

## **Chapter 4**

### **Heterologous expression of a Polygalacturonase gene from *Colletotrichum lupini* in *Pichia pastoris***

## Abstract

A cDNA copy of the PG gene of *Colletotrichum lupini* SHK 2148 was successfully constructed by switching an internal genomic fragment flanking the intron with the corresponding internal cDNA fragment. This cDNA copy was used as a template for designing several PG gene constructs for expression in *Pichia pastoris*. The mature PG protein with its own signal peptide as well as the mature protein with the  $\alpha$ -MF signal peptide was expressed in *P. pastoris*. In addition to this, two hybrid PGs, the PG from *C. lupini* SHK 2148 containing the N-terminal part of the mature *Fusarium moniliforme* PG and the latter containing the N-terminal part of the *C. lupini* SHK 2148 PG were also transformed into *P. pastoris*. None of the transformants had PG activity. Western blot analysis with an antibody directed against the *F. moniliforme* PG gave no signal while hybridisation was observed with an antibody directed against the *Aspergillus niger* PG. However, the size of the hybridising bands was very large which might be due to extensive post translational modifications such as glycosylation. Conclusive evidence could not be provided for expression of the *C. lupini* SHK 2148 PG in *P. pastoris*.

## 4.1 Introduction

Several expression systems are currently available for heterologous protein expression. They vary from simple bacterial systems to more complex mammalian cells (Anderson *et al*, 2002; Rai *et al*, 2001). Although bacterial systems are very easy and inexpensive to use, they do lend themselves to some limitations especially in regard to translational and post translational modifications of eukaryotic proteins (Rai *et al*, 2001).

A favourable alternative to bacterial expression systems has been presented in the form of yeasts such as *Saccharomyces* strains. The latter presented itself as a system that could be grown with ease on simple media to high cell densities (Rai *et al*, 2001), and was supported by the complete genomic sequence of the yeast, which made it easy to manipulate on a molecular level. Although *Saccharomyces* were a good alternative to the bacterial systems, it had some disadvantages; it was a fermentative system in which metabolic by-products could build up to toxic levels, fermentative conditions, such as pH levels and carbon source feed rate could become expensive and demanding to control and the secretion level of the desired proteins were sometimes very low (Rai *et al*, 2001).

*Pichia* is one of the genera of the methylotrophic yeasts that is able to utilize methanol as sole carbon source (Houard *et al*, 2002). *Pichia pastoris*, isolated from an exudate of oak, has been developed during the past 15 years for heterologous protein expression and is a better alternative to *Saccharomyces* (Cereghino *et al*, 1999; Houard *et al*, 2002). This system is fairly easy to manipulate, expresses proteins at high levels both intra-and extra cellular and provides higher eukaryotic protein modification functions (Cereghino *et al*, 1999). Furthermore, the *Pichia* system is a respiratory system, which is not limited by the accumulation of toxic fermentative products as in the *Saccharomyces* system (Cereghino *et al*, 1999). Apart from this, genes carried on transformation vectors are stably integrated into the genome of *Pichia* (Houard *et al*, 2002). So far, more than 400 proteins have been expressed successfully in *P. pastoris* including two fungal pectinases; *Fusarium solani* pectate lyases (*pelC* and *pelD*) (Cereghino *et al*, 2000). Several vectors and

strains are available for the expression of foreign genes (Cereghino *et al*, 2000). The expression is mainly driven under the influence of the methanol induced AOX1 (alcohol oxidase gene 1) or constitutive induced GAP (glyceraldehyde 3-phosphate dehydrogenase) promoters (Cereghino *et al*, 2000; Houard *et al*, 2002). The use of different promoters allows the constitutive or induced expression of recombinant proteins. The GAP promoter provides a constitutive production of the recombinant protein to levels comparable to that obtained with the AOX1 promoter. With the GAP promoter it is unnecessary to transfer cultures from one growth media to another and it is not necessary to build up and maintain the methanol concentrations in the media (Cereghino *et al*, 2000).

Yeast signal sequences are often used for expression of foreign proteins in yeast systems. Several signal sequences are available to secrete foreign proteins in yeast; so far the  $\alpha$ -MF factor ( $\alpha$ -mating factor signal sequence) from *S. cerevisiae* has given the best results. In some cases protein production using the  $\alpha$ -MF signal gave better success than the native protein signal sequence (Cereghino *et al*, 2000).

This chapter describes attempts to express a polygalacturonase (PG) gene cloned from *Colletotrichum* (chapter 3) in *Pichia pastoris*. The native genomic PG gene copy of *Colletotrichum* contains an intron (chapter 3). Therefore, it was necessary to obtain a cDNA copy without the intron for the heterologous expression in *P. pastoris*. Attempts to obtain a full length PG cDNA copy without the intron through RT-PCR failed, and only a partial internal fragment of the PG gene without the intron could be obtained. Therefore, a synthetic cDNA construct of the *Colletotrichum* PG gene was constructed by restriction digestion the genomic gene copy to remove the region containing the intron, followed by ligation of the partial cDNA fragment without the intron (obtained through RT-PCR), yielding a synthetic intronless cDNA copy of the full length PG gene. Several PG gene constructs for expression in *P. pastoris* were constructed from this cDNA construct, transformed into a *Pichia pastoris* and their PG activity evaluated. The work was performed in the laboratories of Prof. F. Cervone, La Sapienza University, Rome.

## 4.2 Materials and Methods

### 4.2.1 Ligation and cloning reactions

DNA and cDNA fragments were purified from agarose gels using Qiagen PCR purification kit (Qiagen, Germany), the Qiagen gel extraction kit (Qiagen) or Nucleospin PCR purification kit (Nucleospin, Macherey Nagel).

Ligation reactions were performed using 1U T4 ligase (Roche Diagnostics, Mannheim, Germany) and 50ng pGEM-T-Easy (Promega, Madison, USA) or 5ng pGAPZ $\alpha$ A and pPICZA respectively. All ligation reactions were performed overnight at 5 °C. Ligation mixtures were mixed with 40 $\mu$ l of competent *E. coli* cells, placed on ice for 30 minutes, heat shocked for 45 sec. at 42 °C, transferred to 1ml LB medium and incubated for 1 hour at 37 °C. Transformants were plated onto LB agar plates containing 100mg/ml ampicillin, 40 $\mu$ l IPTG (100mM) and 40 $\mu$ l X-gal (100mM) for pGEM-T-Easy transformants or LSLB (low salt luria broth) agar plates containing 25 $\mu$ g/ml zeocin for pGAPZ $\alpha$ A and pPICZA transformants. Alternatively ligation mixes were electroporated into cells using a Biorad electroporator at 1.8kV, 25m Faradys and 200 $\Omega$ .

### 4.2.2 RNA extractions

*C. lupini* SHK 2148 was grown in Czapek Dox medium (Difco, Detroit, USA) and transferred to pectin containing media as described in chapter 3. Total RNA was isolated from fungal mycelia (collected at day 3 after inoculation) using the RNeasy kit from Qiagen (Hilden, Germany). A quick check for the presence of RNA was done by running RNA samples on a 1% TAE agarose gel. From the total RNA, mRNA was isolated using the Oligotex mRNA isolation kit (Qiagen). The concentration of RNA samples was determined with a photometer (Eppendorf Biophotometer, Brinkmann Instruments, Westbury, NY, USA).

#### 4.2.3 RT-PCR amplification

A range of gene specific primers (Table 4.1) was used to amplify the PG cDNA copy from mRNA, using the Ready-To-Go RT-PCR bead kit (Amersham Biosciences, Buckinghamshire, UK). RT-PCRs were performed with four different PG primers sets: (1) a complete primer set, C. lupini\_Cmpl F I and C. lupini\_Cmpl R; (2) complete primer set C. lupini\_Cmpl F II and C. lupini\_Cmpl R II), (3) internal primer set PG-COLL-F2 and PG-COLL-R; and (4) internal primer set C. lupini\_Int F I and C. lupini\_Int R I. The RT-PCRs with the Ready-To-Go Beads were set up by first dissolving the dried pellet in the appropriate amount of RNase free water, followed by addition of the various primer pairs (20 $\mu$ M each) and 230ng of mRNA as template per bead tube. The reagents added to each Ready-To-Go Bead tube were mixed thoroughly, where after the reverse transcription reaction was done by incubating the tubes at 42 °C for 30 min. Following reverse transcription, PCR cycles were conducted that consisted of an initial denaturation step at 96 °C for 2 min, followed by a ten-cycle repeat of a denaturation step at 94 °C for 20 sec., an annealing step at 55 °C for 45 sec. and an elongation step at 72 °C for 45 sec. This was followed with another thirty cycles that each consisted of a denaturation step at 94 °C for 30 sec.; an annealing step at 55 °C for 40 sec. and an elongation step at 72 °C for 45 sec. The final elongation step was carried out at 72 °C for 7 minutes in a GeneAmp 2700 thermal cycler (ABI Advanced Biotechnological Institute, Perkin-Elmer Corporation, Foster City, USA). The PCR cycles for the four different primers were identical except for the annealing temperatures: the internal primer sets and the complete primer set I was annealed at 55 °C, while the complete primer set II was annealed at 65 °C in a PCR cycle that consisted of an initial denaturing step of 5 minutes at 95 °C, a thirty-three cycle repeat of three steps of 94 °C for 1 minute, the relevant annealing temperature for 1 minute and 72 °C for 1 minute. A final elongation step of 72 °C for 5 minutes completed the PCR reaction.

Each RT-PCR reaction was accompanied by a positive control PCR that contained the full length cloned plasmid PG gene copy (pPGcompl1) as template. The PCR reaction consisted of 40ng of pPGcompl1 (Chapter 3), 0.5 $\mu$ l Biotaq (1.25U/ $\mu$ l, Bioline, Germany), 5mM MgCl<sub>2</sub>, 200 $\mu$ M of each dNTP and 10 $\mu$ M of

C. lupini\_Cmpl F I and C. lupini\_Cmpl R. The PCR amplification cycles were the same as those used in the RT-PCR.

A control RT-PCR of the  $\beta$ -tubulin gene was also included in each RT-PCR with the PG gene primers to ensure that the mRNA was of good quality and that reverse transcription was taking place. The size product amplified from genomic DNA with the  $\beta$ t1 primers, are approximately 540 bp for ascomycetes (Glass *et al*, 1995). This region is interrupted with a 59 bp intron in *Neurospora crassa*, but the size of the intron might vary between the different ascomycetes (Glass *et al*, 1995). The RT-PCR of the  $\beta$ -tublin gene consisted of 2 $\mu$ l of each  $\beta$ -tublin primer (Table 4.1)  $\beta$ t1a and  $\beta$ t1b (10mM respectively) and 230ng m-RNA per bead reaction in a final volume of 50 $\mu$ l. A control  $\beta$ -tublin PCR reaction with genomic DNA as template was also included. The  $\beta$ -tublin PCR reactions consisted of 30ng of genomic DNA, 0.25 $\mu$ l Biotaq (1.25U/ $\mu$ l), 1.5mM MgCl<sub>2</sub> and dNTPs (200 $\mu$ M of each).

**Table 4.1 Primers used for RT-PCRs, sequencing reactions and the construction of the PG constructs.**

Primer	Sequence	Annealing Temp
$\beta$ t1a	5' TTCCCCCGTCTCCACTTCTTCATG 3'	58 °C
$\beta$ t1b	5' GACGAGATCGTTCATGTTGAACTC 3'	
C. lupini_Compl F	5' CCACGTGTTGATCACATACC 3'	55 °C
C. lupini_Compl R	5' CCCACCATCAAGCATTTAGC 3'	
C. lupini_Compl FII	5' CGATGAAGTTCCTCTCCGTTGTC 3'	65 °C
C. lupini_Compl RII	5' TTTAGCACTTGGCACCGGAGCCGGAAGG 3'	
PG-COLL-F2	5' CGC GCC TCG TGC ACC TTC 3'	55 °C
PG-COLL-R	5' GCT GAC GCC GGA CCA CTT CCA 3'	
C. lupini_Int F I	5' CCTTCGGCTACAAGGAGT 3'	55 °C
C. lupini_Int R I	5' AACGGAACCGATGGACAG 3'	
T7	5' TAATACGACTCACTATAGGG 3'	55 °C
SP6	5' ATTTAGGTGACACTATAG 3'	
FMPG FWeco_pichia	5-ATGCGAATTCGATCCCTGCTCCGTGACT-3	55 °C
FMPG RVXba_pichia	5-ATGCAGATCTCTAGCTGGGGCAAGTGTG-3	55 °C
CollyPGEcoFWII	5' ACGTAGAATTCGCCCCCGCCGAGCTC 3'	58 °C
CollyPGEcoFWIII	5' ACGTAGAATTCGCCTCGTGCACCTTCAC 3'	58 °C
CollyPGXbaRV	5' ATCGATCTAGATTAGCACTTGGCACCGGA 3'	58 °C
SOE a.sense CLFMPG	5' CTGTTAGAACCTTTGCCATCCCACCAGCGCTCACCT 3'	55 °C
SOE sense CLFMPG	5' AGGGTGAGCGCTGGTGGGATGGCAAAGGTTCTAACAG3'	55 °C
SOE Forward FMCLPG	5' GGTCAGGCGTACTGGGACGGCAAGGGAGGCAA 3'	55 °C
SOE Reverse FMCLPG	5' TTGCCTCCCTTGCCGTCCCAGTACGCCTGACC 3'	55 °C

#### 4.2.4 Cloning and characterization of an internal cDNA PG gene fragment

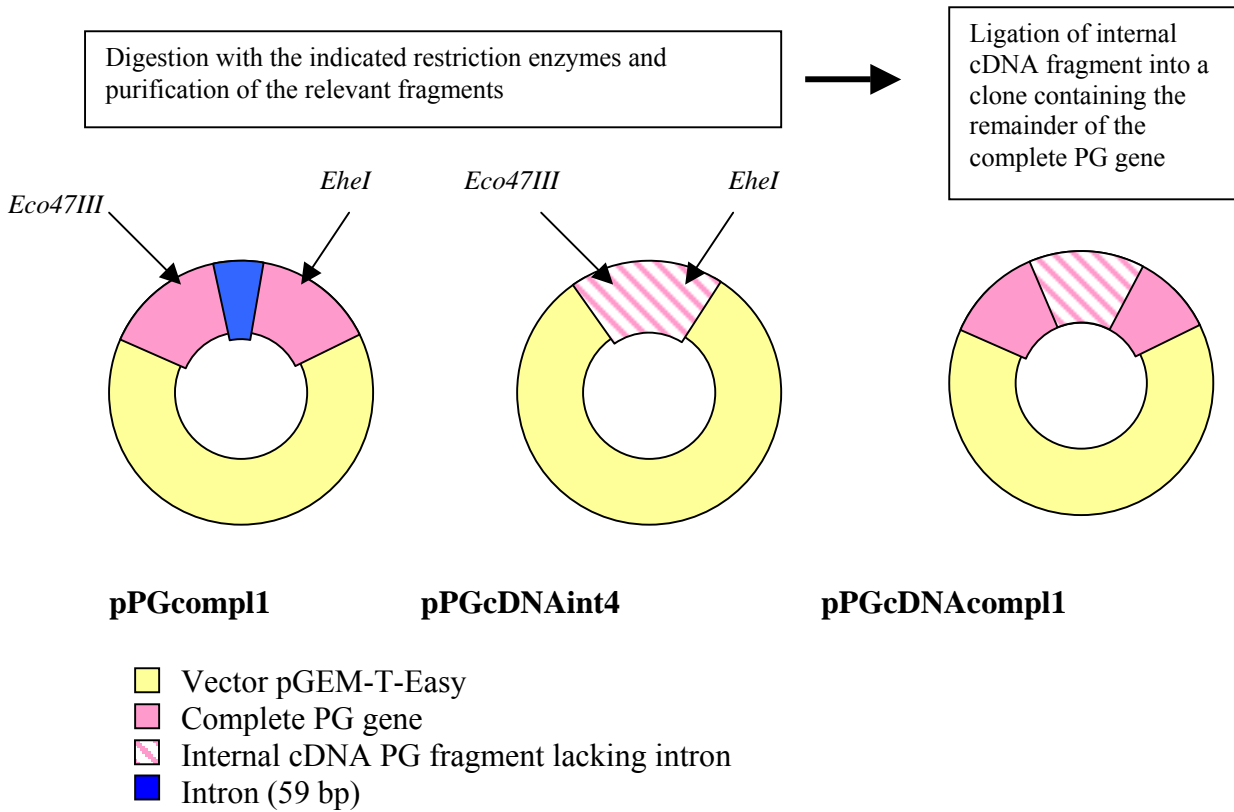
40ng purified cDNA PG fragment was cloned into pGEM-T-Easy and transformed into *E.coli* JM 109 competent cells as described before (Section 4.2.2). Plasmid DNA was isolated from clones according to the method of Sambrook *et al* 1989. An aliquot of 5 $\mu$ l of each plasmid preparation was subjected to restriction analysis with 0.3 $\mu$ l



*EcoRI* (10U/ $\mu$ l, Roche) in a 10 $\mu$ l reaction volume for 1h at 37 °C. Restriction reactions were evaluated on an EtBr containing 1% (w/v) TAE agarose gel. Clones were subjected to sequence analysis with T7 and SP6 primers (Table 4.1). The sequence reaction was set up as described in Chapter 3 (3.2.4).

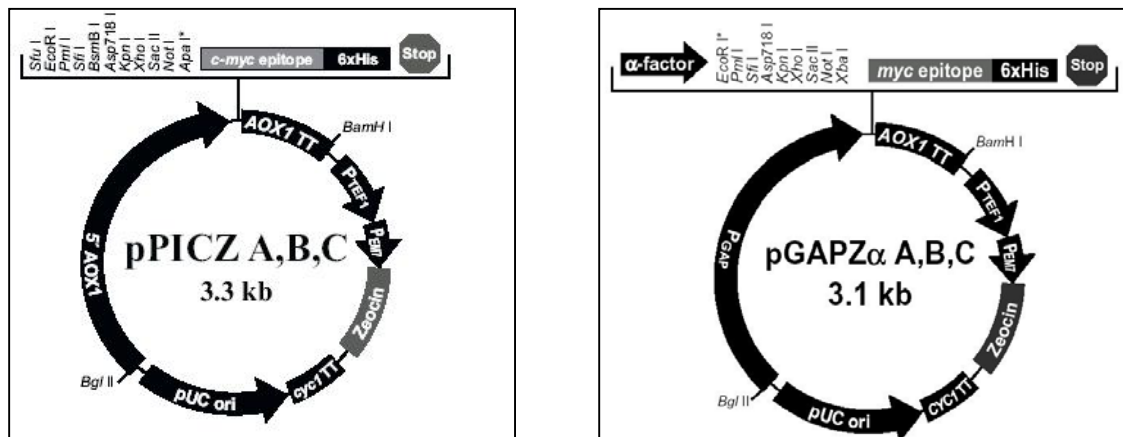
#### **4.2.5 Construction of a full-length PG cDNA copy**

pPGcompl1 (100ng) and pPGcDNAint4 (100ng) were transformed into *E.coli* JM110 (dam<sup>-</sup> strain) via electroporation using a Biorad electroporator as described above. Clones were screened using colony PCR with the complete primer set C. lupini\_Comp1 FII and C. lupini\_Comp1 RII and the internal PCR primer set II (C. lupini\_Int F I and C. lupini\_Int R I) and previously described PCR parameters (Section 4.2.5). Plasmid DNA was isolated and subjected to *EcoRI* analyses as described previously (section 4.2.6). pPGcompl1 ( $\approx$ 1 $\mu$ g) and pPGcDNAint4 ( $\approx$ 1 $\mu$ g) were digested with 2.5 $\mu$ l *EheI* (10U/ $\mu$ l) and *Eco47111*(10U/ $\mu$ l) (Fermentas, Maryland, USA) respectively in a 50 $\mu$ l reaction volume. The reaction was evaluated on a 1.2% agarose gel. The *EheI* and *Eco47111* restricted internal cDNA PG fragment, was gel-purified and ligated to *EheI* and *Eco47111* digested pPGcompl1 (Fig. 4.1). The ligation reaction was set up as previously described and chemically transformed into JM109. Transformants were screened via colony PCR with the C. lupini\_Int F I and C.lupini\_Int R I primers. Plasmid constructs were isolated from clones, subjected to restriction enzyme analysis with *EheI* and *Eco47111* and sequenced with the universal primer sets SP6 and T7.



**Fig. 4.1** Schematic presentation of the construction of a cDNA clone of the PG gene from *Colletotrichum lupini* SHK 2148.

#### 4.2.6 Expression vectors



**Fig. 4.2** *Pichia pastoris* expression vectors: (www.invitrogen.com)

Heterologous expression of the PG gene was performed with the easy select *Pichia* expression kit from Invitrogen (Invitrogen Research Corporation Technologies, Inc. Tucson, Arizona, Cat. No. K174001).

Two vectors, pGAPZ $\alpha$ A and pPICZA (Fig. 4.2) were used to design the PG gene constructs for expression in *P. pastoris*. The vectors are provided in three reading frames (A, B, and C), contains a MCS (multiple cloning site) as well as a C-terminal myc epitope (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) (Evans *et al*, 1985) and a C-terminal polyhistidine tag (encoding six histidine residues that form a metal-binding site) that facilitates the detection and affinity purification of the recombinant protein respectively (Fig. 4.3 and Fig. 4.4). The selectable marker for the vectors is derived from the zeocin resistance gene that is encoded by the *Sh ble* gene (*Streptoalloteichus hindustanus ble* gene). Two different promoters drive protein expression; in pGAPZ $\alpha$ A, the GAP promoter allows constitutive expression of the foreign protein, while the expression in pPICZA is under the regulation of the methanol induced AOX1 promoter (Fig. 4.4 and Fig. 4.3 respectively). Another significant difference between the two vector systems is the  $\alpha$ -Factor secretion signal that is present in pGAPZ $\alpha$ A (Fig. 4.4). This is the native *Saccharomyces cerevisiae*  $\alpha$ -factor, which has proven to be very successful for the secretion of recombinant proteins produced in *P. pastoris*. The AOX1 transcription termination (TT) region is the native transcription termination and polyadenylation region from the AOX1 gene, which ensures correct processing of the foreign gene to provide a more stable mRNA transcript (Fig. 4.3 and Fig. 4.4). Both vectors are shuttle vectors that are able to replicate in *E. coli* (with the pUC origin) and be integrated into the *P. pastoris* genome via a homologous recombination between the GAP and AOX1 promoter region and loci on the genome respectively.

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5' end of AOX1 mRNA
811 AACCTTTTTT TTTATCATCA TTATTAGCTT ACTTTCATAA TTGCGACTGG TTCCAATTGA
5' AOX1 priming site
871 CAAGCTTTTG ATTTTAACGA CTTTAAACGA CAACTTGAGA AGATCAAAAA ACAACTAATT
Sfu I EcoR I Pml I Sfi I BsmB I Asp718 I Kpn I Xho I
931 ATTTCGAAACG AGGAATTCAC GTGGCCAGC CGGCCGTCTC GGATCGGTAC CTCGAGCCGC
Sac II Not I Apa I myc epitope
991 GGC GGCCGCC AGCTT GGGCCC GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG
Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu
Polyhistidine tag
1042 AAT AGC GCC GTC GAC CAT CAT CAT CAT CAT CAT TGA GTTTTAGCCT TAGACATGAC
Asn Ser Ala Val Asp His His His His His His ***
1098 TGTTCCTCAG TTCAAGTTGG GCACCTACGA GAAGACCGGT CTTGCTAGAT TCTAATCAAG
3' AOX1 priming site
1158 AGGATGTCAG AATGCCATTT GCCTGAGAGA TGCAGGCTTC ATTTTGTATA CTTTTTATT
3' polyadenylation site
1218 TGTAACCTAT ATAGTATAGG ATTTTTTTTG TCATTTTGTT

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Fig. 4.3 pPICZA multiple cloning site.

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361 GATTATTGGA AACCACCAGA ATCGAATATA AAAGCGAAC ACCTTTCCCA ATTTTGTTTT
pGAP forward priming site
421 CTCCTGACCC AAAGACTTTA AATTTAATTT ATTTGTCCCT ATTTCATCA ATTGAACAAC
481 TATTCGAAA CG ATG AGA TTT CCT TCA ATT TTT ACT GCT GTT TTA TTC GCA
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala
532 GCA TCC TCC GCA TTA GCT GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG
Ala Ser Ser Ala Leu Ala Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr
α-factor signal sequence
583 GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT TTA GAA GGG GAT
Ala Gln Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp
634 TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC AGC ACA AAT AAC GGG TTA TTG
Phe Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
685 TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA GAA GAA GGG GTA TCT
Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val Ser
Xho I Kex2 signal cleavage EcoR I Pml I Sfi I
736 CTC GAG AAA AGA GAG GCT GAA GC GAATTCAC GTGGCCA GCCGGCCGTC TCGGATC
Leu Glu Lys Arg Glu Ala Glu Ala
Ste13 signal cleavage
Asp718 I Kpn I Xho I Sac II Not I Xba I myc epitope
793 GGTACCTCGA GCCGCGGCGG CCGCCAGCTT TCTA GAA CAA AAA CTC ATC TCA GAA GAG
Glu Gln Lys Leu Ile Ser Glu Glu
polyhistidine tag
851 GAT CTG AAT AGC GCC GTC GAC CAT CAT CAT CAT CAT CAT TGA GTTTTAGC CTTA
Asp Leu Asn Ser Ala Val Asp His His His His His His ***
905 GACATGACTG TTCCTCAGTT CAAGTTGGGC ACTTACGAGA AGACCGGTCT TGCTAGATTC TAAT
3' AOX1 priming site
969 CAAGAGGATG TCAGAATGCC ATTTGCTGTA GAGATGCAGG CTTTATTTTT GATACTTTTT TATT
1033 TGTAACCTAT ATAGTATAGG ATTTTTTTTG TCATTTTGTT TCTTCTCG

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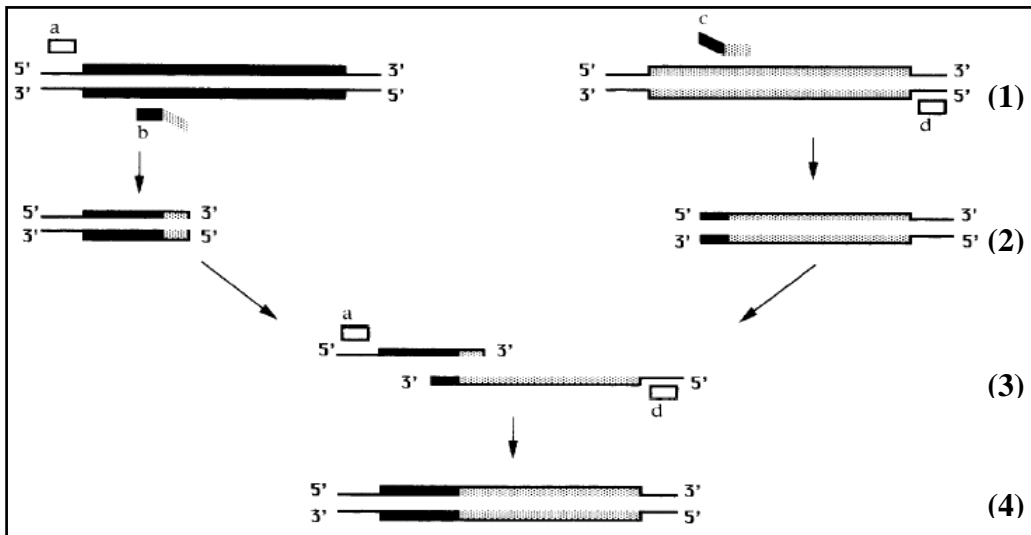
Fig. 4.4 pGAPZαA multiple cloning site.

#### 4.2.7 PG gene constructs for expression in *P. pastoris*

##### 4.2.7.1 PCR amplification

The PG fragment for PG constructIII was amplified from pPGcDNAcompl7 using primer pair CollyPGEcoFWIII (50 $\mu$ M) and CollyPGXbaRV (50 $\mu$ M) (Fig. 4.8).

The N- terminus sequence of the *Fusarium moniliforme* mature PG gene (Fig. 4.7) sequence was switched with the N- terminus sequence of the *C. lupini* SHK 2148 PG gene (Fig. 4.8) and vice versa via Splicing by Overlap Extension by PCR (SOE PCR) (Fig. 4.5 and Fig. 4.6). SOE PCR involves three separate PCR reactions. In the first two PCR reactions (Fig. 4.5 (1)) two hybrid primers (Fig. 4.5 b & c) are involved, which are designed from the known sequence and are tipped with overlapping sequence data. In this study SOE sense CLFMPG and SOE a.sense CLFMPG are the hybrid primers for the first hybrid construct while SOE Reverse FMCLPG and SOE Forward FMCLPG are for the second hybrid construct (Fig. 4.6). SOE sense CLFMPG and SOE Reverse FMCLPG are designed from *F. moniliforme* PG gene sequence and tipped with *C. lupini* SHK 2148 sequence, while SOE a.sense CLFMPG and SOE Forward FMCLPG are designed from *C. lupini* SHK 2148 gene sequence and tipped with sequence data from *F. moniliforme* PG gene (Fig. 4.6). These hybrid primers are used with two terminal primers (Fig. 4.5 a & d) to create PCR products, one which contains sequence data 5' to the splice point and one which contain sequence data 3' to the splice point (Fig. 4.5 (2)). In this case FMPG RVXba\_pichia creates SW1, CollyPGEcoFWIII creates SW2III, FMPG FWeco\_pichia delivers SW3 and CollyPGXbaRV produces SW4 (Fig. 4.6). These templates are denatured and will partly anneal at the overlapping sequence (Fig. 4.5 (3)) that was incorporated by the hybrid primers. The partly annealed DNA serves as template for the third PCR reaction in which the terminal primers are used to produce the final hybrid product ((Fig. 4.5 (4)). In this study SWAIII produces the *F.moniliforme* PG with the *Colletotrichum* N terminus using the SWIII and SW1 PCR products, while SWB produces the *Colletotrichum* PG with the *F. moniliforme* N terminus from the SW3 and SW4 products (Fig. 4.6).



a & d: Terminal primers

b & c: Hybrid primers

**SW1 (Swop 1):**

SOE sense CLFMPG



FMPG RVXba pichia

**SW2 III (Swop 2- Mature PG *C. lupini*)**

CollyPGEcoFWIII



SOE a.sense CLFMPG

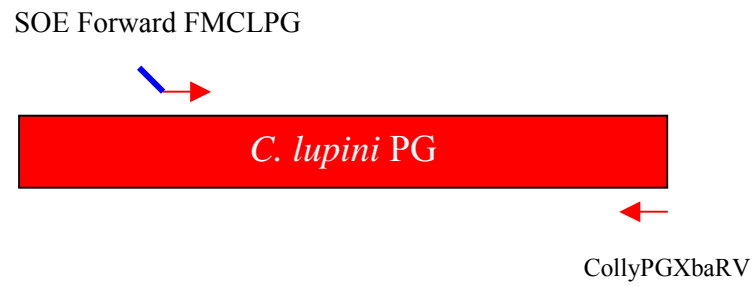
**SW3(Swop 3):**

FMPG FWeco\_pichia

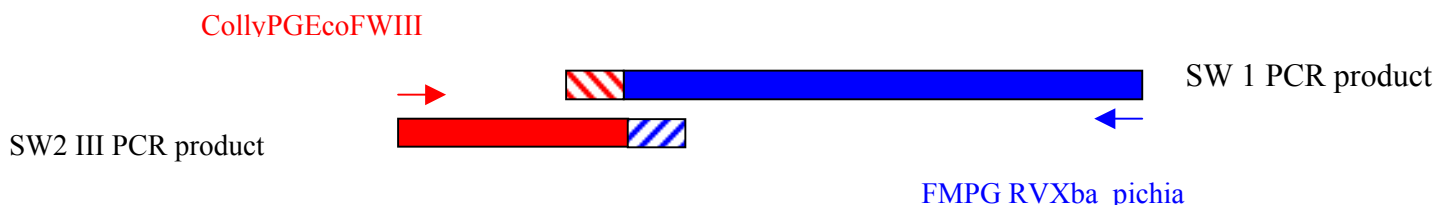


SOE Reverse FMCLPG

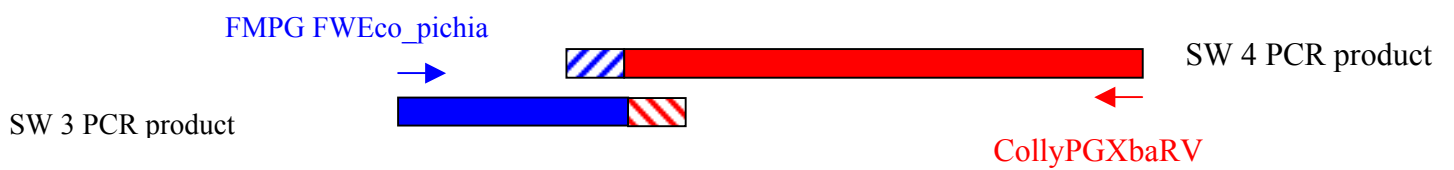
**SW4(Swop 4):**



**SW A III:**



**SW B:**



**Fig. 4.6 SOE PCR amplification steps creating the hybrid PG gene construct.**

University of Pretoria etd – Lotter, H C (2005)

M V R N I V S R L C S Q L F A L P  
5' ATG GTT CGA AAC ATC GTA TCG CGG CTT TGC TCC CAG CTG TTT GCT CTG CCC

S S S L Q E R  
TCA AGC TCT CTC CAG GAA CGA

FMPG FWeco\_pichia  
1 GAT CCC TGC TCC GTG ACT GAG TAC TCT GGC CTC GCC ACC GCT GTC TCA TCC  
1 D P C S V T E Y S G L A T A V S S  
52 TGC AAA AAC ATC GTG CTC AAC GGT TTC CAA GTC CCG ACA GGC AAG CAA CTC  
18 C K N I V L N G F Q V P T G K Q L  
103 GAC CTA TCC AGC CTC CAG AAT GAC TCG ACC GTT ACC TTC AAG GGC ACG ACC  
35 D L S S L Q N D S T V T F K G T T  
154 ACT TTT GCC ACC ACT GCT GAT AAC GAC TTT AAT CCT ATC GTC ATT AGT GGA  
52 T F A T T A D N D F N P I V I S G  
205 AGT AAC ATC ACT ATC ACT GGT GCA TCT GGC CAT GTC ATT GAT GGC AAC GGT  
69 S N I T I T G A S G H V I D G N G  
SOE PCR splice point  
256 CAG GCG TAC TGG GAT GGC AAA GGT TCT AAC AGC AAT AGC AAC CAA AAG CCC  
86 Q A Y W D G K G S N S N S N Q K P  
307 GAT CAC TTC ATC GTT GTT CAG AAG ACC ACC GGC AAC TCA AAG ATC ACA AAC  
103 D H F I V V Q K T T G N S K I T N  
358 CTA AAT ATC CAG AAC TGG CCC GTT CAC TGC TTC GAC ATT ACA GGC AGC TCG  
120 L N I Q N W P V H C F D I T G S S  
409 CAA TTG ACC ATC TCA GGG CTT ATT CTT GAT AAC AGA GCT GGC GAC AAG CCT  
137 Q L T I S G L I L D N R A G D K P  
460 AAC GCC AAG AGC GGT AGC TTG CCC GCT GCG CAT AAC ACC GAC GGT TTC GAC  
154 N A K S G S L P A A H N T D G F D  
511 ATC TCG TCC AGT GAC CAC GTT ACG CTG GAT AAC AAT CAT GTT TAT AAC CAA  
171 I S S S D H V T L D N N H V Y N Q  
562 GAT GAT TGT GTT GCT GTT ACT TCC GGT ACA AAC ATC GTC GTT TCT AAC ATG  
188 D D C V A V T S G T N I V V S N M  
613 TAT TGC TCC GGC GGC CAT GGT CTT AGT ATC GGA TCT GTT GGT GGA AAG AGC  
205 Y C S G G H G L S I G S V G G K S  
664 GAC AAT GTC GTT GAT GGT GTT CAG TTC TTG AGC TCG CAG GTT GTG AAC AGT  
222 D N V V D G V Q F L S S Q V V N S  
715 CAG AAT GGA TGT CGC ATC AAG TCC AAC TCT GGC GCA ACT GGC ACG ATC AAC  
239 Q N G C R I K S N S G A T G T I N  
766 AAC GTC ACC TAC CAG AAC ATT GCT CTC ACC AAC ATC AGC ACG TAC GGT GTC  
256 N V T Y Q N I A L T N I S T Y G V  
817 GAT GTT CAG CAG GAC TAT CTC AAC GGC GGC CCT ACT GGA AAG CCG ACC AAC  
273 D V Q Q D Y L N G G P T G K P T N  
868 GGA GTC AAG ATC AGC AAC ATC AAG TTC ATC AAG GTC ACT GGC ACT GTG GCT  
290 G V K I S N I K F I K V T G T V A  
919 AGC TCT GCC CAG GAT TGG TTT ATT CTG TGT GGT GAT GGT AGC TGC TCT GGA  
307 S S A Q D W F I L C G D G S C S G  
970 TTT ACC TTC TCT GGA AAC GCT ATT ACT GGT GGT GGC AAG ACT AGC AGC TGC  
324 F T F S G N A I T G G G K T S S C  
FMPG RVXba\_pichia  
1020 AAC TAT CCT ACC AAC ACT TGC CCC AGC TAG 3'  
341 N Y P T N T C P S STOP

Fig. 4.7 *F. moniliforme* PG gene sequence (Dr. C. Caprari).



**CollyPGEcoFWII**

1 M K F L S V V A G L S A L A A A A P A E  
 1 5' ATG AAG TTC CTC TCC GTT GTC GCG GGC CTC AGC GCC CTG GCT GCC GCC GCC CCC GGC GAG

**CollyPGEcoFWIII**

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

**SOE PCR splice point**

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 STOP  
 1141 GGT GCC AAG TGC TAA 3'

**CollyPGXbaRV**

**Fig. 4.8 Complete PG gene sequence from *C. lupini* SHK 2148.**

*Italics* Signal peptide

Regions from which primers were designed

Splice point region for the SOE PCR

----- Intron

#### 4.2.7.2 PCR parameters and cycle

Each PCR reaction was carried out in a final volume of 50 $\mu$ l, using 10ng of pPGcDNAcompl7 template (or less than 10ng for the second set of PCR reactions of the SOE PCR method), dNTPs (final concentration 0.2mM), 1.25 units of high fidelity PWO taq (Roche, Mannheim, Germany) and the relevant primer pairs (final concentration 1 $\mu$ M). As a positive control, CollyPGEcoFWII (50 $\mu$ M) and CollyPGXbaRV (50 $\mu$ M) primer pair was used to amplify a PG fragment from the pPGcDNAcompl7.

The PCR cycle, performed in a Gene pulser x-cell (Biorad) consisted of a denaturing cycle of 4 minutes at 94 $^{\circ}$ C, followed by a three cycle repeat of 94 $^{\circ}$ C for 1:30 minutes; the relevant annealing temperature of the primers (table 4.1) for 1:30 minutes, an extension temperature of 72  $^{\circ}$ C for 1:30 minutes. The cycle was finished by a final elongation step of 72 $^{\circ}$ C for 7 minutes.

A tenth of the PCR reactions were evaluated on a 1% TAE agarose gel containing EtBr. The PCR products were excised, purified from the gel and electrophoresed with a dilution series of  $\lambda$  DNA to determine their concentration.

#### 4.2.7.3 Construction of the PG gene constructs

pPGcDNAcompl7 was digested with *EcoRI*, while amplified PG fragments were restricted with *EcoRI* and *XbaI*. Digested PG fragments were gel-purified and subjected to concentration determination on a 1% TAE agarose gel with a dilution series of  $\lambda$  DNA.

*Pichia* expression vectors, approximately 200ng of each, were digested with the 10U of the relative restriction enzymes; pPICZA were restricted with *EcoRI* while pGAPZ $\alpha$ A were restricted *EcoRI* and *XbaI* for 1h at 37  $^{\circ}$ C. The restriction reactions were evaluated on a 1% TAE agarose gel, purified and subjected to concentration determination with a dilution series of  $\lambda$  DNA on a 1% TAE agarose gel. Purified vector and PG fragments were ligated and transformed into *E. coli* top 10 F' as previously described (section 4.2).

#### 4.2.7.4 Screening of transformants

Colony PCR was used to screen several transformants (table 4.2)

**Table 4.2 PG clones screened with colony PCR with different primer pairs and annealing conditions.**

Construct	Number of colonies screened	Primer pair	Annealing temperature
PG construct I	60	CollyPGEcoFWIII (50µM)	58 °C
PG construct III	10	CollyPGXbaRV (50µM)	
SWAIII	5	CollyPGEcoFWIII (50µM) FMPGRVXba_pichia (50µM)	55 °C
SWB	5	FMPG FWeco_pichia (50µM) CollyPGXbaRV (50µM)	55 °C

Positive clones were inoculated into 5ml LSLB media with 25µg/ml zeocin incubated overnight at 37 °C whereafter plasmid isolations were performed with the Nucleospin plasmid extraction kit (Nucleospin plasmid DNA purification kit, Macherey Nagel).

Plasmid DNA was subjected to restriction enzyme analysis; PG construct I clones were digested with *EcoRI*, *KpnI* or *XhoI* respectively, PG construct III clones were digested with *EcoRI* and *XbaI* respectively as well as with *EcoRI* and *KpnI* in double digest restriction reactions and SWAIII and SWB clones were digested with *EcoRI* and *XbaI* in a double digestion reaction. Restriction reactions were performed in a 10 µl reaction volume for 1h at 37 °C.

#### 4.2.8 Transformation of *P. pastoris*

*P. pastoris* strain X-33, with the wild-type genotype and methanol utilization (mut+) phenotype were prepared for electroporation according to the suppliers instructions (Invitrogen) :

The *P. pastoris* strain was shake-incubated overnight in 5ml YPD at 28 °C. The 5ml culture was used to inoculate 500ml fresh YPD medium. The culture was grown overnight until it reached an OD<sub>600</sub> between 1.3 – 1.5. *Pichia* cells were collected by centrifugation at 1500 x g for 5 minutes at 4°C. The pellet was resuspended in 500ml ice-cold sterile water. This step was repeated and cells were resuspended in 250ml ice-cold sterile water. Cells were centrifuged again and resuspended in 20ml ice-cold sorbitol (1M). After a final centrifugation step, cells were resuspended in 1 ml ice-cold sorbitol (1M) and kept on ice for the electroporation procedure.

Clones were prepared for *Pichia* transformation by linearising pGAPZαA constructs with 5 U *AvrII* or pPICZA constructs with 5U *BstXI*. Linearised DNA were precipitated for 30 minutes at –80 °C with 2 volumes isopropanol and ammonium acetate (final concentration of 2M). The precipitated DNA were centrifuged for 30 minutes at 4 °C at 18 000 rpm and vacuum dried for 5 minutes. The concentrations of the linearised product were determined before electroporation of *P. pastoris*.

Between 50ng/μl and 200ng/μl of DNA were added to 80μl of *Pichia* cells, cells were pulsed under the predetermined Gene Pulser Xcell Biorad electroporator conditions: C = 25 uF; PC = 200ohm; V = 2.0kV, (Biorad, Life Science Research Group 2000 Alfred Nobel Drive Hercules, CA) and immediately transferred to 1 ml ice-cold sorbitol (1M). The transformed cells were incubated for 3-4 h at 28 °C without shaking, where after 20 – 100μl were plated onto LSLB plates with zeocin (100μg/ml). Plates were incubated for 2- 3 days at 28 °C.

Colonies transformed with pPICzA were inoculated into 3ml BMGY containing zeocin (100μg/ml) and incubated for 24-36h before they were transferred to BMMY light containing methanol (0.5 –3%) (Appendix A), while colonies harbouring pGAPzAα constructs were inoculated into 3ml BMMY light (Appendix A), with zeocin (100μg/ml). Clones in the BMMY light medium were grown for approximately four days

#### 4.2.9 PG activity assay

The cup plate method was adapted from the agarose diffusion assay of Taylor and Secor (1987). Four *P. pastoris* clones of each construct were analysed on 90mm diameter cup plates containing 30 ml of a solution of polygalacturonic acid from citrus spp. (0.5%), agarose (0.8%) (Invitrogen) both dissolved in Sodium acetate (100mM, pH 4.6).

Wells were pressed into the cup plate and filled with 60µl crude culture extract from each *P. pastoris* clone. The crude culture extract was obtained from an aliquot of the culture that was grown BMMY light media with methanol (for the pPICzA clones) or BMMY light media (for the pGAPzAα clones). Purified *Fusarium moniliforme* PG (0.5ng) were included as a positive control on each plate. Plates were incubated overnight at 28 ° C and stained with HCl (6M).

#### 4.2.10 SDS PAGE analysis

*P. pastoris* clones were grown for 4 days in BMMY light media with methanol (for the pPICzA clones) and BMMY light media (for the pGAPzAα clones) at 28 °C with shaking at 280 rpm. Supernatant were obtained from these cultures by spinning 1ml of culture at 5 000 rpm for 1 min. Proteins were precipitated from 500µl of the supernatant using TCA (trichloroacetic acid). TCA was added to the supernatant (0.11v/v) and mixed well. The samples were incubated at 4 °C for 30 min and centrifuged at maximum speed (12 000 rpm) for 30 min. TCA was discarded and the pellets were washed three times with 70% ice-cold ethanol. Pellets were air dried and resuspended in 10µl SDS loading buffer (3×). Approximately a third of the samples were electrophoresed on the SDS PAGE gel (See Appendix for preparation). A purified PG sample from *Fusarium moniliforme* (1ng/µl) and the LMW ladder from the LMW calibration kit from Amersham were included on the gels.

Electrophoresis proceeded for approximately 1h at 170V and 300mA in the Biorad Mini Trans-Blot system. The SDS-PAGE gel was silver stained by first fixating the gel for 30 minutes in a fixation buffer (50%) mercaptoethanol, 12% Acetic acid and 0.0185% of a 37 % formamide stock solution. Subsequently, the gel was washed

twice for 10 minutes in a 50% ethanol solution, pre-treated for 1 minute in a 200mg/10ml sodium tiosulphate ( $\text{Na}_2\text{S}_2\text{O}_3 \times 5 \text{H}_2\text{O}$ ) solution, and then briefly washed three times in ultra pure water where after it was incubated for 10 minutes in the silver nitrate solution (0.2%  $\text{AgNO}_3$ , 0.0280% of the 37% Formamide stock solution). After the incubation period, two brief wash steps in ultra pure water removed the excess silver nitrate. The gel was developed in a developing solution (6%  $\text{Na}_2\text{CO}_3$ , 0.0185% of the 37% formamide stock solution and 200mg/10 ml sodium tiosulphate) until clear bands were visible on the gel. The gel was briefly washed in ultra pure water and the development reaction terminated by a solution containing 50% mercaptoetanol and 12% acetic acid.

#### 4.2.11 Western blot analysis

One clone for each construct expressed in *P. pastoris* was chosen for a Western blot analysis. The analysis was performed on the supernatant of each chosen clone as well as the boiled cells themselves. Two antibodies were used in the study, the first set of blots were performed with the antibody directed towards the *F. moniliforme* PG while the another set of blots were probed with an antibody directed towards the *A. niger* PG (both antibodies were provided by Dr. Caprari, University of Rome). The positive controls of each blot included purified PG from *F. moniliforme* and *A. niger* respectively (1ng/ $\mu\text{l}$  each). 3 $\mu\text{l}$  of each sample was boiled for 5 minutes and loaded onto a SDS-PAGE gel. This was performed in duplicate. The electrophoresis proceeded as described before, where after one gel was subjected to silver staining while the other gel was used for the western blot.

The blot was set up as follows:

A sponge, soaked in the transfer buffer, were placed in the transfer “cassette” of the TRANS-BLOT system (Biorad), followed by two soaked filter papers, the SDS-PAGE gel a Nitrocellulose membrane and finally another soaked filter paper and sponge. The cassette was closed, placed into the TRANS\_BLOT system, filled with transfer buffer and transfer of the proteins to the membrane was allowed to proceed at 100V (100-200mA) for 3h.

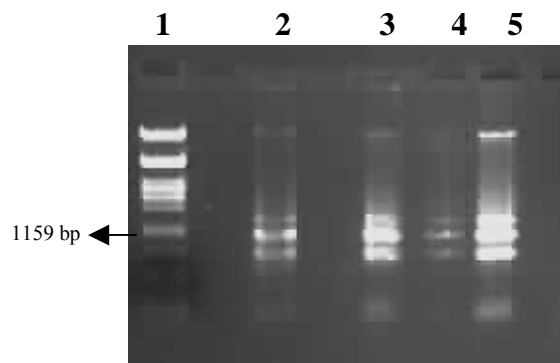
After the transfer procedure was completed, the membrane was incubated in blocking solution (Appendix A) for 10 minutes and then twice for 5 minutes. The membrane was incubated for 12h in a solution containing 0.5% BSA with the *FmPG* antibody or *A. niger* antibody (1:1000 dilution).

The membrane was washed with washing buffer (Appendix A) for one 10 minute and two 5 minute intervals. It was then incubated with the second antibody (anti-rabbit, Amersham, 1:5000 dilution) and 0.5% BSA for 2h. The membrane was washed as described before. The ECL advanced Western blotting detection kit (Amersham) was used to develop the western blots. The membrane was briefly dried between two filter papers and then completely air dried before adding solution 2 (a substrate buffer containing oxidant) and then solution 1 (luminol, enhancer, substrate buffer) from the kit. The membrane was incubated for 1 minute in these solutions and developed with Hyperfilm in a cassette for approximately 1, 5 and 15 minutes.

## 4.3 Results

### 4.3.1 RNA extraction and RT PCR amplification

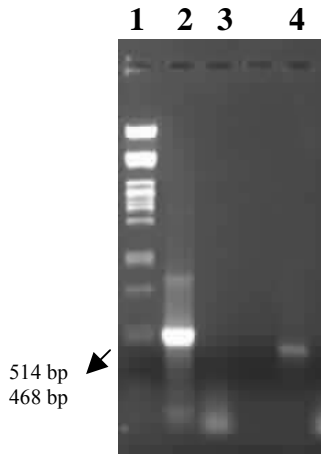
Total RNA was isolated from *C. lupini* SHK2148 mycelia collected after three days of growth in pectin containing media (Fig. 4.9). The concentration of the RNA samples, as determined with a photometer, was 206ng/μl (sample 1), 92ng/μl (sample 2), 98ng/μl (sample 3), and 159ng/μl (sample 5). The total RNA samples were all pooled to isolate mRNA and conduct RT-PCR. RT-PCR of the β-tubulin gene was successful, revealing a RT-PCR band of approximately 450 bp (Fig. 4.10). This falls in the expected size range as reported by Glass *et al* (1995). This showed that the mRNA was of good quality, and could be used for PG gene RT-PCRs.



**Fig. 4.9 RNA extracted from *C. lupini* SHK2148 mycelia collected on day 3 after inoculation in pectin media.**

Approximately 1μg of total RNA isolated from samples 1, 2, 3 and 4 (lanes 2, 3 and 4 respectively) collected from mycelia harvested on day 3 after inoculation in pectin media. λ PstI molecular marker (lane 1, Appendix A)

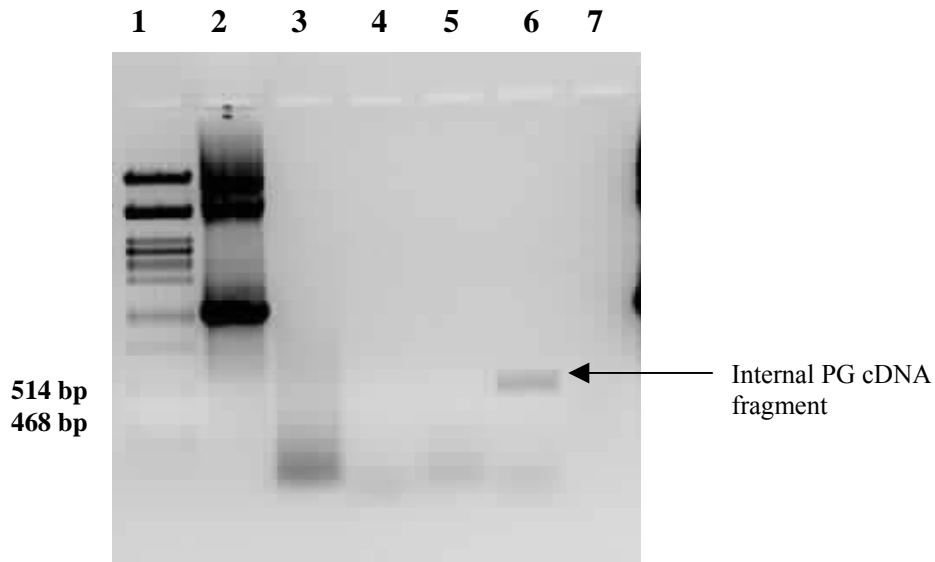




**Fig. 4.10 PCR amplification of the  $\beta$ - tubulin gene from DNA and mRNA template of *C. lupini* SHK 2148.**

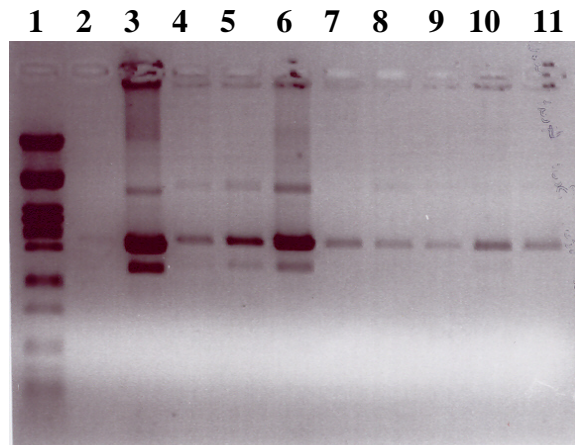
The  $\beta$ - tubulin genomic copy (lane 2) and cDNA copy (lane 4) amplified via PCR and RT-PCR from genomic and mRNA template of *Colletotrichum lupini* respectively. A water control (lane 3 and) and a  $\lambda PstI$  molecular marker (lane 1) were included on the TAE gel.

RT-PCR using four PG gene specific primer sets, were only successful with one of the internal primer sets. A PCR product of approximately 500 bp was obtained only from primer set C. lupini\_Int F I and C. lupini\_Int R I. No other products were obtained for the other primer pairs (Fig. 4.11). The internal cDNA PG fragment was cloned into pGEM-T-Easy, and plasmids successfully isolated from 9 clones pPGcDNAint1 – 9 (Fig. 4.12). Restriction digestion of two selected clones (pPGcDNAint1 and pPGcDNAint4) with *EcoRI* showed that only pPGcDNAint4 (Fig. 4. 13) harboured the expected insert size of 500 bp, whereas the insert size of pPGcDNAint1 was too small ( $\approx$  339 bp) (Fig. 4.14). The larger bands present above these excised fragments represent the partly or undigested plasmid from which these fragments were excised (Fig. 4.14 lane 3 and lane 4). Sequence analyses of pPGcDNAint4 with SP6 and T7 showed that the internal PG fragment formed part of the previously characterised PG gene and that the predicted intron (chapter 3) was spliced out.

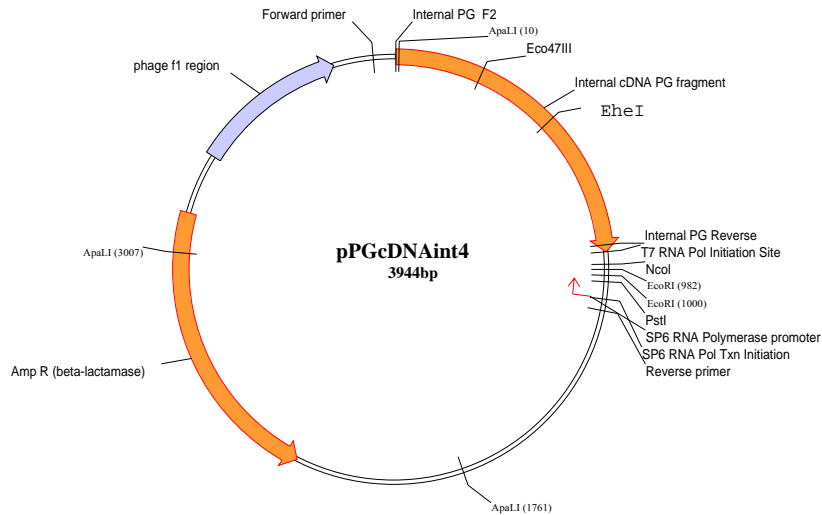


**Fig. 4.11 RT-PCR amplification of an internal PG cDNA fragment with different PG gene specific primers.**

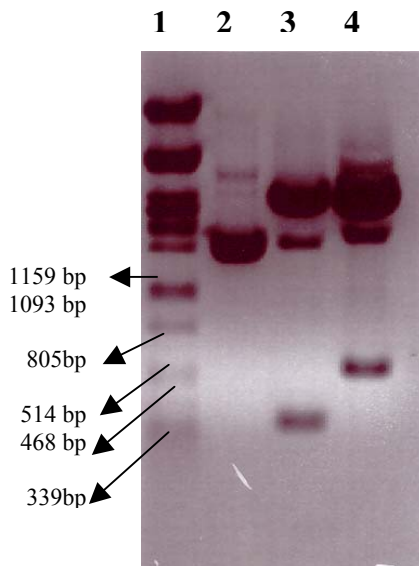
PCR amplification of the complete genomic copy from plasmid pPGcompl1 using primer pair C. lupini\_Compl F I and C. lupini\_Compl RI (lane 1). Unsuccessful RT-PCR amplification of a full cDNA PG copy using PCR primer pairs C. lupini\_Compl F I and C. lupini\_Compl RI (lane 4) as well as a failed amplification of an internal PG cDNA fragment using PG-COLL-F2 and PG-COLL-R (lane 5). Successful amplification of the internal cDNA PG fragment (approximately 500 bp) using RT-PCR with primer pair C. lupini\_Int F I and C. lupini\_Int R I (lane 6). A  $\lambda$  PstI molecular marker (lane1) as well as PCR negative water control (lane 3) were electrophoresed with the PCR products.



**Fig. 4.12 Evaluation of plasmids pPGcDNAint1- pPGcDNAint9, isolated from clones, harbouring the internal cDNA PG fragment cloned into pPGEM-T-Easy.** Plasmid DNA isolations from nine clones desinated as pPGcDNAint1 –9 (lane 3-11), transformed with the putative internal cDNA PG fragment, were isolated and electrophoresed together with a  $\lambda$  PstI molecular marker (lane 1).



**Fig. 4.13** Plasmid map of pPGcDNAint4 harbouring the internal cDNA PG fragment.

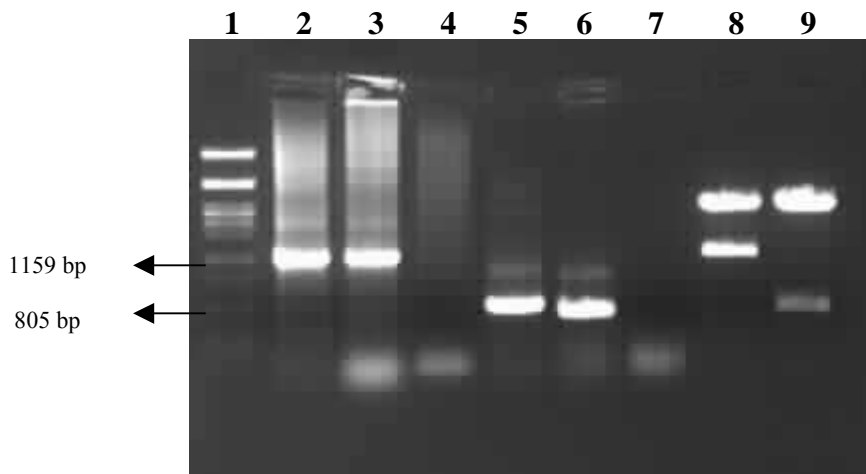


**Fig. 4.14** Restriction analysis of pPGcDNAint1 and pPGcDNAint4 with *EcoRI*. Undigested pPGcDNAint1 (lane 2), *EcoRI* digested pPGcDNAint1 (lane 3), *EcoRI* digested pPGcDNAint4 (lane 4) and  $\lambda$  PstI molecular marker (lane 1).

### 4.3.2 Construction of a full-length cDNA PG clone

The internal fragment of the PG gene without the intron, obtained through RT-PCR, was used as basis to construct a synthetic full length PG gene copy without an intron. Unfortunately no full-length cDNA gene copy could be obtained with RT-PCR,

requiring the construction of a synthetic full length PG gene without the intron using constructs pPGcDNAint4 and pPGcompl1 as basis (section 4.2.5). pPGcDNAint4 and pPGcompl1 was first transformed into *dam*<sup>-</sup> *E. coli* strains (does not methylate DNA), since the cloning strategy required the use of the methylation sensitive enzymes *EheI* and *Eco47111*(section 4.2.5). Colony PCR analyses showed that transformation of pPGcDNAint4 and pPGcompl1 into *dam*<sup>-</sup> *E. coli* strains yielded two positive *E. coli* JM110 *dam*<sup>-</sup> clones containing pPGcompl1-1 and one containing pPGcDNAint4-1 (Fig. 4.15). Restriction analyses of pPGcompl1-1 and pPGcDNA4-1 with *EcoRI* confirmed that the clones contained the correct inserts (Fig. 4.15, lane8 and lane 9).

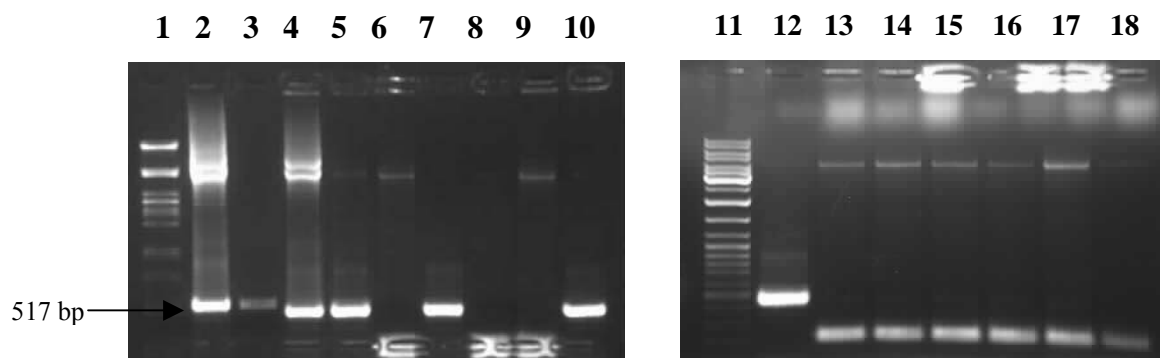


**Fig. 4.15 Colony PCR and *EcoRI* restriction analysis of pPGcompl1-1 and pPGcDNAint4-1 isolated from *E.coli dam*<sup>-</sup> strains.**

PCR amplification from plasmid pPGcompl1 with complete primer set *C. lupini*\_ Compl FII and *C. lupini*\_ Compl RII (lane2) as well as internal primer set *C. lupini*\_ Int F I and *C. lupini*\_ Int R I (lane 5) were included as positive controls to compare with the colony PCR amplification of clone pPGcompl1-1 with primers *C. lupini*\_ Compl FII and *C. lupini*\_ Compl RII (lane 3) and clone pPGcDNAint4-1 with primers *C. lupini*\_ Int F I and *C. lupini*\_ Int R I (lane6). Water controls are included (lane 4 and 7). The *EcoRI* restriction analysis of pPGcDNAint4-1 (lane 8) and pPGcDNAint4 (lane 9) was electrophoretically separated with a  $\lambda$  PstI molecular marker (lane 1).

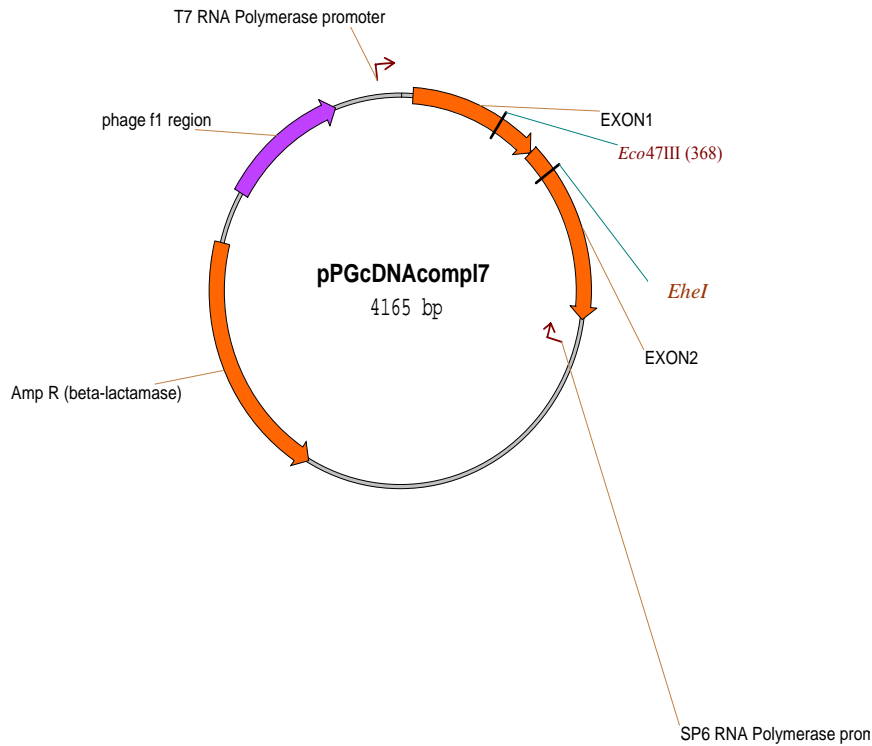
Restriction digestion of pPGcDNAint4-1 and pPGcompl1-1 (obtained from the *dam*<sup>-</sup> *E. coli* strains) with the methylation sensitive enzymes *EheI* and *Eco47111* yielded the expected band sizes (data not shown). The cDNA internal PG fragment band from pPGcDNAint4, and the band from pPGcompl1 from which the genomic internal

fragment was excised, were successfully gel purified (data not shown) and used in a ligation reaction to construct pPGcDNAcompl. Transformation of *E. coli* JM 109 cells with the ligation mix yielded 14 clones designated as pPGcDNAcompl1 – pPGcDNAcompl14. Colony PCR showed that clones 1,2, 4, 7 and 8 contained inserts of the expected size range of approximately 458 bp (59 base pairs smaller than the genomic control of 517 bp) (Fig. 4.16, lanes 4, 5, 7, 10 and 12). Sequence analyses showed that only one, pPGcDNAcompl7 (Fig. 4.17) of the five clones had the insert in the correct orientation (data not shown). Restriction digestion of pPGcDNAcompl7 with *EheI* and *Eco47111* confirmed that the intron was spliced out, seen by the smaller fragment excised from pPGcDNAcompl7 than from pPGcompl1, and that no mutations occurred at the ligation points in pPGcDNAcompl7 (Fig. 4.18).

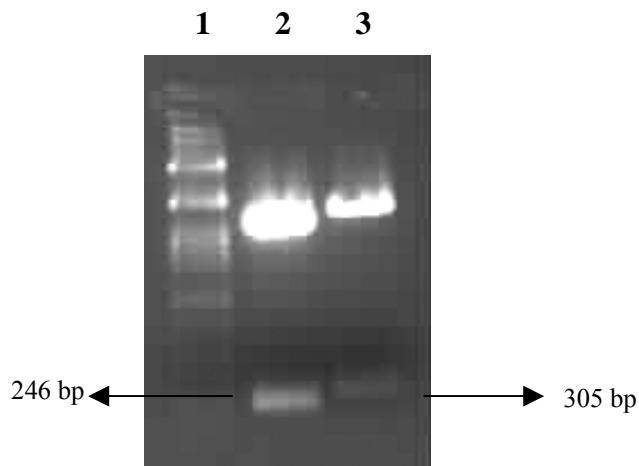


**Fig. 4.16 Colony PCR amplification of clones transformed with the pPGcDNAcompl construct.**

PCR amplification of pPGcompl1 using the internal primer set *C. lupini*\_Int F I and *C. lupini*\_Int R I (lane 2). Colony PCR of clones pPGcDNAcompl1 – pPGcDNAcompl14 (lanes 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16 and 17) using the primer set *C. lupini*\_Int F I and *C. lupini*\_Int R I. Lane 18 represents the water control and a  $\lambda$  PstI molecular marker (lane 1) as well as a 100 bp molecular marker (lane 11, Appendix A) were included during the electrophoresis.



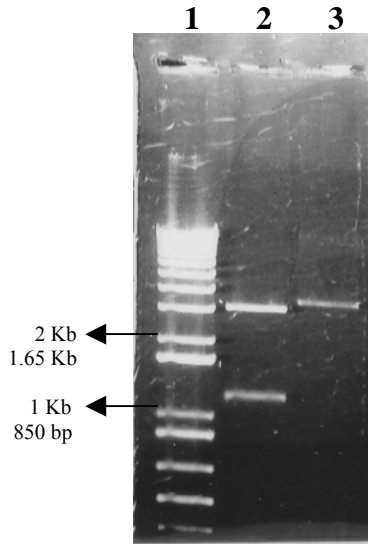
**Fig. 4. 17** Plasmid map of pPGcDNAcompl7 harbouring the constructed cDNA PG copy.



**Fig. 4.18** Restriction digestion of pPGcDNAcompl7 and pPGcompl1 with *EheI* and *Eco47111*. pPGcDNAcompl7 and pPGcompl1 digested with *EheI* and *Eco47111* (lane 2 and lane 3 respectively) electrophoresed with a  $\lambda$  *PstI* molecular marker (lane 1).

### 4.3.3 PG gene constructs used for expression of the gene in *P. pastoris*

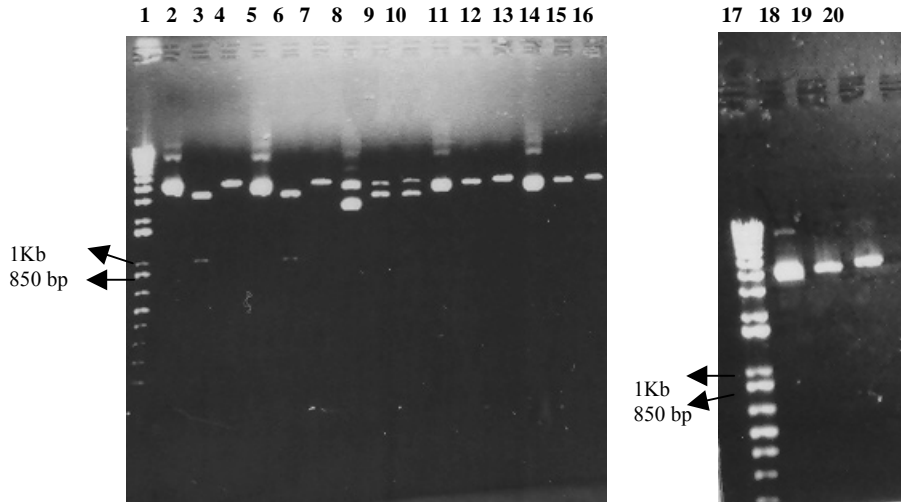
PG construct I was created by excising the cDNA fragment of the PG gene from pPGcDNAcompl7 through *EcoRI* restriction digestion, and subcloning the fragment into pPICZA. Restriction digestion of pPGcDNAcompl7 with *EcoRI* yielded an approximately 1.2 Kb and 3 Kb band (Fig. 4.19, lane 2), whereas pPICzA was only linearized by *EcoRI* (Fig. 4.19, lane 3).



**Fig. 4.19** *EcoRI* restriction of pPGcDNAcompl7 and pPICzA. pPGcDNAcompl7 digested with *EcoRI* releasing the cDNA PG fragment (lane 2), pPICzA linearised with *EcoRI* (lane 3) and a 1 Kb plus molecular marker (lane 1, Appendix A) were electrophored on a 1% TAE gel.

Transformation of *E. coli* cells with a ligation mixture of the 1.2 Kb band of pPGcDNAcompl7 and linearized pPICzA yielded many transformed clones, from which 60 were selected for colony PCR. Colony PCR of the *E. coli* clones revealed several positive clones of which six clones designated as PG construct I- 15, PG construct I- 19, PG construct I- 20, PG construct I- 23, PG construct I- 24 and PG construct I- 28, were digested with *XhoI* and *KpnI*. *XhoI* was used to show successful linearization of the plasmid (Fig. 4.20 lanes 4, 7, 10, 13, 16 and 20) whereas *KpnI* was used to determine the orientation of the ligated fragment by evaluating the size of the band excised by *KpnI* (Fig. 4.20, lanes 3, 6, 9, 12, 15 and 18). PG construct I- 19, PG construct I- 20 and PG construct I- 28 contained the fragment in the correct orientation; a fragment of approximately 200 bp was excised from the clones with *KpnI* (The small fragment excised was visible on the Agarose gel, but not in the photo) (Fig. 4.20, lanes 12, 15 and 18). PG construct I- 23 and PG construct I- 24

contained the cloned fragment in the incorrect orientation, since an approximately 1Kb band was excised from the clones by *KpnI* digestion (Fig. 4.20, lanes 3 and 6). Restriction of PG construct I- 15 was unsuccessful (Fig. 4.20 lanes 9 and 10).

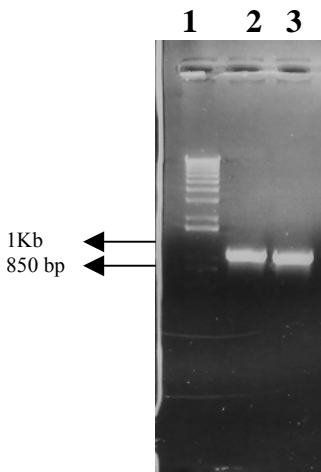


**Fig. 4.20 Restriction analysis of PG construct I clones with *KpnI* and *XhoI*.**

Uncut PG constructs I – 23, PG constructs I – 24, PG construct I – 15, PG construct I – 19, PG construct I – 20 and PG construct I – 28 (lanes 2, 5, 8, 11, 14 and 18), *KpnI* restricted PG constructs I-23, PG constructs I-24, PG constructs I-15, PG constructs I-19, PG constructs I-20 and PG constructs I-28 (lanes 3, 6, 9, 12, 15 and 19). *XhoI* restricted PG constructs I-23, PG constructs I-24, PG constructs I-15, PG constructs I-19, PG constructs I-20 and PG constructs I-28 (lanes 4, 7, 10, 13, 16 and 20). 1Kb plus molecular marker were electrophoresed in lanes 1 and 17.

PG construct III was constructed by cloning the PCR fragment, amplified from pPGcDNAcompl7 (Fig. 4.21, lane 3) into pPGAPzA $\alpha$ . Transformation of the ligation mixture into *E. coli* Top 10 yielded numerous transformants, however colony PCR was only performed on 10 clones (Fig. 4.22). Expected PCR amplification products in the size range of a 1000 bp are evident. The PG fragments were cloned in frame with the  $\alpha$ - signal factor.

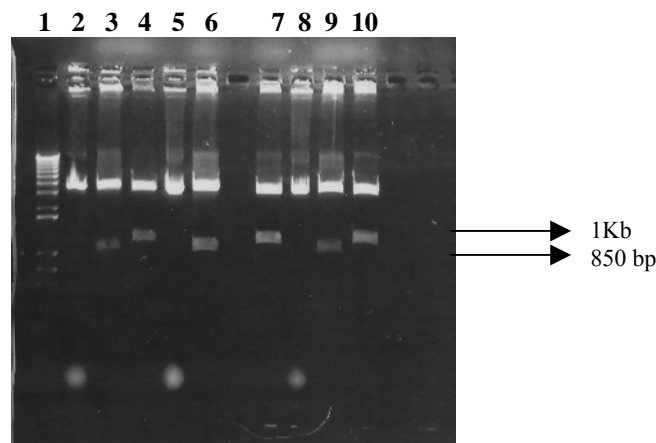




**Fig. 4.21 PCR amplification of the PG fragment from pPGcDNAcompl7 for the construction of the PG construct III.**

PCR amplification of a PG fragments that will represent PG construct III (lane 3) were electrophoresed with 1 Kb plus molecular marker (lane 1). A positive PCR amplification with primer pairs CollyPGEcoFWII and CollyPGXbaRV using pPGcDNAcompl7 as template as were included during gel electrophoresis (lane2)

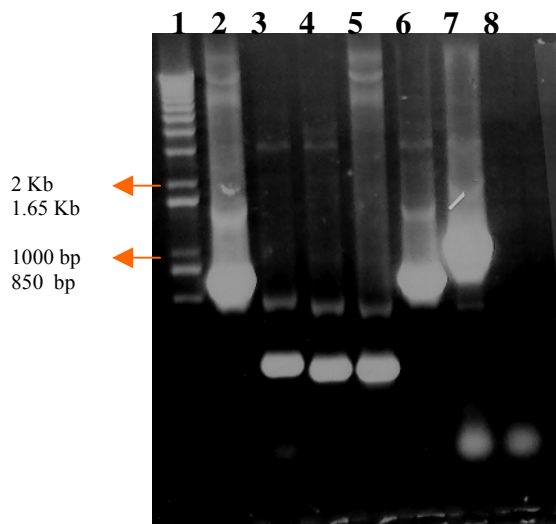
*E. coli* clones potentially harbouring PG construct III were subjected to restriction enzyme analysis. Three clones of PG construct III, designated as PG construct III –4, PG construct III –5 and PG construct III –6, were restriction digested with *EcoRI* and *KpnI* and yielded a fragment of approximately 850 bp (Fig. 4.22), confirming cloning of the correct fragment. Similarly, restriction digestion of the three clones with *EcoRI* and *XbaI* revealed the expected excised PG fragment of approximately 1Kb (Fig. 4.22).



**Fig. 4.22 *EcoRI* –*KpnI* and *EcoRI* –*XbaI* digestion of PG construct III clones.** Uncut plasmid DNA from PG construct III –4, PG construct III –5 and PG construct III –6 uncut (lanes 2, 5 and 8 respectively), *EcoRI* –*KpnI* restricted PG construct III –4, PG construct III –5 and PG construct III –6 (lanes 3, 6 and 9 respectively) and *EcoRI* –*XbaI* restricted PG construct III –4, PG construct III –5 and PG construct III –6 (lanes 4, 7 and 10 respectively). A 1Kb plus molecular marker were loaded in lane1.

#### 4.3.4 PG hybrid constructs

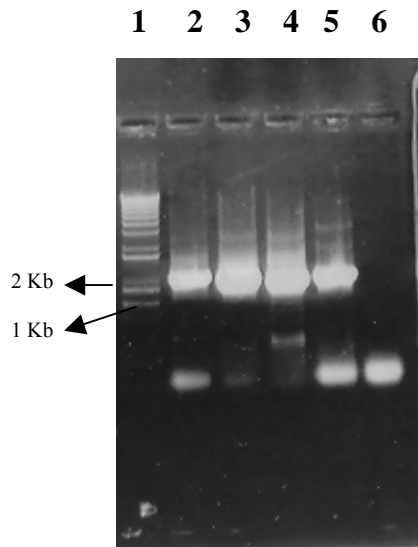
PG hybrid constructs were constructed via SOE PCR (Section 4.2.7). The results of the first set of PCRs SW1, SW2III, SW3 and SW4 are displayed in Fig. 4.23 SW1 (lane 2) represents the PG sequence of *F. moniliforme* excluding the front region from the swap point. SW2III (lane 4) represents the front part of the *C. lupini* SHK 2148 PG to the swap point. SW3 (lane 5) represents the front part of the *F. moniliforme* PG gene to the splice point and SW4 (lane 6) depicts the end part of the *C. lupini* SHK 2148 PG gene to the swap point.



**Fig. 4.23 PCR amplifications for the first set of the splicing overlap by extension PCR (SOE PCR).**

PCR products obtained for SW1, SW2III, SW3 and SW4 SOE PCR reactions (lanes 2, 4, 5 and 6). PCR amplification of pPGcDNAcompl7 using primer pairs CollyPGEcoFWII and CollyPGXbaRV (lane 7). A water control (lane 8) and 1 Kb plus molecular marker (lane 1) were also included. (Non-relevant PCR reaction, lane3).

The second SOE PCR set used the diluted products of the first set as templates (Fig. 4.24). In the PCR reaction of SWAIII (Fig. 4.24 lane 3) SW1 and SW2III were used as template to create a hybrid PG gene with the front part originating from the *C. lupini* SHK 2148 PG gene and the end part from the *F. moniliforme* PG. SWB (Fig. 4.24 lane 4) used SW3 and SW 4 as template to create a hybrid PG with the front part from *F. moniliforme* PG and the end part from the *C. lupini* SHK 2148 PG.

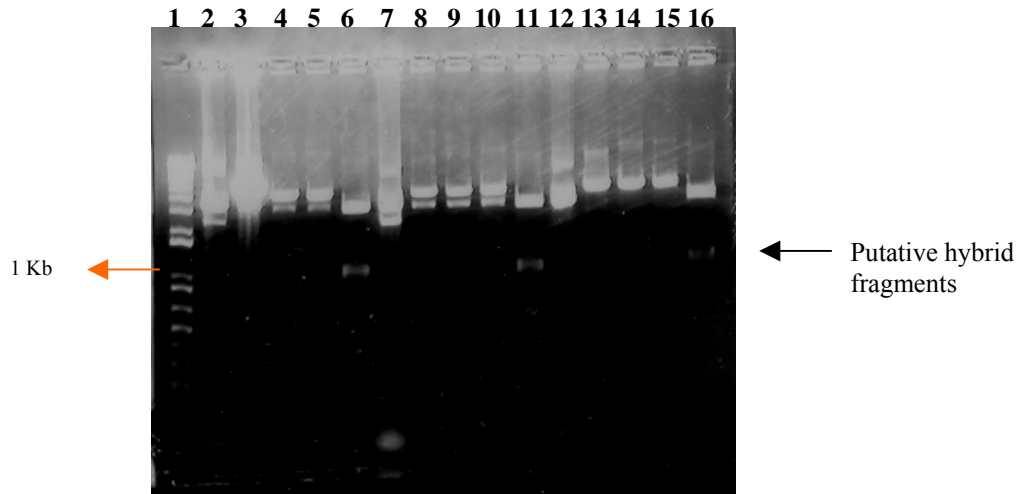


**Fig. 4.24 PCR amplifications for the second set of the splicing overlap by extension PCR (SOE PCR) resulting in hybrid PG fragments.**

PCR amplification of SWAIII (lane 3) and SWB (lane 4) were electrophoresed with the PCR product of pPGcDNAcomp17 using primer set CollyPGEcoFWII and CollyPGXbaRV (lane 5), a water control (lane 6) and a 1 Kb plus molecular marker (lane 1). Lane 2 contains a PCR reaction not relevant to this study.

Following transformation, 5 *E. coli* clones of each hybrid construct were subjected to colony PCR (results not shown). PG hybrid clones SWAIII-4 and SWB-1 were selected and digested with *EcoRI* (Fig. 4.25 lanes 8 and 13), *XbaI* (Fig. 4.25 lanes 9 and 14) and an *EcoRI-XbaI* combination (lanes 11 and 16). The constructs harboured an insert of approximately 1000bp as revealed by the *EcoRI-XbaI* double digestion. The plasmids were linearised with *AvrII* (Fig. 4.25 lanes 10 and 15).

Table 4.3 illustrates the different constructs and their key properties that were prepared for transformation into *P. pastoris*.



**Fig. 4.25 Restriction analysis of hybrid PG clones.**

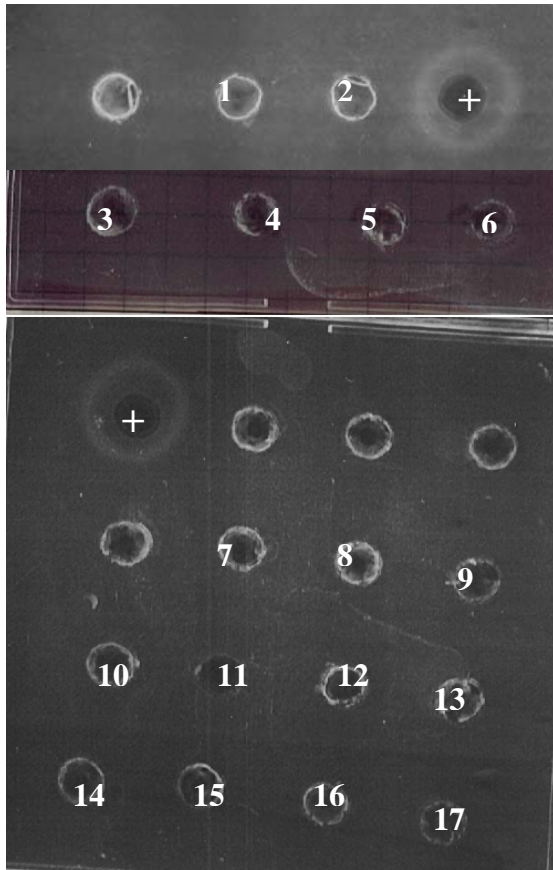
Plasmid DNA of SWAIII-4 and SWB-1 which were undigested (lanes 7 and 12 respectively), subjected to *EcoRI* restriction (lanes 8 and 13 respectively), digested with *XbaI* (lanes 9 and 14 respectively), linearised with *AvrII* (lanes 10 and 15 respectively) and double digested with *EcoRI* and *XbaI* (lanes 11 and 16 respectively). Compared to the 1Kb molecular marker (lane 1), the clones contained a fragment of approximately 1000 bp as revealed by the double digestion with *EcoRI-XbaI*. Lanes 2-6 represents work that is not discussed in this study.

**Table 4.3 Key features of PG constructs analysed prior to transformation of *P. pastoris*.**

PG construct	Clones analysed	Key feature
PG construct I	PG construct I-23, PG construct I-24, PG construct I-15, PG construct I-19, PG construct I-20 and PG construct I-28.	Mature PG protein with native peptide signal.
PG construct III	PG construct III-4, PG construct III-5 and PG construct III-6.	Mature PG protein with $\alpha$ -MF signal peptide.
SWAIII	SWAIII-4	PG protein from <i>C. lupini</i> SHK 2148 containing the N-terminal part of the mature PG protein from <i>F. moniliforme</i> with the $\alpha$ -MF signal peptide.
SWB	SWB-1	PG protein from <i>F. moniliforme</i> containing the N-terminal part of the mature PG protein from <i>C. lupini</i> SHK 2148 with the $\alpha$ -MF signal peptide.

#### 4.3.5 Transformation of *P. pastoris* and PG activity analysis

Electroporation of the various PG constructs into *P. pastoris* electrocompetent cells, yielded time constants between 5.2 and 5.5, indicating that the electroporation was successful. Small colonies only appeared after 4 days of incubation. Several colonies were inoculated into the appropriate media and incubated for 6 days at 28 °C. No growth however was observed. Since the colonies were very small, the selection was repeated, however this time they were plated onto a master plate, allowed to grow to an appropriate size before inoculation in the media. Colonies grew well after 6 days at 28 °C. Cup plate analysis were performed on each clone, a positive control of purified *F. moniliforme* PG was included on each cup plate (Fig. 4.26). No zones were observed for any of the clones harbouring the PG constructs; the only zone observed was for the positive control. The lack of PG activity for each clone selected for further analysis was confirmed by a repeat of the cup plate analysis (results not shown).



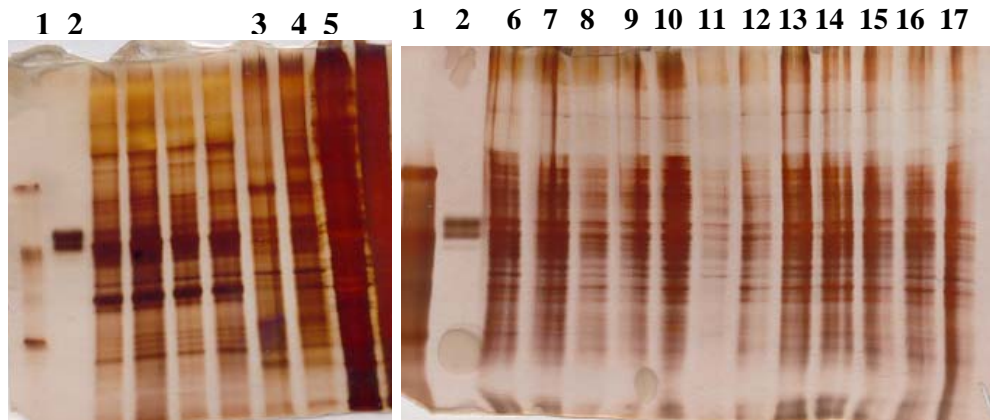
**Fig. 4.26 PG activity analysed with an agarose diffusion assay, of *Pichia* cultures transformed with the PG constructs and PG hybrid constructs.**

Crude culture extract of *Pichia* clones harbouring several PG gene constructs were evaluated for PG activity using the agarose diffusion assay. Clones evaluated for their PG activity included PG construct I-28.1, PG construct I-28.2, SWAIII-2.1, SWAIII-2.2, SWAIII-2.3, SWAIII-2.4, SWB-1.1, SWB-1.2, SWB-1.3, SWB-1.4, PG construct III- 5.1, PG construct III- 4.2 and PG construct III- 4.1. (Numbers 1, 2, 3, 4, 5, 6,7, 8, 9, 10, 11, 12, 13, 14, 15, 16 and 17 respectively). Purified *F. moniliforme* PG (0.5 ng) were included as a positive control.

#### **4.3.6 SDS-PAGE analysis of *Pichia* transformants**

Clones selected for SDS-PAGE and western blot analysis included two clones from PG construct I-28, designated PG construct I-28.1 and PG construct I-28.2; three clones from PG construct III, designated PG construct III-4.1, PG construct III-4.2 and PG construct III-5.1 and four clones from each hybrid construct SWAIII-2 and SWB-1, designated as SWAIII-2.1, SWAIII-2.2, SWAIII-2.3, SWAIII-2.4, SWB-1.1, SWB-1.2, SWB-1.3 and SWB-1.4.

Total protein from the supernatant of several clones, as outlined above, grown for four days were isolated and concentrated. An aliquot of 3  $\mu$ l of each sample were evaluated with SDS-PAGE analyses (Fig. 4.27). There is a high concentration of proteins present in all the clones investigated in the size range of reported PGs (30- 40 kDa). The size of the *F. moniliforme* PG is approximately 40kDa. The SDS PAGE gel for the PG construct III clones are not presented here, however during Western blot analysis the proteins of these clones were visualised on a separate SDS PAGE gel. The profiles for the different clones appeared to be similar; unfortunately it is difficult to compare these profiles with the protein profile of the untransformed *Pichia* clone (Fig. 4.27, lane 5), since the latter was present as darker smear from which distinct bands could not be identified.



**Fig. 4.27 SDS PAGE analysis for *Pichia pastoris* clones transformed with different PG constructs.**

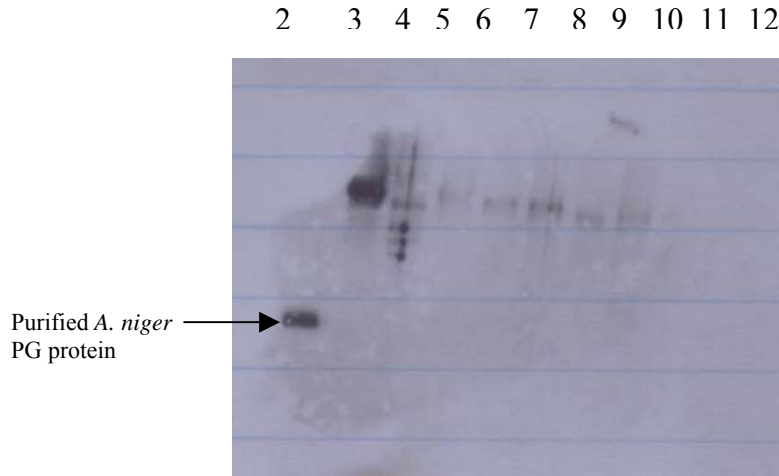
Purified PG protein from *Fusarium moniliforme* (lane 2) were separated on the SDS-PAGE gel with total protein isolations from clones transformed with PG constructI-28.1, PG constructI-28.2, SWAIII-2.1, SWAIII-2.2, SWAIII-2.3, SWAIII-2.4, SWB-1.1, SWB-1.2, SWB-1.3 and SWB-1.4 (lanes3, 4, 10, 11, 12, 13, 14, 15, 16 and 17). A protein molecular marker (lane 1, Appendix A) as well as an untransformed *pichia* clone (lane 5) was included in the analyses. (Lanes 6, 7, 8 and 9 contain clones that are not relevant to this study).

#### 4.3.7 Western Blot analysis of *Pichia* transformants

From the SDS PAGE analysis, one clone for each construct, which displayed a high protein concentration in the desired size range, were chosen for Western blot analysis. To ensure that proteins were present on the SDS page gel used in Western analysis,

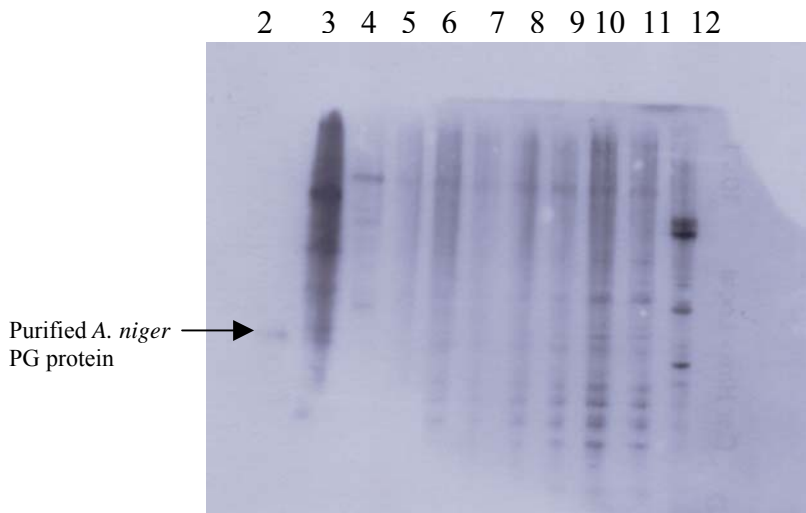
samples were separated on two SDS PAGE gels; one was used for silver staining to confirm the presence of proteins and the other for the Western blot (SDS-PAGE gels not presented). An untransformed *Pichia* clone was included in the analysis to determine any false positive hybridisation to other proteins present in *P. pastoris*. Membranes containing proteins from the supernatant of *P. pastoris* transformants harbouring the PG construct I and hybrid constructs, did not reveal any positive hybridising signal with the antibody directed against the *F. moniliforme* PG (results not shown). The only positive signal obtained was with the purified *F. moniliforme* PG protein that was included as a positive control in the blot (results not shown). Further analysis of the selected clones included a Western blot analysis of the boiled transformed *Pichia* cells, this blot revealed non specific hybridisation to all the clones including the untransformed *Pichia* clones (results not shown). Since the PG proteins of *F. moniliforme* and *Colletotrichum lupini* were only 42 % identical a Western blot analysis with an antibody directed towards the *A. niger* PG, which is more similar to the PG of *Colletotrichum lupini* (50%), was performed on both the supernatant of the selected clones of PG construct I and the PG hybrid constructs (Fig. 4.28) as well as the boiled cells (Fig. 4.29). The Western blot analysis of the supernatant of the clones revealed the presence of a high molecular weight band in all the clones except for the untransformed control of wild type *P. pastoris* (Fig. 4.28, lane 12). *Pichia* cells were boiled on order to release any protein (PG) that is not secreted. Analysis of the boiled *Pichia* cells resulted in non-specific hybridisation (Fig. 4.29). In both analyses the positive control (of *A. niger*) gave a signal (Fig. 4.28 & 4.29 lanes 2).





**Fig. 4.28** Western blot analysis of the supernatant of *P. pastoris* clones containing different PG constructs. The blot was detected with an antibody directed against the PG of *A. niger*.

The western blot contained purified PG protein from *A. niger* (lane 2), proteins isolated from *Pichia* cells that were transformed with PG construct I-28.1, PG construct III-4.1, SWAIII-2.1 and SWB-1.1 (lanes 4, 6, 8 and 9 respectively) as well as an untransformed control of wild type *P. pastoris* (lane 12). A protein molecular marker (lane 1) was included in the analyses. Lanes 3, 5, 7, 10 and 11 represent additional clones not applicable to this study.



**Fig. 4.29** Western blot analysis of boiled *P. pastoris* cells containing different PG constructs. Blots were detected with an antibody directed against a PG of *A. niger*.

The blot contained proteins isolated from *Pichia* cells that was transformed with PG construct I-28.1, PG construct III-4.1, SWAIII-2.1 and SWB-1.1 (lane 4, 6, 8 and 9 respectively). Purified *A. niger* PG protein (lane 2), an untransformed wild type *P. pastoris* (lane 12) as well as a molecular marker (lane 1) were included on the blot. Lanes 3, 5, 7, 10 and 11 represents additional clones not applicable to this study.

## 4.4 Discussion

A full-length cDNA PG copy, without an intron, was constructed by restriction digestion and ligation reactions. This construct was used to construct several PG gene constructs suitable for expression in *P. pastoris*. One construct (PG construct I) included the complete gene sequence to allow potential expression under the native signal peptide. Another construct (PG construct III) with the mature protein sequence was constructed by comparing previously reported sequence data for the *A. niger* PG and the PG genes from *C. lindemuthianum* with the PG sequence data of *Colletotrichum lupini*. This was used to determine the putative signal sequence and to construct a vector containing only the mature PG protein sequence or one where the N-terminal part of the mature *C. lupini* PG protein sequence was exchanged with that of the *F. moniliforme* PG. In addition to this, several other constructs were made, which were not discussed in this chapter. These included a construct which contained the mature protein sequence with additional upstream propeptide sequence, since it was reported that a propeptide sequence from a plant PG might play a role in expression and secretion (Dal Degan *et al*, 2001). Two tagged PG constructs, one containing the mature protein sequence and the other containing the additional propeptide sequence, were also included in the expression studies but not discussed in this study.

Even though clones selected for transformation of *P. pastoris* appeared to harbour the desired fragments, no PG activity was observed for any of the constructs expressed in *P. pastoris*. SDS-PAGE analysis revealed high protein concentrations in the reported size range for PGs, it was however difficult to determine if these proteins were only present in the transformed *Pichia* clones, since the presence or absence of these bands could not be clearly distinguished in the untransformed *Pichia* control. Western blot analysis was performed to determine if any protein, active or inactive was produced, intracellular or extra cellular. No clear positive signal was obtained for any of the clones and analysis of the boiled cells resulted in non-specific hybridisation. Since the PG of *F. moniliforme* is only 42 % identical to that of *Colletotrichum lupini*, it was decided to do a Western blot analysis of the clones with the antibody directed against *A. niger* which had a higher identity (50 %) to the PG of *Colletotrichum lupini*. With this antibody a putative positive signal was observed for the supernatant

of the clones, the size of the band was however very large. Glycosylation was considered as a reason for the increase in the protein size. Several post translational modifications including glycosylation, might increase the size of a protein. However since only a single N-glycosylation site was predicted for the PG protein of *C. lupini* SHK 2148 (<http://www.cbs.dtu.dk/services/NetNGlyc/>), it is unlikely that the size of the protein will increase dramatically to yield such a large band. Four potential glycosylation sites were predicted for the *F. moniliforme* PG (Caprari *et al*, 1993) and one for the *A. niger* PG (van Santen *et al*, 1999). Non-specific hybridisation was again observed when the boiled cells were analysed. Although the PG of *Colletotrichum lupini* could not be expressed in *P. pastoris* several other cell wall degrading enzymes (CWDE) of fungi have been expressed in *P. pastoris* or other yeast systems. Previous CWDE that were successfully expressed in *P. pastoris* included an endoPG gene of *Sclerotinia sclerotiorum* that has been successfully expressed in *Kluyveromyces lactis* under the control of the constitutive *pgk* promoter. The expressed protein was 37.5 kDa and not heavily glycosylated (Cotton *et al*, 2002). The PG gene of *F. moniliforme* was expressed in *S. cerevisiae* under the control of a galactose inducible promoter and the signal peptide sequence of the *Kluyveromyces lactis* killer toxin gene (Caprari *et al*, 1996). The *F. moniliforme* PG was in the size range 40-50 kDa (Caprari *et al*, 1996). *Fusarium solani* pectate lyase (*pelC*), *Fusarium solani* pectate lyase (*pelD*) were expressed and in *Pichia pastoris*, *pelC* and *PelD* were both secreted out of the cells and expressed under the *P. pastoris* acid phosphatase signal peptide (PHO) and native signal peptide respectively (Gonzalez-Candelas Guo *et al*, 1995 and Gonzalez-Candelas Guo *et al*, 1996).

Although results from this study suggest that the *Colletotrichum* PG is not expressed in *P. pastoris*, several additional controls need to be included in future studies to confirm these results that are only tentative; a *P. pastoris* isolate that has previously been shown to express the *F. moniliforme* PG should be included in the transformation of both the *E. coli* and *P. pastoris* system. Unfortunately this control was not included in the present study since the *P. pastoris* expression system, of the lab of Prof. Cervone in Rome, has been established and has always been very effective. However, recent communications with the lab suggest that they might currently be experiencing problems with the *P. pastoris* expression system, emphasizing the need to always include a *P. pastoris* control that expresses a known

protein should this be available. This would serve as a positive control in the PG activity assay in addition with purified PG protein of *F. moniliforme*. Apart from this a clear untransformed *Picha* clone as well as PG(s) extracted from *Colletotrichum* SHK 2148 should be included in both the SDS-PAGE and Western blot analysis to determine what proteins are unique to the transformed clones and similar to the extracted PG(s). Should these positive controls have been included it could have revealed whether there is a problem with the expression constructs or the *P. pastoris* expression system.

Furthermore, due to time constraints, none of the PG constructs were sequenced to confirm that no mutations occurred during their construction. PG construct I was subcloned while the others were constructed using PCR, which renders them more likely to mutations than PG construct I. Future studies should include a full sequence analysis of the plasmid DNA constructs that were isolated from the *E. coli*, prior to the transformation of *P. pastoris*. However, since the cDNA PG fragment was sequenced and had no mutations and the recently isolated complete cDNA PG displayed a 100% homology to the constructed cDNA PG gene (pPGcDNAcomplA, chapter 3) it was unlikely that mutations could have been introduced in all the constructs that were expressed in *P. pastoris*.