Chapter 3

Cloning and characterization of a full-length Polygalacturonase gene from *Colletotrichum lupini*

Abstract

Several plant pathogenic fungi produce endopolygalacturonases (endoPGs) to facilitate the degradation of the plant cell wall and many of the encoding genes of these enzymes have been isolated and characterized. *Colletotrichum lupini* SHK 2148 is the causal agent of lupin anthracnose in South Africa. PG activity has previously been reported for this fungus and was confirmed with an agarose diffusion assay. The highest PG activity was observed when the fungus was grown for three days on pectin. An internal fragment of a PG encoding gene was isolated from the genome of *C. lupini* SHK 2148, while inverse PCR was used to resolve the remaining sequences of the gene. The complete PG gene was subsequently isolated and characterized from genomic DNA of *C. lupini* SHK 2148. The isolated PG gene displayed typical filamentous fungal gene characteristics, it was 1153 bp in size and was interrupted by a single intron of 59 base pairs. Furthermore, the PG gene of *C. lupini* SHK 2148 was present as a single copy in the genome and had the highest similarity to the *CmpgII* and *ClpgII* genes of *Colletotrichum gloeosporioides* f.sp. *malvae* and *Colletotrichum lindemuthianum*, respectively. The encoded protein sequence of the PG gene from *C. lupini* SHK 2148 contained all four conserved regions reported for PGs. The cDNA copy of the PG gene confirmed the predicted intron position and splice sites and *in vitro* expression studies revealed that the gene was expressed after 3, 4, 5, and 7 days after inoculation in the pectin media.

3.1 Introduction

Endopolygalacturonases (EndoPGs) are ubiquitous in plants, fungi, bacteria and insects (Markovič *et al*, 2001). In these organisms they have different functions of which many have not been resolved yet. Therefore, several PG genes have been isolated and characterized to elucidate their biological function (Markovič *et al*, 2001). Characterizations of EndoPG genes from various organisms have shown that several residues are conserved amongst all PGs, while other residues are only conserved in a species relationship (Markovič *et al*, 2001).

Endopolygalacturonases are one of the first of a series of cell wall degrading enzymes (CWDEs) that are produced in fungal pathogens when the pathogen contacts the host cell wall (Albersheim and Anderson, 1971; Collmer and Keen, 1986; Cooper *et al*, 1981; Esquerré-Tugayé *et al*, 2000). Fungal EndoPGs along with some other CWDEs, are being studied for their role as pathogenicity factors. However, while several studies have addressed this issue; only pectic enzymes appear to be key role players as pathogenicity factors (reviewed by D'Ovidio *et al*, 2004). One of the aspects of EndoPGs as pathogenicity factors that has been studied, is their interaction with plant polygalacturonase inhibiting proteins (PGIPs). Plant PGIPs are leucince rich proteins that are associated with plant cell walls (De Lorenzo *et al*, 2001; De Lorenzo *et al*, 2001). PGIPs are thought to be part of the plant's defense response against pathogens, since they inhibit pathogen EndoPGs that are produced to degrade the host cell wall. Studies investigating the specific interactions between PGIPs and PGs have indicated that certain domains and residues present in both play an important role in their interaction (Federici *et al*, 2001; Di Matteo *et al*, 2003; Leckie *et al*, 2003).

Several EndoPGs have been cloned and characterized from fungi. In fungi, EndoPGs are present as gene families of various sizes, ranging from as little as one family member in *Colletotorichum gloeosporioides* f.sp. *malvae*, two members in *C. lindemuthianum* (Centis *et al*, 1996 and 1997), six members in *Botrytis cinerea* (Ten Have *et al*, 1998; Ten Have, 2000) and up to seven family members in *Aspergillus niger* (Bussink *et al*, 1990 and 1992; Pařenicová *et al*, 2000 a, b). Most of

these EndoPG genes display characteristics common to filamentous fungal genes as described by Gurr *et al* (1987).

Lupin, an important crop in the animal feed, the flower and numerous other industries, are threatened by lupin anthracnose, which is considered to be the most devastating disease of lupin world-wide. Lupin anthracnose is caused by *Colletotrichum* and has been recorded in several countries including South Africa (Koch, 1996).

The aim of this study was to first establish whether *Colletotrichum lupini* isolates obtained from lupin plants with anthracnose symptoms had PG activity. The presence of PG activity would suggest that the *Colletotrichum* isolates also contain the genes encoding these enzymes. After establishing PG activity a putative EndoPG gene was cloned from one of the *Collectotrichum* isolates. The expression of the mRNA EndoPG copy was investigated by northern and reverse transcriptase polymerase chain reaction (RT-PCR) analyses.

3.2 Materials and Methods

3.2.1 Determining PG activity

C. lupini SHK 2148 was inoculated into Czapek Dox medium (Difco, Detroit, USA) and grown for a period of 7 days. The mycelia was harvested; resuspended in pectin containing media (Appendix A), whereafter 2.5ml of the suspension was inoculated into 25ml of similar pectin-containing media. This was performed in three replicates. Mycelia and supernatant were collected on day 3, 4, 5, 6, 7 and 12 after inoculation by vacuum filtration. Protein was precipitated from the filtrate with ammonium sulphate at a final concentration of 80%. Precipitation proceeded overnight followed by a 30 minute centrifugation step at 4 °C. The pellet was resuspended in 40mM Sodium-acetate pH 5 (1/10 of the original volume). Protein concentrations were determined for a dilution series of each day of each replicate using the Bradford assay method, which is based on the binding of an acidic dye, to the basic and aromatic amino acid residues of the protein, which absorbancy is measured at 595nm. BSA was used as a standard to construct a BSA curve to facilitate protein concentration determination (Appendix C). PG activity was evaluated with the agarose diffusion assay (ADA) (Taylor and Secor, 1988). The medium consisted of 100mM citrate, 200mM Na_2HPO_4 (pH 5.3), 0.5% ammonium oxalate, 0.01% polygalacturonic acid (PGA) and 1% molecular grade agarose. The mixture was dissolved in a microwave, whereafter 20ml aliquots were poured into petri dishes (9cm diam.). 30µl of precipitated protein sample from each day of each replicate was pipetted into wells (punched with a #1 cork borer) in the ADA plates. The plates were incubated overnight at 25 °C, stained with 0.05% Ruthenium red (Sigma) for 1h at 37 °C whereafter the zone diameters were determined. The PG activity of each day was expressed as zone diameter/ng protein.

3.2.2 Genomic DNA extraction

Isolation of genomic DNA was performed according to the method of Raeder and Broda (1985). Mycelium-covered agar blocks were transferred to 250ml visualized flasks containing 25ml Czapek-Dox media amended with 50mg/ml Streptomycin and 50mg/ml Chloramphenicol. The flasks were shake-incubated for approximately 7

days at 25 °C and 150 rpm. Mycelium was collected on Whatman qualitative filter paper (# 1) and frozen in liquid nitrogen. Frozen mycelia were ground to a powder and transferred to 15ml PP-Tubes (Greiner Labortechnik, Germany) to which 10µl/mg genomic DNA extraction buffer (Appendix A) was added. An equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added to the suspension and mixed. The phases were separated by centrifuging for approximately 40 minutes at 10 000 rpm. The upper aqueous phase was transferred to a new sterile Polypropylene-Tube to which 20 mg/ml RNAseA (Roche Diagnostics, Mannheim, Germany) was added and tubes incubated for 10min at 37 °C. Two chloroform extractions were performed, whereafter the phases were separated during a centrifugation period of 10min at 10 000 rpm. The clear upper phase was transferred to a new Polypropylene –tube in which DNA was precipitated with 0.54 volume of isopropanol and pelleted by centrifuging for 10 min at 10 000 rpm. The pellet was washed with 70% Ethanol, centrifuged for 5 min, air-dried and resuspended in 100µl TE, pH 8. The concentration of the DNA samples was determined with a Hoefer® DyNA Quant ® 200 Fluorometer (Hoefer, Germany). The fluorometer was calibrated with 1× TNE buffer, pH 7.4 (Appendix A) containing 1µg/ml Hoechst 33258 DNA binding buffer, 100ng/µl Calf thymus DNA (Sigma) was included as a DNA standard. Alternatively, concentrations were determined using agarose gel-electrophoresis with a λ DNA standard.

3.2.3 Cloning an internal fragment of a *Colletotrichum* PG gene

PCR amplification on the internal PG gene fragment

PCR reactions were performed in a final PCR volume of 50µl, using Biotaq (1.25U/µl) (Bioline, Germany), 1.5mM $MgCl_2$, 1× PCR reaction Buffer, dNTPs (200µM of each), DNA template (30ng) and two PCR primer pairs (information of primers presented in Table 3.1); PCR primer set1 containing PG-COLL-F1 and PG-COLL-R and PCR primer set 2 containing PG-COLL-F2 and PG-COLL-R, at a concentration of 0.2µM each. PCR reactions were performed with genomic DNA from all three *Collectotrichum* isolates as DNA template. The PCR cycle were performed as previousy described (Chapter 2, section 2.2.4). PCR products were

separated on an EtBr bromide containing 1% (w/v) agarose gel (TAE) together with a λ- Pst generated molecular marker. Bands were visualized on a UV transimmulator. Where multiple bands were present, the desired band was excised and gel purified with the Gene Clean kit from Southern Cross Biotechnology, and single PCR bands were purified using the Qiagen PCR purification kit (Qiagen, Germany). The concentration of the purified samples was determined via agarose gel electrophoresis on a 1% (w/v) agarose EtBr containing gel with a λ DNA standard.

Table 3.1 Information of primers used in this chapter.

Primer	Sequence	Annealing Temp
PG-COLL-F1 PG-COLL-F2 PG-COLL-R	5' CGC GCC AGC TGC ACC TTC 3' 5' CGC GCC TCG TGC ACC TTC 3' 5' GCT GAC GCC GGA CCA CTT CCA 3'	55 °C
InversePCR_FII InversePCR_RII	5' TCCATCGGTTCCGTTGGTGGAACGTAG 3' 5' AGGTCTTCTTGGACATGGCCGTCTTG 3'	58 °C
C. lupini_Compl F C. lupini_Compl R	5' CCACGTGTTGATCACATACC 3' 5' CCCACCATCAAGCATTTAGC 3'	55 °C
C. lupini_Compl FII C. lupini_Compl RII	5' CGATGAAGTTCCTCTCCGTTGTC 3' 5' TTTAGCACTTGGCACCGGAGCCGGAAGG 3'	65 °C
C. lupini_Int F I C. lupini_Int R I	5' CCTTCGGCTACAAGGAGT 3' 5' AACGGAACCGATGGACAG 3'	55 °C
T7 SP6	5' TAATACGACTCACTATAGGG 3' 5' ATTTAGGTGACACTATAG 3'	55 °C
M13 R	5' TGAGCGGATAACAAT TTCACA CAG 3'	55 °C

Cloning and sequencing of the internal PG fragments

The purified putative internal PG fragments, obtained by PCR amplification of genomic DNA from the three *Colletotrichum lupini* islolates, SHK 788, SHK 1033 and SHK 2148, were ligated separately into pGEM-T-Easy (Promega, Madison,USA), in a 15µl reaction volume containing 1µl of T4 ligase (1U/ µl) (Roche), 40ng – 50ng of insert and 50ng pGEM-T-Easy, to construct pPGint788, pPGint1033 and pPGint2148 respectively. Ligations were incubated overnight at 5 °C. A positive control (50ng pGEM-T-Easy vector with 8ng control insert, supplied by the manufacturer) a background control (50ng pGEM-T Easy vector) as well as 50ng of a pUC plasmid were included in the transformation reactions. Competent *E. coli* JM 109 cells (Promega) were prepared according to the $CaCl_2$ method (Dugert *et al*

Ehrlich, 1974; Seideman *et al*, 1997) and transformed with pUC-vector, positive control, the background control as well as with the vector constructs containing the putative internal PG fragments (half of the volume of each ligation mix was used), by heat shocking cells for 40 sec. at 42 °C. Cells were incubated in LB for 1h at 37 °C. Transformants were plated onto Luria agar plates containing 100mg/ml Ampicillin (Sigma) as well as 40µl of 100mM IPTG and 40 µl X-gal. The plates were incubated overnight at 37 °C. Positive colonies (white) were inoculated into 5ml LB broth with 100mg/ml ampicillin, grown for 16h, whereafter plasmid DNA was isolated from the clones according to the method of Sambrook *et al* (1989). The plasmid constructs were digested in a reaction volume of 10µl using 1µl *EcoRI* (10U/µl) and 1× restriction buffer for 1h at 37 °C. The result of the restriction reaction was visualized on a 1% (w/v) TAE agarose gel.

Positive plasmid clones as well as PCR products that were not cloned, were sequenced using the PCR BigDye Terminator v 3.1 cycle sequencing kit (Applied Biosystems) and 10µM each of the universal primers T7 and SP6 (Table 3.1). All sequence reactions were performed in a 10 µl reaction volume, containing 4 µl Big Dye v 3.1, 1µl primer (10µM) and DNA template (50 –100ng). The PCR sequencing cycles consisted of a denaturation step at 96 °C for 1min., followed by 25 cycles consisting of three steps; 96°C for 10 sec., and annealing step carried out at 50°C for 5 sec. and an elongation step at 60°C for 4 min. The sequencing reactions were purified in 0.5ml Eppendorf tubes by adding 2µl NaOAc (3M, pH 4.6) and 50µl ice cold Ethanol (96%) to the sequencing mixture and centrifuging it at maximum speed for 30 minutes. The pellet was washed twice with 250µl Ethanol (70%) and air-dried. The sequencing reactions were performed in an ABI prism 3000 sequencer (ABI Advanced Biotechnological Institute, Perkin-Elmer Corporation, Foster City, USA).

3.2.4 Southern blot analysis of *Colletotrichum lupini* SHK 2148

Probe and marker labeling and quantification

The cloned internal PG fragment of SHK 788 was used for labeling with DIG, and subsequent Southern analysis. The PG fragment was first excised from pPGint788 by restriction digestion of the plasmid with 10U *EcoRI* (1µl) in 1× reaction buffer in a total a reaction volume of 25µl. The digestions were carried out at 37 °C for 1h. In total approximately 1.3ug of pPGint788 were restriction digested and run on a 1% (w/v) TAE agarose gel. The 950 bp internal PG fragments were excised from the gel and purified using the Qiagen gel purification kit. The concentration of the gel-purified sample was determined via electrophoresis with a λ DNA standard as well as the Fluorometer as previously described. For the labeling reaction approximately 360ng excised purified internal PG gene fragment and 1µg of lambda *PstI* marker were heat denatured, for 10 minutes, snap frozen on ice, whereafter 4µl of the DIG-High prime was added (DIG High Prime DNA Labeling and Detection Starter Kit I, Roche Biochemicals). The probe and marker mix were incubated overnight at 37 °C. The reaction was terminated with 2µl EDTA (0.2M, pH 8.0).

The quantity of labeled probe and molecular marker was determined by the spot test. A series of dilutions, of known DIG labeled concentrations (DIG High Prime DNA Labeling and Detection Starter Kit I, Roche Biochemicals), were prepared and 1µl of each dilution was spotted onto a nitrocellulose membrane (Hybond, Amersham Biosciences, Buckinghamshire, UK) together with 1µl of a dilution series of the samples. The DNA was fixed onto the membrane via UV-cross linking. The membrane was washed in maleic acid buffer (Appendix A) for 2min at room temperature and transferred into blocking solution (Elite 2% fat-free milk powder, added to 50ml Maleic acid buffer) for 30 min at room temperature. A 1:5000 dilution of anti-DIG-alkaline phosphatase antibiotics (Roche) was added to the blocking solution and the membrane was incubated for 30 min at room temperature. The membrane was washed twice for 15 min with washing buffer (Appendix A) and incubated for 2 minutes in detection buffer (Appendix A). The color development proceeded in the dark after the detection buffer was replaced with 10ml fresh detection buffer containing 45µl NBT and 35µl BCIP. Sterile distilled water was

added to terminate the reaction. Spot intensities were compared to determine the probe and marker concentrations.

Southern membrane preparation

Approximately 3.6µg of genomic DNA from isolate *C. lupini* SHK 2148 were each digested with 2µl *AvaI* (Roche, 5U/µl), *BamHI* (Roche, 10U/µl), *EcoRI* (Roche, 10U/µl), *EcoRV* (Roche, 10U/µl) and *HindIII* (Roche, 10U/µl) in separate reactions. The restricted DNA was separated on a 1% TAE agarose gel together with an aliquot (100ng) of the pPGint788 internal fragment construct to serve as a positive control. A non-labeled λ Pst marker was included on the gel. The gel was visualized under a UV transilluminator whereafter it was subjected to two 15 minute denaturing steps with 1.5M NaCl and 0.5M NaOH buffer, followed by two 15 minute neutralization washes with 1M Tris (pH 7.8), 1.5M NaCl. The DNA was fixed to the membrane on a UV transilluminator (Vacutec) for 5minutes.

Prehybridisation, hybridisation and detection of Southern membrane

The blot was set up according to the method in Current protocols, first described by Southern (1975). The membrane was prehybridised for 3-4 h in a 50% formamide buffer (Appendix A). Subsequently, the membrane was visualized overnight at 42 °C with the labeled probe (10ng/µl) in a LASEC hyb-oven. Following hybridization, the membrane was subjected to two stringency washes for 5 minutes each with a 2 × wash solution (2 X SSC/0.1% SDS) at room temperature and three washes for 15minutes with a 0.5 × wash solution (0.5 X SSC/0.1% SDS) at 65 °C. The membrane was rinsed in a wash buffer (Appendix A) and subjected to the blocking buffer (Maleic acid buffer containing 2% fat-free milk powder) for 45min at room temperature. The antibody was added to the blocking buffer in a dilution ratio of 1: 20 000 and allowed to interact with the hybridized probe during an incubation period of 30 minutes at room temperature. The unbound antibody was removed by washing the membrane twice for 15 minutes with a washing buffer (Appendix A). The membrane was equilibrated with the detection buffer (Appendix A) for 2 minutes whereafter the color development step proceeded with the NBT/ BCIP solution (200µl/10ml Detection

buffer) in the dark for 60 hours. The reaction was terminated by the addition of distilled water.

3.2.5 Resolving the complete PG gene sequence through inverse PCR

The complete PG gene sequence was obtained through inverse PCR. A schematic representation of the inverse PCR method using genomic DNA is presented in Fig. 3.1. The inverse PCR method was done using genomic DNA of *C. lupini* isolate SHK 2148, as well as pPGint778 that was used as a positive control. The steps used in the inverse PCR method for genomic DNA and the positive plasmid control is summarized in Table 3.2.

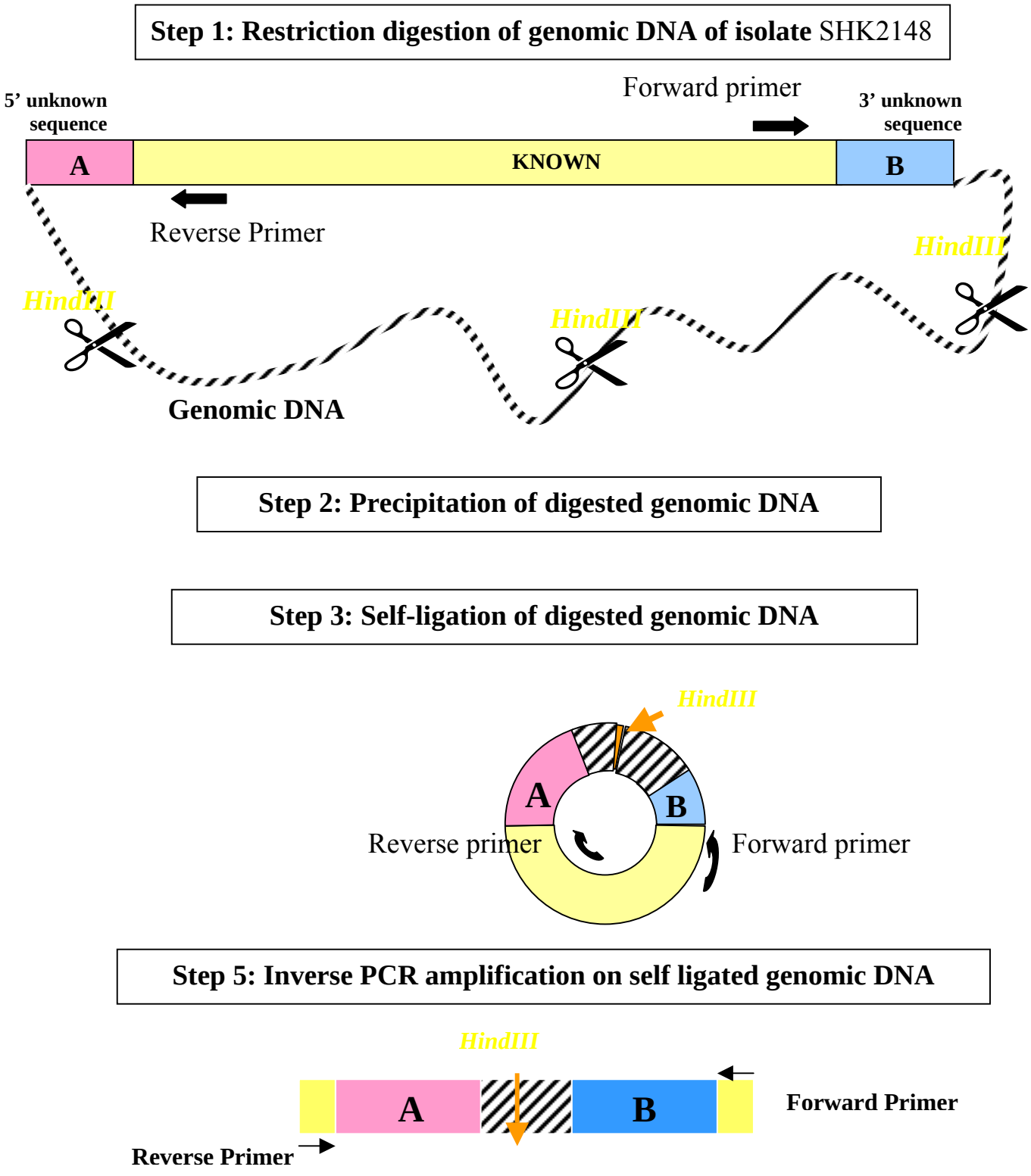


Fig. 3. 1 Schematic presentation of the inverse PCR method used for isolation of a full-length genomic PG copy from *C. lupini* SHK2148.

Table 3.2 Steps used in the inverse PCR method for isolation of the full-length genomic PG copy from *C. lupini* SHK 2148.

	Positive Control	Genomic DNA
Source	pPGint788	Genomic DNA isolated from SHK2148
Inverse PCR step1	Restrict construct with *SalI*	Restrict genomic DNA with *AvaI*, *HindIII*, *SacI* or *ClaI*
Inverse PCR step2	Precipitation of restricted DNA construct	Precipitation of DNA fractions
Inverse PCR step3	Self ligation of vector construct	Self ligation of DNA fragments
Inverse PCR step4	Linearise construct, using restriction enzyme with unique cutting site in the PG gene fragment (*BstEII*)	Bypassed this step, proceeded with the inverse PCR reaction
Inverse PCR step5	Proceed with Inverse PCR	Proceed with inverse PCR

Restriction digestion and religation of inverse PCR positive control

The vector pPGint788, was used as positive control during the inverse PCR reaction and subjected to all the steps of an inverse PCR reaction (Table 3.2). At first, an aliquot of 120ng of the positive control was restricted with 2.5µl *SalI*, which cuts outside the internal PG fragment to linearise the construct. The restriction was confirmed with agarose gel electrophoresis on a 1% (w/v) TAE agarose gel with EtBr. The restricted construct was subjected to the same clean-up procedure used for the genomic DNA samples as described by Arendse *et al.* (1999). The linearised construct (40ng) was religated overnight, using T4 Ligase (1U/µl) and an aliquot was used in the inverse PCR reaction. The rest of the religated sample was restricted with 2.5µl *BstEII*, which restricts the PG fragment only once to linearise the construct for the inverse PCR reaction. The pPGint788 positive controls were used as templates in the inverse PCR; the uncut pPGint788 template (12ng), pPGint788 *SalI* linearised and relegited (15ng) and pPGint788 *SalI* linearised, religated and *BstEII* linearised (25ng).

Restriction digestion and religation of *C. lupini* SHK 2148 genomic DNA

Genomic DNA extraction and concentration determination were performed as previously described. Approximately 10µg of genomic DNA from *C. lupini* SHK 2148 were restriction digested in a 110µl reaction volume with 1× restriction buffer

using 5 – 10U of the following enzymes that had no restriction sites in the internal PG fragment: *AvaI*, *BamHI*, *HindIII*, *SalI*, *SacI*, *EcoRV*, *ClaI* and *BssHII*. Restriction reactions were carried out at 37 °C for 1h. Aliquots of the restricted DNA samples were evaluated on a 1% agarose gel to determine if the restrictions were successful. Digested DNA samples were precipitated by using 3 M Sodium acetate (1/10 volume) and 2.5 volumes absolute Ethanol (as performed by Arendse *et al*, 1999). Restricted fragments were religated overnight, at 5 °C in a 50µl volume, using 1U of T4 DNA ligase (Roche).

Inverse PCR components and parameters

The inverse PCR reaction was set up with primers InversePCR_FII (0.2µM) InversePCR_RII (0.2µM) (Table 3.1), dNTPs (0.2mM), $mgCl_2$ (1.5mM), PCR buffer (1×) and 0.3µl Biotaq in a total volume of 50µl. Aliquots of the ligation mixes were used as template, the concentration of the DNA used in each PCR varied and were as follow:

AvaI Inverse PCR	4.8ng/µl
BamHI Inverse PCR	4.1ng/µl
ClaI Inverse PCR	13ng/µl
EcoRV Inverse PCR	2.9ng/µl
BssHI Inverse PCR	1.7ng/µl
HindIII Inverse PCR	4.4ng/µl
SalI Inverse PCR	4.8ng/µl
SacI Inverse PCR	0.7ng/µl

The inverse PCR cycle consisted of an initial denaturation step of 96 °C for 2min., followed by 25 cycles that each contained a denaturation step, 94 °C for 20 sec., an annealing step, 65 °C for 45 sec. and an elongation step of 72 °C for 45 sec. The final PCR cycle was completed with two hold steps, one at 4 °C and another final hold step at 25 °C. A small aliquot of the PCR reactions were visualized on a 1% agarose gel. PCR DNA bands were excised from the agarose gel and gel purified using the Qiagen gel purification kit. Purified fragments (50ng) were ligated overnight at 5 °C into

pGEM-T-Easy (50ng) using 1U of T4 ligase (Roche) in a 10µl reaction volume. An aliquot (2µl) of each of the ligation mixtures as well as 10ng pPGint788 were electroporated into *E. coli* DH10β in a BIORAD electroporator at 1.8kV, 25m Faradys and 200Ω. Transformants were selected as previously described.

Screening of positive transformants

Positive transformants were inoculated into 5ml LB containing 100mg/ml ampicillin and incubated with shaking overnight at 37 °C. Plasmid DNA was isolated as described previously. An aliquot of 28ng of the plasmid sample was restricted with *EcoRI* (1µl) in a 10µl reaction volume to verify the presence of an inverse PCR product. Uncut plasmid as well as the restriction reaction was electrophoresed on a 0.8% agarose gel. The cloned inverse PCR fragment was sequenced with T7, SP6 and M13 primers (10µM each, Table 3.1) using the Big Dye system as described before.

3.2.6 Isolation and characterization of the complete PG gene using sequence data obtained from inverse PCR analyses

The inverse PCR data were used to compile a composite PG gene sequence. This sequence was used to design primers for the isolation of the complete PG gene from the genome of isolate *C. lupini* SHK 2148 using 10ng of template together with primer pair, C. lupini_Compl F and C. lupini_Compl R (Table 3.1) at a final concentration of 1µM, 1.5mM $mgCl_2$, 0.2mM dNTPs and 0.2µl Biotaq. The cycle included a 94 °C denaturation step for 2 minutes, followed by a three-step cycle, consisting of 94 °C for 20 sec., 55 °C for 45 sec. and 72 °C for 45 sec., this cycle was repeated thirty-three times. A final elongation step at 72 °C for 4 minutes completed the PCR step. 10µl of each reaction were electrophoresed on an EtBr containing, 1% (w/v) TAE agarose gel. The PCR bands were excised and gel purified as described previously. The purified fragment's concentration was determined whereafter it was cloned into pGEM-T-Easy as previously described using approximately 50ng of purified insert, 50ng pGEM-T-Easy and 1µl T4 ligase (Roche, 1U/µl). The ligation reaction proceeded overnight at 5 °C. A third of the ligation solution was chemically transformed into DH5α competent cells as well as a pUC positive control (300ng) as previously described. Positive colonies were inoculated into 5ml Luria broth with

100mg/ml Ampicillin, plasmid isolations were performed after 18h of incubation using the Qiagen plasmid isolation kit. The plasmid constructs (3µl) were restricted with *EcoRI* (1µl) for 1h at 37 °C to verify the presence of an insert with the correct size. Positive clones were sequenced with T7 and Sp6 primers as described before.

3.2.7 RNA extractions

RNA was extracted from mycelia grown in pectin media for 12 days using the TRI reagent (Chomczynski *et al*, 1987) (Sigma). Approximately 50mg of fungal tissue, from day days 3, 4, 5, 6, 7 and 12 after the inoculation in the pectin media (described previously), was crushed in liquid nitrogen. TRI reagent was added to the fungal starting material (1ml per 50mg of tissue), mixed and the homogenate was centrifuged for 10 minutes, 12 000 × g at 4 °C. The clear supernatant was transferred to a sterile 15ml falcon tube (Greiner Bio-One); left for 5 minutes at room temperature whereafter chloroform was added (0.2ml/ml TRI reagent used). The samples were mixed vigorously for 15 seconds and left at room temperature for 10 minutes, samples were then centrifuged for 15 minutes at 4 °C and 12 000 × g. The resulting upper phase was transferred to a sterile falcon tube to which isopropanol (0.5ml per ml of TRI reagent used) was added. The samples were mixed and left at room temperature for 8 minutes. The RNA was precipitated by a centrifugation step of 10 minutes at 12 000 × g and 4 °C. The pellet was washed with 75% ethanol (1ml per 1ml TRI reagent used) vortexed and centrifuged for 5 minutes at 4 °C and 7500 × g. The washed RNA pellet was briefly air dried for 5 minutes and resuspended in RNAse free water. The concentration of the RNA samples was determined with a photometer (Eppendorf BioPhotometer).

3.2.8 Northern Blot analysis

Probe labeling

The complete PG gene was PCR amplified from plasmid pPGcompl1 using primers C. lupini_Compl F II and C. lupini_Compl R II (Table 3.1) (20µM of each). The PCR product was cleaned up using the Qiagen PCR clean-up kit according to manufacturer's instructions. The purified PCR product was used as a probe for the

Northern blot analysis on RNA isolated from mycelia of isolate *C. lupini* SHK 2148 grown for 12 days in pectin media. The mycelia were harvested on days 3, 4, 5,6,7 and 12 after inoculation. The probe was labelled with the HexaLabel Plus DNA labeling Kit (Fermentas Inc. 7520 Connelley drive, Unit A, Hanover, MP 21076, USA); approximately 100ng of probe was mixed with the hexanucleotide 5× reaction buffer and Millipore water in a reaction volume of 40µl. The mixture was incubated for 10 minutes in a boiling water bath and cooled on ice whereafter 4µl [α^{-32} P] labeled dATPs and 5U of Klenow fragment were added and the reaction was incubated at 37 °C for 60 minutes. The unincorporated labelled nucleotides were removed by eluting the solution through a PCR clean-up column (Qiagen) in a final volume of 50µl Millipore water using manufacturer's instructions.

Northern gel preparation and blotting

A 1.2% agarose formaldehyde (FA) gel (Appendix A) was prepared. 20µg of RNA, isolated from mycelia grown for 3, 4, 5, 6, 7 12 days in pectin containing media, was resuspended in 1 × RNA loading buffer (Appendix A), denatured at 65 °C for 10 minutes and snap frozen. RNA concentrations were determined with a spectrophotometer. The denatured RNA samples were loaded on the formaldehyde gel and run in 1 × FA gel running buffer (Appendix A) at 120V until the loading dye front was two thirds from the top of the gel. After electrophoresis, the gel was removed and washed three times for 10 minutes in DEPC treated water. The gel was then washed in 10 × SSC, pH 7 (DEPC prepared) for 45 minutes. Subsequently, RNA on the gel was transferred overnight onto a Hybond nitrocellulose membrane (Amersham) in 10 × SSC. After transfer the membrane was rinsed in 2 × SSC and cross-linked in the UV cross linker (Whitehead Scientific, Brakenfell, Cape Town, South Africa) at 0.167 joules.

Prehybridisation, hybridization and detection

The Northern membrane was prehybridised in 10ml HYB-9 hybridisation solution (Gentra system) at 65 °C for 10 minutes in the TECHNE Hybridiser HB-1D hybridization chamber hybridization oven. Salmon sperm (Sigma) was denatured for

5 minutes and immediately transferred to ice and added to the prehyb. solution to a final concentration of 100µg/ml. The prehybridisation proceeded for another hour. The probe was denatured, immediately cooled on ice and added to the prehyb. solution. The hybridisation continued overnight at 65 °C. The membrane was first washed for 20 minutes in a 2 × SSC/ 0.1% SDS solution, then for 10 minutes in a 1 × SSC/ 0.1% SDS solution and finally for 10 minutes in a 0.1× SSC/ 0.1% SDS solution. The membrane was exposed to an intensifying screen and X-ray film (Hyperfilm, Amersham Biosciences, Buckingshire, England) at –80 °C in an X-ray cassette (Amersham) for three weeks.

3.2.9 RT-PCR amplification

RNA was isolated from day 4 after inoculation, using the Qiagen RNeasy kit. From the total RNA, mRNA was isolated with the Oligotext mRNA kit (Qiagen) according to the manufacturers guidelines. Isolated mRNA samples from day 4 were used for cDNA synthesis using an M-MLV Reverse Transcriptase kit (Sigma); 1µg of mRNA was used with 10mM dNTP mix and 1µl oligo (dT) primer. The mixture was incubated at 70 °C for 10 min. and immediately cooled on ice. M-MLV Reverse Transcriptase buffer (1×), 1µl M-MLV Reverse Transcriptase (200U/µl) and 0.5µl RNAse inibitor (40U/µl) were added to the mixture. The sample was incubated at room temperature for 10min prior to the cDNA synthesis step at 37 °C for 50 min. The M-MLV Reverse Transcriptase was inactivated at 80 °C for 10 min. A PCR reaction was set up using primer set C. lupini_Compl F I and C. lupini_Compl R I (20µM of each), 10mM $MgCl_2$, 10mM dNTPs and 0.5µl Biotaq (25U/µl) with 10ng genomic DNA and 1ng pPGcompl1 as positive controls as well as 10ng cDNA template. The PCR reaction was initiated with a denaturing step at 95 °C for 5 minutes, followed by a 30 cycle repeat of a 94 °C for 1 minute, 60 °C for 1 minute and 72 °C elongation of 2 minutes steps. The cycle was finalized with an elongation step at 72 °C for 5 minutes. The result of the PCR reaction was evaluated on a 2% TAE agarose gel containing EtBr. The bands obtained were excised and gel-purified with the gel purification kit from Qiagen. The purified fragments were subjected to concentration determination and 10ng were ligated to 12.5ng pGEM-T-Easy vector

using 1U T4 ligase (Roche). The ligation mixes were transformed into *E.coli* JM 109 as described previously and selected on LB agar plates containing 100mg/ml Ampicillin (Sigma) as well as 40µl of 100mM IPTG and 40µl X-gal. Colony PCR was performed on 5 clones from each ligation mix using the PCR parameters as previously described with the primer sets C. lupini_Compl F I and C. lupini_Compl R I as well as C. lupini_Int F I and C. lupini_Int R I (20µM of each). Clones containing the desired fragments were subjected to restriction enzyme analysis with 1µl *EcoRI*, 3µl plasmid preparation in a final volume of 10µl at 37 °C for 1 hour. The restriction digestion was evaluated on an EtBr containing 2% TAE agarose gel together with *EcoRI* digested pPGcompl1. Sequence analysis was also performed on selected clones using the universal primer set T7 and SP6 as previously described.

3.3 Results

3.3.1 Polygalacturonase (PG) activity

Culture filtrates of *C. lupini* SHK 2148 grown in pectin media over time produced clear zones in the Agarose diffusion assay (Fig. 3.2). The zones indicated the presence of PG specific activity, which was expressed as zone diameter/ng protein (Fig. 3.3). The PG activity was at its highest on day 3 and 4. The activity decreased for days 5, 6, 7 and 12, however it seems that some basal level of PG activity might be present indicating either a constitutive expression of one or more PGs or different PGs being activated subsequently providing constitutive PG activity.

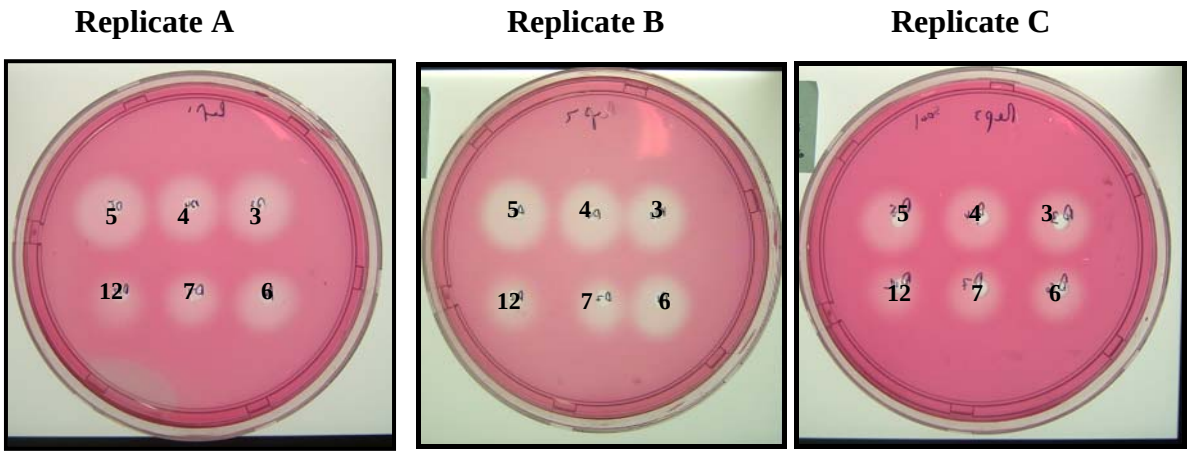


Fig. 3.2 Agarose diffusion assay displaying the PG activity obtained for three replicates A, B and C. The zones indicate PG activity (numbers within zones indicate the number of days that the isolates were grown in the pectin media).

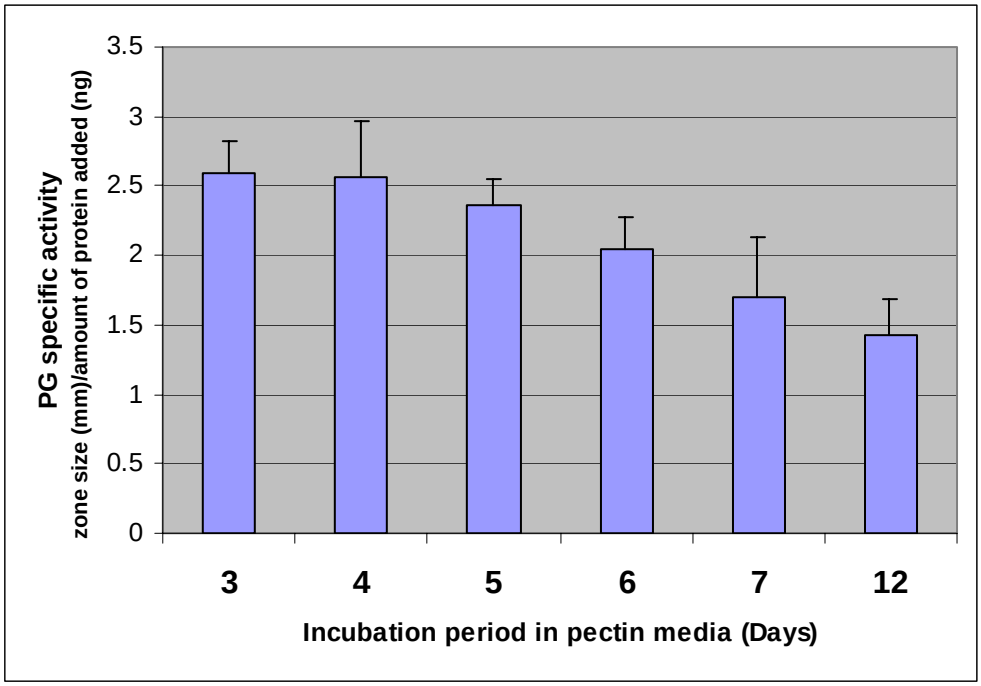


Fig. 3.3 PG specific activity of *C. lupini* SHK 2148, grown in pectin media, expressed as zone size/ amount of protein added. The PG specific activity was initially very high and decreased as the growth period was prolonged.

3.3.2 Genomic DNA Extraction

Genomic DNA was extracted from *Colletotrichum* isolates SHK788, SHK1033 and SHK2148. The concentration of the DNA samples from isolates SHK 788, 1033 and 2148 were 193ng/µl, 78ng/µl and 970ng/µl respectively, according to Fluorometer analyses.

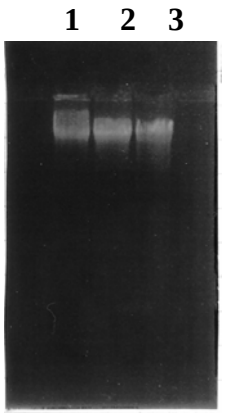


Fig. 3.4 Genomic DNA isolated from *Colletotrichum* strains. Genomic DNA isolated from *Colletotrichum* SHK 788 (lane 1), *Colletotrichum* SHK 1033 (lane 2) and *C. lupini* SHK 2148 (lane 3) was evaluated on a 1% TAE agarose gel

(approximately 5 µg of genomic DNA, isolated from *Colletotrichum* was loaded per lane).

3.3.3 PCR amplification of an internal fragment of the PG gene

PCR primers were designed from alignments of three published *Collectotrichum* PG gene sequences. The PG sequences of *Colletotrichum lindimuthianium clpg1*, *clpg2* and *Colletotrichum gloeosporioides* f. sp *Malvae cgmpg2***,** accession numbers X89370, X95457 and AJ291494 respectively, were downloaded from Genbank. The coding sequences of the PGs were translated and aligned with each other (Fig. 3.5). Three PCR primers, two forward (PG-COLL-F1 and PG-COLL-R) and one reverse primer (PG-COLL-R) (Table 3.1), were designed from conserved regions, RASCTF and WKWSGVS, within the alignment. Subsequently, these primers were for used for PCR amplification of the internal PG gene fragment from three *Colletotrichum* isolates (Fig. 3.6).

The two PCR primer sets both yielded PCR products. Internal PCR primer set 1, consisting of PG-COLL-F1 and PG-COLL-RV, resulted in non-specific amplification, while internal PCR primer set 2, PG-COLL-F2 and PG-COLL-RV, produced a single PCR fragment in all three *Colletotrichum* isolates (Fig. 3.6). The sizes of the internal PG fragments were approximately 950 bp.

Fig. 3.5 Amino acid sequence alignment of the PG genes from *C. gloeosporioides* f.sp. *malvae* (*cmpgII*) and the two PG genes from *C. lindemuthianum* (*clpgII* and *clpgI*). The blocked sequences indicate the conserved region from which the primers were designed. (The DNA sequences of *CmpgII* and *clpgII* are indicated underneath the aminoacid sequence). PG-COLL-R was designed from the *clpgII* sequence.

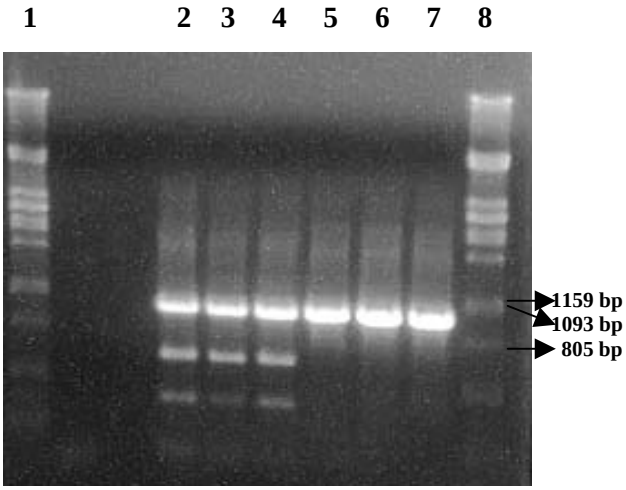


Fig. 3.6 PCR amplification of the internal PG gene fragment from *C. lupini* isolates SHK 788, SHK 1033 and SHK 2148.
Internal PG fragments isolated via PCR with internal PCR primer set 1 for isolates SHK 788, 1033 and 2148 (lanes 2-4 respectively) and the PCR fragments obtained with internal PCR primer set 2 for isolates SHK 788, 1033 and 2148 (lanes 5 –7 respectively). Lane 1 and 8 represents the λ Pst molecular marker.

The 950 bp PCR products obtained from amplifications with internal PCR primer set 2 (Fig 3.6 lanes 5,6 and 7) were successfully gel purified (Fig. 3.7).

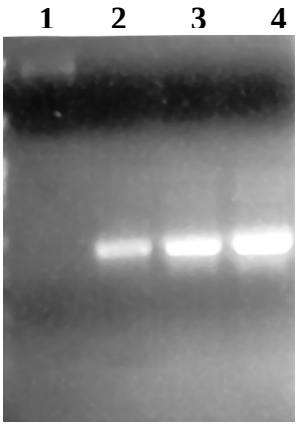


Fig. 3.7 Gel purified internal PG PCR fragments.
The purified PG internal fragment 788, (6 µl), PG internal fragment 1033 (8 µl) and PG internal fragment 2148 ((8 µl) were evaluated on a 1% TAE agarose gel (lane 2,3 and 4 respectively) to determine the quality and quantity with a λ standard 100ng (lane 1).

Ligation of the gel purified PG PCR fragments, PG internal fragment 788, PG internal fragment 1033 and PG internal fragment 2148 (Fig. 3.7) into the pGEM-T Easy vector, followed by transformation of *E. coli* with the ligation mixes, yielded two white colonies each for pPGint788 (pPGint788-1 and pPGint788-2) and pPGint2148 (pPGint2148-1 and pPGint2148-2) (Fig. 3.8) and no white colonies for pPGint1033. No colonies were observed in the background control transformation. Transformation with pUC gave a transformation result of 3.8×10^{7} cfu/ug DNA. Even though the transformation efficiency for pUC was high, only a few transformants (21 colonies) were obtained for the positive control (constructed according to the manufacturers guidelines). The low transformation efficiencies might not be a result of competency as the pUC transformation showed, but rather due to an inefficient ligation reaction, since the pUC vector was not subjected to a ligation reaction, while the others were.

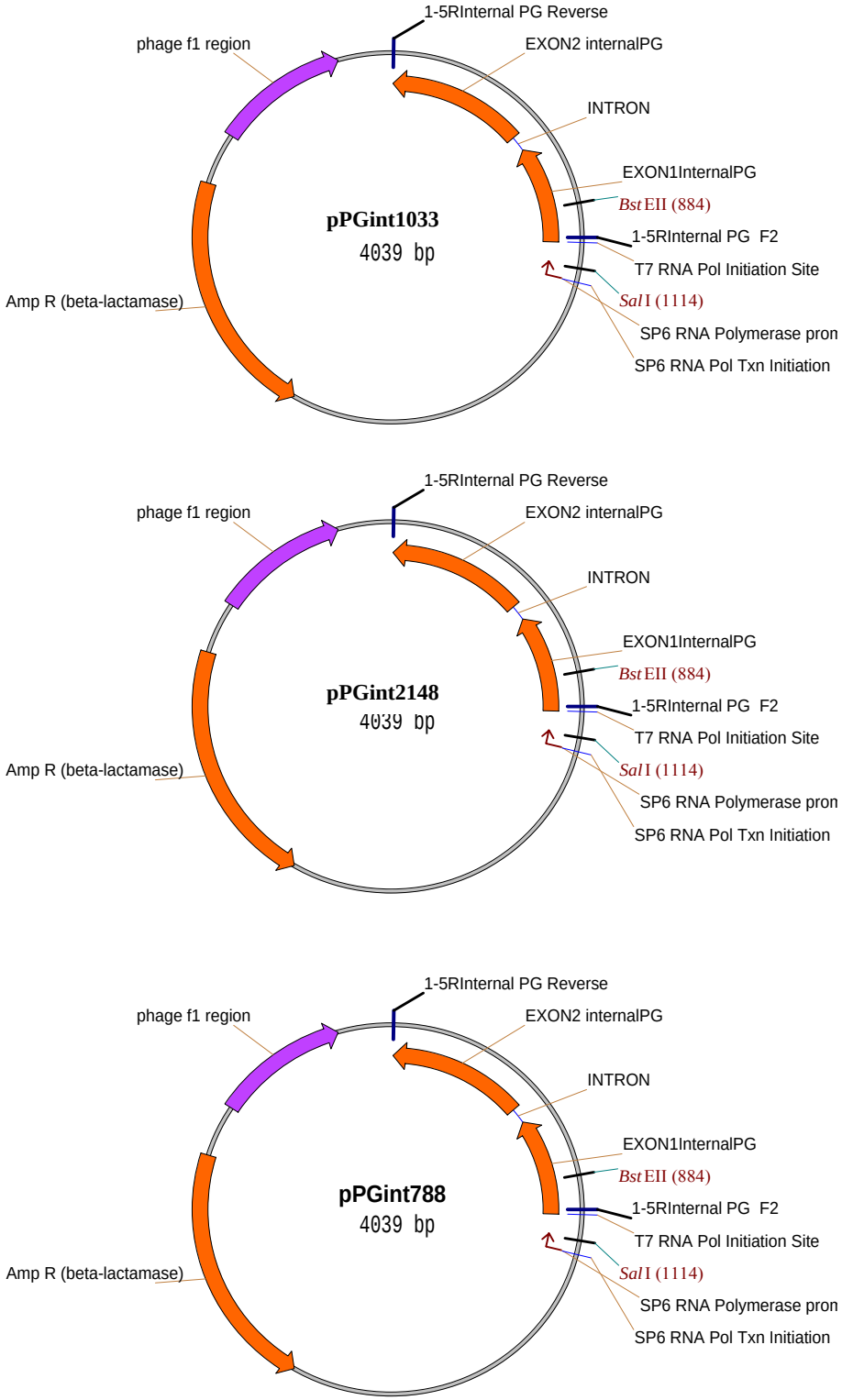


Fig. 3.8 Plasmid maps of pPGint788, pPGint1033 and pPGint2148. (Insert could be cloned in any orientation)**.**

Plasmids were extracted from positive *E. coli* clones containing pPGint788 and pPGint2148. Restriction digestion of the plasmids with *EcoRI* revealed that both clones of pPGint788 (Fig. 3.9, lanes 5 & 6) as well as both clones of pPGint2148 harbored inserts of approximately 900 bp, although the samples ran differently due to overloading (Fig. 3.9, lanes 7 & 8).

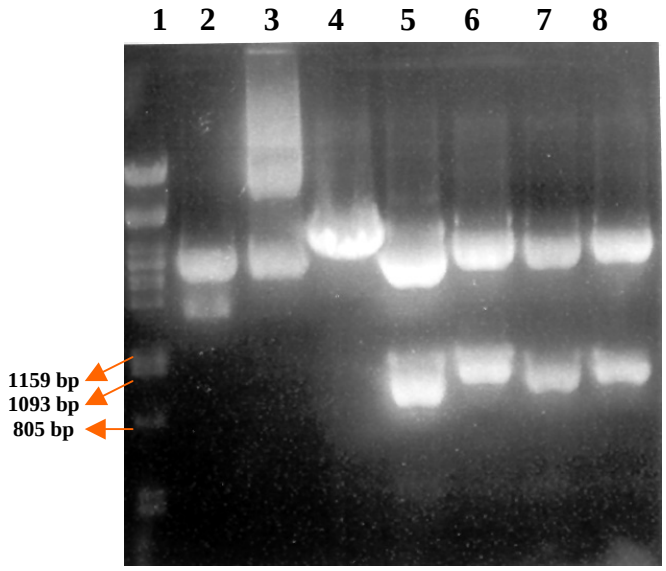


Fig. 3.9 *EcoRI* restriction analysis of both clones from pPGint788 and pPGint2148.
Uncut vectors pUC (lane 2) and pGEM-T-Easy (lane 3) were analysed on a 1% TAE agarose gel with *PstI* digested pGEM-T-Easy, (lane4), *EcoRI* digested pPGint788-1, pPGint788-2, pPGint2148-1 and pPGint2148-2 (lane 5, 6, 7 and 8 respectively). A λ *PstI* molecular marker was included in the electrophoresis (lane 1).

The putative PG fragments were sequenced directly as PCR products obtained from the genomic DNA, as well as from two pPGint788 & two pPGint2148 plasmid clones. Sequence data obtained with PCR primer set 2 were of better quality that that obtained with the first primer pair. All sequences were edited according to their relative chromatograms. Comparison of sequence data obtained directly from PCR products showed that 917 bp of the internal fragment was identical for all three isolates. This core region of 917 bp was aligned to sequence data from the plasmid clones (pPGint778 & pPGint2148) (Fig. 3.10). The alignments showed that a region of 469 bp of the cloned fragments had a good homology to the 917 bp core region. The alignment of the cloned PG sequences and PCR PG sequences differed upstream from this 469 bp region, since the cloned sequences could be more reliable, the front

part of the internal PG fragment was compiled from the alignment of the clones, using the additional sequence data (50 bp) from the forward primer (PG-COLL-F2) to the 469 bp region in the core fragment (Fig. 3.10). The end part of the internal PG fragment was obtained from pPGint788 clone2, which was the only clone providing clear downstream sequence data (48 bp) starting from the reverse primer (PG-COLL-R) to the end of core fragment (Fig. 3.10). The sequence of the compiled internal PG fragment is presented in Fig. 3.11.

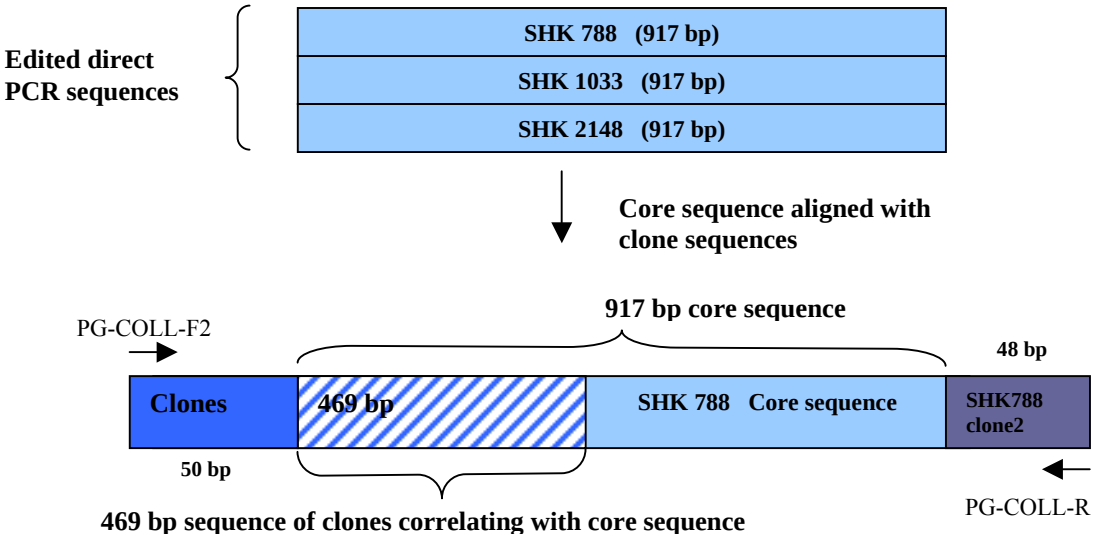


Fig. 3.10 Schematic representation of how sequence data from cloned PG fragments and directly sequenced PCR products were used to obtain the internal PG sequence.

5’ PG-COLL-F2 3’

```
   1  CGCGCCTCGT GCACCTTCAC CGACGCCAAG ACGGCCATGT CCAAGAAGAC
  51  CTCCTGCACC GACATCGTCC TCAACGGCAT CAAGGTTCCC GCCGGCGAGA
 101  CCCTCGACCT CACCGGCCTC AAGGACGGAA CCAAGGTTAC CTTCAAGGGC
 151  ACCACCACCT TCGGCTACAA GGAGTGGGAG GGCCCTCTCA TCGCCATCGG
 201  CGGTAAGAAG GTCAGCGTTG TCGGTGCTTC CGGCAACTCC ATCTCCTGCC
 251  AGGGTGAGCG CTGGTGGGAC GGCAAGGGAG GCAACGGTGG CAAGAAGAAG
 301  CCCAAGTTCT TCAAGGTCAA GATCAACGAC GGTTCCATCT CTGGCCTGAA
 351  CGTCAAGAAC ACCCCGGCCC ACGGATTCTC CATCAGCGGC GTTTCTGGCC
 401  TCAAGGTGTA AGTCACAACT CAATGTTGGG TTTTTGGACA GTAATACTGA
 451  CCCGACTATT CTCTAGCTCC AACATCCTTT TCGACAACAA GGATGGTGAC
 501  TCCAAGGGTG GCCACAACAC CGATGCCTTC GATGTCGGTA CGTCTTCTGA
 551  CGTGACCATC TCCGGCGCCA AGGTCTACAA CCAGGACGAC TGCCTGGCTA
 601  TCAACTCTGG CACCGGTATC ACTTTCGAGA ACGGATACTG CTACGGCTCC
 651  CACGGCCTGT CCATCGGTTC CGTTGGTGGA CGTAGCGACA ACACCGTCCA
 701  GAACGTCATC ATCCGTGACT CCACCATCGA GAAGGCCGAC AACGGTATCC
 751  GCATCAAGAC CATTGCCAAC AAGACTGGCA AGGTCAACGG TATCACCTTC
 801  GAGAACATCA CCCTCAAGAA CATCAACAAG AAGGGCATTG TCATCCAGCA
 851  GGATTACGAG AACGGCAGCC CTACCGGCAA GCCCACCGCT GGTATTCCCA
 901  TCACCGACGT CACCGTCAAG AACGTTAAGG GTACCGTCGC CGCGAAGGGC
 951  ACCAACGTGT ACATCCTTTG CAGCCTTTGC GCCAAGGGTG CCTGCTCCAA
1001  CTGGAAGTGG TCCGGCGTCA GC
```

PG-COLL-R (3’ ACCTTCACCAGGCCGCAGTCG 5’)

Fig. 3.11 Nucleotide sequence of compiled internal PG fragment.

3.3.4 Southern blot analysis of the PG gene from *Colletotrichum lupini*

Gel analysis showed that the genomic DNA of *C. lupini* SHK 2148 and SHK 788 used for Southern analysis were successfully digested with the various restriction enzymes, since a smear was evident after digestion (Fig. 3.12).

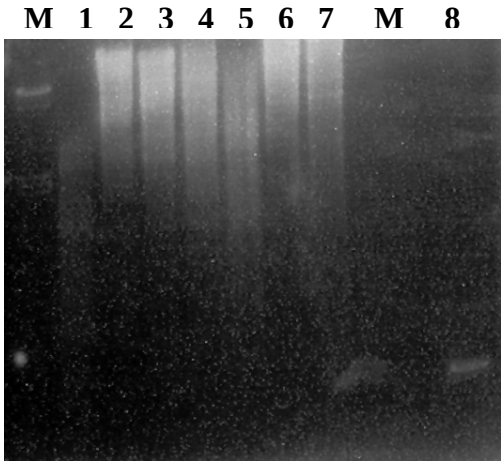


Fig. 3.12 Restriction enzyme digestion and electropoheretic separation of genomic DNA in preparation for Southern blot analysis.
Electrophoretic separation of genomic DNA of *C. lupini* SHK 2148 digested with *Ava1* (lane 1), *BamHI* (lane2)**,** *EcoRI* (lane 3), *EcoRV* (lane 4) and *HindIII*(lane 5) and separation of genomic DNA of *Colletotrichum* SHK 788 digested with *BamHI* (lane 6) and *PstI* (lane 7). As a control 100ng pPGint788 (Lane 8) was included. A λ PstI generated molecular marker (M) was electrophoresed with the samples.

Hybridization of the membrane with the internal PG fragment revealed the presence of a single band for each restriction enzyme used (Fig. 3.13). The positive control was used as a point of reference to determine the sizes of the hybridizing bands. The size of the positive control was approximately 950 bp adding to this an additional 18 bp to accommodate pGEM-T-Easy sequences added after the *EcoRI* digestion. Thus the size of the positive control was 970 bp. The predicted sized of the PG gene fragments were as follows, 1. 8 Kb for *AvaI*, 2.8 Kb for *BamHI*, 10.5 Kb for *EcoRI*, 4.9 Kb for *EcoRV*, 1 Kb for *HindIII* and 2.5 Kb for *Pst*I. Fragments from the *BamHI* generated digestion from both isolates (lanes 2 and 6) appear to be identical in size. The Southern blot provided information to facilitate the inverse PCR, for which one needs an indication of a fragment, that is not too large to amplify, yet is still large enough to provide the missing upstream and downstream sequence data. From the Southern blot results, the fragment from the *HindIII* digestion, which is approximately 1 Kb, falls into a suitable size range for the Inverse PCR. *HindIII* and *EcoRV* were chosen for the inverse PCR analysis as well as additional restriction enzymes, *ClaI*, *BssHI*, *SalI* and *SacI*, which did not digest the internal PG fragment.

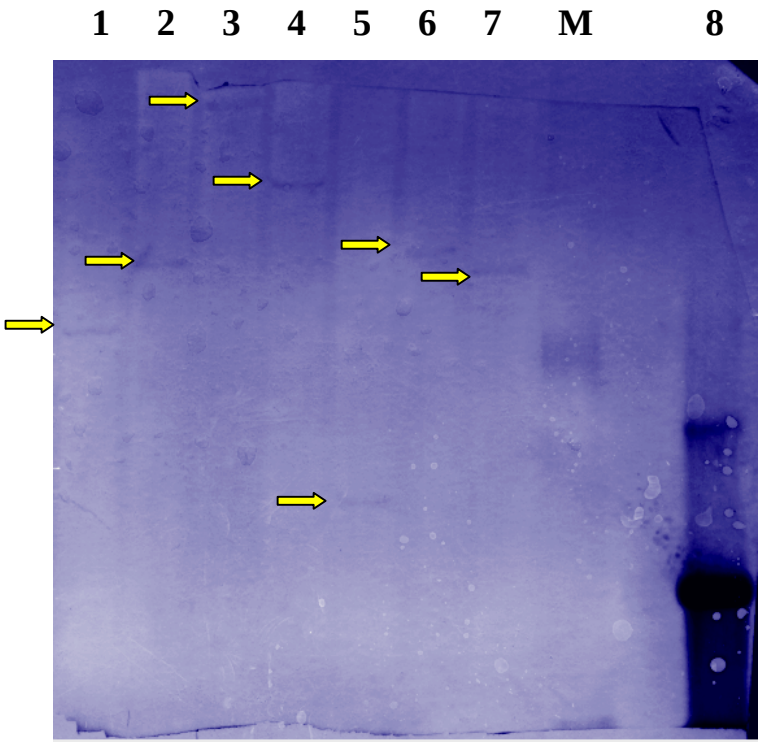


Fig. 3.13 Southern blot analysis of the PG gene from *C. lupini* SHK 2148 and *C. lupini* SHK 788.
Southern blot analysis of *C. lupini* SHK 2148 restricted with *AvaI* (lane 1), *BamHI* (lane2), *EcoRI* (lane 3), *EcoRV* (lane 4), *HindIII* (lane 5) and *C. lupini* SHK 788 restricted with *BamHI* (lane 6) and *PstI* (lane 7) using a 950 bp internal PG fragment, excised from pPGint788, as probe. The same plasmid, pPGint788, was included in the analysis as positive control (lane 8). M represents non-labeled *PstI* generated molecular marker.

Since it was reported that *C. lupini* SHK 2148 was a super virulent strain (Personal communication Dr. S. H. Koch) and that the PG gene sequences of the isolates seem to be identical, it was decided to focus mainly on *C. lupini* SHK 2148 for future research.

3.3.5 Inverse PCR

Genomic DNA Extraction and restriction enzyme digestion

Approximately 10µg of genomic DNA was available for each restriction enzyme digestion with various restriction enzymes (Fig. 3.14). Initially ligation reactions were extracted as previously described by Arendse *et al* (1999). However too much

DNA was lost during the precipitation step and this step was excluded, proceeding directly with the inverse PCR step.

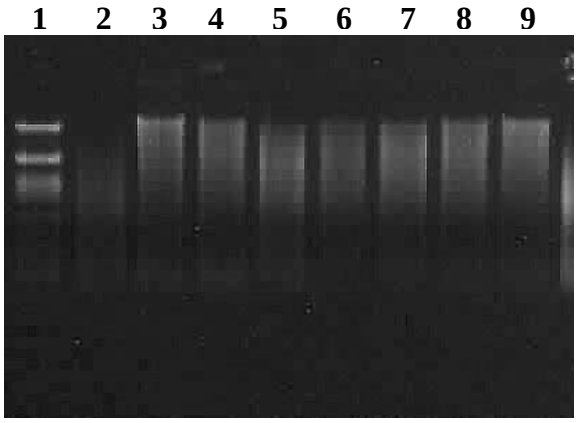


Fig. 3.14 Digestion of genomic DNA from *C. lupini* SHK 2148 in preparation for inverse PCR.
Genomic DNA of isolate *C. lupini* SHK 2148 restricted with *AvaI* (lane 2), *BamHI* (lane 3), *ClaI* (lane 4), *HindIII* (lane 5), *SalI* (lane 6), *SacI* (lane 7), *EcoRV* (lane 8), and *BssHII* (lane 9) electrophoresed with a λ*PstI* molecular marker (lane 1).

A positive control, pPGint788 containing the internal PG fragment, was subjected to all the inverse PCR steps. All three phases of the positive control gave a band signal during the inverse PCR with the primers InversePCR_FII and InversePCR_RII (Fig. 3.15, lanes 8-10), which confirm that the ligation of the genomic fragments should be successful and that the primers used in the inverse PCR were adequate to produce a PCR product. The size of the positive control included the vector size of 3015 bp and some PG gene sequence included by the inverse PCR primers, which is approximately 338 bp. Thus the expected size of the positive control was approximately 3353 bp.

Faint PCR bands were observed for *EcoRV* (Fig. 3.15, lane3), *BssHI* (Fig. 3.15, lane 4) while brighter bands were visible in the *HindIII* (Fig. 3.15, lane 5) and *ClaI* (Fig. 3.15, lane 2) inverse PCR reactions, which were approximately 1 Kb and 3 Kb respectively (Fig. 3.15). No bands were visible for the other reactions with *SalI* (lane 6) and *SacI* (Fig. 3.15, lane 7).

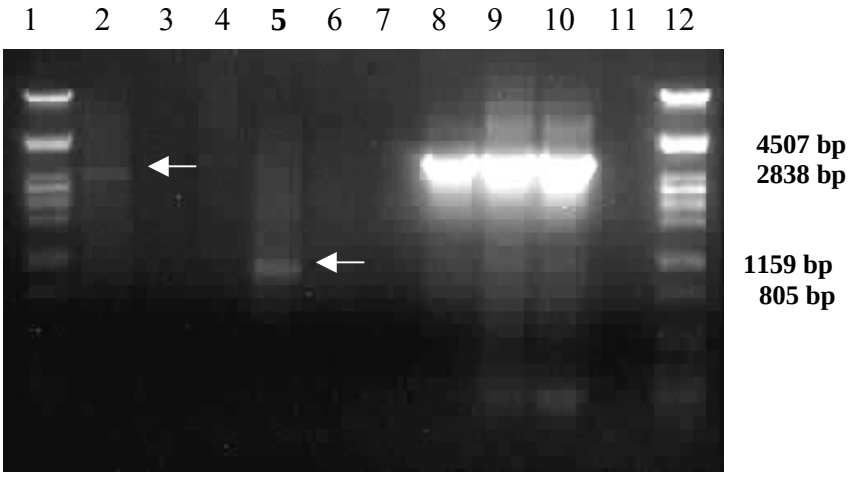


Fig. 3.15 Inverse PCR performed on religated genomic DNA fragments from *Colletotrichum* SHK2148 and pPGint788
Inverse PCR reactions, performed with ligation mixes containing religated molecules that were restricted with *ClaI* (lane 2), *EcoRV* (lane3), *BssHI* (lane 4), *HindIII* (lane 5), *SalI* (lane 6) and *SacI* (lane 7) digestions, were evaluated with agaorse gel electrophoresis. PCR amplification reactions from pPGint788 (lane 8); pPGint788 *SalI* restricted, religated and *BstEII* linearised (lane 9); pPGint788 *SalI* restricted and religated (lane 10) as well as a PCR water control (lane 11) and λ*PstI* molecular marker (lane 1 and 12) were included in the electrophoresis.

Cloning of the inverse PCR *HindIII* fragment

The gel-purified inverse PCR fragments of the *HindIII* sample were ligated into pGEM-T-Easy and electroporated into *E.coli* DH10B cells. pPGint788 was also included in the electroporation event as a positive control. The presence of a lawn of bacteria for the positive control transformation confirmed that the cells were very competent (data not presented). Several colonies were also obtained for the transformation with the *HindIII* inverse PCR-pGEM-T-Easy ligation reaction. An average of 28 colonies were obtained per plate of which approximately 72% were white colonies. Two positive controls, two negative controls and 12 white colonies were selected and inoculated for plasmid isolations and restriction enzyme digestion verification. From the 12 selected positive transformants, 7 contained the plasmid. Restriction digestion with *EcoRI* showed that one clone had the predicted (1.1 kb) sized insert (Fig. 3.16). This clone was designated pPGinvHind10, and was used for a larger scale plasmid preparation that was then sequenced.

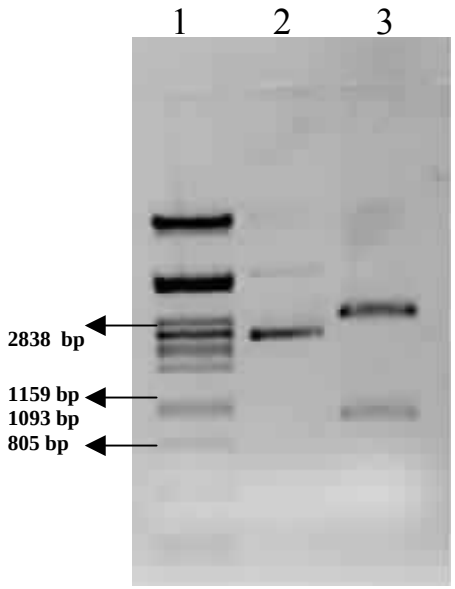


Fig. 3.16 *EcoRI* restriction analysis of plasmid pPGinvHind10
Uncut pPGinvHind10 (lane2) were separated on an agarose gel together with pPGinvHind10 restricted with *EcoRI* (lane3) from which an insert of approximately 1 Kb were excised. A λ*PstI* marker was loaded as well (lane1).

Three sequence data sets obtained from sequencing with the universal primers SP6, T7 and M13, were used to solve the sequence of the complete PG gene cloned in pPGinvHind10 (Fig. 3.17). The reverse compliment of the SP6 and M13 reverse sequence revealed the missing sequence of region A (the 5' region of the PG gene), while the T7 sequence data resolved region B (the 3' region of the PG gene) (Fig. 3.17).

In the T7 sequence data neither the sequence corresponding to the Reverse or the Forward primer could be identified with certainty, however the *HindIII* site was located and used as a point of reference. Sequence data from the start of the T7 sequence to the *HindIII* site were aligned with the internal fragment and with the PG gene sequence from *Colletotrichum gloeosporioides* f. sp *malvae* (the PG gene sequence which showed the highest similarity to the internal PG fragment of *C. lupini* SHK 2148). From the alignments the end part of the gene was resolved, the remaining 3' sequence included the TAA stop codon as well as an additional 21 bp to the *Hind III* site.

The same approach was followed to obtain the front part of the gene by using the Sp6 and M13 R sequence information. From these data, a putative *HindIII* site was determined and confirmed with alignments with a previously sequenced SP6-set1 as well as the T7 sequence. Sequence data from the reverse primer location to the putative *HindIII* site were aligned with a corresponding reverse compliment sequence of the PG gene from *Colletotrichum gloeosporioides* f. sp *malvae.* A very good alignment was obtained and the front part of the gene was resolved. In addition to the ATG start, approximately 400 bp of upstream sequence was also resolved.

Thus a complete PG gene sequence was constructed by using sequence data of B for the end part, the internal fragment (previously constructed) and the A for the front part. Fig. 3.18 represents the sequence of the compiled PG gene sequence.

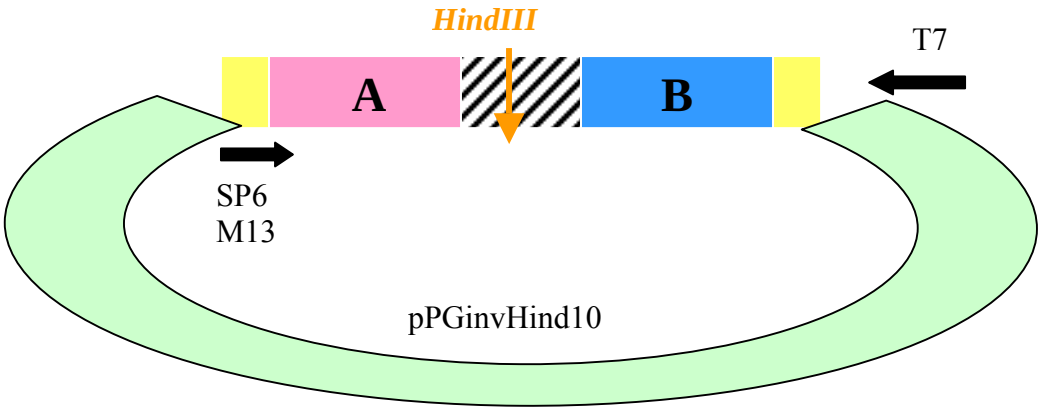


Fig. 3.17 Schematic representation of pPGinvHind10 showing primer annealing sites and the different regions that were resolved by sequence analyses.

Known region of the PG gene

Unknown front region of PG gene

Unknown end region of PG gene

PGEM-T- Easy vector

Additional genomic DNA included from the restriction reaction

HindIII restricted point – relegated

3.3.6 Isolation and characterization of the complete PG gene

PCR primers, C. lupini_Compl F and C. lupini_Compl R, were designed from the compiled PG gene sequence (Fig. 3.18). These primers amplified a product of approximately 1Kb (Fig. 3.19). The size predicted from the composite gene sequence was 1210 bp. The fragment was gel purified and cloned into pGEM-T-Easy and transformed into *E.coli* strain DH5α together with pUC DNA as a positive control. Numerous colonies were obtained for the positive control. Five clones, pPGcompl1 – pPGcompl5, were screened for the presence of the complete PG gene insert via *EcoRI* digestion (Fig. 3.20). One of these clones was sequenced with T7 and SP6 and it was revealed that the clone harboured the complete PG gene; this construct was depicted as pPGcompl1 (Fig. 3.21). Although proofreading Taq was not used for the PCR reactions, it did not seem as if mutations were introduced during subsequent PCR reactions, since the internal PG fragment, the compiled PG sequence as well as the complete PG gene sequence did not display any sequence discrepancies. The

complete PG gene sequence was aligned with the PG cDNA from *Colletotrichum gloeosporioides* f. sp *malvae* since this gene had the highest sequence similarity to the compiled PG gene (Fig. 3.22). This alignment together with the characteristics of filamentous fungal genes described by Gurr *et al* (1987) was used to characterize the complete PG gene and determine the position of a putative intron. Fig. 3.23 displays the complete nucleotide and predicted amino acid sequence of the PG gene from *C. lupini* SHK 2148. Furthermore, the nucleotide sequence of the PG gene from *C. lupini* SHK 2148 was aligned with PG genes from several other fungal sources (results not shown). As mentioned, the PG gene from *C. lupini* SHK 2148 had the highest identity to the PGs of *Colletotrichum gloeosporioides* f. sp *malvae* (*cmpgII*) and *C. lindemuthianum* (*clpgII*), 87% and 86% respectively. The intron positions of these genes were very similar, however the sequence of the introns themselves differed significantly (results not shown).

The predicted amino acid sequence of the PG gene from *C. lupini* SHK 2148 was aligned with amino acid sequences from several other fungal PG genes which was obtained from Genbank using the accession numbers provided by Markovič *et al* (2001) (Fig. 3.24); *ClpgII* and *ClpgI* (*Colletotrichum lindemutianum*), *pecB*, and *pecA* (*Aspergillus flavus*), *PgaII* (*A. niger*), *FmPG* (*Fusarium moniliforme*), *BcPG1*, *BcPG2*, *BcPG3*, *BcPG4*, *BcPG5* and *BcPG6* (*Botrytis cinerea*) and *CmpgII* (*C. gloeosporioides* f. sp. m*alvae*). The predicted PG protein had the highest homology to the *CmpgII* PG protein of *C. gloeosporioides* f. sp. m*alvae* (87. 3%) and *ClpgII* of *C. lindemutianum* (85. 5%) and contained the four domains (Fig. 3.24) that are conserved in all PGs (Markovič *et al*, 2001).

HindIII

AAGCTTAGGGGAATAAATTACCAGGGGGAGGCCTCTTTTGGTCTTGGGGAAACTCGGGGGCAGGCAAATAAGGGTTTGAATCAGGGCCTCTAGTCCTTCAAAGACGGGAGATAACAACGGATGCCGCTATCCGGGTGGAACCGCA

CCAACCCAGTTGGGGAATTCAACGAGCTTCCACAAGTAGCATCGAACGAAAGGGGAGGAGGAGGCCTAGAGTCTTGCATGTTCGA**CAAAT**TGA**TATAA**GTATCATCTTTTCTGCCCACCTCTTTTGCTTCTGTTCTTGAT

C. lupini_Compl F

ACCCCATCGTGCTTCTTCACTCACACTCTTTTCAACCACATTCTTTTTTTCCAACTCTAGTCTCTCATTCGACAATTTTTGTACATACTCGAAGTATTCCACGTGTTGATCACATCCAAAACAAATCAAGAAACG

```
 1    M   K   F   L   S   V   V   A   G   L   S   A   L   A   A   A   A   P   A   E   L   D   T   R   A   S   C   T   F   T
 1    ATG AAG TTC CTC TCC GTT GTC GCG GGC CTC AGC GCC CTG GCT GCC GCC GCC CCC GCC GAG CTC GAT ACC CGT GCC TCG TGC ACC TTC ACC
```

PG-COLL-F1 and F2

```
31    D   A   K   T   A   M   S   K   K   T   S   C   T   D   I   V   L   N   G   I   K   V   P   A   G   E   T   L   D   L
91    GAC GCC AAG ACG GCC ATG TCC AAG AAG ACC TCC TGC ACC GAC ATC GTC CTC AAC GGC ATC AAG GTT CCC GCC GGC GAG ACC CTC GAC CTC
```

InversePCR_RII

```
61    T   G   L   K   D   G   T   K   V   T   F   K   G   T   T   T   F   G   Y   K   E   W   E   G   P   L   I   A   I   G
181   ACC GGC CTC AAG GAC GGA ACC AAG GTT ACC TTC AAG GGC ACC ACC ACC TTC GGC TAC AAG GAG TGG GAG GGC CCT CTC ATC GCC ATC GGC

91    G   K   K   V   S   V   V   G   A   S   G   N   S   I   S   C   Q   G   E   R   W   W   D   G   K   G   G   N   G   G
271   GGT AAG AAG GTC AGC GTT GTC GGT GCT TCC GGC AAC TCC ATC TCC TGC CAG GGT GAG CGC TGG TGG GAC GGC AAG GGA GGC AAC GGT GGC

121   K   K   K   P   K   F   F   K   V   K   I   N   D   G   S   I   S   G   L   N   V   K   N   T   P   A   H   G   F   S
361   AAG AAG AAG CCC AAG TTC TTC AAG GTC AAG ATC AAC GAC GGT TCC ATC TCT GGC CTG AAC GTC AAG AAC ACC CCG GCC CAC GGA TTC TCC

151   I   S   G   V   S   G   L   K   V                    Putative intron of 59 bp
451   ATC AGC GGC GTT TCT GGC CTC AAG GT GTA AGT CAC AAC TCA ATG TTG GGT TTT TGG ACA GTA ATA CTG ACC CGA CTA TTC TCT AG

159   V   S   N   I   L   F   D   N   K   D   G   D   S   K   G   G   H   N   T   D   A   F   D   V   G   T   S   S   D   V
541   C TCC AAC ATC CTT TTC GAC AAC AAG GAT GGT GAC TCC AAG GGT GGC CAC AAC ACC GAT GCC TTC GAT GTC GGT ACG TCT TCT GAC GTG
```

```
189   T   I   S   G   A   K   V   Y   N   Q   D   D   C   L   A   I   N   S   G   T   G   I   T   F   E   N   G   Y   C   Y
631   ACC ATC TCC GGC GCC AAG GTC TAC AAC CAG GAC GAC TGC CTG GCT ATC AAC TCT GGC ACC GGT ATC ACT TTC GAG AAC GGA TAC TGC TAC

219   G   S   H   G   L   S   I   G   S   V   G   G   R   S   D   N   T   V   Q   N   V   I   I   R   D   S   T   I   E   K
721   GGC TCC CAC GGC CTG TCC ATC GGT TCC GTT GGT GGA CGT AGC GAC AAC ACC GTC CAG AAC GTC ATC ATC CGT GAC TCC ACC ATC GAG AAG
                          InversePCR_FII

249   A   D   N   G   I   R   I   K   T   I   N   K   T   G   K   V   N   G   I   T   F   E   N   I   T   L   K   N   I   N
811   GCC GAC AAC GGT ATC CGC ATC AAG ACC ATT AAC AAG ACT GGC AAG GTC AAC GGT ATC ACC TTC GAG AAC ATC ACC CTC AAG AAC ATC AAC

279   A   K   K   G   I   V   I   Q   Q   D   Y   E   N   G   S   P   T   G   K   P   T   A   G   I   P   I   T   D   V   T
991   GCC AAG AAG GGC ATT GTC ATC CAG CAG GAT TAC GAG AAC GGC AGC CCT ACC GGC AAG CCC ACC GCT GGT ATT CCC ATC ACC GAC GTC ACC

                                                                                                PG-COLL-R
309   V   K   N   V   K   G   T   V   A   A   G   T   N   V   Y   I   L   C   A   K   G   A   C   S   N   W   K   W   S   G
1081  GTC AAG AAC GTT AAG GGT ACC GTC GCC GCG GGC ACC AAC GTG TAC ATC CTT TGC GCC AAG GGT GCC TGC TCC AAC TGG AAG TGG TCT GGC

339   K   V   S   V   T   G   G   K   S   S   S   E   C   S   G   I   P   S   G   S   G   A   K   C   *
1171  AAG GTC TCC GTT ACC GGT GGC AAG TCC TCT TCT GAG TGC TCT GGC ATC CCT TCC GGC TCC GGT GCC AAG TGC TAA ATG CTT GAT GGT TGG GAA GCT T
                                                                                                C. lupini_Compl R      HindIII
```

Fig. 3.18 Compiled nucleotide and predicted amino acid PG gene sequence of *Colletotrichum lupini* SHK 2148. The sequence presents 420 bp of the 5' untranscribed region of the gene, the sequence of the compiled PG gene from the inverse PCR data as well as the *HindIII* site that was used during the inverse PCR reaction. The putative signal peptide of the PG gene is indicated in red, while the predicted intron is underlined and the CAAT and TATA boxes are presented in bold. Furthermore the regions from which primers were designed are highlighted.

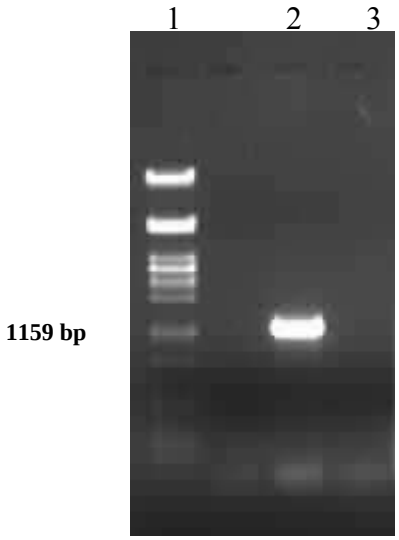


Fig. 3.19 PCR amplification of the complete PG gene from the genome of *C. lupini* SHK 2148.
A band, representing the putative complete PG gene, was amplified using primers C.lupini _Compl F, C. lupini_Compl R and genomic DNA of isolate *C. lupini* SHK 2148 (lane 2). A PCR water control (lane 3) and λ*PstI* marker (lane1) were included on the agarose gel.

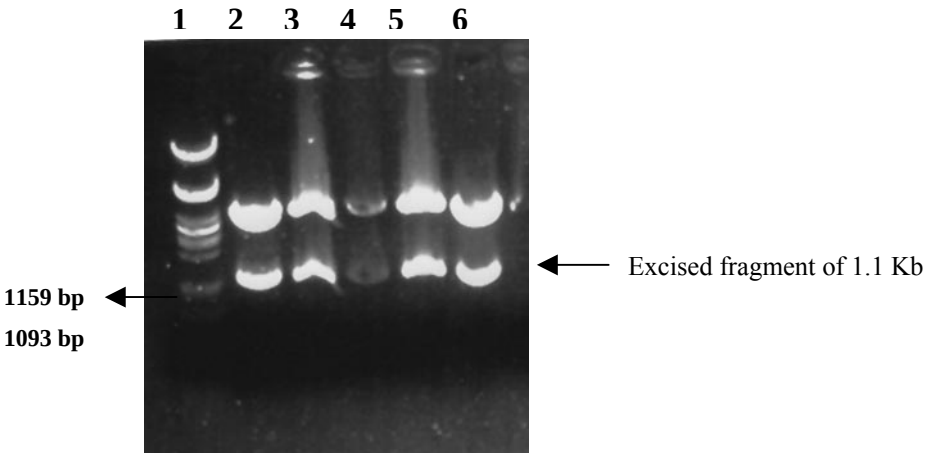


Fig. 3.20 *EcoRI* restriction analysis of pPGcompl1 – pPGcompl5, harboring the putative complete PG gene of *C. lupini* SHK 2148.
Plasmids pPGcomplete1 – pPGcomplete5 were restricted with *EcoRI* (lanes 2 – 6 respectively), revealing the presence of a 1 Kb putative complete PG gene fragment. λ*PstI* generated molecular marker (lane 1) were electrophoresed with the digested plasmids.

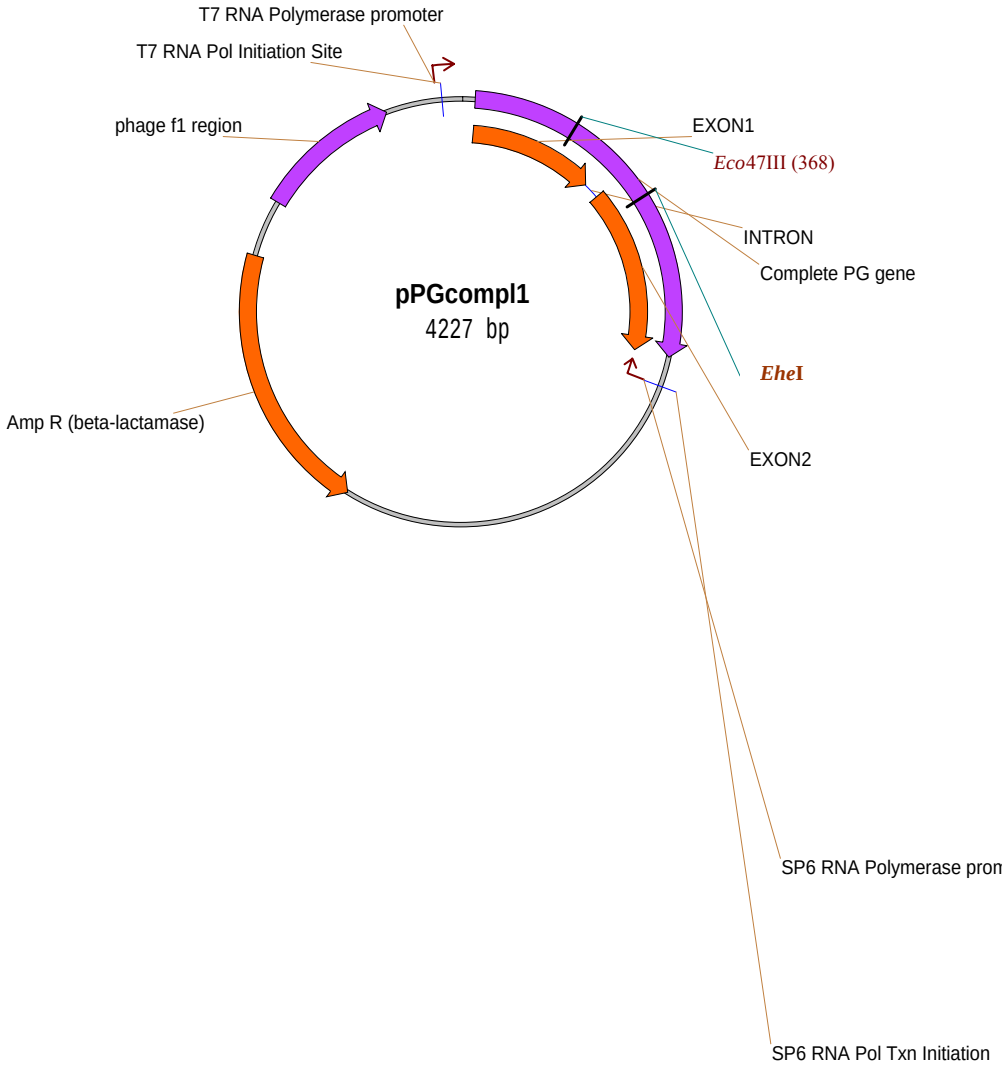


Fig. 3.21 Plasmid map of pPGcompl1 harbouring the complete PG gene.

```
PG COLLETOTRICHUM          (451) ATCAGCGGCGTTTCTGGCCTCAAGGTGTAAGTCACAACTCAATGTTGGGT
C.gloeosporioides(Malvae)(451) ATCAACAGCGTCAAGGGCCTCAAGGT----------------------

PG COLLETOTRICHUM          (501) TTTTGGACAGTAATACTGACCCGACTATTCTCTAGCTCCAACATCCTTTT
C.gloeosporioides(Malvae)(477) ----------------------------------CGTCAACGTCGAGTT

PG COLLETOTRICHUM          (551) CGACAACAAGGATGGTGACTCCAAGGGTGGCCACAACACCGATGCCTTCG
C.gloeosporioides(Malvae) (492) CAACAACAAGGACGGCGACACCAAGGGCGGCCACAACACGGACGTCTTTG
```

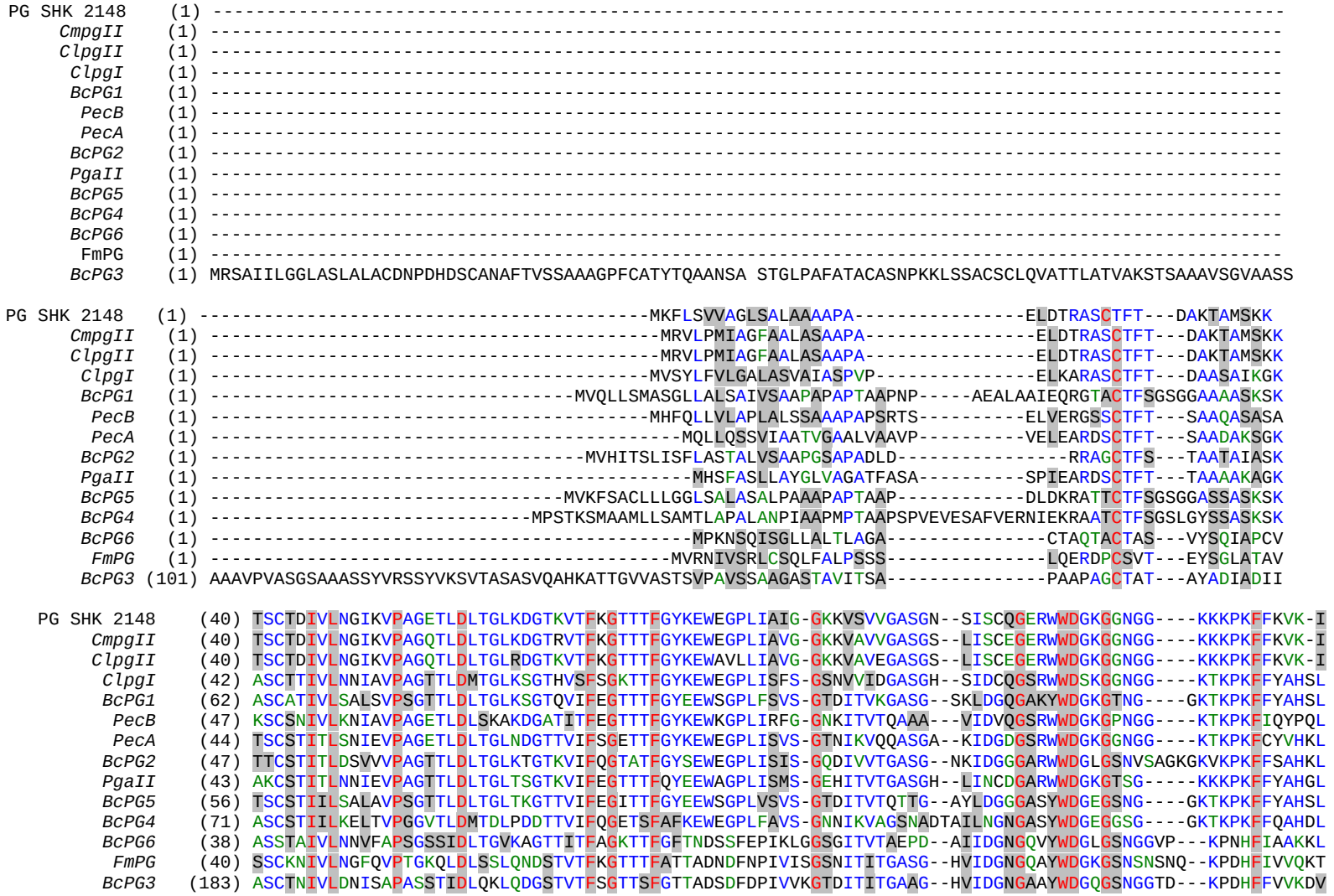
Fig. 3.22 Partial nucleotide alignment between the PG cDNA of *C. gloeosporioides* f. sp. *Malvae* and the complete PG gene cloned from genomic DNA of *C. lupini* SHK 2148. This alignment revealed a predicted intron of 59 bp with typical border sequences, indicated in red bold letters, as described by Gurr *et al* (1987).

```
 1    M  K  F  L  S  V  V  A  G  L  S  A  L  A  A  A  A  P  A  E
 1   ATG AAG TTC CTC TCC GTT GTC GCG GGC CTC AGC GCC CTG GCT GCC GCC GCC CCC GCC GAG
21    L  D  T  R  A  S  C  T  F  T  D  A  K  T  A  M  S  K  K  T
61   CTC GAT ACC CGT GCC TCG TGC ACC TTC ACC GAC GCC AAG ACG GCC ATG TCC AAG AAG ACC
41    S  C  T  D  I  V  L  N  G  I  K  V  P  A  G  E  T  L  D  L
121  TCC TGC ACC GAC ATC GTC CTC AAC GGC ATC AAG GTT CCC GCC GGC GAG ACC CTC GAC CTC
61    T  G  L  K  D  G  T  K  V  T  F  K  G  T  T  T  F  G  Y  K
181  ACC GGC CTC AAG GAC GGA ACC AAG GTT ACC TTC AAG GGC ACC ACC ACC TTC GGC TAC AAG
81    E  W  E  G  P  L  I  A  I  G  G  K  K  V  S  V  V  G  A  S
241  GAG TGG GAG GGC CCT CTC ATC GCC ATC GGC GGT AAG AAG GTC AGC GTT GTC GGT GCT TCC
101   G  N  S  I  S  C  Q  G  E  R  W  W  D  G  K  G  G  N  G  G
301  GGC AAC TCC ATC TCC TGC CAG GGT GAG CGC TGG TGG GAC GGC AAG GGA GGC AAC GGT GGC
121   K  K  K  P  K  F  F  K  V  K  I  N  D  G  S  I  S  G  L  N
361  AAG AAG AAG CCC AAG TTC TTC AAG GTC AAG ATC AAC GAC GGT TCC ATC TCT GGC CTG AAC
141   V  K  N  T  P  A  H  G  F  S  I  S  G  V  S  G  L  K  V
421  GTC AAG AAC ACC CCG GCC CAC GGA TTC TCC ATC AGC GGC GTT TCT GGC CTC AAG GT

481  GTA AGT CAC AAC TCA ATG TTG GGT TTT TGG ACA GTA ATA CTG ACC CGA CTA TTC TCT AG

161   V  S  N  I  L  F  D  N  K  D  G  D  S  K  G  G  H  N  T  D
541   C TCC AAC ATC CTT TTC GAC AAC AAG GAT GGT GAC TCC AAG GGT GGC CAC AAC ACC GAT
181   A  F  D  V  G  T  S  S  D  V  T  I  S  G  A  K  V  Y  N  Q
601  GCC TTC GAT GTC GGT ACG TCT TCT GAC GTG ACC ATC TCC GGC GCC AAG GTC TAC AAC CAG
201   D  D  C  L  A  I  N  S  G  T  G  I  T  F  E  N  G  Y  C  Y
661  GAC GAC TGC CTG GCT ATC AAC TCT GGC ACC GGT ATC ACT TTC GAG AAC GGA TAC TGC TAC
221   G  S  H  G  L  S  I  G  S  V  G  G  R  S  D  N  T  V  Q  N
721  GGC TCC CAC GGC CTG TCC ATC GGT TCC GTT GGT GGA CGT AGC GAC AAC ACC GTC CAG AAC
241   V  I  I  R  D  S  T  I  E  K  A  D  N  G  I  R  I  K  T  I
781  GTC ATC ATC CGT GAC TCC ACC ATC GAG AAG GCC GAC AAC GGT ATC CGC ATC AAG ACC ATT
261   N  K  T  G  K  V  N  G  I  T  F  E  N  I  T  L  K  N  I  N
841  AAC AAG ACT GGC AAG GTC AAC GGT ATC ACC TTC GAG AAC ATC ACC CTC AAG AAC ATC AAC
281   A  K  K  G  I  V  I  Q  Q  D  Y  E  N  G  S  P  T  G  K  P
901  GCC AAG AAG GGC ATT GTC ATC CAG CAG GAT TAC GAG AAC GGC AGC CCT ACC GGC AAG CCC
301   T  A  G  I  P  I  T  D  V  T  V  K  N  V  K  G  T  V  A  A
961  ACC GCT GGT ATT CCC ATC ACC GAC GTC ACC GTC AAG AAC GTT AAG GGT ACC GTC GCC GCG
321   G  T  N  V  Y  I  L  C  A  K  G  A  C  S  N  W  K  W  S  G
1021 GGC ACC AAC GTG TAC ATC CTT TGC GCC AAG GGT GCC TGC TCC AAC TGG AAG TGG TCT GGC
341   K  V  S  V  T  G  G  K  S  S  S  E  C  S  G  I  P  S  G  S
1081 AAG GTC TCC GTT ACC GGT GGC AAG TCC TCT TCT GAG TGC TCT GGC ATC CCT TCC GGC TCC
361   G  A  K  C  *
1141 GGT GCC AAG TGC TAA
```

Fig. 3.23 Nucleotide and predicted amino acid sequence of the complete PG gene of *C. lupini* SHK 2148. The green block indicates the initiation codon while the asterisk indicates the termination codon. A putative signal peptide is indicated in red and the predicted intron is underlined.


PG SHK 2148 (1)
CmpgII (1)
ClpgII (1)
ClpgI (1)
BcPG1 (1)
PecB (1)
PecA (1)
BcPG2 (1)
PgaII (1)
BcPG5 (1)
BcPG4 (1)
BcPG6 (1)
FmPG (1)
BcPG3 (1) MRSAIILGGLASLALACDNPDHDSCANAFTVSSAAAGPFCATYTQAANSA STGLPAFATACASNPKKLSSACSCLQVATTLATVAKSTSAAAVSGVAASS
PG SHK 2148 (1)
CmpgII (1)
ClpgII (1)
ClpgI (1)
BcPG1 (1)
PecB (1)
PecA (1)
BcPG2 (1)
PgaII (1)
BcPG5 (1)
BcPG4 (1)
BcPG6 (1)
FmPG (1)
BcPG3 (101) AAAVPVASGSAAASSYVRSSYVKSVTASASVQAHKATTGVVASTSVPAVSSAAGASTAVITSA
PG SHK 2148 (40)
CmpgII (40)
ClpgII (40)
ClpgI (42)
BcPG1 (62)
PecB (47)
PecA (44)
BcPG2 (47)
PgaII (43)
BcPG5 (56)
BcPG4 (71)
BcPG6 (38)
FmPG (40)
BcPG3 (183)

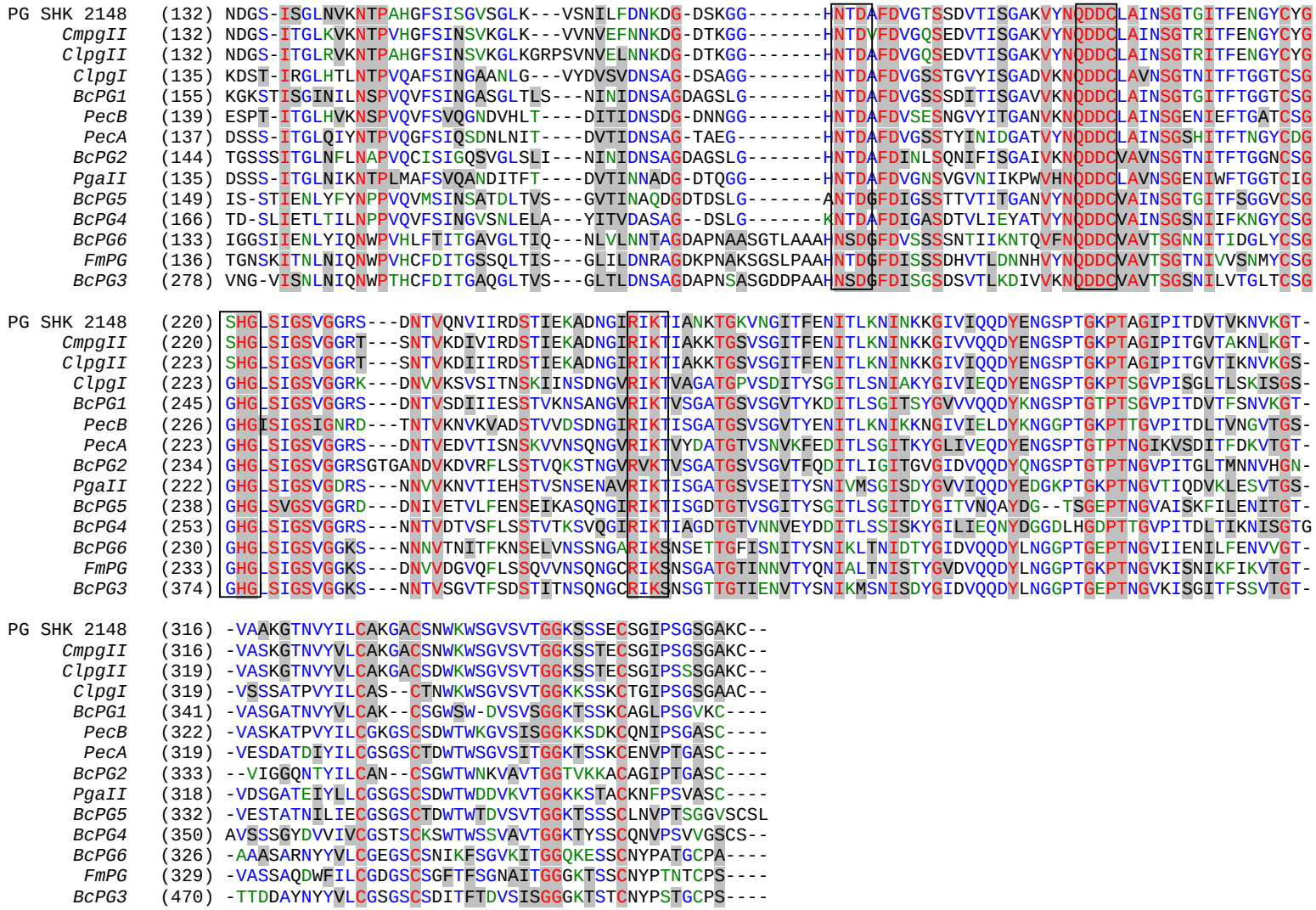


Fig. 3.24 Amino acid sequence alignment of the PG gene from *Colletotrichum* SHK 2148 with the amino acid sequences of various other fungal PG genes indicating the four conserved domains found in polygalacturonases. (The four most conserved domains, present in PGs, are indicated in blocks).

2.3.7 Northern blot analysis

Electrophoretic analyses of RNA on the gel that was used for Northern blotting showed that the RNA samples on the gel were not equally loaded, even though concentrations were determined with the spectrophotometer. Day 12 had the lowest RNA concentration and day 5 the highest according to gel analyses (Fig. 3.25). The RNA samples from days 6 and 12 seem to be slightly degraded (Fig. 3.25). Hybridization of the northern membrane with the full length cloned PG gene showed that the PG transcript was present on day 3, 4 and 7 after inoculation. The transcript was absent for days 5, 6 and 12 (Fig. 3.26).

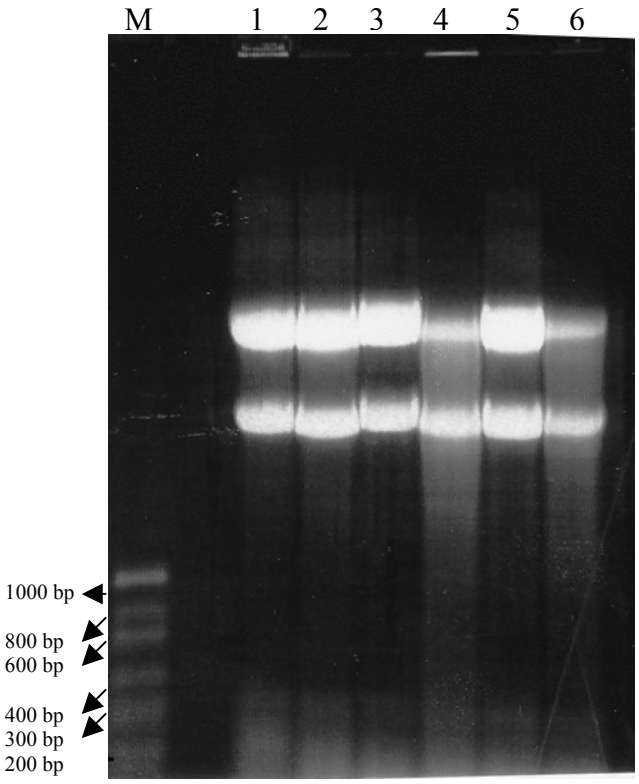


Fig. 3.25 RNA extractions of *C. lupini* SHK 2148 grown on pectin from different time periods. Total RNA extracted from mycelia of the fungus *C. lupini* SHK 2148 grown for 3, 4, 5, 6, 7 and 12 days (lanes 1, 2, 3, 4, 5 and 6 respectively) on pectin. M indicates the RNA molecular marker included on the gel.

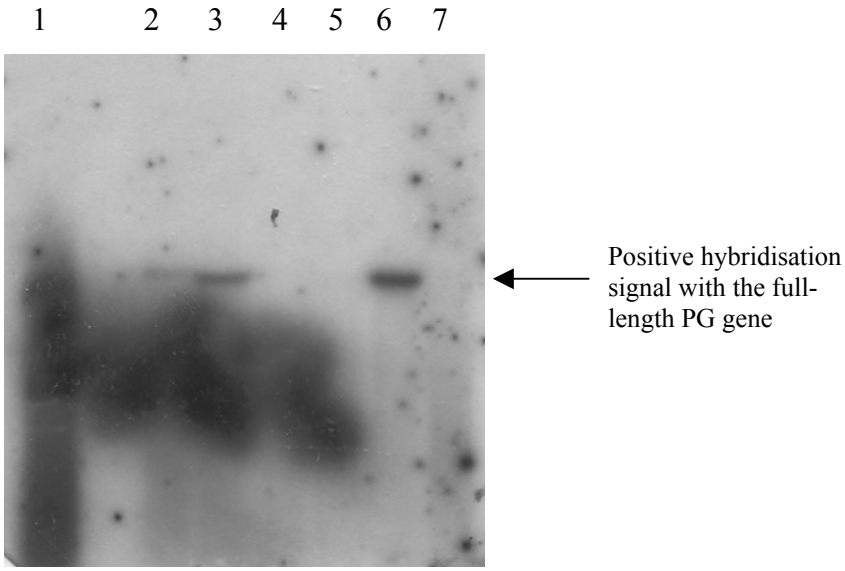


Fig. 3.26 Northern blot analysis of *C. lupini* SHK 2148 RNA samples, harvested from the fungus grown on pectin for different time periods, using the full-length PG gene as probe. Bands displayed of the full length PG gene hybridising to RNA samples obtained from mycelia of *C. lupini* SHK 2148 grown on pectin as sole carbon source for 3, 4, 5, 6, 7 and 12 days (lanes 2, 3, 4, 5, 6 and 7). A molecular marker was included in the analyses (lane1).

3.3.8 RT PCR amplification

RT-PCR analyses were done on RNA extracted from day 4 since this day displayed PG activity and expression according to the agarose diffusion assay and northern blot analyses respectively. The expected PCR product size of 1.1 kb was obtained with PCR amplification of cDNA from day 4 using primers C. lupini_Compl F I and C. lupini_Compl R I (Fig. 3.27 lane 4). A size difference of the cDNA copy vs. the genomic copy was evident on the gel (Fig. 3.27 lanes 2 and 3 vs. lane 4). The complete cDNA PG gene fragment was cloned into pGEM-T-Easy and transformed into *E. coli*. Five transformants, pPGcDNAcomplA- pPGcDNAcomplE, were subjected to colony PCR screening using both a complete PG gene primer set, C. lupini_Compl F I and C. lupini_Compl R, as well as an internal PG gene primer set, C. lupini_Int F I and C. lupini_Int R I. From the colony PCR results using both the complete primer set (Fig. 3.28) and internal primer set (Fig. 3.29), a slight size difference can be seen between the genomic and the cDNA fragments (Fig. 3.28 lane 2 vs. lanes 3 – 7 and Fig. 3.29 lane 7 vs. lanes 2- 6). One of the clones, pPGcDNAcomplA (Fig. 3.30), was subjected to restriction enzyme analysis with

EcoRI and compared to the *EcoRI* restricted genomic equavalent pPGcompl1 on a 2% TAE gel (Fig. 3.31). There is a slight size difference visible between the excised cDNA PG fragment (Fig. 3.31 lane 3) and the excised genomic PG fragment (Fig. 3.31 lane 5). Sequence analyses of pPGcDNAcomplA revealed that the size difference was due to the presence of a single intron of 59 bp, as predicted in the genomic copy that was spliced out in the cDNA copy (Fig. 3.32).

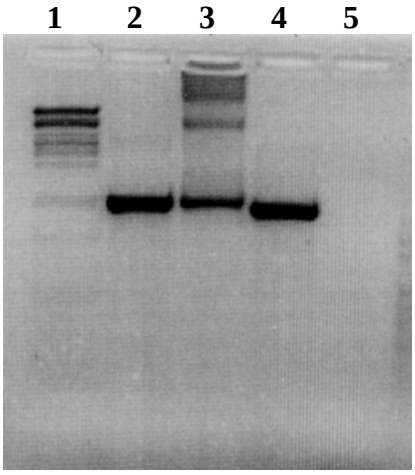
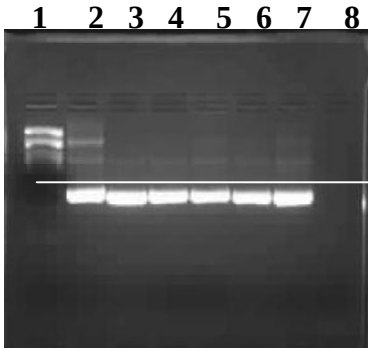


Fig. 3.27 RT-PCR analysis of *C. lupini* SHK 2148 cDNA sample grown on pectin for a time period of 4 days.
Lane 1: λPst molecular marker, genomic PG copy amplified from genomic DNA (lane 2), genomic DNA PG copy amplified from pPGcompl1 (lane 3), putative cDNA PG copy amplified from cDNA template of the Day 4 sample (lane 4) and water control (lane 5).



Line indicating size difference between the amplified genomic and cDNA PG fragments.

Fig. 3.28 Colony PCR of analyses of clones, pPGcDNAcomplA-pPGcDNAcomplE, containing the putative complete cDNA PG copy using a complete primer set.
The amplified genomic PG fragment from pPGcompl1 (lane2) was electrophoresed with the colony PCR products of clones pPGcDNAcomplA, pPGcDNAcomplB, pPGcDNAcomplC, pPGcDNAcomplD and pPGcDNAcomplE (lanes 3, 4, 5, 6 and 7 respectively). A water control (lane 8) and λPst molecular marker (lane1) were included in the electrophoresis.

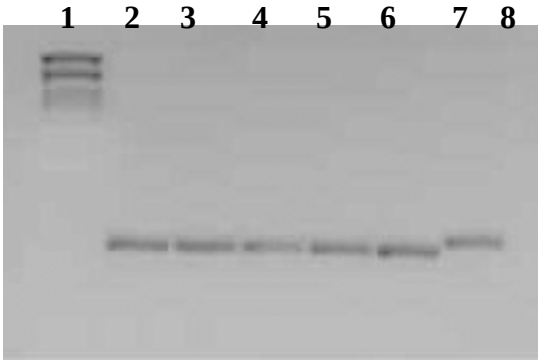


Fig. 3.29 Colony PCR of analyses of clones, pPGcDNAcomplA-pPGcDNAcomplE, containing the putative complete cDNA PG copy using an internal primer set.
The internal genomic PG fragment amplified from pPGcompl1 (lane7) were separated on an agarose gel with the colony PCR products of clones pPGcDNAcomplA, pPGcDNAcomplB, pPGcDNAcomplC, pPGcDNAcomplD and pPGcDNAcomplE (lanes 2, 3, 4, 5, and 6 respectively) as well as a water control (lane 8) and λPst molecular marker (lane1).

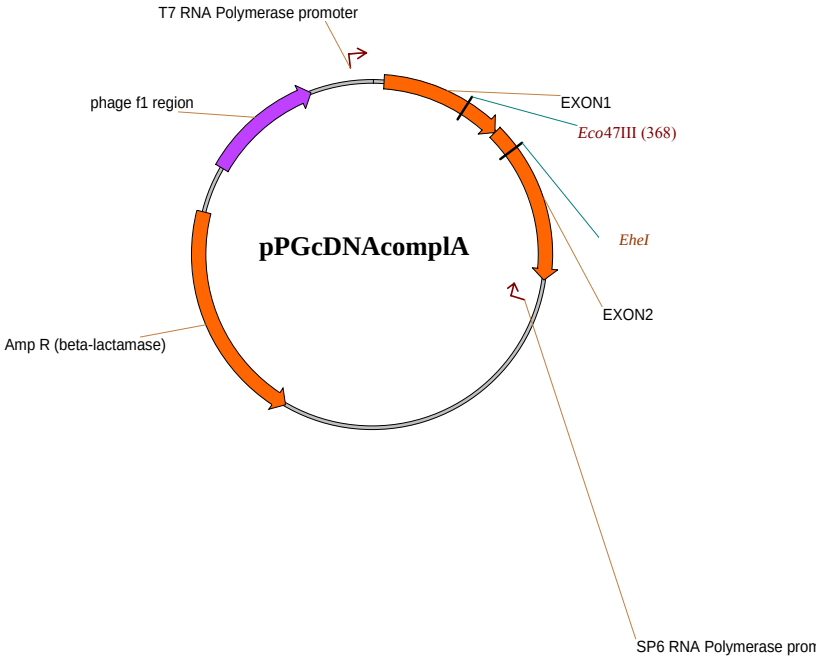


Fig. 3.30 Plasmid map of pPGcDNAcomplA, harbouring the full cDNA PG fragment, indicated by filled red arrows (Exon1 and Exon2).

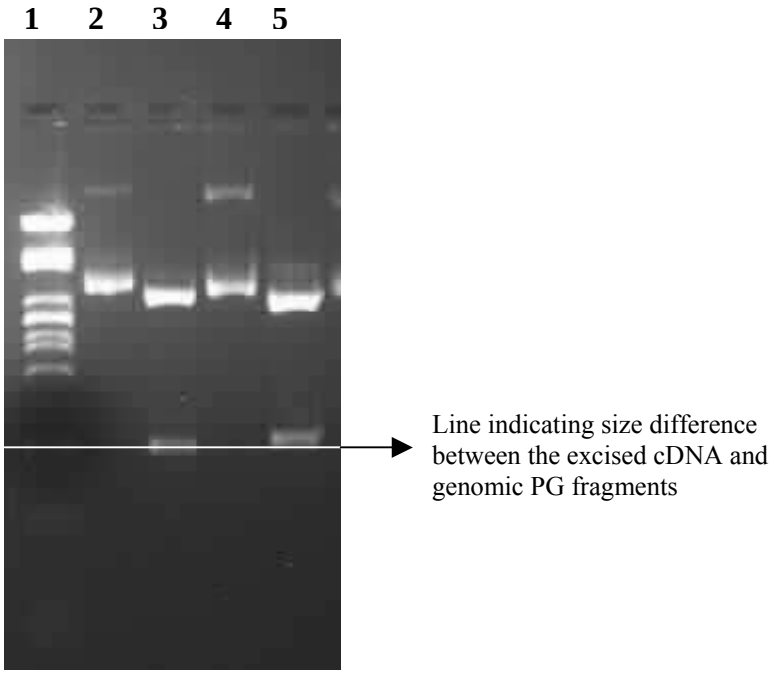


Fig. 3.31 *EcoRI* restriction analysis of pPGcDNAcomplA and pPGcompl1 on a 2% TAE agarose gel.
Unrestricted pPGcDNAcomplA and pPGcompl1 (lanes 2 and 4 respectively) were separated on a 2% agarose gel with *EcoRI* restricted pPGcDNAcomplA and pPGcompl1 (lanes 3 and 5 respectively). A λPst molecular marker was loaded in lane 1.

```
Genomic PG fragment     (451) ATCAGCGGCGTTTCTGGCCTCAAGGTGTAAGTCACAACTCAATGTTGGGT
cDNA PG fragment        (225) ATCAGCGGCGTTTCTGGCCTCAAGGT------------------------

Genomic PG fragment     (501) TTTTGGACAGTAATACTGACCCGACTATTCTCTAGCTCCAACATCCTTTT
cDNA PG fragment        (251) ----------------------------------CTCCAACATCCTTTT

Genomic PG fragment     (551) CGACAACAAGGATGGTGACTCCAAGGGTGGCCACAACACCGATGCCTTCG
cDNA PG fragment        (266) CGACAACAAGGATGGTGACTCCAAGGGTGGCCACAACACCGATGCCTTCG
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Fig. 3.32 Nucleotide alignment of a section of the genomic and full cDNA PG gene confirming the predicted intron (indicated in red).

3.4 Discussion

A PG gene was cloned and characterised from a lupin anthracnose fungus *C. lupini* SHK 2148. Culture filtrates of this pathogenic fungus, grown on pectin medium, displayed PG activity in an agarose diffusion assay, suggesting the presence and expression of one or more PG genes. A full-length copy of an endoPG gene as well as 420 bp of the 5 ' UTR was cloned from *C. lupini* SHK 2148 using internal PCR primers and inverse PCR. Northern and RT-PCR analysis confirmed that the gene was expressed using pectin as sole carbon source. Furthermore, sequence analysis of the cDNA copy revealed that a single putative intron is spliced out post-transcriptionally (Fig. 3.30).

Colletotrichum had previously been reported to have the highest PG activity for days 4, 5, 6 and 7 with slightly less activity for day 3 after inoculation in the pectin containing media (Oelofse, 2003). In this study the PG activity from a crude extract of *C. lupini* SHK 2148 grown in pectin media for 12 days were evaluated on days 3, 4, 5, 6, 7 and 12. PG activity was stronger during the early growth period, day 3 and 4 after inoculation, and decreased towards the end of the growth period. This was similar to the PG activity observed for *C. gloeosporioides* f.sp. *malvae*, in which the authors observed the highest PG activity 3 days after inoculation, which declined towards day 9 (Li *et al*, 2002).

A full length PG gene was cloned and characterised from *C. lupini* isolate SHK 2148, that has been reported has highly virulent on lupins (Personal communication, Dr. S.H. Koch). The full-length PG gene was cloned by first cloning an internal PG fragment from three *C. lupini* isolates (SHK 788, SHK 1033 and SHK 2148) using degenerate primers. Sequence analysis revealed that the internal PG gene fragment in all three isolates was identical, suggesting that the PG genes in the isolates also will have very high sequence homology. The complete PG gene cloned from *C. lupini* SHK 2148 is 1153 bp and contains a single intron of 59 bp that displays the typical 5' GT and AT 3' border sequences (Fig. 3.23 and Fig. 3.30). The intron was similar in size and position compared to the introns of the PG genes of *C. gloeosporioides* f. sp. *malvae* and *C. lindemuthianum* (*CmpgII* and *ClpgII* respectively). Splicing of the intron predicted from sequence analyses was confirmed by cloning the cDNA copy of

the PG gene by RT-PCR. The cloned PG from *C. lupini* SHK 2148 has a potential signal peptide of 23 amino acids. This suggests that it will be exported, similar to other fungal endoPGs. The 5' untranscribed region of the PG gene displays several characteristics of filamentous fungal genes such as the conserved border sequences of the intron, a putative TATAA signal and a CAAT box (Chapter 1; Gurr *et al*, 1987).

The predicted protein product, after intron splicing, consists of 363 amino acids, which falls in the size range of other predicted endoPG proteins. Further comparison of the predicted amino acid sequence of the PG gene from *C. lupini* SHK 2148 with other fungal PG genes revealed that the gene had the highest similarity to the *CmpgII* gene of *C. gloeosporioides* f. sp. *malvae* and the *ClpgII* gene of *C. lindemuthianum*. The *C. lupini* SHK 2148 predicted PG protein furthermore displayed all four domains present in fungal PGs as described by Markovič *et al* (2001) as outlined in Fig. 3.3.20; the NTD and DD domain of the *C. lupini* SHK 2148 PG protein was identical to the majority of the fungal PGs compared with variations present in *BcPG6* and *BcPG3* of *B. cinerea*. The third domain of *C. lupini* SHK 2148 PG protein was similar to that of *cmpgII* (*C. gloeosporioides* f. sp. *malvae*) and *clpgII* (*C. lindemuthianum*), while the fourth domain, RIK, was identical to all the other fungal PGs with the exception of *BcPG2* of *B. cinerea.*

The Southern blot membrane was UV fixed before storage, this enhanced the background colour of the membrane leading to very faint bands. Only single bands were detected on the membrane, which could indicate that this particular PG gene is present as a single copy in the genome. However, due to the poor quality of the developed membrane and the fact that fungi usually produce more than one PG, it is difficult to support this observation.

The PG cloned from *C. lupini* SHK 2148 is very similar to *CmpgII* of *Colletotrichum* f.sp. *malvae*, which is present as a single copy in the genome, has a similar size and contains a single intron of 64 bp (Li *et al*, 2002). These two genes are also very similar to the *Clpg2* gene of *C. lindemuthianum*, which is expressed during the early stages of infection (Dumas *et al*, 1999).

Northern blot analysis evaluated the *in vitro* transcription of the fungus grown at pH 5, on pectin media as sole carbon source. Transcripts were only detected for days 3, 4, and 7. The slight degradation of the RNA from days 6 and 12 might explain why no transcript was present for those days, yet no transcript was obtained from the RNA from day 5, which were not degraded. It could be that the regulation of the PG is pH dependant as is the case for the PG gene from *C. gloeosporioides* f.sp. *malvae*, transcripts for the PG gene were only detected when the fungus was grown on mallow cell wall extracts at pH 3.5 and not at pH 7.5 or other carbon source like pectin (Li *et al*, 2002). Thus, it might be possible that the growth of the fungus alters the pH and the transcription of the gene. This would however not explain the presence of a transcript at day 7. Another explanation for the transcripts at day 7 could be that more than one PG are involved. The first PG is expressed during the early growth period at pH5, while the other PG(s) are only activated later on at another pH level. There are reports of differentially regulated PGs that are activated at different time periods such as the PGs from *C. lindemuthianum* (Centis *et al*, 1997; Dumas *et al*, 1999) where both genes are induced by pectin during the early growth phase, while only *Clpg1* is expressed later on. There have also been reports of pH dependant expression of other CDWE; transcripts of the *pel* (pectate lyase) gene of *C. gloeosporioides* on avocado fruits were only detected if the pH of the pectin containing media was pH 5.8 and higher (Yakoby *et al*, 1999).

Future research needs to be done address the expression and regulation of the PG gene(s) from *C. lupini* SHK 2148 on different carbon sources and under different pH conditions.