed markets. Lupins are mainly used as feedstock for poultry and livestock, however, a human food market, which includes lupin flour, lupin pasta and hulls for dietary fiber, is also being developed in the USA (www.ext.nodak.edu/extpubs/alt-ag/lupin.htm). The annual turnover from the lupin industry in South Africa is approximately 60 million rands.

Lupin *spp.* however are highly susceptible to anthracnose. This disease is considered to be the most devastating disease of lupin industries, causing crop losses that range from 50% in narrow leafed lupins (*Lupinus angustifolius*) to 100% in white lupins (*Lupinus albus*) (Oelofse *et al*, 2003). The identity of the anthracnose causing *Colletotrichum* isolates is still a much-debated issue. Thus, one of the aims in this study was to resolve the current taxonomic placing of the South African lupin anthracnose isolates.

Plant pathogenic fungi produce several extracellular enzymes that facilitate the invasion and colonization of the pathogens. Endopolygalacturonases are only one of these enzymes that are produced by the pathogen when it is cultivated on isolated plant cell walls as well as during the infection process (Albersheim and Anderson, 1971; Cooper *et al*, 1981). The literature review (Chapter 2) discusses the importance of these enzymes as role players in pathogenicity and elicitors of the plant defense response. However, endo-PGs are inhibited by polygalacturonase inhibiting proteins (PGIP), which are leucine-rich repeat proteins associated with plant cell wall (Caprari *et al*, 1993; Rha *et al*, 2001; Di Pietro *et al*, 1996). By inhibiting fungal PGs, PGIPs directly interfere with host cell wall degradation and provide a first line of defense against a fungal attack. Thus, PGIP genes provide a potential tool that can be used to increase the resistance of lupin plants through genetic modification.

Apple PGIP1, expressed in transgenic tobacco plants at the ARC-Roodeplaat, was able to inhibit PG activity from the *Colletotrichum* SHK pathogens (Oelofse *et al*, 2003). Thus the aim of the study was to isolate the gene(s) responsible for the PG activity and characterize them in terms of their copy number, similarity to other fungal PG genes and their *in vitro* expression on pectin. The subsequent aim was to express and purify the protein in a yeast system and then test the inhibition effectiveness of the purified apple PGIP1 against the purified *Colletotrichum* PG.

In this research project, Chapter 1 provides a literature review, which includes information on the characterization of the casual agent of lupin anthracnose, the important fungal endoPG gene families, the role that some of these endoPGs play in pathogenicity, the ability of PGs to elicit a plant defense response and the structural domains in both PGs and PGIPs and their inhibition interaction with each other are discussed. In chapter 2 the taxonomic identity of the South African lupin anthracnose isolates are re-evaluated and compared with the recent classifications of lupin anthracnose causing *Colletotrichum* isolates. The rest of the project, Chapter 3 and Chapter 4, includes molecular studies. Chapter 3 describes the *in vitro* PG activity of the fungus, the isolation and characterization of a single PG gene from *C. lupini* as well as the *in vitro* transcription of this gene. Chapter 4 discusses the construction and expression of a cDNA copy of the PG gene in *Pichia pastoris*. Chapter 5 contains the concluding discussion of the results obtained in this project.