Isolation and characterisation of two chitinase and one novel glucanase genes for engineering plant defence against fungal pathogens

by

Susana M. E. Severgnini

B.Sc. (Biotechnology), Murdoch University, Perth
Honours (Biotechnology), Murdoch University, Perth

This thesis is presented for the degree of
Doctor of Philosophy
of Murdoch University

March, 2006

Division of Science and Engineering
School of Biological Sciences and Biotechnology
Murdoch University, Perth, Western Australia

DECLARATION

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

Susana Severgnini

ABSTRACT

Hydrolytic enzymes such as chitinases and glucanases are implicated in plant defense responses against fungal pathogens. These enzymes are responsible for the breakdown of chitin and glucan, two major components of the fungal cell walls. Genes encoding these enzymes have been used to genetically engineer plants to enhance their protection against fungal pathogens.

Western Australia has over 4000 endemic plant species and a largely unknown fungal biota. Given that fungi possessing chitinases and glucanases with novel activities have been isolated in other parts of the world, we propose that fungi from Western Australian soils may possess novel biochemical/enzymatic activities.

The aims of this research project were to isolate chitinolytic and glucanolytic fungi from soil and to clone the genes encoding for chitinase and glucanase enzymes. To achieve these aims, fungi with activity against chitin and glucan were isolated, the activity quantified by colorimetric and inhibition assays and gene fragments with homology to known chitinase and glucanase genes were isolated and their sequences determined.

Soil fungi were isolated from five locations in and around the Perth Metropolitan area of Western Australia with the use of a medium containing Rose Bengal that eliminates all actinomycetes and most bacteria and reduces the growth of fast growing mold colonies. Forty-one isolates were obtained by this method. Twenty-four chitinolytic and glucanolytic fungal isolates were identified by growing them on chitin-containing media to select for those species that utilised chitin/glucan as a carbon source. These were assayed for production of exo- and endochitinolytic and glucanolytic enzymes.

Enzyme activity was compared between crude and dialysed supernatants. Exochitinase activity was determined in the supernatants of 4-day old fungal cultures by the release

of *p*-nitrophenol from *p*-nitrophenyl-*N*-acetyl-*β*-D-glucosaminide. The supernatants were measured for endochitinase activity determined by the reduction of turbidity of suspensions of colloidal chitin. Glucanase activity was determined by release of reducing sugars (glucose) from laminarin. Supernatants from eleven of the twenty four isolates showed significant levels of enzyme activity.

Eleven isolates were assayed for activity against purified cell walls of phytopathogenic fungi. Activity was determined by measuring reducing sugars in the fungal supernatants against cell wall preparations of six economically important plant pathogens. Chitinolytic activity was detected in seven isolates against cell wall preparations of *Botrytis cinerea* and *Rhizoctonia solani*, in four isolates against *Fusarium solani* and *Sclerotinia sclerotium*; in five isolates against *Ascochyta faba* and in six isolates against *Leptosphaeria maculans*. Similarly glucanolytic activity was detected in eight isolates against *B. cinerea*, in seven against *R. solani*, in two against *F. solani*, in three against *S. sclerotium* and *A. faba* and in one against *L. maculans.*

The supernatants derived from the isolates were used in a bioassay to determine growth inhibition against live *B. cinerea* spores by measuring turbidity reduction. Growth inhibition was measured against a control (*B. cinerea,* grown in medium with no added supernatant). Boiled supernatant did not inhibit the growth of *B. cinerea* spores but there was 100% inhibition by the crude supernatant from ten of the twenty four isolates. Similarly, supernatants were used to assess growth inhibition against live mycelia cultures of *F. solani* and *S. sclerotium.* Growth inhibition of *F solani* ranged from 9-59%, boiled and crude supernatants respectively whilst growth inhibition of *S. sclerotium* ranged from 46-75%, boiled and crude supernatants respectively.

Two partial chitinase genes from the soil filamentous fungus *Trichoderma asperellum*, (*ChiA* and *ChiB*) and a novel glucanase gene from the filamentous fungus *Aspergillus*

(*Glu1*) were cloned. *ChiA*, was 639 bp long, encoding 191 amino acids with identity to other chitinase genes. Two highly conserved regions, characteristic of glycosyl hydrolases from family 18, were present.

ChiB, was 887 bp long and encoded a 293 amino acid sequence that was closely related to an endochitinase gene from the filamentous fungus *Trichoderma asperellum.* The two highly conserved regions corresponding to the substrate binding and active sites that characterise the glycosyl hydrolases from family 18, also found in *ChiA*, were found in this gene.

Glu1 was 2844 bp long and encoded a 948 amino acid sequence that shared high identity with a β-1, 3-glucanase from the filamentous fungus *Aspergillus oryzae.* The sequence contained conserved regions found in glycosyl hydrolases from family 17 that encode for substrate binding, N-terminal sequences and putative asparagine-linked glycosylation sites.

The partial putative sequence *ChiA* is probably a pseudogene because it has two in-frame stop codons. However, once the entire sequence of *ChiB* is known, both *ChiB* and the novel glucanase gene *Glu1* could be useful contenders for engineering resistance in crop plants.

TABLE OF CONTENTS

DEDICATION

I dedicate this thesis to my dearest children Marina and Matias and to my husband Paul for showing patience and understanding while I was undertaking this work. Without your help and support I could not have achieved this.

ABBREVIATIONS

3’	hydroxyl-terminus of DNA molecule
5’	phosphate-terminus of DNA molecule
α	alpha
β	beta
λ	lambda
bp	base pairs
CTAB	hexadecyltrimethylammonium bromide
cDNA	complementary DNA
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
g	gram
Kb	kilobase
Lb/in^2	pounds per square inch
L^{-1}	per litre
LB	Luria-Bertani
µL	microlitre
M	molar
mg	milligram
min	minutes
mL	millilitre
µM	micromolar
mM	millimolar
mm^2	millimetre square
mRNA	messenger ribonucleic acid

MW	molecular weight
ng	nanograms
N-terminus	amino terminus
nm	nanometre
°C	degrees Celsius
ORF	open reading frame
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
PVP	polyvinylpyrrolidone
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
sec	seconds
spp	species (plural)
TAE buffer	tris-acetate acid-EDTA electrophoresis buffer
TE buffer	tris-EDTA buffer
Tris	tris(hydroxymethyl)aminomethane
UV	ultra violet
vol	volume
X-gal	5-bromo-4-chloro-3-indonyl-β-galactopyranoside

ACKNOWLEDGEMENTS

I wish to express my deepest gratitude to my supervisors Dr. Philip O'Brien and Dr. Stephen Wylie for their supervision and for providing me with a challenging and interesting project to develop my own investigative and analytical skills. I am also indebted to them for their helpful discussions and constructive criticism of this manuscript.

I am greatly indebted to the Australian Research Council, Murdoch University and industry partner Grain Biotech Australia (GBA) for their financial contribution without which, this project would not have eventuated.

Many thanks to:

Dr. Robert Mead for sharing his extensive knowledge in biochemistry and for his continuous encouragement during this project.

Dr. Susanna Driessen for her invaluable contribution in putting this thesis together.

Mr. William Kenworthy from CBBC for helping me with the bioinformatics chapter of this manuscript.

Ms. Trudi Papps who helped to identify and photograph the fungal isolates.

Dr. Giles Hardy for his advice and sharing his knowledge on fungal biota.

My laboratory buddies Susanna, Morag, Pranjal, Nari, Katherine, Michaela, Cornelia and Mee Hua for their support and friendship.

CHAPTER 1

LITERATURE SURVEY

1.1 FUNGAL DISEASES AND THEIR IMPACT

Fungal pathogens cause significant crop losses amounting to several billion dollars per annum . Control of diseases in agricultural and horticultural crops is vital with food shortages experienced in many regions as well as a demand for improved efficiency in food production coupled with environmental protection in others (Johnson 1992). Loss of fertile soils due to improper management and erosion threatens to limit production of vital food crops in many areas of the world (Logemann and Schell 1993). In addition some plant pathogens, including many causing root diseases, such as *Pythium* spp.or *Rhizoctonia* spp.*,* infest soil to a point where it is not economical to grow a principal crop unless the soil is fumigated or crop rotations are established. Debilitating diseases weaken crops, resulting in depressed yields and/or poor quality. Plant diseases caused by fungal pathogens may also wipe out a crop completely (Lucas *et al.* 1992).

Disease management expenses constitute one of the major costs associated with crop production (Bridge *et al.* 2004). Several general approaches taken to control fungal diseases are: (a) management/quarantine of agricultural land, (b) use of fungicides and (c) breeding of resistant crop varieties.

Management includes chemical fallow, or soil cultivation that are expensive to implement and may cause undesirable side effects such as soil erosion (Leong 2004).

Some diseases are only able to be kept in check by the repeated applications of fungicides. Environmental pollution and death to many non target organisms, chemical residues in the food and health issues resulting from high and repeated exposure to these chemicals are of considerable concern (Faize *et al.* 2003; Lucas *et al.* 1992).

Furthermore, despite the great advances in chemical management of fungal diseases, some of the important plant pathogens causing vascular wilt, anthracnoses, take-all of wheat and other root infections remain uncontrolled by current fungicidal chemicals (Knight *et al.* 1997). In addition fungicides may become less effective due to the evolution of resistance among the pathogens (Faize *et al.* 2003).

Breeding for disease resistance is one method of protecting crops. Inherited resistance is a valuable attribute because it is easy for the grower to use and reduces the need for other methods of control. However, it is subject to significant biological and financial constraints and in many instances it may not be an option because there are no sources of resistance for breeding. For example, no useful levels of exploitable resistance have been identified for take-all of wheat, caused by *Gaeumannomyces graminis* var. *tritici* (Johnson 1992). In addition, only a few resistance-genes have been shown to provide pathogen control for an extended period of time such as the cases of wheat stem rust and rice blast caused by *Puccinia graminis* and *Magnaporthe grisea* respectively (Rommens and Kishore 2000). Another problem associated with using resistance genes is the emergence in some cases of new virulent pathotypes which are able to overcome a previously effective resistance gene (Keen *et al.* 1992).

1.2 DISEASE RESISTANCE THROUGH GENETIC ENGINEERING

Pathogenic fungi are continually becoming resistant to existing resistance genes and fungicides therefore other methods of disease control are highly desirable (Rommens and Kishore 2000). One such alternative is the identification of biological agents in combination with molecular biology for the control of plant diseases. Microorganisms with inhibitory activity against pathogens are a potential source of genes to confer disease resistance in plants (Herrera-Estrella and Chet 1999).

Thus, genetic engineering of plants for resistance has become an attractive alternative. Molecular techniques have facilitated the introduction of beneficial traits into model organisms to produce potential biocontrol agents (Herrera-Estrella and Chet 1999). Also, the introduction of genetically determined traits by transformation eliminates the species boundaries that have traditionally limited germplasm sources. Research with pathogen avirulence genes has suggested that functionally similar disease resistance genes are shared between taxonomically diverse plants (Kobayashi and Keen 1985; Kobayashi *et al.* 1989). This theory led researchers to believe that disease resistance genes from a certain plant may function when introduced into an unrelated plant. Thus, cloned disease resistance genes as well as defence response genes such as those encoding for chitinases and glucanases, have important uses against pathogens that attack plant species with little or no currently available resistance. For example, *Phytophthora cinnamomi* is a serious pathogen on cultivated avocado trees (*Persea americana*), primarily because little naturally occurring resistance has been identified. However, several *Persea* species that cannot be intercrossed or successfully intergrafted with *P. americana* exhibit high level resistance to *P. cinnamomi*. If resistance genes could be cloned from these species and transformed into the cultivated avocado, resistance to *P. cinnamomi* might be obtained (Keen *et al.* 1992).

Genes involved in disease resistance and defence response have been cloned from a variety of plants and used to engineer resistance. A number of these genes have been tested for their ability to control fungal pathogens in transgenic plants grown in the laboratory and to some extent in the field (Leong 2004). In a study by Oldach *et al* (2001), a barley class II chitinase and a barley type I ribosome inactivating protein (RIP) were expressed in transgenic wheat. When the plants were infected with mildew spores (*Erysiphe graminis*), the researchers observed a 32-42% reduction of mildew

colonies in the lines carrying the RIP and the chitinase. Furthermore, it was found that the fungal lesions were bigger on the controls, resulting in stronger sporulation than the colonies on leaves of RIP and chitinase II plants. Similarly, when the transgenic lines were inoculated with leaf rust (*Puccinia recondita*), a reduction of between 30 and 50% of rust colonies were observed (Oldach *et al.* 2001).

A number of studies have successfully demonstrated that chimeric genes can protect plants against infection by fungal pathogens (Broglie *et al.* 1991; Jach *et al.* 1995) (Table 1.1). Plants expressing these chimeric genes have shown increased resistance but not total resistance to the pathogens. This may be due to variation in expression of the transgene which is determined mainly by the site of insertion or promoter strength (Zhu *et al.* 1994). Greater levels of resistance can be achieved using combinations of genes (Jach *et al.* 1995; Zhu *et al.* 1994). The discovery of new and more efficient enzymes particularly from sources such as fungi has become paramount to the success of engineered resistance against plant pathogens.

Table 1.1- Defence response-related-proteins cloned genes introduced in transgenic plants.

Protein genes	Transgenic crop	References
Cell-wall degrading proteins (chitinase and glucanase) Origin:barley, tobacco	Tobacco, rice, wheat, peanut	(Bliffeld *et al.* 1999; Broglie *et al.* 1991; Jach *et al.* 1995; Mauch *et al.* 1988; Rohini and Sankjara Rao 2001; Schaffrath *et al.* 2000)
Ribosome inactivating protein Origin:barley, maize	Rice, wheat	(Bieri *et al.* 2000; Kim *et al.* 1999)
Phenyl alanine ammonia lyase (PAL) Origin:bean	Tobacco	(Blount *et al.* 2000)
Osmotin (PR5)	Tobacco, potato	(Liu *et al.* 1994; Zhu *et al.* 1996)
Plant defensin Origin: alfalfa	Potato	(Gao *et al.* 2000)
Cystatin gene	Rice	(Irie *et al.* 1996)
H_2O_2-generating glucose oxidase	Potato	(Wu *et al.* 1995)
Cowpea trypsin inhibitor gene	Rice	(Xu *et al.* 1996a)
Thaumatin-like PR-5 gene	Rice, wheat	(Chen *et al.* 1999; Datta *et al.* 1999)

1.3 FUNGAL CELL WALL

Chitin and β-glucan are the main components of fungal cell walls of filamentous fungi. Chitin forms the backbone and laminarin (β-1, 3-glucan) is the filling material (Cohen-Kupiec *et al.* 1999).

Chitin is a linear polysaccharide composed of β-1, 4- linked *N*-acetylaglucosamide units and is found in nature as α and β-chitin (Fig 1.1).

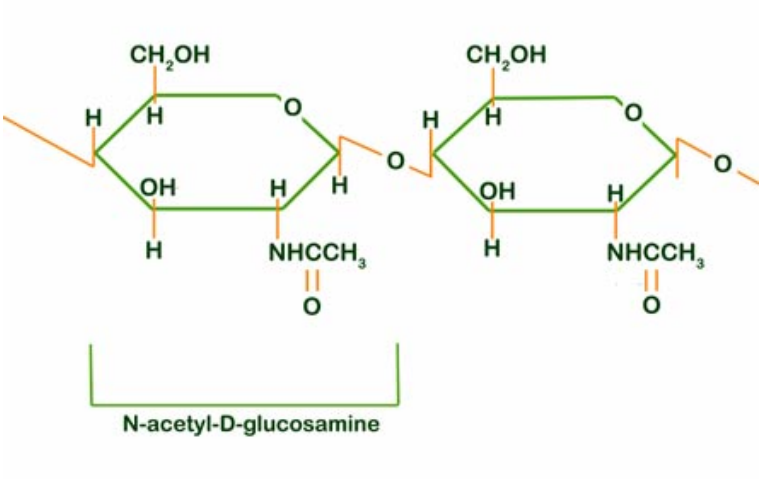


Figure 1.1- Chitin molecule. The diagram shows the N-acetyl-glucosamine monomers with $\beta(1\rightarrow4)$ linkages. Chitin is found in the exoskeleton of insects, crustaceans, worms and nematodes and in the cell wall of most fungi except Phycomycetes and Mucorales (Rosenberger 1976).

Laminarin is a polymer of D-glucose in a β-1, 3 configuration arranged as helical coils, from which minor polymers of β-1, 6 –D-glucose branch (Fig 1.2). Fungal cell walls contain more than 60% laminarin which is hydrolysed mainly by β-1, 3-glucanases (Cohen-Kupiec *et al.* 1999).

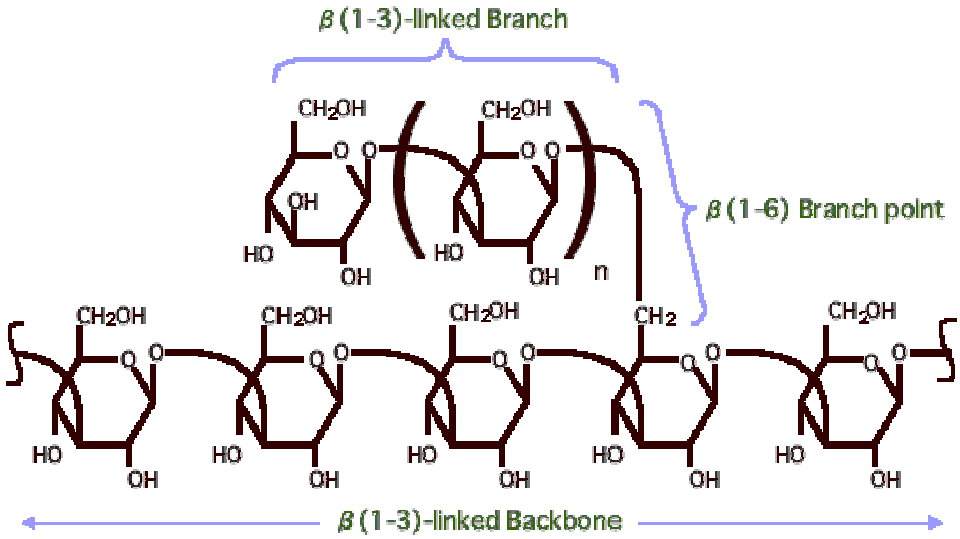


Figure 1.2- Laminarin molecule is composed of glucose monomers with $\beta(1\rightarrow3)$ and $\beta(1\rightarrow6)$ linkages. Laminarin, also known as R-glucan is found in the cell wall of most fungi except Mucorales (Rosenberger 1976).

In fungal cell walls, chitin is arranged in regularly ordered layers, parallel to each other in the β-form and antiparallel in the α-form, whilst laminarin fibrils are arranged in an amorphic manner (Cohen-Kupiec *et al.* 1999).

1.4 CELL WALL DEGRADING ENZYMES

Chitinases, glucanases and other hydrolytic enzymes have many roles in a wide range of different biological systems. These enzymes are usually extracellular, of low molecular weight and highly stable. In addition they may be produced in multiple forms or isozymes that differ in charge, size, regulation, stability and ability to degrade cell walls (Koga *et al.* 1999). Pathogens and predators of chitinous organisms produce chitinases whereas hosts to chitinous pathogens, including plants and humans produce chitinases to defend themselves (Gooday 1999). The involvement of chitinases and other cell wall-degrading enzymes and their genes in penetration, pathogen ramification, plant defence induction and symptom expression has been studied extensively, however, conclusive evidence for or against a role for any particular enzyme activity in any aspect of pathogenesis has been difficult to discern (Walton 1994).

1.4.1 Chitinases

These enzymes are extensively distributed among plants, fungi, bacteria and viruses. In higher plants, chitinases are used as defence against plant pathogens (Koga *et al.* 1999). These enzymes are found at low levels in healthy plants, however, their expression is increased during pathogen attack. The production of chitinases elicits other plant responses including the synthesis of antifungal phytoalexins (Gooday 1999). The antifungal activity of chitinases and β-1, 3-glucanases cause rapid lysis of fungal hyphal tips and germinating spores. These enzymes are an effective tool for the complete degradation of mycelial or conidial walls of phytopathogenic fungi (De la Cruz *et al.* 1995a; Flach *et al.* 1992).

Chitinases are found in two families of glycohydrolases, 18 and 19. Family 18 contains many conserved repeats of amino acids. The enzyme core of these enzymes is eight strands of parallel β sheets forming a barrel laid down with α helices forming a ring

toward the outside. Family 19 includes mainly enzymes from plant sources. The enzyme has a mixture of secondary structures, including 10 α-helical segments and one three-stranded β- sheet (Gooday, 1999).

Three classes of chitinases have been identified as ubiquitously found in plants. Class I chitinases are basic and contain an *N*-terminal *Cys*-rich domain believed to participate in chitin binding. Class II chitinases are acidic, excreted into the extracellular space and lack Cys-rich domains. Class III chitinases are distinguished by their lysozyme activity and can be considered as molecular markers of the systemic acquired resistance (SAR) response (Busam *et al.* 1997).

1.4.2 Glucanases

These enzymes are widely distributed among bacteria, fungi and higher plants. Many of these proteins have been purified and characterised. There are 2 types of glucanases. The first type, exo-β-1,3-glucanases hydrolyse laminarin by sequentially cleaving glucose residues from the non-reducing end of polymers or oligomers. Consequently, the sole hydrolysis products are glucose monomers. The second type, endo-β-glucanases cleave β-1,3-linkages at random sites along the polysaccharide chain releasing smaller oligosaccharides (Cohen-Kupiec *et al.* 1999).

Plant glucanases are a fundamental part of the defence mechanism against fungal pathogens, however, it is thought that they have a role in cell differentiation as well (Donzelli *et al.* 2001). In fungi, they play a part in morphogenetic-morpholytic processes during development and differentiation. They are involved in the mobilization of β-glucans when carbon and energy sources have been exhausted, acting as autolytic enzymes (De la Cruz *et al.* 1995b). Buchner *et al* (2002) identified a new β-1,3-glucanase in pea. This gene has a very specific pattern of expression related mainly to seed development. β-1, 3-glucanases have been found throughout the plant kingdom

whilst β-1, 3-1, 4-glucanases have only been identified in monocotyledons (Buchner *et al.* 2002).

β-1,3-glucanases are also involved in fungal pathogen-plant interaction degrading callose (β-D-1, 3-glucan), a component of papilla, in the host's vascular tissues when under attack by fungal pathogens. The exclusive substrate of these enzymes is 1, 3-glucans found as callose and laminarin in fungal cell walls. They are induced in response to pathogen attack or environmental stress. Together with chitinases they have a defence-related biological function by inhibiting growth of pathogenic fungi (Jach *et al.* 1995). These enzymes have an important nutritional role in saprophytes and mycoparasites. Another proposed role of these enzymes is the release of elicitors from pathogen cell walls for the induction of the defence response (Keen and Yoshikawa 1983). β-1,3-glucanases genes have also been part of tissue specific and developmentally regulated non-pathogen induced expression (Hird *et al.* 1993). In growing plant tissues, these enzymes, participate in the dissolution of the tetrad callose wall and the release of the young microspores into the anther locules (Kotake *et al.* 1997).

The multiplicity of function of these enzymes provides higher plants with the advantage of several lines of defence against invading microorganisms. Diversity, organ specificity and developmental and differential expression patterns show that β-1,3-glucanases have biological functions in plant growth and development as well as their role in the defence mechanism of plants (Jin *et al.* 1999).

1.5 CHITINASE AND GLUCANASE GENES USED FOR GENETIC ENGINEERING

1.5.1 Plant genes

Genetic engineering represents a powerful tool for the improvement of existing cultivars and for the introduction and expression of genes outside the scope of conventional breeding (Pappinen *et al.* 2002). The most attractive candidates for manipulation of the single gene defence mechanism approach are genes encoding chitinases or β-glucanases because these two enzymes hydrolyse chitin and β-1, 3-glucans which are structural components of cell walls of fungi.

Plant chitinases and glucanases often act synergistically to enhance antifungal activity by inhibiting the growth of many pathogenic fungi (Busam *et al.* 1997). Different types of cell-wall degrading chitinases and glucanases have been successfully transferred into crops such as tobacco, barley and rice (Table 1.2)

Table 1.2- Cell wall degrading enzyme genes transferred to plants.

PR Genes transferred	Transgenic crop	References
Chitinase	Potato	(Chye *et al.* 2005)
Chitinase (sugarbeet)	Silver birch	(Pappinen *et al.* 2002)
Class I chitinase (rice)	Grapevine	(Yamamoto *et al.* 2000)
Chitinase	Rose	(Marchant *et al.* 1998)
Chitinase (rice)	Cucumber	(Tabei *et al.* 1998)
Chitinase (ChiI)	Tobacco	(Terakawa *et al.* 1997)
β-1, 3-glucanase	Alfalfa	(Masoud *et al.* 1996)
1, 3-1, 4- β-glucanase	Barley	(Jensen *et al.* 1996)
Class II chitinase (peanut)	Tobacco	(Kellmann *et al.* 1996)
Basic chitinase (RC24)	Rice, rose	(Xu *et al.* 1996b)
Chitinase	Rice	(Lin *et al.* 1995)
Class II chitinase, β-1, 3-glucanase, type I	Tobacco	(Jach *et al.* 1995)

Detailed analysis of rice, *Oryza sativa*, infected with *R. solani* (causal agent of sheath blight) showed that, chitinases and β-glucanases acted directly on fungal cell walls in a synergistic manner to cause lysis of hyphal tips. Also, their action on the fungal cell walls resulted in the release of oligosaccharide-signal molecules bringing about a variety of plant defences (Anuratha *et al.* 1996). Different chitinases were expressed in different tissues at different developmental stages. Transcripts of chitinases increased substantially in the infected rice plants (Anuratha *et al.* 1996). Chitinases and glucanases from tobacco and petunia (Lindhorst *et al.* 1990); bean (Benhamou *et al.* 1993); barley (Jach *et al.* 1995); peanut (Kellmann *et al.* 1996); pine tree (Wu *et al.* 1997); sweet orange (Nairn *et al.* 1997); soybean (Yeboah *et al.* 1998); are a few of the many enzymes that have been studied for their antifungal effect. In an experiment carried out by Jach and colleagues (Jach *et al.* 1995) a chitinase gene and a glucanase gene from barley were used to transform tobacco plants and then exposed to the fungus *R. solani*. The tobacco plants co-expressing the chitinase and glucanase genes showed enhanced protection against fungal attack compared to plants expressing a single chitinase transgene, with up to 60% reduction in disease symptoms. It was concluded that multigene resistance was more effective than single-gene resistance and could reduce the probability of the emergence of resistance-breaking strains of phytopathogenic fungi (Jach *et al.* 1995). This conclusion was supported by a second study in which tobacco was transformed with a rice chitinase and an alfalfa glucanase. Combination of the two transgenes gave greater protection against *Cercospora cotianae* than either chitinase or glucanase alone. The size of the lesions, 5 days after inoculation were 35mm^2 for plants with the combined transgenes; around 70mm^2 for either chitinase or glucanase and 80mm^2 for the wild types (Zhu *et al.* 1994).

Response to fungal pathogens by transgenic carrot and cucumber plants, was studied by Punja and Raharjo (1996) who concluded that: a) chitinase over-expression increased tolerance to pathogen infection in carrot but not cucumber; b) plant cultivar or gene integration may be a factor in disease response; and c) that different chitinase genes differ in antifungal activity in planta and that pathogenic fungi differ in susceptibility to chitinases *in vivo* (Punja and Raharjo 1996). The transgenic cucumber and carrot plants expressing chitinase genes were found to be less susceptible to infection by pathogens such as *Alternaria cucumerina, Botrytis cinerea, Colletotrichum lagenarium* and *Rhizoctonia solani* though not to the same degree (Punja and Raharjo 1996). Other studies examining transgenic tobacco (*Nicotiana tabacum*) showed high expression of the transferred *Serratia marcescens* chitinase gene when exposed to *R.solani*. The reduction in *R.solani* disease incidence ranged from between 25 and 60% for the chitinase transgene expressing plants (Howie *et al.* 1994). Broglie and colleagues (1991) studied transgenic tobacco constitutively expressing a bean chitinase gene under the control of a CMV 35S promoter. The transgenic plants showed a higher level of survival in soil infested with the fungal pathogen *R.solani* and delayed development of disease symptoms.

1.5.2 Fungal genes

An alternative to plant genes is the use of fungal genes. Fungal glucanases and chitinases are more active (up to 100-fold) and have a wider spectrum of antifungal activity than their plant counterparts (Lorito *et al.* 1998). In addition, many novel forms of these enzymes have been extracted and characterised from fungi (De la Cruz and Llobell 1999; De la Cruz *et al.* 1995a; De la Cruz *et al.* 1995b; De la Cruz *et al.* 1993; Fuglsang *et al.* 2000; Lora *et al.* 1995)

Filamentous fungi, especially mycoparasitic fungi, are prolific producers of chitinases and glucanases (Lorito *et al.* 1998). Mycoparasitic fungi such as *Trichoderma* spp., *Rhizopus oligosporus* and *Aspergillus* spp have been studied for the production of hydrolytic enzymes (Takaya *et al.* 1998a; Takaya *et al.* 1998b). There are described, at least 20 fungal chitinases and 1, 3-β-glucosidases (glucanases) that can potentially be sources of genes for genetic engineering of plants against fungal disease (Donzelli *et al.* 2001).

Harman *et al* (1993) showed that there are a number of chitinolytic enzymes produced by *T. harzianum* and that there are multiple forms of chitinases. The chitinases purified were found to be potent inhibitors of a range of chitin-containing fungi including species of *Fusarium, Botrytis, Ustilago, Uncinula* and other *T. harzianum* strains (Harman *et al.* 1993; Lorito *et al.* 1993). In addition, the enzymes isolated from *T. harzianum* were found to be substantially more active and effective against a wider range of fungi than the chitinolytic enzymes from plants and other microorganisms (Harman *et al.* 1993) and showed increased activity when used in combination with each other (Lorito *et al.* 1993). Similar enzymes have been purified from other strains of *Trichoderma* spp. Usui *et al* (1990) purified an endochitinase that was larger (58 kDa) than the enzyme purified by Harman *et al* (1993) (41 kDa). Uloha and Peberdy (1991) purified a 118 kDa chitinolytic enzyme from *T. harzianum*

Several plant species expressing the *Trichoderma* cell-wall degrading enzyme genes have been generated. Transgenic grapevine transformed with this gene reduced the growth of *Botrytis cinerea* under laboratory conditions (Kikkert *et al.* 2000). Similarly, the *Trichoderma* gene was used to transform two cultivars of apple (Galaxy and Ariane) against scab caused by the pathogen *Venturia inaequalis*. The transgenic lines showed enhanced resistance and a reduction of up to 80% of scab symptoms (Bolar *et al.* 2000).

When the apple plants were transformed with endo and exo-chitinase genes from *T. harzianum*, synergistic activity was observed that resulted in higher resistance to the pathogen (Bolar *et al.* 2001). Rice sheath blight caused by *Rhizoctonia solani*, and rice blast caused by *M. grisea*, are important plant diseases in China (Liu *et al.* 2004). An endochitinase *ech*42, an exochitinase *nag*70 and an exo-1, 3-β-glucanase gene *gluc*78 from *T. atroviride* were used to transform rice. Evaluation of disease resistance to sheath blights resulted in a significant negative correlation between endochitinase activity and lesion length with plants carrying the *ech*42 gene alone (R^2= 93%, P=0.02). The line with the *nag*70 showed only mild resistance to the pathogen (R^2= 48.8%, P= 0.122). The correlation between glucanase transgenic activity and disease resistance was not analysed because high glucanase activity resulted in reduced vigour. Transgenic plants co-expressing the two chitinase genes were significantly less affected by fungal diseases than the control. Evaluation of disease resistance to rice blast showed increased resistance to blast in all populations of transgenic plants. However, the transgenic lines carrying the *ech*42 and the *nag*70 genes performed best in resistance to blast disease (Liu *et al.* 2004).

In a study by Lorito *et al* (1998) an endochitinase gene from *T. harzianum* was transferred to tobacco and potato where its expression represented 0.01-0.5% of total protein content. Plants transformed with a genomic copy of the gene and tobacco with the cDNA fused to a tomato secretion sequence showed 10 and 400-fold increase in endochitinase activity than the control plants, in roots and leaves respectively. The transgenic plants with high chitinase activity showed no developmental difference to the control plants or wild types. In addition, transgenic tobacco and potato plants showed high to complete resistance to the fungal pathogen *Alternaria alternata* (Lorito *et al.* 1998).

1.6 PROJECT AIMS

In view of the promising results of previous studies, fungal glucanases and chitinases appear extremely useful for engineering disease resistance in plants. It is likely that novel forms of the enzymes have not yet been discovered.

Western Australia is a region of great biodiversity in fungal species (Bougher and Syme 1998). Approximately 500 species of larger fungi have been recorded, most from the South West (Bougher and Syme 1998). It is believed that only a small proportion of south-western fungi has been discovered. In a survey of the larger fungi, in a nature reserve on the south coast of WA, a total of 441 species mostly unidentified fungal species were recorded over two years, with an estimated 365 of these probably not yet known to science (Bougher and Syme 1998). Thus, Western Australia presents a unique opportunity for the isolation of yet undescribed fungal species that could be potential sources of novel forms of antifungal enzymes. Therefore, the aims of this project thesis were to:

1. Isolate soil fungi from a number of different soil types in WA.

2. Screen the fungal isolates for chitinase and glucanase production.

3. Evaluate the fungal chitinases and glucanases for their ability to inhibit the growth of phytopathogenic fungi.

4. Clone chitinase and glucanase genes from these isolates.

1.6.1 Thesis approach:

1.6.1.1 Isolation of novel enzymes from fungi residing in soil

A variety of soil fungi will be isolated and screened for their chitinase and glucanase production and fungal enzymes active against plant pathogenic fungi will be identified.

Soil will be collected from five study sites that reflect distinct vegetation and soil types. One site belongs to a natural forest in the southwest of Western Australia. Soil will also be collected from a bauxite mine rehabilitation site in the southwest of Western Australia. Other sites include a market garden, a compost heap in suburban Perth (W.A.) and chitin-baited soil. To recover chitinase and glucanase-producing fungi from the chitin-baited soil, the soil is left *in situ* for several months. These conditions have been shown to lead to an increase in the number of chitinolytic/glucanolytic isolates in the soil (Kong *et al.* 2001; Krsek and Wellington 2001). The supernatant of the soil/water slurry from these soil samples is then plated on selective media containing laminarin or chitin as sole carbon source that would create optimal conditions for induction of chitinases and glucanases (Noronha *et al.* 2000).

1.6.1.2 Screening of the isolated fungi for enzyme activity

Screening of chitinolytic and glucanolytic isolates will be carried out by plating on colloidal chitin agar.

Reducing sugar assays for glucanases and *p*-nitrophenyl-*N*-acetyl-*ß*-D-glucosaminide assays for chitinases will be performed to measure of extracellular enzyme activity of the isolated fungi.

1.6.1.3 Cloning the enzyme genes

The approaches to cloning the genes will be to (1) identify conserved regions within the proteins by alignment of sequences from the sequence databases; (2) design degenerate primers to these regions; (3) amplify and sequence the amplicons; (4) obtain the sequence of the flanking regions using methods such as vectorette PCR.

Vectorette-PCR method where the vectorette is a partially double-stranded DNA cassette that is phosphorylated in its 5'-ends, and after ligation of the vectorette unit to a mixture of chromosomal DNA fragments each strand has a vectorette unit attached to both ends. This library is used as a template for amplification with a genome-specific primer together with a vectorette-specific primer which is identical to a region in the vectorette cassette (Kilstrup and Kristiansen 2000; Siebert *et al.* 1995)}.

1.6.1.4 Characterisation of cloned genes

The cloned genes will be characterised by: (1) sequencing; (2); genome walking to sequence the flanking sequences, and (3) sequence analysis.

CHAPTER 2

MATERIALS AND GENERAL METHODS

2.1 FUNGAL STRAINS, GROWTH MEDIA AND CULTURE CONDITIONS

2.1.1 Isolation of fungal species from soil

Fungal isolates from soil were grown on modified synthetic medium (SM) 15 g chitin agar (per litre: colloidal chitin from crab shells (Sigma-Aldrich, Australia); 5 g yeast extract (BBL Becton Dickinson and Co., USA); 1 g $(NH_4)_2SO_4$; 0.3 g $MgSO_4.7H_2O$; 1.36 g KH_2PO_4; pH adjusted to 5.5; 20 g agar (BBL Becton Dickinson and Co., USA) (Dana *et al*, 2001). To prevent bacterial growth, the culture medium was amended with 50 mg/L ampicillin (PROGEN Industries Ltd, Australia), 15 mg/L Tetracycline (Sigma-Aldrich, Australia) and 30 mg/L Spectromycin (Boehringer Mannheim GmbH, Germany). The soil isolates were subsequently maintained on Potato Dextrose Agar (PDA) (Difco Laboratories, Australia) prepared according to manufacturer's instructions.

Sphaerollopsis filum, a mycoparasite of *Puccinia boroniae* was provided by Dr Susanna Driessen (Murdoch University) and the plant pathogens, *Botrytis cinerea, Sclerotinia sclerotium, Fusarium solani, Rhizoctonia solani, Leptosphaeria maculans* and *Ascochyta faba* were obtained from the CPSM collection at Murdoch University. These cultures were maintained on PDA at 26 °C in the dark and subcultured as necessary.

All media were sterilised by autoclaving at 121 ºC for 25 minutes.

2.1.2 Colloidal chitin preparation

Colloidal chitin was prepared by the method of Roberts and Selitrennikoff (1988). A 20 g sample of crab-shell chitin (Sigma) was dissolved in 350 mL cold concentrated HCl

and incubated at 4 ºC for 24 hours. The mixture was filtered through glass wool into 2 litres of ethanol at -20 ºC with rapid stirring and the filtrate was then centrifuged at 10,000 g for 20 minutes. The chitin pellet was washed repeatedly with water until the pH was neutral, lyophilised and stored at -20 ºC.

The isolates grown on chitin agar (Section 2.1) were subcultured in synthetic medium (SM) chitin broth by cutting a $5mm^2$ agar plug from the growing edge of the culture and inoculating 100 mL of broth (per Litre: Colloidal chitin 15 g; yeast extract 0.5g, $(NH_4)_2SO_4$ 1g; $MgSO_4.7H_2O$ 0.3 g; KH_2PO_4 1.36 g. pH adjusted to 5.5) (Rodriguez-Kabana *et al*, 1983).

2.1.3 Laminarin medium:

Per Litre: 5 g Laminarin (Sigma-Aldrich, Australia); 0.25 g glucose; 1g $(NH_4)_2NO_3$; 0.3 g $MgSO_4$; 0.8g KH_2PO_4; 0.2 g KNO_3. pH adjusted to 5.5.

2.1.4 Potato Dextrose Broth:

Per Litre: Potato Dextrose Broth (PDB) (Difco Laboratories, Australia) 24 g at pH 5.5.

2.1.5 Czapek Dox Broth

Per Litre: Czapek Dox Broth (CDOX) (Oxoid, Hampshire, England) 19 g at pH 5.5.

All media were sterilised by autoclaving at $121lb/in^2$.

2.2 MEASUREMENT OF GLUCANASE AND CHITINASE ACTIVITY

Colour reagents for measuring chitinase and glucanase activity were prepared according to manufacturer's instructions.

For chitinase activity the colour reagent was prepared with 5.3 M sodium potassium tartrate solution (12 g of sodium potassium tartrate tetrahydrate were dissolved in 8 mL of 2 M NaOH and heated in a boiling water bath to dissolve) and 96 mM 3, 5-dinitrosalicylic acid (438 mg 3, 5-dinitrosalicylic acid were dissolved in 20 mL of deionised water and heated in a boiling water bath). The two solutions were mixed with stirring and diluted with 40 mL of deionised water.

For glucanase activity the dinitrosalicylic acid solution was prepared according to the method by Miller (1959) .

2.2.1 Protein concentration assay

Protein concentration was determined using BioRad protein assay kit (BioRad Laboratories, Richmond, Calif. USA) based on the Bradford (1976) method, with bovine serum albumin (BSA) as a standard. A standard curve was prepared using 0, 2, 8, 12, 20 and 25 µg of protein per mL in PBS (phosphate buffered saline) buffer pH 7.4 (Sambrook *et al*, 1989). The test was carried out using 160 µL of fungal filtrate or standard and 40 µL of supplied dye reagent (BioRad). The standards and tests were replicated three-fold. The absorbance was read at 595nm on a microtrite reader (BioRad, Model 3550-UV)). The PBS was made with 8 g of NaCl; 0.2 g of HCl; 1.44 g of Na_2HPO_4; 0.24 g of KH_2PO4 dissolved in 800 mL of distilled water, the pH adjusted to7.4 with HCl and adding water to 1 lt. The buffer was sterilised by autoclaving for 20 minutes at 12l ºC (Sambrook *et al.* 1989).

2.3 ISOLATION OF FUNGAL DNA

Mycelia were grown in colloidal chitin medium from a 5 mm^2 plug excised from a fungal culture for 4 days at 26 ºC. Genomic DNA from each fungal species outlined in Section 2.1, was extracted either using DNeasy Plant Mini Kit (Qiagen, Australia)

following manufacturer's instructions or by the method of Graham (Graham *et al.* 1994). This method consists of grinding the fungal mycelia in liquid nitrogen using a mortar and pestle and adding 1 ml of extraction buffer (2% CTAB, 100mM Tris HCL pH 8.0; 1.4 M NaCl; 2% PVP- 40) per gram of mycelium. The suspension was incubated at 55 ºC for 20 min, followed by centrifugation on a bench-top microcentrifuge at 14000 g for 20 min. The supernatant was incubated with 200 µg/mL RNAse A (Sigma, Aldrich, Australia) at 37 ºC for 20 min and extracted with 24 parts chloroform to 1 part isoamylalcohol. The suspension was centrifuged at 14000 g for 5 minutes and the upper aqueous layer transferred to a clean microcentrifuge tube. The DNA was precipitated with 0.7 vol isopropanol at room temperature for 5 minutes and collected by centrifugation at maximum speed for 20 minutes. The resulting pellet was air dried and resuspended in 50 µL of TE buffer. DNA isolated using this protocol was further purified using the Wizard™ DNA Cleanup System (Promega, Madison, USA) according to manufacturer's instructions and resuspended in 20 µL of water.

2.4 AMPLIFICATION OF CHITINASE AND GLUCANASE GENES FROM FUNGAL DNA

PCR reactions were carried out using approximately 100 ng of total genomic DNA, 0.5µM of each primer, 4 µL of 1x DNA polymerisation buffer containing 67 mM Tris HCl (pH 8.0) 16.6 mM $[NH_4]_2SO_4$; 0.45% Triton X-100; 0.2 mg/mL gelatin; 0.2 mM dNTPs (Fisher Biotec, Australia), 1 U of *Taq* DNA polymerase (Fisher Biotec, Australia), 2.5mM $MgCl_2$, and sterile distilled water to 20 µL. The PCR was carried out in a Hybaid Omnigene thermal cycler. The PCR conditions were as shown in Table 2.1.

Table 2.1- PCR conditions applied to fungal genomic DNA using chitinase degenerate and specific primers or glucanase degenerate primers.

Initial denaturation step	Denaturation	Annealing	Extension	No cycles	Final elongation step
94 ºC, 2 min	94 ºC, 30 sec	54 ºC – 60 ºC, 45 sec	72 ºC, 60 sec	25	72 ºC, 7 min

Table 2.2- PCR conditions applied to DNA vectorette library.

Initial denaturation step	Denaturation	Annealing	Extension	No cycles	Final elongation step
94 ºC, 2 min	94 ºC, 30 sec	55 ºC – 60 ºC, 45 sec	72 ºC, 60 sec	45	72 ºC, 15 min

2.4.1 Agarose gel electrophoresis

PCR products were examined using agarose gel electrophoresis carried out in Bio-Rad Mini Sub™ cells, or Super 120 High Performance Gel System (Fisher Biotec, Australia). TAE buffer contained 40mM Tris-acetate, 1mM EDTA, pH8.0, as described by Sambrook *et al* (1989). The agarose (PROGEN) concentration used was 1% to separate fragments between 500bp-2000bp. Gels were electrophoresed at 10V/cm until the loading dye (50% glycerol, 50 mM EDTA, 0.1% bromophenol blue) had migrated between half and two thirds of the way down the gel. The sample was electrophoresed beside an appropriate molecular weight marker. Gel images were digitally captured under UV light (EDAS 120, Kodak Digital Science™) after staining with 0.5 $\mu g.ml^{-1}$ EtBr and fragment sizes determined by comparison to molecular weight standards (100 bp DNA ladder or 1 Kb DNA ladder- Promega, (Madison, USA) using Kodak Digital Science™ ID (v 3.0.2) software.

2.4.2 Recovery of DNA

Purification of amplified fragments from agarose gels was carried out with QIAquick gel extraction kit (Qiagen, Australia) according to manufacturer's instructions.

2.5 CLONING OF THE AMPLIFICATION PRODUCTS

The DNA extracted from the agarose gel (approximately 10 ng) was cloned in pCR®2.1-TOPO® (Invitrogen, USA) vector (10 ng) followed by transformation in *Escherichia coli* One shot TOP 10 chemically competent cells, F' *mcr*AΔ (*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec* A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rsp*L (Str^R) *end*A1 *nup*G. (Invitrogen, USA). The transformed cells were plated on Luria Broth (LB) agar with Ampicillin (50 mg/L) and X-gal (PROGEN, Australia) (40 mg/mL) for blue/white colony selection. The cells were grown at 37 °C overnight.

2.5.1 Plasmid purification of cloned fragments

A single bacterial white colony was selected and inoculated into 20 mL LB broth containing suitable antibiotics and grown overnight at 37 °C. Plasmid DNA was isolated on a small scale for analysing or sequencing using a modification of the method developed by Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981) . The overnight culture was centrifuged at 20,000 g for 15 minutes; the pellet resuspended in 200 µL of Solution I (50mM glucose; 25 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0). Then 200 µL of freshly prepared Solution II (0.2 N NaOH and 1% SDS) were added. The contents of the tube were mixed by gently inverting the tube several times. The tube was then stored at room temperature for 5-10 min. 150 µL of ice-cold Solution III (60 mL of 5M potassium acetate; 11.5 mL of glacial acetic acid, 28.5 mL of H_2O) was added and the contents of the tube were mixed by inverting several times and

incubating on ice for 10 min. The bacterial lysate was centrifuged at 20000 g for 15 min at 4 °C. The resulting supernatant was transferred to a fresh tube and 2 vol of 100% ethanol was added. The solution was allowed to stand at room temperature for 2 min. Recovery of nucleic acids was by centrifugation at 20000 g for 15 min at room temperature. The nucleic acid pellet was washed with 70% ethanol and dried at room temperature. The pellet was resuspended in 90 µL of water and 10 µL of a 2 µg/µL water solution of RNAase A (Promega, Australia). The reaction was incubated at 37 °C for 15 min followed by phenol:chloroform:isoamyl-alcohol (49.5:49.5:1) extraction using 1 vol of phenol:chloroform:isoamyl-alcohol, mixing and centrifuging at 20000 g for 2 min. The upper aqueous layer was transferred to a fresh tube where one volume of chloroform was added and mixed. The tube was centrifuged as before and the top layer transferred to a new tube. The DNA was recovered by adding 2.5 vol of 100% ethanol and 0.1 vol of sodium acetate pH 5.2. The tube was incubated on ice for 20 min and centrifuged at 20000 g for 20 min. The pellet was rinsed with 70% ethanol and dried in a vacuum for 20 min. The pellet was resuspended in 25 µL of sterile distilled water and stored in aliquots at -20 ˚C or -80 ˚C.

Glycerol stocks were made of bacterial clones for long-term storage. For storage of clones, 0.5 mL aliquots of cells from an overnight culture were mixed with 1 vol sterile 30% glycerol, snap frozen in liquid nitrogen and stored at -80 ˚C.

2.5.2 Analysis of transformants by PCR

White colonies were picked and resuspended directly in 50 µL of PCR master mix as in Section 2.4 using M13 Forward and M13 Reverse primers. The PCR conditions were the same as in Table 2.1 except that the initial denaturation step was 10 minutes.

2.6 SEQUENCING REACTIONS

Standard half reactions were completed, composed of 4 µL ABI PRISM® BigDye Terminator Ready Reaction Cycle Sequencing Kit mix (version 3 or 3.1) (Applied Biosystems), 1.6 ρmoles primer (3.2 ρmoles for version 3.1), 150–300 ng plasmid DNA and made up to a final volume of 10 µL with H_2O. Sequencing reactions were completed in a heated-lid thermocycler programmed as follows: 96 °C for 4 min, followed by 25 cycles of 96 °C (10 sec), 50–55°C (5 sec) and 60 °C (4 min). Post-sequence reactions were purified by ethanol precipitation according to Applied Biosystems recommendation. Purified samples were submitted to the Western Australian State Agricultural Biotechnology Centre (SABC) for sequencing, completed on an ABI373XL automated sequencer (Applied Biosystems).

The primers used for all pCR®2.1-TOPO® sequencing were M13 forward and M13 reverse, the sequences are as follows:

M13-forward: 5'-TGTAAAACGACGGCCAGT-3'

M13-reverse: 5'-CAGGAAACAGCTATGACC-3'.

2.6.1 Sequence analysis

The resulting sequences were analysed using SeqEd™, version 1.0.3, Applied Biosystems Industries (Foster City, California).

Sequence searches were carried out using NCBI (National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD) BLAST (Basic Local Alignment Search Tool) programme and ANGIS (Australian National Genomic Information Service).

2.7 QUANTIFICATION OF DNA

For accurate measure of the amount of genomic and plasmid DNA in a sample, the fluorescent dye, Hoechst 33258 in 1x TNE (Tris-C 10mM, NaCl 100mM, EDTA 1mM, pH 7.4, Hoechst 33258 solution, 0.1 mg/mL) was mixed with a sample of DNA and the fluorescence emitted at 460nm was measured and compared to the emission of a known amount of standard DNA. Fluorescence was measured in a Hoefer TKO 100 DNA Fluorometer.

2.8 cDNA SYNTHESIS

Fungal RNA was extracted using RNeasy Plant Mini Kit (Qiagen, Australia) following manufacturer's instructions. cDNA synthesis was carried out using SuperScript™ Plasmid System with Gateway® Technology for cDNA synthesis and cloning (Invitrogen, USA) following manufacturer's instructions.

2.9 SELECTIVE AGENTS

Selective agents were added to autoclaved media when it had cooled to 45 ˚C. All sterile antibiotic stock solutions were stored in aliquots at -20 ˚C as described by Sambrook *et al* (1989), or by the manufacturers.

List of selective agents showing working concentrations:

i) antibiotics

Ampicillin	50 µg/mL (PROGEN Industries Ltd, Australia)
Streptomycin	30 µg/mL (Boehringer Mannheim GmbH, Germany)
Tetracycline	15 µg/mL (Sigma-Aldrich, Australia)

ii) dyes

Rose bengal sodium salt	0.03 g/L (Sigma-Aldrich, Australia)

CHAPTER 3

ISOLATION OF GLUCANOLYTIC AND CHITINOLYTIC FUNGI FROM SOIL

3.1 INTRODUCTION

Soil fungi that are prolific producers of chitinolytic and glucanolytic enzymes have been studied extensively. Some soil fungi thus far studied include *Rhizopus oligosporus* (Sahai and Monacha 1993), *Metarhizium anisopliae* (Valadaris-Inglis *et al.* 1997) and *Trichoderma* spp (Limon *et al.* 1995; Lorito *et al.* 1993). However, these studies have focused particularly on species of *Trichoderma,* a soil-borne mycoparasite (Lorito *et al.* 1993). Among the chitinases and glucanases produced by soil fungi, investigators have found novel forms of the enzymes (Cohen-Kupiec *et al.* 1999; De la Cruz *et al.* 1992; De la Cruz *et al.* 1995b; Garcia *et al.* 1994; Lima *et al.* 1997; Lorito *et al.* 1993; Lorito *et al.* 1998; Vasseur *et al.* 1995). There have been at least 20 separate chitinases and 1,3-β-glucosidases (glucanases) described from these fungi (Lorito *et al.* 1998). In knockout studies with putative genes encoding similar endochitinases in different *Trichoderma* strains, every possible result (an increase, a decrease and no effect) upon pathogen control was obtained (Baek *et al.* 1999; Woo *et al.* 1999). This apparent inconsistency in the results, may reflect different roles of the enzymes in different strains or against different pathogens, or perhaps there are so many antifungal factors that the loss of a single one makes little difference in the outcome of pathogen control. In view of the successful isolation of novel forms of chitinolytic and glucanolytic enzymes, it is likely that novel forms of the enzymes exist but remain unexplored.

Therefore, one of the main aims of this project was to isolate fungi from soil that

produce chitinases and glucanases. Since Australia has an estimated 250,000 fungi of which only 5-10% have been discovered and named to date (Bougher 1994; Letcher *et al.* 2004) this study set out to isolate soil fungi that produce enzymes that inhibit growth of phytopathogenic fungi. Furthermore, Western Australia is likely to have a unique fungal diversity as a result of its long isolation from the rest of Australia by the Nullabor Plain (Nelson 1981).

Having special environmental characteristics, and being rich in soil fungi populations, the microbiology of Western Australian soils has not been very well explored, constituting an excellent source for the search of new enzymes. The soil samples in this study were selected from a range of locations in Western Australia including a natural forest, a compost heap, chitin-baited garden soil, a mine rehabilitation site and a market garden.

Commonly, chitinolytic fungi are isolated from soil samples by plating soil on media containing Rose Bengal to inhibit bacterial growth (Newsham *et al.* 1995). Chitin is provided in the medium as the sole carbon source (Chernin *et al.* 1995) to encourage the selection of chitinolytic and glucanolytic fungi.

This chapter describes experiments to isolate chitinolytic fungi from local soils and the subsequent identification of novel forms of glucanases and chitinases whose genes could be used in plant protection.

3.2 MATERIALS AND METHODS

3.2.1 Collection sites

For the isolation of soil fungi, soil samples were obtained from five sites around Perth including a natural forest, a mine rehabilitation site, a market garden, chitin-baited garden soil and a compost heap. These sites reflect distinct vegetation types of central-

western and southern Western Australia. The five collection sites were located within a 180-km radius of Perth, W.A and were at least 10 km apart. Soils were collected in a National Park in Margaret River or private land with permission of the owners. Average annual precipitation for all collection sites is 800 mm and is distributed irregularly through the year and between years. The climate of the region is temperate Mediterranean with an average minimum temperature of 12 ºC and an average maximum temperature of 21 ºC (Brown *et al.* 1997).

The native woodland is a dry sclerophyll forest with dark grey to dark brown loamy sand, mildly to strongly acidic (Northcote *et al.* 1975). The vegetation consists of Jarrah and Banksia trees.

The mine rehabilitation site was located 140 km south of Perth. Rehabilitation consisted in seed treatment, seed application, adding of topsoil, native plant propagation and the use of smoke to stimulate seed germination. Rehabilitation of the site resulted in plant species equal to the surrounding jarrah forests (Alcoa 2004).

Compost serves as an ideal food base to attract biocontrol agents such as chitinolytic and glucanolytic fungi (Hoitink and Boehm 1999). The residential compost used for this study consisted of a wide variety of plant-derived organic matter at various stages of decomposition. The site was located within Perth metropolitan area.

Agricultural soil from the market garden consisted of the top 10 cm of a sandy soil from a tilled and irrigated agricultural field located at 40 km from Perth City.

In order to isolate fungi responsible for chitinolytic activity in soil, an *in situ* baiting approach was used. The method involved inoculating garden soil with silk bags containing 30 g of crab-shell chitin (Sigma) that had been autoclaved and buried for a

period of 3 months. A 10 cm-diameter soil-core surrounding the chitin bag was collected for analysis. The garden was located 18 km from Perth City.

3.2.1.1 Isolation from soil

Soil from test sites was mixed with sterile water (2 g soil in 10mL water) and shaken for 1 hour and centrifuged at 12000 rpm for 15 minutes. The supernatant was used as inoculum in three dilutions, neat, 10-fold and 100-fold. The medium used to grow the soil organisms was Martin's agar (Martin 1950) (per litre: KH_2PO_4 1 g; K_2HPO_4 0.5 g; $MgSO_4$ 5 g; peptone 5.0 g; dextrose 10.0 g; yeast extract 0.5 g; Rose Bengal 0.03 g; pH 5.5 with HCL; agar (BBL Becton Dickinson and Co.) containing Ampicillin (50 mg/L)(Sigma), Tetracycline (15 mg/L)(Sigma) and Streptomycin (30 mg/L) (Sigma) to prevent growth of bacteria. Each fungus was subsequently isolated on modified synthetic medium (SM) chitin agar made as per instructions in Chapter 2, Section 2.1.1.

The isolates growing on chitin agar were sub-cultured in liquid chitin synthetic medium (SM) by cutting an agar plug (5mm) from the growing edge of the culture and inoculating 100 mL of liquid SM chitin. Per Litre: Chitin 15 g; yeast extract 0.5g, $(NH_4)_2SO_4$ 1g; $MgSO_4.7H_2O$ 0.3 g; KH_2PO_4 1.36 g. pH adjusted to 5.5 (Rodriguez-Kabana *et al.* 1983).

Sphaerellopsis filum (telemorph *Eudarluca caricis*) was provided by Dr Susanna Driessen (Murdoch University) and maintained on Potato Dextrose Agar (Difco Laboratories).

3.2.2 Measurement of chitinase and glucanase activity

Commercial enzymes were prepared according to manufacturer's instructions. Stock solutions and dilutions of chitinase from *Serratia marcescens* (Sigma Aldrich. Australia) were made in 200 mM potassium phosphate buffer, pH 6.0 with 2 mM

calcium chloride. The 0.5 mL solutions contained 0.625, 0.5 and 0.375 units of enzyme in 160 mM potassium phosphate, 2 mM calcium chloride and 2 mL of a 1.0% chitin (Poly (1→4) β-*N*-acetyl-D-glucosamine) (Sigma) suspension in 200mM potassium phosphate buffer at pH 6.0. The reactions were incubated for 2 hours at 25 ˚C. The reactions were stopped by boiling for 5 minutes. After cooling to room temperature, 1 unit of β-*N*-acetylglucosaminidase was added to each reaction. The tubes were incubated at 25 ºC for 30 minutes. The tubes were centrifuged at 20,000 x g for 5 minutes. One millilitre of supernatant was mixed with 2 mL of deionised water and 1.5 mL of colour reagent made as per instructions in Section 2.2. Absorbances were measured in a spectrophotometer at 540 nm.

Unit definition: one unit will liberate 1 mg of *N*-acetyl-D-glucosamine from chitin per hour at pH 6.0 at 25 ºC in a two-step reaction with β-*N*-acetylglucosaminidase from *Aspergillus niger*.

Glucanase from Sigma Aldrich (Australia) was prepared in cold deionized water according to manufacturer's instructions, containing 1 mL of an enzyme solution containing 0.15, 0.1 and 0.075 units of enzyme. The assay was carried out in 3 mL of a 100 mM sodium acetate buffer, adjusted to pH 5.0 with 1 M HCl and 1 mL of a 2.5% (w/v) laminarin substrate solution. The reactions were incubated at 37 ºC for 1 hour. The reactions were stopped by boiling and 1 mL of a colour reagent made up as per instructions in Section 2.2 was added to each tube. The absorbances were measured in a spectrophotometer at 540 nm.

Unit definition**:** 1 unit liberates 1 mg of reducing sugar (measured as glucose) from laminarin per minute at pH 5.0 at 37 ºC.

Exochitinases are enzymes that release monomeric units and are also referred to as *N*-

acetyl-β-glucosaminidase or just glucosaminidase and enzymes that cleave chitin randomly are called endochitinases. In the case of the fungus *T. harzianum*, an extra term is needed as these fungi produce enzymes that release dimeric units and are called chitin 1,4-β-chitobiosidase or chitobiosidase (Harman *et al.* 1993).

3.2.3 Production of fungal enzymes

Chitinases and glucanases were assayed in culture filtrate from the isolated fungi. The fungi were grown in 250 mL conical flasks containing 100 mL of medium (SM) supplemented with 0.15% chitin (Rodriguez-Kabana *et al.* 1983) with antibiotics as described in Section 2.1. The fungi were grown for 4 days on a rotary shaker at 225 rpm at 26 ºC. Filter sterilisation of the filtrates was carried out to purify extracellular enzymes from the fungal filtrates using mixed cellulose ester membrane (Advantec MFS, Inc) with a pore size of 0.45 microns.

After filtration, the supernatants were dialysed against 100 mM sodium acetate buffer, pH 5.5 using a cellulose membrane (Sigma) that retains most proteins of molecular weight of 12,000 Da or greater

Exochitinases or *N*-acetyl-β-glucosaminidase were assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma) using a modification of the method of Roberts and Selitrennikoff (1986) and Ohtakara (1988) (Ohtakara 1988; Roberts and Selitrennikoff 1986). Test samples of 30 µL were added to wells in a flat bottom well microtitre test plate. Fifty microlitres of the substrate solution containing 300 µg/mL dissolved in 50 mM potassium phosphate (pH 6.7) was added. The plates were incubated at 50 ºC in a water bath for 60 minutes. Reactions were terminated by addition of 50 µL 0.4M Na_2CO_3 to each well, which also served to enhance the color of *p*-nitrophenol formed by the enzymatic cleavage of the substrate. Absorbance at 405 nm was measured with a BioRad microplate reader. Substrate and

enzyme blanks were also included. Substrate blanks contained 30 µL of the supernatant crude or dialysed and 50 µL of the buffer. Enzyme blanks contained 30 µL of the buffer and 50 µL of the substrate. All tests and blanks were represented three-fold.

Unit definition: One unit of enzyme activity was defined as the amount of enzyme which released 1 nmol of nitrophenol per minute per mililitre of enzyme.

Endochitinase activity was measured by the reduction of turbidity of suspensions of colloidal chitin. Enzyme tests were carried out in a microtitre plate in which 100 µL of a colloidal chitin solution (1% w/v of moist purified chitin in 50 mM potassium phosphate at pH 6.7 and 0.02% NaN_3), 50 µL of potassium phosphate (pH 6.7), and 50 µL of enzyme solution were added to each well and incubated for 24 h at 28 ºC with agitation. Turbidity of the suspensions was determined at 405 nm (Tronsmo and Harman 1993). Activity was calculated as the percentage of reduction in turbidity relative to that of a similar suspension that contained water rather than enzyme solution.

Unit definition: One unit of enzyme was defined as the amount of enzyme required to reduce turbidity of a chitin suspension by 5%.

3.2.4 Enzyme activity on fungal cell wall preparations

The fungal isolates with glucanase and chitinase activities were used to study enzyme activity by measuring the release of reducing sugars from cell-wall preparations of phytopathogenic fungi as substrate. Enzyme solution was obtained as previously described in 3.2.3.

The reaction mixture consisted of 0.5 mL each of filtrate and 100mM sodium acetate buffer pH 5.5 mixed with 1 mL of a 1% cell-wall preparation. The reactions were incubated for 30 minutes at 30 ºC. The reactions were stopped by boiling for 5 minutes. Four millilitres of a colour reagent were added to each tube (Chapter 2, Section 2.2) and

measured on a spectrophotometer at 540 nm, units were determined as per relevant section.

3.2.4.1 Preparation of fungal cell walls

Some of the target fungi used in this study belong to the phylum Ascomycota *(B. cinerea, F. solani, S. sclerotium, A. faba* and *L. maculans)* while *R. solani* belongs to the phylum Basidiomycota. The two phyla were chosen because members have chitinous cell walls that also contain R-glucan, S-glucan and heteropolymers such as mannose, galactose, xylose and glucose (Rosenberger 1976).

The plant pathogens *B. cinerea, R. solani, F. solani, S. sclerotium. A. faba* and *L. maculans* were grown without shaking on PDB (Difco) for 6 days at 26 ˚C. All mycelia were collected by filtration.

To prepare the target species cell-wall substrate, hyphae or sclerotia were crushed in liquid N_2, and washed three times with deionised sterile water (Inglis and Kawchuck 2002). The cell-wall preparations were suspended in 100mM sodium acetate buffer, pH 5.5 in a concentration of 1g per 100 mL of buffer.

3.2.4.2 Protein concentration

Protein concentration to determine total amount of protein in lysates was measured according to the method described in Section 2.2.1.

3.2.5 Identification of fungal isolates

Fungal material was examined microscopically and identified using the mycological keys of Ellis (1971, 1976), Sutton (1980) and Barnett and Hunter (1998). Spore size, shape and the manner in which spores are produced were considered to identify fungal species.

3.3 RESULTS

3.3.1 Collection sites

The soil samples for this study came from broadly different environments. Forty one pure cultures were obtained on Martin's agar that selects preferentially for fungi (Martin 1950).

Six of the original forty one isolates came from native woodland in the Margaret River District. Six isolates were obtained from the mine rehabilitation site. Eighteen isolates were cultured from compost. Seven isolates came from the chitin-baited garden soil. The lowest number of isolates came from a market garden. Only four isolates were obtained from this site (Table 3.1).

To identify chitinase and glucanase producers, the isolates were tested for their ability to grow on chitin as a carbon source. Of the 41 isolates, 24 hydrolysed colloidal chitin after 96 hours of growth on synthetic medium supplemented with chitin as the sole carbon source. These results suggested that chitinolytic and glucanolytic enzymes may be secreted by the strains into the culture medium. The chitinolytic and glucanolytic activity in the culture supernatant was analysed using appropriate substrates as shown in experiments presented below.

Table 3.1-Fungal isolates collected from five sites in the central and south areas of Western Australia, Australia

Isolate numbers	Collection sites				
	Natural woodland	**Rehabilitation site**	**Compost heap**	**Chitin baited soil**	**Market garden**
01-025	+				
01-026	+				
01-027	+				
01-028	+				
01-029	+				
01-030	+				
02-018		+			
02-019		+			
02-023		+			
02-033		+			
02-038		+			
02-039		+			
03-002			+		
03-003			+		
03-006			+		
03-007			+		
03-008			+		
03-009			+		
03-012			+		
03-014			+		
03-015			+		
03-016			+		
03-020			+		
03-021			+		
03-022			+		
03-031			+		
03-034			+		
03-035			+		
03-036			+		
03-037			+		
04-000				+	
04-001				+	
04-004				+	
04-005				+	
04-013				+	
04-032				+	
04-040				+	
05-010					+
05-011					+
05-017					+
05-024					+

* the prefixes indicate the sites of origin of the soil samples

3.3.2 Optimisation of activity assay

Initially an experiment was carried out to determine how the activity of the enzyme varied with time of incubation of the assay mixture. A time course for each of the commercial enzymes was prepared according to manufacturers instructions. The activity of the chitinase enzyme (Sigma-Aldrich) reached its maximum after 60 minutes of incubation. 0.5 U of chitinase had produced 0.062 mg of *N*-acetyl-D-glucosamine from chitin per minute (Fig 3.1 A). The commercial chitinase is a mixture of exo and endochitinases.

After 60 minutes a commercial glucanase (Sigma-Aldrich, Australia) produced 0.4 mg of reducing sugars from laminarin per minute (Figs 3.1 B). Enzyme activity in the commercial enzyme preparations reached its maximum at 60 minutes and this time period was chosen for all later assays presented here.

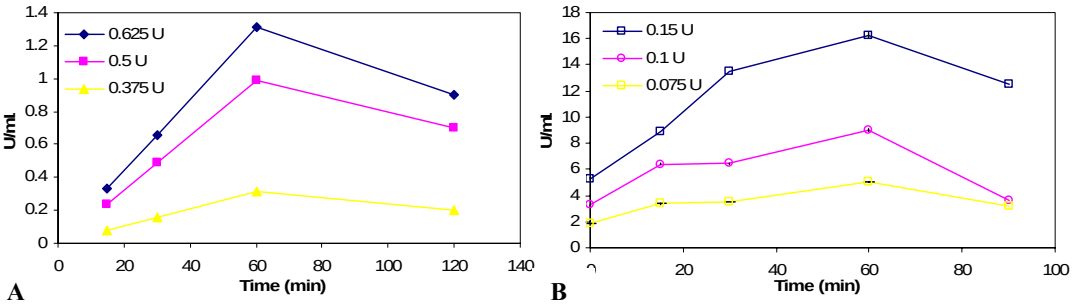


Figure 3.1- A-Time course of commercially available chitinase (Sigma Aldrich. Australia) The units of activity are the amount of enzyme required to release 1 mg of *N*-acetyl-D-glucosamine from chitin. The bars represent the standard errors of the mean (n=3).

B-Time course of commercially available laminarinase (Sigma Aldrich. Australia). The units of activity are the amount of enzyme required to release 1 mg of reducing sugar expressed as glucose equivalent. The bars represent the standard errors of the mean (n=3).

3.3.3 Chitinase and glucanase production by fungal isolates

Isolates were inoculated into SM medium with chitin or laminarin and after 4 days incubation, chitinase and glucanase activities in the cell free supernatant were measured.

Glucanase activity ranged from 0 to 7.57 U/mg of protein in the crude supernatant from the studied strains (Table 3.2). The highest glucanase specific activity was 7.57 U/mg of protein in the crude supernatant of isolate 04-001.

The specific activity of the exochitinase was calculated to range from 0 to 10.30 U of enzyme per mg of protein in the crude supernatant with the most active filtrate belonging to isolate 04-040 (Table 3.2).

Endochitinase activity was calculated to be between 0 to 0.96 U/mg of protein in the crude supernatant with the turbidity reducing assay. The most active filtrate was from isolate 04-013 (Table 3.2).

3.3.3.1 Effect of dialysis on enzyme activity

The activity measurements in the supernatants may not be an accurate representation of the true amount of activity due to accumulation of chitin-breakdown products during growth. These products can have the effect of inhibiting the enzyme activity (Pinto *et al.* 1997). To overcome this, the supernatant was dialysed to remove these products before measuring activity. Activity was higher in the crude supernatant than in the dialysed filtrate for most of the isolates. The filtrate was dialysed with a cellulose dialysis membrane (molecular weight cutoff, 12,000), which excluded lower molecular weight substances such as antibiotics and free sugars.

Glucanase activity in the dialysed supernatant range from 0 to 5.58 U/mg of protein. The highest activity shown by isolate 04-001, however, it was 37% lower than in the crude supernatant.

Exochitinase activity range from 0 to 5.93 U/mg of protein in the dialysed supernatant (isolate 03-008) (Table 3.2). Exochitinase activity was lower in the dialysed supernatant than the crude except for 04-001 and 03-002, 03-003, 04-004, 03-016, 05-017, 03-022, 05-024 and 02-033.

Endochitinase activity range from 0 to 0.43 U/mg of protein in the dialysed supernatant with the most active filtrate belonging to isolate 04-013. The specific activity of the commercial enzyme preparation was 0.45 U/mg of protein.

Table. 3.2- Glucanolytic and chitinolytic activities in extracellular proteins of fungal strains grown for 96 hours with chitin as the sole carbon source. The values represent the mean of triplicates. The fungal species were given arbitrary numbers as at this stage they were unknown species, except for *S. filum*. Activities lower thanb the commercial enzyme activity are indicated with the letter a. Activities higher than the commercial enzyme activity are indicated with the letter b.

	Glucanase				**Exo-chitinase**				**Endo-chitinase**			
	Crude		Dialysed		Crude		Dialysed		Crude		Dialysed	
	U/mg		U/mg		U/mg		U/mg		U/mg		U/mg	
Comm enz	2.2				0.45				0.45			
Isolates		SE		SE		SE		SE		SE		SE
01-028	1.83a	0.00	1.79a	0.00	4.60b	0.06	0.33	0.00	0.92b	0.00	0.32	0.01
01-029	0.00	0.00	0.00	0.00	1.01b	0.01	0.29	0.01	0.33	0.01	0.11	0.01
02-019	0.00	0.00	0.00	0.00	1.20b	0.03	0.60a	0.23	0.00	0.00	0.00	0.00
02-023	3.53b	0.00	2.44b	0.00	6.67b	0.00	0.51a	0.02	0.72a	0.02	0.28	0.01
02-033	0.00	0.00	0.00	0.00	0.00	0.00	0.85b	0.03	0.71a	0.00	0.34	0.00
02-039	0.00	0.00	0.00	0.00	1.57b	0.02	0.61a	0.03	0.37	0.01	0.00	0.00
03-002	4.04b	0.00	3.88b	0.01	1.55b	0.02	2.49b	0.01	0.40	0.00	0.27	0.01
03-003	2.91b	0.00	2.70b	0.01	0.59a	0.02	0.75a	0.00	0.00	0.00	0.40	0.01
03-006	0.00	0.00	0.00	0.00	0.31	0.01	0.00	0.01	0.00	0.00	0.00	0.00
03-008	3.71b	0.00	2.76b	0.00	6.57b	0.05	5.93b	0.06	0.78a	0.01	0.30	0.01
03-012	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.45a	0.01	0.05	0.00
03-016	0.00	0.00	0.00	0.00	0.30	0.01	0.63a	0.04	0.00	0.00	0.00	0.00
03-021	0.00	0.00	0.00	0.00	1.46b	0.03	0.25	0.00	0.40	0.01	0.00	0.00
03-022	0.00	0.00	0.00	0.00	0.13	0.01	0.46a	0.01	0.00	0.00	0.11	0.01
03-034	0.00	0.00	0.00	0.00	0.06	0.00	0.03	0.00	0.00	0.05	0.00	0.00
04-000	3.01b	0.01	2.66b	0.00	0.49	0.01	0.33	0.01	0.45a	0.01	0.23	0.01
04-001	7.57b	0.00	5.50b	0.01	2.12b	0.01	3.90b	0.02	0.66a	0.02	0.33	0.00
04-004	5.05b	0.01	4.76b	0.01	0.00	0.00	4.31b	0.14	0.76a	0.01	0.17	0.01
04-005	0.00	0.00	0.00	0.00	1.42b	0.01	0.00	0.00	0.00	0.00	0.00	0.00
04-013	4.27b	0.00	2.92b	0.00	0.00	0.00	0.00	0.00	0.96b	0.01	0.43	0.01
04-032	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.18	0.00	0.00	0.00
04-040	2.24b	0.00	1.96a	0.00	10.30b	0.07	3.83b	0.05	0.44	0.00	0.19	0.01
05-017	0.00	0.00	0.00	0.00	0.53a	0.01	0.77a	0.05	0.00	0.00	0.00	0.00
05-024	0.00	0.00	0.00	0.00	0.00	0.00	0.59a	0.02	0.00	0.00	0.00	0.00
S.filum	3.55b	0.01	3.36b	0	2.44b	0.02	0.44a	0.02	0.67a	0.01	0.13	0.00

Enzyme activity was related to the areas where the isolates came from to establish the soil harbouring fungi with the most chitinolytic and glucanolytic activity. Interestingly, the natural woodland only produced one isolate with chitinolytic enzyme activity, yet, it was the most active (Fig 3.2).

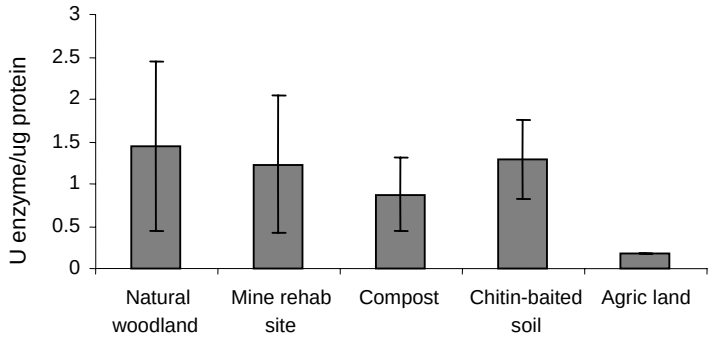


Figure 3.2- Average enzyme activity from isolates collected at each site. The natural woodland had only one representative, 01-028, with highest enzyme activity. The compost heap had the greatest number of representatives, nine isolates, the average enzyme activity was not as high as the natural woodland. Chitin-baited soil, with seven representatives showed higher average enzyme activity than the compost. The values represent the mean of triplicates.

Eleven of these isolates showed chitinolytic and glucanolytic enzyme activities. The highest activity was shown by 03-008 in chitin and fungal cell-walls however, the broadest spectrum of activity belonged to isolates 04-000, 04-001 and 01-028. The eleven isolates were chosen for further analyses. The isolates were: 04-000, 04-001, 03-002, 03-003, 04-004, 03-008, 04-013, 02-023, 01-028, 04-040 and *S. filum*.

3.3.4 Assays of the fungal supernatants using fungal cell-walls as substrates

The structure and organisation of chitins and glucans in the fungal cell walls varies with

species (Hunsley and Burnett 1970; Rosenberger 1976). It is therefore to be expected that the ability to degrade cell walls will also vary from species to species. To test this, enzyme activity measurements were carried out using fungal cell walls as substrate. The dyalised filtrate activities were significantly different when analysed against pathogen cell walls (Tables 3.3 and 3.4). While isolates 04-000, 04-001, 03-003, 04-004 and 03-008 showed glucanase and chitinase activities in their supernatant against *B. cinerea* there was no activity measured in the filtrates from 02-023, 01-028 and 04-040. Isolates 04-000, 04-001, 03-003, 04-004, 03-008 and 02-023 showed chitinase and glucanase activities against the pathogen *R. solani*. Only extracts from isolates 04-000 and 04-001 and 01-028 displayed chitinase and glucanase activities against *F. solani*, while filtrates from 04-040 and from *S. filum* showed only chitinase activity against *F. solani* cell walls. Filtrates from isolates 04-000, 04-001 and 01-028 showed chitinase and glucanase activity against the cell wall preparations from *S. sclerotium* and *A. faba* and only 01-028 showed both chitinase and glucanase activity against *L. maculans*.

Table 3.3- Chitinase activity measured as the reducing sugar equivalents released from 1% solution of fungal cell walls by test fungi per mg of total protein. Standard errors of the mean (n=3) are included. Units are defined as nmoles reducing sugar equivalents per minute.

Chitinase activity						
Target fungi	***B. cinerea***	***R. solani***	***F. solani***	***S. sclerotium***	***A. faba***	***L. maculans***
Specific activity	U/mg	U/mg	U/mg	U/mg	U/mg	U/mg
Isolates						
01-028	0	0	0	0.36±0.001	0.19±0.004	0.176±0.02
02-023	0	0.18±0.001	0	0	0	0
03-002	0.44±0.005	0.41±0.004	0	0	0	0
03-003	0.19±0.002	0.25±0.002	0	0	0	0
03-008	0.21±0.003	0.19±0.001	0	0	0	0.08±0.002
04-000	0.18±0.001	0.25±0.01	0.3±0.001	0.255±0.01	0.18±0.001	0.1±0.002
04-001	0.16±0.01	0.31±0.003	0.42±0.004	0.352±0.002	0.14±0.001	0.125±0.01
04-004	0.9±0.02	0.85±0.007	0	0	0	0
04-013	0.22±0.05	0	0	0	0	0
04-040	0	0	0.21±0.007	0.21±0.001	0.14±0.001	0.123±0.01
S. filum	0	0	0.195±0.02	0.189±0.02	0.2±0.003	0.125±0.02

Table 3.4- - Glucanase activity measured as the reducing sugar equivalents released from 1% solution of fungal cell walls by test fungi per mg of total protein. Standard errors of the mean (n=3) are included. Units are defined as nmoles of reducing sugar equivalents per minute.

Glucanase activity						
Target fungi	***B. cinerea***	***R. solani***	***F. solani***	***S. sclerotium***	***A. faba***	***L. maculans***
Specific activity	U/mg	U/mg	U/mg	U/mg	U/mg	U/mg
Isolates						
01-028	0	0	0	0.51±0.001	0.22±0.005	0.22±0.001
02-023	0	0.25±0.002	0	0	0	0
03-002	0.38±0.001	0.30±0.001	0	0	0	0
03-003	0.67±0.001	0.53±0.004	0	0	0	0
03-008	0.3±0.001	0.23±0.02	0	0	0	0
04-000	0.35±0.01	0.35±0.001	0.38±0.001	0.38±0.003	0.17±0.002	0
04-001	0.44±0.002	0.39±0.002	0.44±0.002	0.43±0.002	0.19±0.001	0
04-004	1.4±0.01	1.32±0.004	0	0	0	0
04-013	0.24±0.002	0	0	0	0	0
04-040	0	0	0	0	0	0
S. filum	0.26±0.005	0	0	0	0	0

3.3.5 Identification of the fungi

Although identification of the fungi was not the objective of this study, the isolates that will be used in later experiments were identified by microscopy using spore size, shape and manner of production (T. Paap, pers. comm). The fungal isolates identified were: *Trichoderma* spp. (04-000, 04-001, 01-028), *Aspergillus* spp. (03-002, 03-003, 03-008, 04-013), *Rhizopus* spp. (04-040) and a *Penicillium* spp (04-004) (Barnett and Hunter 1998; Ellis 1971; Ellis 1976; Sutton 1980) Microscopic photographs of eleven of these fungi are shown in Fig 3.3.

3.4 DISCUSSION

The aim of this study was to isolate soil fungi, and to identify those that display glucanolytic and chitinolytic activity, keeping in mind that both enzyme groups sometimes work together to elicit an effect. A number of locations were explored for fungi of interest and a baiting strategy was tested to enrich the soil flora for fungi of interest. Initially 41 pure cultures were obtained from the different locations. Twenty four were able to grow on chitin and of those, eleven showed enzyme activity against chitin, laminarin and fungal cell walls.

Composted organic material provides nutrients to soil and reduces their predisposal to soil borne pests and disease (Muhammad and Amusa 2003). *A. niger* and *T. harzianum* as well as the bacteria *Bacillus cereus* and *Bacillus subtilis* have been found in composted material. These organisms are believed to be responsible for the decrease in plant diseases by inhibiting the growth of plant pathogens such as *F. oxysporum, S. rolfsii, Pythium aphanidermatum* and *R. solani* amongst others (Muhammad and Amusa 2003). Other studies have also found that compost prepared from substances high in sugar such as fruit and vegetable waste, attracts *Penicillium* and *Aspergillus* spp.

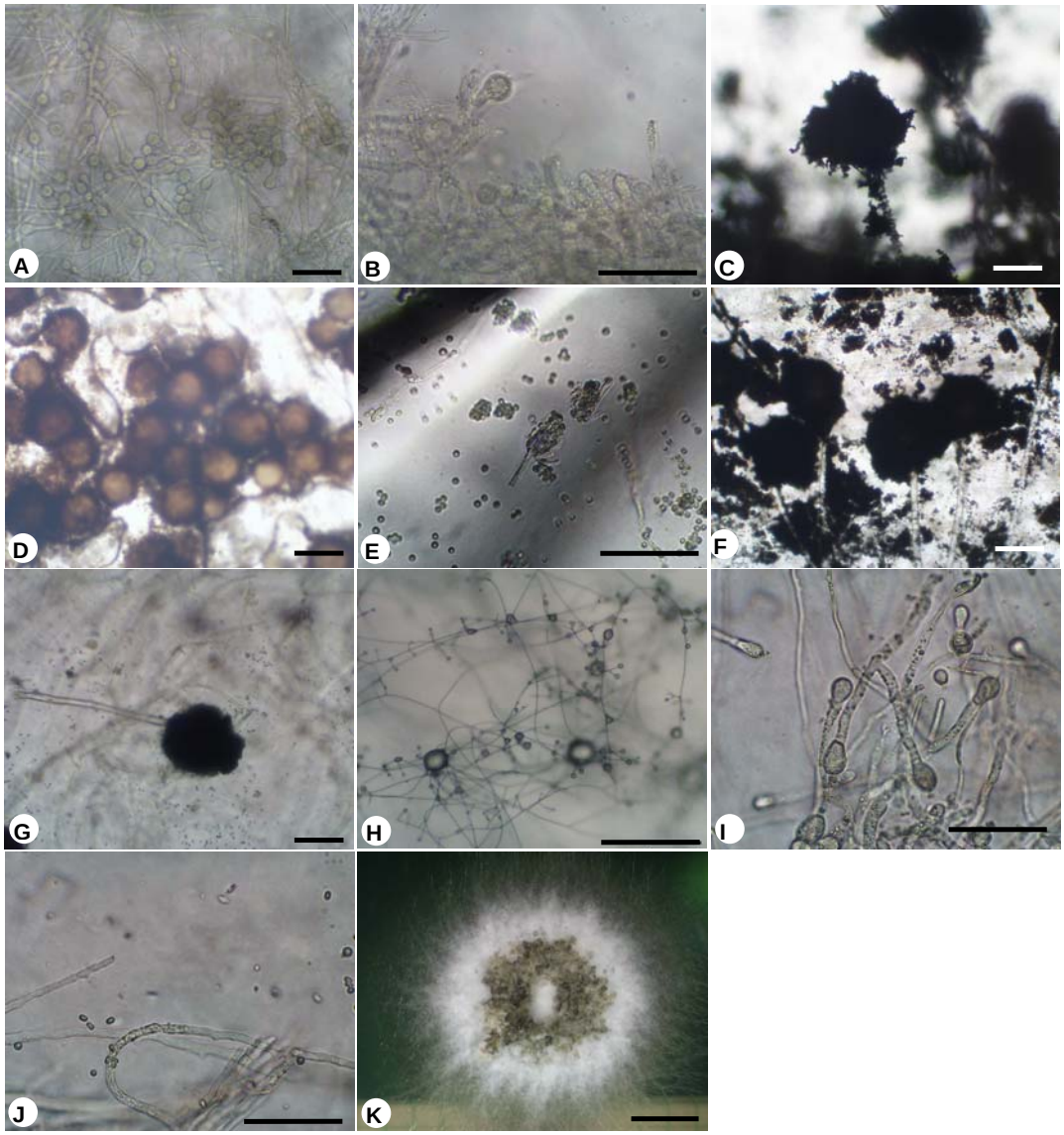


Figure 3.3-A selection of the fungal species isolated from soil**. Bars A–J = 50 µm, K = 2 mm.**

A. *Trichoderma* sp. **B.** *Trichoderma* sp. **C.** *Aspergillus* sp. **D.** *Aspergillus* sp.**E.** *Penicillium* sp. **F.** *Aspergillus* sp.**G.** *Aspergillus* sp.**H.** Unknown **I.** *Trichoderma* sp.**J.** *Rhizopus* sp. **K.** *Sphaerellopsis filum.*

(Hoitink and Boehm 1999). In this study it was found that the compost heap yielded the greatest total number of fungal isolates, but only three isolates, 03-002, 03-003 and 03-008 showed chitinolytic and glucanolytic activities, all three were identified as *Aspergillus*. However glucanase activity was two and a half times higher than exochitinase activity and 10 times higher than endochitinase activity in filtrate from isolate 03-002 and almost five times higher than exochitinase activity in filtrate from isolate 03-003 (Table 3.2). In contrast, filtrate from isolate 03-008 showed highest exochitinase activity (6.57 U/mg protein) compared to glucanase activity (3.71 U/mg protein). Species of *Aspergillus* have already been shown to produce glucanases for the degradation of α-1, 3-glucan, a fungal cell wall component (Wei *et al.* 2001).

Baiting proved to be a productive method for enriching the surrounding soil flora for chitinolytic and glucanolytic species. Seven isolates identified from chitin-enriched peat, from a total of 41 isolates, showed chitinase enzyme activity (Table 3.2). Another group reported that a similar baiting technique gave higher cell numbers of the chitinase-producing bacterium *Bacillus subtilis* AF1 (5.0 log units) than soil with no chitin supplements (Manjula and Podile 2001). The authors also found that chitin containing materials showed better control of *A. niger* (causal agent of crown rot of groundnut) and *Fusarium udum* (causal agent of wilt of pigeon pea) (Manjula and Podile 2001).

Soil from a dry sclerophyll forest located in the south west of Western Australia was chosen because of the many unique Australian fungi believed to exist in natural woodlands (Glen, 2001). However, only six isolates were obtained from this soil. Although, we were selecting for a subset of chitinolytic fungi, this result is surprising as it has been shown that there is high species richness in eucalypt ecosystems (Glen, 2001). In a study carried out by Glen (2001) using molecular tools such as PCR and

RFLP analyses, the author found 154 unidentified types as well as 109 species of basidiomes in an area of 3 hectares (Glen, 2001). Only one of the six fungi that grew on chitin medium, showed chitinolytic and glucanolytic activities (01-028). 01-028 was identified as belonging to the genus *Trichoderma.*

The mine rehabilitation site yielded six isolates, one of which produced chitinolytic and glucanolytic enzymes. This bauxite mine site has been successfully rehabilitated with plant species richness equal to the surrounding jarrah forests (Alcoa 2004).

Agricultural soils are often subjected to the use of fungicides and fertilizers and their effect on fungal population can be detrimental, hence the market garden yielded the lowest number of isolates none of which showed chitinolytic or glucanolytic activities.

Since glucanase and chitinase activities are required in order to utilize chitin as a carbon source (De la Cruz *et al.* 1993) the forty one fungal isolates were grown on chitin selective media. This selection resulted in the isolation of 24 pure cultures. The isolation technique used in this study was selective for fast growers and discriminatory for slow growers, therefore, a true representation of the soil flora was not obtained. In future studies this can be addressed by allowing longer incubation period for the soil isolates.

Some of the isolates were characterised to the genus level by observation of taxonomic features. There were *Trichoderma* spp. (04-000, 04-001, 01-028), *Aspergillus* spp. (03-002, 03-003, 03-008, 04-013), *Rhizopus* spp. (04-040) and a *Penicillium* spp (04-004). These fungi are commonly found in soil (Domsch *et al.* 1993). *Aspergillus* is ubiquitous in nature; *Penicillium* is the most abundant genus of fungi in soil; *Trichoderma* is found in forests or agricultural soils at all latitudes and *Rhizopus* is distributed world-wide with high prevalence in tropical and subtropical regions (Domsch *et al.* 1993). The species status of the isolates was not determined so it is unclear whether any are

endemic to the area collected.

A number of different hydrolytic enzymes may act in synergism to degrade the cell walls of fungi. Synergism of the enzymes has been demonstrated by various studies that found that the chitinolytic system of mycoparasites such as *Trichoderma* spp. consists of glucanases, chitobiosidases and chitinases (De la Cruz *et al.* 1992; Lorito *et al.* 1993; Lorito *et al.* 1994; Lorito *et al.* 1998; Tronsmo and Harman 1993). These enzymes degrade fungal cell walls that contain chitin, which appears to be protected by β–glucans, and is not readily accessible to chitinases. Thus it has been shown that chitinase activity is preceded by, or coincides with the hydrolytic activity of other enzymes, especially β-1,3- and β-1,6-glucanases (Cherif and Benhamou 1990).

T. harzianum produces several extracellular enzymes including *β*-1, 3-glucanases, a protease and a chitinase when grown on liquid culture on laminarin, chitin or host cell walls (Ridout *et al.* 1986; Ulhoa and Peberdy 1991). *T. harzianum* (strain IMI 206040) has been shown to produce at least two β-1, 3-glucanases and one constitutive neutral isozyme as well as one acidic form which was subject to both carbon catabolite repression and induction by laminarin, pustulan (β-1, 6-glucan) or *R. solani* cell-walls (Geremia *et al.* 1991). Lorito *et al* (1993) studied an endochitinase and a chitobiosidase from *T. harzianum* finding that, in combination, antifungal activity against several target fungi including *F. solani* and *B. cinerea*, was four times higher than the activity of the endochitinase on its own and 10 times higher for the chitobiosidase. Furthermore, chitinases and glucanases act synergistically to inhibit the growth of several genera of fungi *in vitro* (Bolar *et al.* 2001; Lorito *et al.* 1994). Other fungi also produce a number of enzymes that may degrade components of the host cell wall. Extracellular β-1, 3-glucanase, lipase and protease but not chitinase were detected *in vitro* when *Pythium oligandrum* was grown on laminarin and isolated host cell-walls. Glucanase and

protease production was repressed in the presence of glucose (Lewis *et al.* 1989). Pathogenic isolates of *M. anisopliae, Beauveria bassiana* and *Verticillium lecanii* produce a range of extracellular enzymes, including *N*-acetyl glucosaminidase (exochitinase), chitinases, lipases and proteases in sequence (Goettel *et al.* 1989; St Leger *et al.* 1986). It may be possible that synergism of the hydrolytic enzymes in the fungal supernatant had a marginal effect on the results of the assays, particularly when trying to measure the activities of the individual enzymes.

The fungal isolates were grown in synthetic chitin liquid medium and the filtrate, containing extracellular enzymes, was harvested. Subsequent treatment of the supernatant affected the apparent enzymatic activity. Crude filtrates showed greater glucanase activity than dialysed filtrates and in most cases, chitinase activity was also greater in the crude supernatant than in the dialysed filtrates (Table 3.2). Dialysis eliminates small molecules such as free sugars reducing the effect that those molecules could have on the results of the assays.

The reducing sugar assay did not distinguish between the reducing sugars resulting from substrate hydrolysis and the reducing sugars accumulated from the degradation of complex carbohydrates such as chitin and laminarin in the medium from when the fungi were actively growing (Inglis and Kawchuck 2002). This is significant because an accumulation of free sugars in the filtrates can result in a decrease of enzyme activity due to feedback inhibition. Chitinase inhibition by accumulation of the end-product of chitin hydrolysis, has been reported by Pinto *et al* (1997). This is consistent for exo-chitinase activity in the crude supernatants of isolates 03-002, 03-003, 04-001 and 04-004 where the activity was lower than in the dialysed filtrate.

In contrast, endochitinase activity decreased after dialysis. Activity was higher in the crude supernatant for all isolates except for isolate 03-003 which increased activity in

the dialysed supernatant compared to no activity in the crude. This result seems to suggest that dialysis diluted and/or denatured the enzyme solution resulting in decrease activity. Yet, specific activity was comparable (0.72 to 0.96 U/µg of protein)(Table 3.2) to the activity reported for an endochitinase enzyme isolated from *T. harzianum* (0.86 U/µg of protein) with the turbidity-reducing assay (Harman *et al.* 1993), the same method used in this study.

Although different substrates were used to distinguish between the different enzyme activities in the complex enzyme systems of the fungal isolates, assaying activity in the supernatant is a relatively crude method of measuring enzyme activity. Assaying enzyme activity from the purified enzymes rather than from the fungal supernatants that contain various enzymes, secondary metabolites and metabolic waste would result in a more accurate estimation of individual enzyme activity. This point is illustrated by a study by Di Pietro *et al* (1993) who found that a purified endochitinase from *Gliocladium virens* was substantially less active against fungi than when the endochitinase from *T. harzianum* was measured in a mixture of enzymes, even though the two fungi are closely related and the enzymes are of similar size and similar specific activity against colloidal chitin (Di Pietro *et al.* 1993).

The release of reducing sugars into the medium was assayed as a direct measure of cell-wall degradation. The difference in the activity of the enzymes in relation to each substrate cell-wall preparation from the six target species was expected given that the supernatants contained a combination of enzymes. Furthermore, fungal cell–wall is a complex mixture of polymers where the spectrum of polymers present can vary over the lifecycle of the organism, as well as between species (Bartnicki-Garcia 1968; Bartnicki-Garcia and Nickerson 1962). The physical composition of the fungal cell-walls is also complex. Fungal walls contain a network of fibrils with the spaces in the net filled by

matrix polymers (Hunsley and Burnett 1970). This model of walls as fibre-matrix composites, in which different polymers can fulfill the functions of matrix and fibres and whose mechanical properties can be modified by altering parameters such as fibre concentration, is one on which this work was based. The complexity of the cell-walls as substrates together with the multiple enzymes present in the supernatants might explain the differences in enzyme activities displayed by the isolates that, *in vivo*, possess multiple hydrolytic enzymes to facilitate degradation of fungal cell-walls (Tables 3.3 and 3.4). Therefore, variability in enzyme activity against the different fungal species used in this study was expected. In *Schizophyllum* chitin dry weight represents 5% of the cell walls (Wessels 1965) while in *Sclerotium,* chitin content is 60% (Rosenberger 1976). β (1→3)-glucan (syn: S-glucan) is a straight-chain polysaccharide and a major component in the walls of Ascomycetes and Basidiomycetes (Wessels *et al.* 1972). It comprises 15-25% of the wall polysaccharides. Only β(1→3)-glucans have a wider distribution than chitin among fungi.

Several researchers have indicated a synergistic interaction among cell-wall lytic enzymes. This study attempted to determine quantitatively the importance of a complex of enzymes in the degradation of plant pathogen cell-walls. Furthermore, the results suggest that *B. cinerea* and *R. solani* cell-walls were more vulnerable to degradation by chitinases and glucanases than the other pathogenic fungi used in this study (Tables 3.3 and 3.4). Previous studies have shown that extracellular glucanases and chitinases from *Trichoderma* spp. were induced in the presence of *R. solani* cell walls (Ridout *et al.* 1986). Moreover, results presented by Lima *et al* (1997) provided evidence of the hydrolytic action of an enzyme solution from *Trichoderma* spp. on cell walls of *R. solani* and *S. rolfsii.* The study also suggested the synergistic action of a combination of enzymes in the fungal filtrate when the purified chitinase failed to affect the cell walls

of *R. solani* (Lima *et al.* 1997). *B. cinerea* cell walls have also been used in other studies to induce the production of chitinolytic and glucanolytic enzymes in *Trichoderma* spp. by Lorito *et al* (1993) and de la Cruz *et al* (1993).

3.5 CONCLUSIONS

Even though there are a large number of unidentified fungi in Western Australian soils, this study found that only six out of forty fungal isolates originated in a natural forest. In addition and despite the fact that twenty four soil isolates were able to grow on chitin, only eleven produced significant levels of chitinolytic and glucanolytic enzymes. Of the eleven, five were isolated from chitin-baited or amended soil.

Isolates 04-000, 04-001 and 01-028 showed the most consistent results. These three isolates appear to belong to *Trichoderma* spp. and tests to confirm this, such as sequencing the ITS regions, should be carried out. If they belong to a *Trichoderma* spp. the results of this experiment would correlate those obtained by other groups who have found that fungi of this genus produce the most efficient system of enzymes to control plant pathogenic fungi (De la Cruz *et al.* 1992; De la Cruz *et al.* 1995a; Lorito *et al.* 1998). The lytic activity of these enzymes has been implicated in disease control for several years.

There were challenges involved in identifying the hydrolytic enzymes responsible for enzyme activity. These challenges were overcome by the use of different substrates that distinguish between exo-, endo-chitinases and glucanases. However, to eliminate any elements that could influence the outcome of the activity measurements, future work should be carried out using purified enzymes rather than crude supernatants.

Similar levels of chitinase and glucanase production were obtained against fungal cell walls with some of the isolates showing strong activity particularly against *B. cinerea*

and *R. solani*. This result has major implications as both pathogens are responsible for high economical losses in agriculture (Stirling and Stirling 1997).

CHAPTER 4

BIOASSAY TO ASSESS FUNGAL GROWTH INHIBITION BY FUNGAL ENZYMES

4.1 INTRODUCTION

Most fungi contain chitin and β-glucans in their cell walls and degradation of these structural polymers adversely affects the development and differentiation of fungi (Rosenberger 1976). Different chitinolytic enzymes act synergistically in the control of pathogenic fungi as discussed more fully in Chap 3.

Many studies have investigated the effect of chitinolytic enzymes on the germination of spores and development of hyphae of mycoparasitic fungi. Lorito *et al* (1993) used chitinolytic enzymes from *T. harzianum* to inhibit spore germination and germ tube elongation from a wide range of fungi including *B. cinerea*, *F. solani*, *Ustilago avenae, Erysiphe necator* and *F. graminearum.* In 1994 Lorito and colleagues found that cell wall degrading enzymes produced by *T. harzianum* and *Gliocladium virens* inhibited spore germination of *B. cinerea* in a bioassay *in vitro*. A chitinase produced by *T. harzianum* was shown to hydrolyse the cell wall of *S.rolfsii* but had no effect on the cell wall of *R. solani* (Lima *et al.* 1997).

The purpose of this study was to assess the effect of the supernatants of the fungi isolated in Chapter 3 on the growth of three pathogenic fungi, *F. solani* and *S. sclerotium* and *B. cinerea.*

Growth inhibition of the pathogens by the enzymes present in the fungal supernatants was quantified using two different methods:

- by measuring turbidity as Absorbance at 595nm of a spore suspension of *B. cinerea* grown in the presence and absence of the fungal enzyme solutions.
- by comparison of dry weight of mycelia of *F. solani* and *S. sclerotium* grown in the presence and absence of the fungal enzyme solutions.

4.2 MATERIALS AND METHODS

4.2.1 Growth media

Growth media containing chitin or laminarin were prepared as described in 2.1.2 and 2.1.3, 2.1.4 and 2.1.5.

4.2.2 Strains

The plant pathogens*, S. sclerotium*, *F. solani* and *B. cinerea* were obtained from the CPSM collection at Murdoch University and maintained on Potato Dextrose Agar (PDA) (Difco Laboratories) (39g/L).

The soil isolates from Chapter 3 and *S. filum* were also maintained on PDA. An agar plug of 5mm of diameter was excised and used to inoculate the media containing either laminarin, chitin or Potato Dextrose Broth (PDB).

4.2.3 Enzyme production

All fungal isolates were grown in, either chitin, laminarin or PDB liquid media for 4 days at 26 °C. The supernatant from each isolate was filter-sterilised and dialysed. Half of the dialysed supernatant was boiled for 15 minutes and half was left untreated.

Filter sterilisation was carried out using mixed cellulose ester membrane (Advantec MFS, Inc) with a pore size of 0.45µm. The dialysis was carried out in Sigma dialysis tubing, against 4 litres of a 100 mM sodium acetate buffer pH 5.5.

The target fungal species were: *S. sclerotium* and *F. solani.* These fungi were grown and maintained on PDA (39 g/L) and incubated at 26 °C for 10 days.

Inhibition was tested by adding 5 mL of fungal supernatant to 10 mL of V8 medium inoculated with an agar plug taken from the leading edge of a culture of the target species (either *F. solani* or *S. sclerotium*). Because glucose inhibits chitinase and glucanase activities (Schirmbock *et al.* 1994), the amount of glucose in the V8 juice was tested at the Division of Veterinary and Biomedical Sciences at Murdoch University and found to be 17.5 mM or 0.32 %. For controls, 5 mL of water was used instead of the fungal supernatant.

After incubation of 6 days at 26 °C, the fungal mycelia were collected by filtration on 0.45 μm pore-size membrane filters (Advantec MFS, Inc). The filters were dried in a drying oven at 110 °C for 2 hours and the dry weights determined. The inhibition activity was expressed relative to controls. The tests were carried out with three-fold replication.

4.2.4 *B. cinerea* spore production

B. cinerea was grown for five days on potato dextrose agar in the dark at 27 °C before being incubated at room temperature for a further 10 days also in the dark. Spores were collected by adding 4 mL 0.1% v/v Tween 20 solution to the agar plate and scraping the spores into a filter with three layers of muslin and diluting to 2 x 10^4 spores per mL in CDOX.

The isolates were grown on colloidal chitin as described in Section 2.1.2. The supernatants were collected and treated as described in Section 4.2.3.

Inhibition was tested by the method described by Broekaert *et al* (1990) using microtitre plates. Each well contained 15 μL of fungal supernatant and 100 μL of spore suspension

with a concentration of 2 x 10^4 spores in 100 µL of CDOX. Control wells contained 100 µL of spore suspension and 15 µL of sterile distilled water. All plates had wells of just CDOX to check for sterility. Spore germination was assessed after 0, 24, 48 and 72 hours by measuring the absorbance of the medium at 595nm. The zero hour absorbances were subtracted from the subsequent readings to allow for slight differences in the colour of the supernatants. Each assay was performed six times on the same plate and mean results were used. Five wells were randomly chosen from each microtitre plate to check for sterility. Sterility was checked by transferring the samples to PDA plates. The cultures were then grown for five days at 27 °C in the dark and visually checked to ensure sterility.

The values obtained for the control were taken as 0% inhibition and all other values were divided by these values and multiplied by 100 to obtain percentage inhibition.

4.3 RESULTS

4.3.1 Inhibition of *B. cinerea*

B. cinerea or grey mold-rot is a foliar pathogen that affects most vegetable and fruit crops, as well as a large number of shrubs, trees and flowers (Domsch *et al.* 1993). To evaluate the ability of the fungal proteins in the supernatants to inhibit spore germination of *B. cinerea*, a bioassay was carried out that measured optical density (turbidity) as a measure of inhibition. A typical inhibition curve of *B. cinerea* is shown in Figure 4.1. When compared with the control, the fungal filtrates inhibited spore germination of *B. cinerea.*

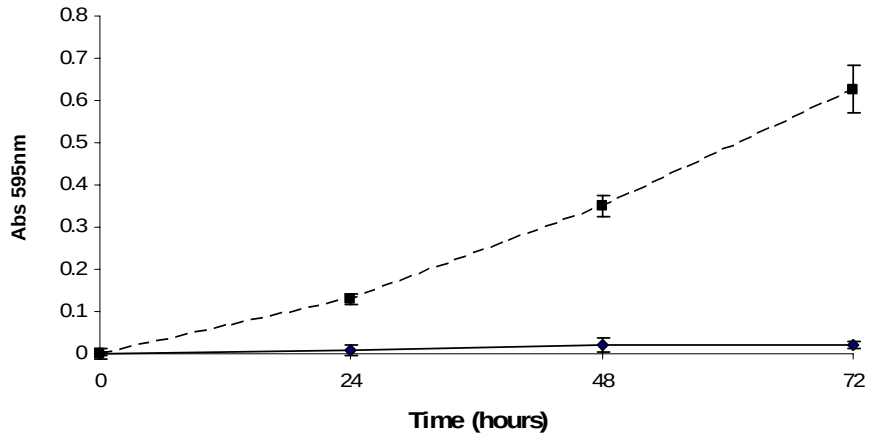


Figure 4.1-Typical growth curve of *B. cinerea* when grown with fungal supernatant from an inhibitory isolate (black solid line) compared to the control (dotted line). Each point represents an average of six tests in the same microtitre plate. The bars represent the standard errors of the mean (n=6).

A supernatant was not considered inhibitory if the OD of the culture closely matched the control's OD. When *B. cinerea* spores were grown in the supernatant from isolate 05-017, inhibition was not detected, compared to the control. Isolate 05-017 was chosen to illustrate non-inhibitory activity against spore germination (Fig 4.2).

When *B. cinerea* was grown in the presence of fungal supernatant, inhibition was seen in eleven cases when compared to the controls after 24 hours of incubation (Fig 4.3). Filtrates from isolates 01-028 and 03-003 showed the most inhibition. However, filtrates from isolates 02-023, 03-002, 03-008, 04-000, 04-001, 04-004, 04-013, 04-040 and from *S. filum* caused inhibition of spore germination.

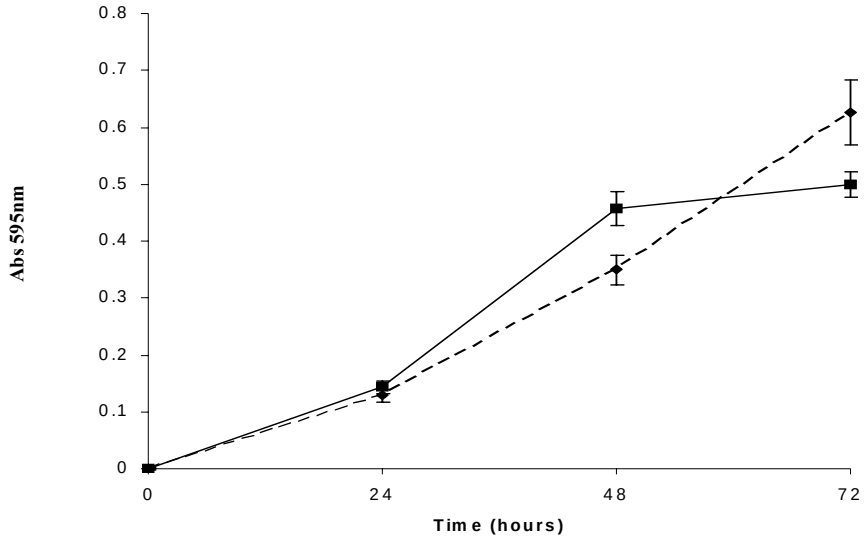


Figure 4.2-Growth curve of *B. cinerea* when grown with the fungal supernatant from a non-inhibitory isolate (05-017) (black solid line) compared to the control (dotted line). Each point represents an average of six tests in the same microtitre plate. The bars represent the standard errors of the mean (n=6).

One hundred per cent inhibition was shown by filtrate from isolate 03-003, followed by 97% inhibition by filtrate from isolate 01-028 and 90% by filtrate from isolate 03-002.

In order to test whether inhibition was due to enzymes or other elements in the supernatant, boiled filtrates were added to the growth medium (Fig 4.4). When *B. cinerea* was grown in the absence of enzymes, none of the filtrates showed inhibitory activity against the spores. All supernatants appear to encourage growth compared to the control.

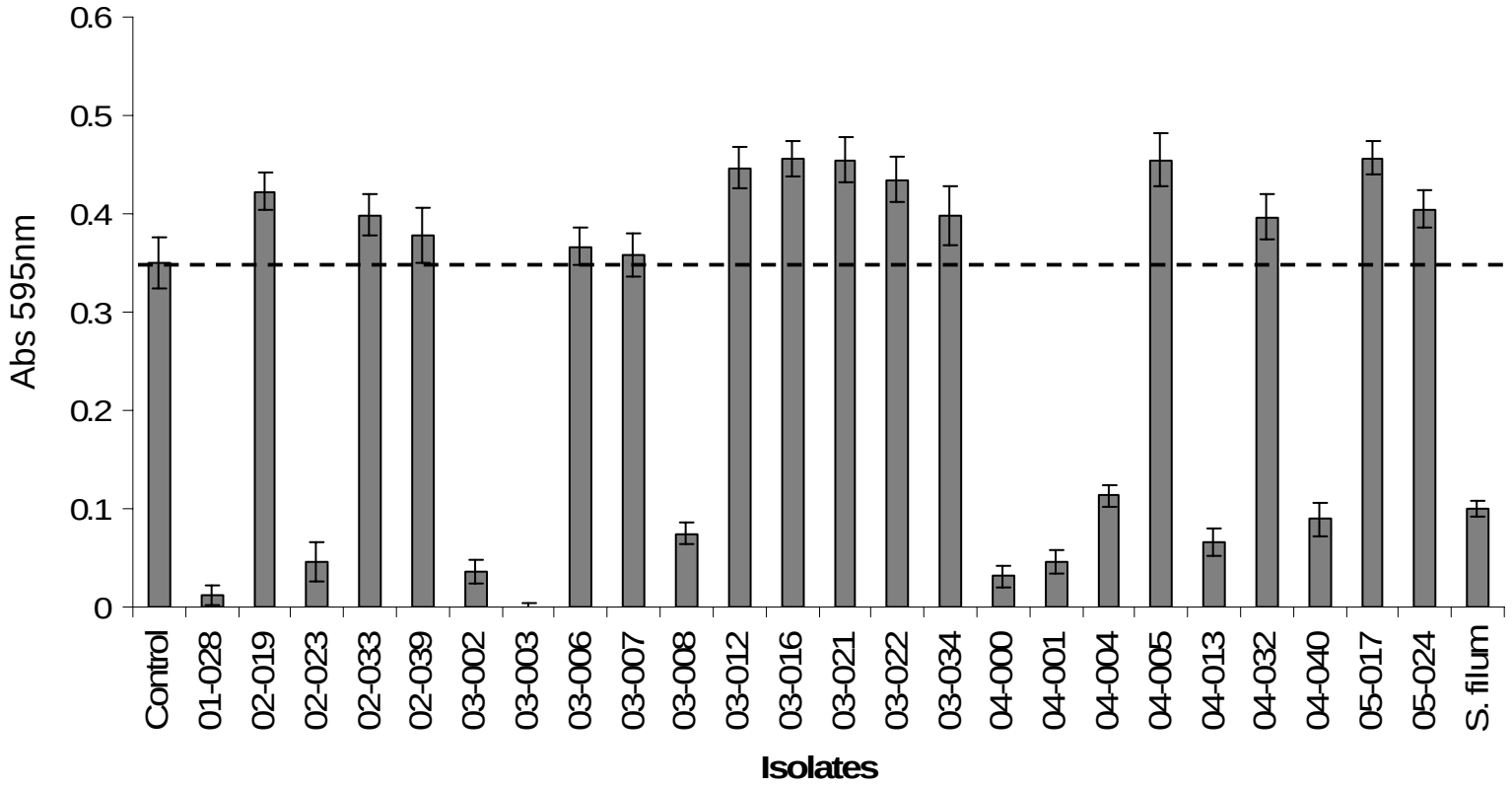


Figure 4.3-Relative growth of *B. cinerea* after two days, when grown in the presence of native supernatant, containing enzymes. Error bars represent the standard error of six tests in the same microtitre plate. The columns with values below the control baseline show inhibition.

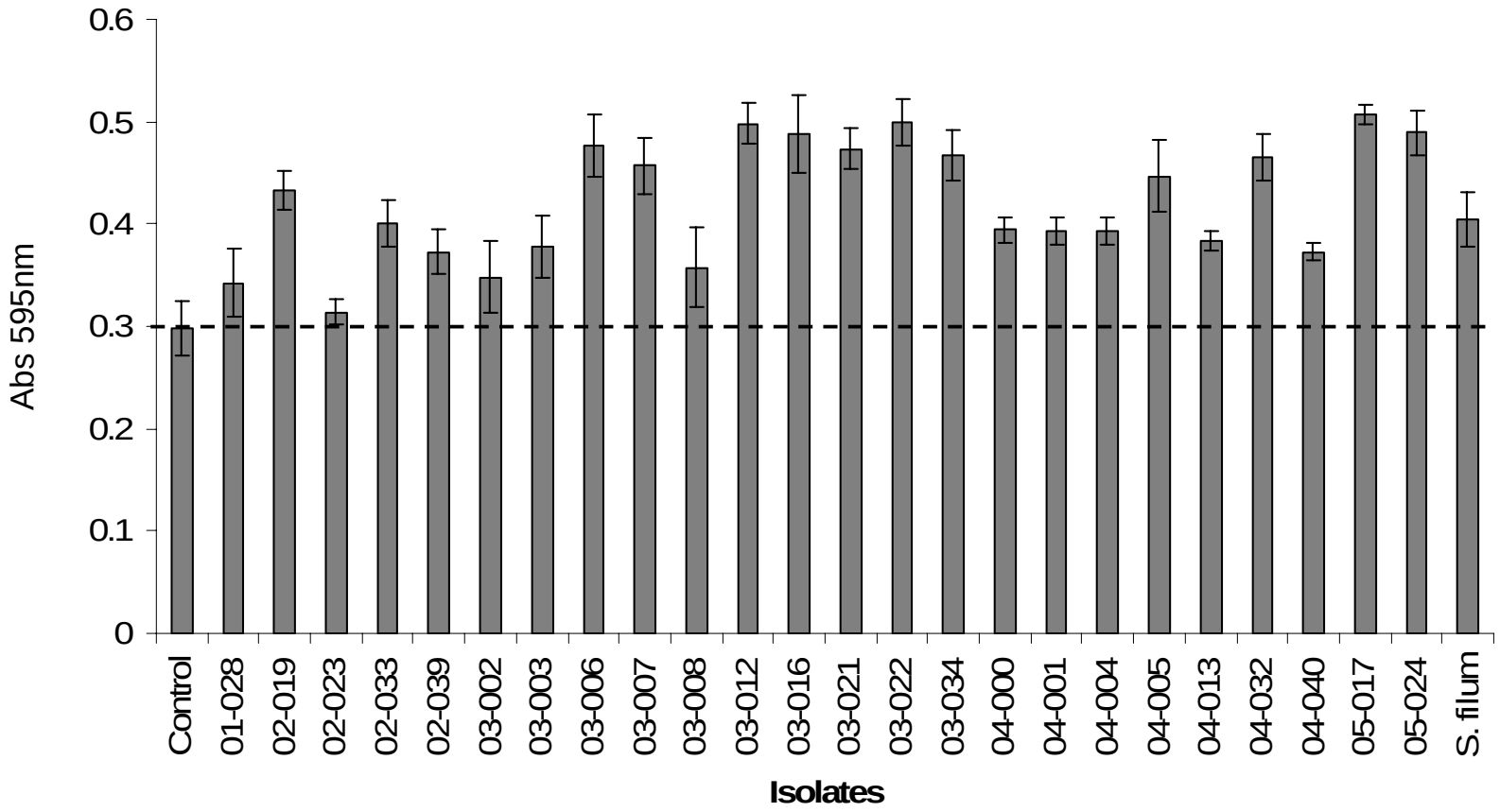


Figure 4.4-Relative growth of *B. cinerea* after two days when grown with boiled supernatant. Error bars represent the standard error of six tests in the same microtitre plate. The columns with values above the control baseline signify growth enhancement.

4.3.2 Inhibition of *F. solani* and *S. sclerotium*

To gain a more quantitative measure of the ability of antifungal proteins produced by the fungal isolates to inhibit growth of the target pathogens, the isolates were grown on media containing laminarin; chitin or PD Broth. The extracellular supernatant from a chitin, laminarin or PDB-induced culture of each isolate was either left untreated or boiled (to confirm inhibition by enzymes as opposed to antibiotics) and used as medium to grow the plant pathogens *F. solani* or *S. sclerotium* supplemented with V8 juice.

The pathogens *F. solani* and *S. sclerotium* were chosen for their economic importance in agriculture. The genus *Fusarium* is a soil-borne necrotrophic plant pathogen that causes serious plant diseases worldwide. *F. solani* attacks legumes including alfalfa, beans, clover and groundnuts that causes root and stem rots. It is also a well-known parasite of stored potatoes in which it causes dry rot (Agrios 2005). *S. sclerotium* is a plant parasite distributed across temperate zones across the world and causes damping off of seedlings, stem canker, crown blight and root, crown, bulb and tuber rot and fruit rots. Its most important host plants are bean, potato, lettuce, sunflower and rape (Agrios 2005; Domsch *et al.* 1993).

4.3.2.1 Fusarium solani

The filtrates inhibited growth of *F. solani* compared to the control dry weight (36 mg) (Table 4.1). Evidence from the results seemed to suggest that all twenty-five isolates produced enzymes that inhibited growth of the pathogen. Enzyme production by the isolates was not greatly affected by growth medium as all twenty-five isolates showed similar inhibitory ability when grown on different media. Isolate 04-004 supernatant inhibited the growth of *F solani* to a greater extent when grown on laminarin-containing medium (14 mg) than when grown on chitin or PDB (15.6 mg). Likewise, isolate 04-

000 showed higher inhibitory activity when grown on chitin-containing (14.6 mg) than when grown on laminarin-containing medium (15 mg) or PDB (17.3 mg) (p=0.089). Isolate 03-003 showed highest inhibitory activity when grown on PDB (15.3 mg) compared to all other isolates however, it showed higher inhibitory activity when grown on laminarin (14.6 mg).

In a number of cases there was a slight increase in inhibition by boiling the supernatant (turquoise shading). This effect was not consistent, but appeared when the isolate was grown on a particular medium, eg. isolate 03-006 grown on laminarin was affected by boiling but not when grown on chitin or PDB. Also isolates 01-028 and 04-032 exhibited increase inhibition when grown on laminarin and PDB but not on chitin. Isolate 05-024 supernatant showed increased inhibition after boiling when it was grown on PDB but not on laminarin or chitin.

There were instances where the degree of inhibition was greater when the culture was treated with the crude supernatant (yellow shading) compared to the boiled. The results shown for the pathogen *F. solani,* suggest that laminarin and chitin-containing media had minor effect on growth as in the case of *S. sclerotium* (Table 4.2). PDB showed the highest inhibitory effect when the supernatant was untreated. In addition, these effects did not correlate with the effects of boiled supernatants on growth of *S. sclerotium*, eg. boiling the supernatant of 05-024 grown on chitin increased inhibition of *S. sclerotium* but not of *F. solani*.

Table 4.1-Inhibition of *F. solani* by supernatants of the isolates.

***F.solani* mycelia dry weight (mg)**

	Laminarin crude		Laminarin boiled		Chitin crude		Chitin boiled		PDB crude		PDB boiled	
Isolate												
Control	36	0.58	36	0.58	36	0.58	36	0.58	36	0.58	36	0.58
01-028	20.3	0.88	20	1.15	22.8	0.00	23	0.58	22.6	0.33	22.3	1.53
02-019	22.6	0.33	23	1.15	23.6	0.88	24.6	0.33	25.6	1.86	25.6	1.86
02-023	15.3	1.20	22.6	0.33	24.6	0.33	25.3	0.88	19.6	0.88	22.3	0.88
02-033	20.3	0.88	21	0.58	19.6	0.33	18	0.58	28	1.53	28.3	0.33
02-039	19.3	0.33	20	0.58	17	0.00	19	0.58	21	1.00	22.3	1.33
03-002	14.6	0.33	24.6	0.33	16.6	0.33	17.3	1.20	17	1.15	26	1.15
03-003	14.6	0.33	19.6	1.20	15.6	0.33	22.6	0.33	15.3	0.33	26.6	1.20
03-006	23.3	0.88	21.3	1.33	25.6	0.58	26	0.58	25	0.58	25.3	0.33
03-007	21	1.00	22.6	0.88	22	0.58	23	0.58	24	0.00	25	1.00
03-008	15	0.00	18	1.53	17	0.58	25.6	0.33	17.3	1.20	24.3	0.88
03-012	21	0.88	21.6	1.20	22.3	1.86	18.3	0.33	28.3	0.88	29.3	0.33
03-016	17.3	0.33	17.3	0.33	17.6	1.33	19	1.15	24	2.00	24.3	1.76
03-021	25.6	0.33	25	1.73	22.6	1.15	21	0.58	21.3	1.85	21.3	1.45
03-022	18	0.58	18.3	0.88	23	0.58	18	0.58	33.3	1.33	33.3	0.88
03-034	22.3	0.33	22.3	0.88	18.6	0.33	19.6	0.67	24.3	0.88	25	0.58
04-000	15	0.58	19.6	1.20	14.6	0.33	18	0.58	17.3	0.33	23.6	0.88
04-001	15	0.58	22.6	0.33	16.6	0.33	19	0.58	17.3	1.20	24.3	1.20
04-004	14	0.58	19	0.58	15.6	0.88	20	0.58	15.6	0.33	22	1.73
04-005	27.6	0.33	28	1.00	21.6	0.88	22.3	1.45	30	0.58	30.6	0.33
04-013	15	0.00	19	0.00	17.3	0.88	19.3	0.33	17.3	1.20	20.3	0.33
04-032	22.6	1.45	19.6	0.33	19.6	1.20	19.3	0.33	27.6	0.88	27	1.53
04-040	15	0.58	19.3	0.88	15.6	0.33	25.3	0.33	18.3	0.33	25	1.73
05-017	23.3	0.33	24	1.52	24	0.58	25.3	0.33	28	1.15	28.3	1.76
05-024	25	0.58	25.6	0.67	47	0.58	27	0.58	25.6	0.33	24.6	0.33
S. filum	15.6	0.88	21	0.58	15.3	0.67	22.6	0.88	19.6	0.67	27.3	1.20

The data summarised in this table shows dry weight (in mg) of the pathogen *F. solani* growing on extracellular culture supernatant from fungal isolates incubated for 96 hours in three different media. The values shown represent the means of replicates (n=3) with standard errors. Yellow shading indicates isolates that inhibited the growth of pathogenic fungi. Turquoise represents higher inhibition after boiling supernatant. Control represents the weight of the pathogen when grown V8 juice supplemented with water instead of supernatant. The prefixes indicate the sites the isolates originate from, 01-natural woodland; 02-mine rehabilitation site; 03-compost heap; 04-chitin-amended soil; 05-agricultural land.

4.3.2.2 Sclerotinia sclerotium

A group of isolates (yellow shading) are inhibitory to *S. sclerotium* (Table 4.2). *S. sclerotium* dry weight was 55 mg for the control and between 13.3 and 20 mg when grown on the filtrates. As in the experiment with *F. solani* the growth substrate did not have a significant effect on the ability of the isolates to inhibit the growth of the

pathogen. Isolates 04-000 and 04-004 showed greater inhibiting ability when grown on laminarin-containing medium (14 mg) than when grown on chitin-containing medium (15 mg and 16.6 mg respectively) or PDB (16.3 mg or 15 mg, respectively). In chitin-containing medium, isolate 02-023 showed the highest inhibiting ability (13.3 mg) compared to 15 mg in laminarin-containing medium and 14 mg in PDB. Isolate 02-023 had the highest inhibiting ability compared to all other isolates when grown on PDB but compared to its own growth on chitin-containing medium, inhibition was lower (13.3 mg). There was little difference in dry weight when *S. sclerotium* was grown on untreated or boiled filtrates (between 0.4 and 2.7 mg). Boiling the supernatant did not have a significant effect except in cases highlighted in turquoise. In these cases boiling caused further reduction in growth compared to the untreated filtrate. This, however, depended on the growth medium. Boiling increased inhibition mostly when isolates were grown on laminarin and PDB as substrates but not when grown on chitin, this is the case for isolates 03-006, 03-007, 03-016 and 01-028 for the laminarin-containing medium and isolates 04-013 and 01-028 for PDB. In contrast boiling increased inhibition of the 05-024 supernatant from the chitin cultures but not the laminarin or PDB cultures. In general, laminarin-containing medium showed an insignificant difference when it was untreated or boiled. Chitin appeared to discourage growth of the pathogen when it was untreated whilst PDB appeared to have the highest inhibitory effect on the pathogen when it was untreated. The remaining examples where boiling increased inhibition of *S. sclerotium* were not mirrored in their effect on *F. solani.*

Table 4.2-Inhibition of *S. sclerotium* by supernatants of the isolates.

	***S. sclerotium* mycelia dry weight (mg)**											
	Laminarin crude		**Laminarin boiled**		**Chitin crude**		**Chitin boiled**		**PDB crude**		**PDB boiled**	
Isolate												
Control	55	±0.58	55	±0.58	55	±0.58	55	±0.58	55	±0.58	55	±0.58
01-028	22.6	±1.20	20	±1.53	19.6	±0.67	20	±0.58	20	±0.58	19.6	±1.00
02-019	22	±0.00	22.6	±0.33	20	±0.58	20	±1.15	18.6	±0.33	20	±0.58
02-023	15	±0.58	25.6	±0.33	13.3	±0.33	18	±0.00	14	±0.58	20	±0.58
02-033	25.3	±0.33	28.3	±1.45	20.3	±0.33	20.6	±0.67	21	±0.58	21.3	±0.88
02-039	27.6	±0.33	29.3	±0.33	20	±0.58	20	±0.33	29.6	±0.33	29.6	±0.33
03-002	15	±0.58	14.6	±0.33	17	±0.58	17	±0.00	14.6	±0.33	15	±0.58
03-003	15	±0.00	15	±0.00	15.3	±0.33	15.3	±0.88	14.6	±0.67	15.3	±0.88
03-006	20	±1.15	19.6	±0.33	20	±1.53	20.3	±2.03	30.3	±2.08	30.3	±0.33
03-007	20	±0.58	19.3	±0.33	21.3	±0.33	21.6	±1.33	17.6	±0.67	18.6	±0.33
03-008	14.6	±0.33	24.6	±0.33	15.6	±0.33	20.3	±0.88	15.3	±0.33	19.3	±1.20
03-012	19.6	±0.67	20	±0.58	20.6	±0.67	21.6	±0.33	19.6	±0.33	18.6	±0.33
03-016	18.3	±0.33	17.3	±0.88	16	±1.00	16.3	±0.88	16.3	±0.88	16.6	±0.88
03-021	18	±0.58	18.3	±0.33	15	±0.58	15.6	±0.33	18.6	±0.33	18.6	±0.33
03-022	18	±0.00	22	±0.58	15.6	±1.20	15.3	±0.33	14.3	±0.33	15.3	±0.33
03-034	25.3	±0.33	29.3	±1.20	25	±0.00	26.6	±1.20	28	±0.58	29.3	±0.88
04-000	14	±0.58	18.6	±0.33	15	±0.00	20	±0.58	16.3	±0.88	21.3	±0.88
04-001	15	±0.00	25	±0.58	14.6	±0.33	24.6	±1.20	15.3	±0.88	19	±1.15
04-004	14	±0.00	16	±0.58	16.6	±0.88	18	±0.58	15	±0.00	15.3	±0.33
04-005	17	±0.58	17	±0.00	16	±0.58	16.3	±0.88	15.6	±0.88	15.6	±0.88
04-013	15.6	±0.33	15.3	±0.33	15	±0.58	15.6	±0.33	17	±0.00	16.3	±0.67
04-032	20.6	±0.88	22.6	±0.33	27.3	±1.20	27.6	±0.88	26	±0.58	26.6	±3.18
04-040	15.6	±1.20	25.3	±0.33	14.6	±0.88	21.6	±0.33	14.6	±0.33	20.3	±0.33
05-017	18	±0.58	18.3	±0.88	16	±0.58	16.3	±0.33	20.6	±0.33	21.6	±1.20
05-024	18	±0.58	19.6	±0.33	36.3	±0.88	36	±1.53	19.3	±0.88	19.3	±0.33
S. filum	14.6	±0.33	27.6	±0.33	14.3	±0.33	22.3	±0.33	14.6	±0.67	20.0	±0.58

The data summarised in this table shows dry weight (in mg) of the pathogen *S. sclerotium* growing on extracellular culture supernatant from fungal isolates incubated for 96 hours in three different media. The values shown represent the means of replicates (n=3) with standard errors. Yellow shading indicates isolates that inhibited the growth of pathogenic fungi. Turquoise represents higher inhibition after boiling supernatant. Control represents the weight of the pathogen when grown V8 juice supplemented with water instead of supernatant. The prefixes indicate the sites the isolates originate from, 01-natural woodland; 02-mine rehabilitation site; 03-compost heap; 04-chitin-amended soil; 05-agricultural land.

Statistical analysis of these data used a 2-way factorial design with factors of medium and pathogen, with the dependent variable being dry weight crude and dry weight boiled. The tests were replicated 3-fold. A two-way interaction involving these factors and including isolate were also studied. Results of the analysis are presented in Table 4.3.

Table 4.3- Initial MANOVA of data.

Effect	Rao's R (df)	P
Medium	2.087 (4, 90)	0.089
Pathogen	40.53 (2, 45)	0
Isolate x medium	0.796 (92, 90)	0.86
Isolate x pathogen	1.92 (46, 90)	0.004
Medium x pathogen	0.717 (4, 90)	0.58

The medium effect was not significant (Rao's $R_{(4, 90)}$= 2.087, p= 0.089. The pathogen effect was highly significant (Rao's $R_{(2, 45)}$= 40.53, p=0.000

Both pathogens were affected by the same isolates, however, compared to the controls, *S. sclerotium* showed more inhibition, (3.7-fold) than *F. solani* (2.4-fold) when exposed to the supernatants. PDB, chitin-, and laminarin-containing media equally affected pathogen growth, and the results suggest that their effect on the outcome of the experiment was insignificant (Table 4.3). Relationships between isolate and medium, isolate and pathogen and medium and pathogen were not significant (Table 4.3).

4.4 DISCUSSION

4.4.1 Inhibition of *B. cinerea* spore germination

Chitinolytic and glucanolytic enzymes have been implicated as factors contributing to the ability of some fungi to parasitise pathogenic fungi (Lorito *et al.* 1993). This study set out to explore the inhibitory ability of enzymes produced by the soil isolates on *B. cinerea* spore germination. Despite extensive research, the mechanisms by which some

fungi control plant-pathogenic fungi are not well understood. One of the mechanisms proposed is mycoparasitism (Lorito *et al.* 1996; Lorito *et al.* 1993). Suspicions that this intricate process requires the production of enzymes that digest the fungal cell walls have been re-inforced in previous chapters (Chapter 3) and in this study. Of all the filtrates tested, eleven showed inhibitory activity against *B. cinerea* spore germination. Similarly, studies carried out in Chapter 3 showed that *B. cinerea* cell walls were susceptible to fungal proteins when incubated with filtrates from 03-002, 03-003, 03-008, 04-000, 04-001, 04-004, 04-013 and *S. filum*.

Others have found that chitinolytic enzymes produced by *T. harzianum* affected spore germination (cell replication) of *B. cinerea* (Lorito *et al.* 1993). The authors incubated other plant pathogen mycelia such as *F. solani, U. avenae, U. necator* with *T. harzianum* filtrate and found that complete inhibition (100%) of hyphal elongation was achieved with concentrations of 35-135 µg/ mL of a purified endochitinase. In this study, purified enzymes were not used, instead, a mixture of enzymes was likely to exist in the fungal filtrates that caused inhibition of spore germination.

The complexity of this mechanism called mycoparasitism is compounded by the findings that synergism occurs not only between hydrolytic enzymes such as chitinases and glucanases but also between these enzymes and membrane –affecting antibiotics such as peptaibols (Lorito *et al.* 1996). Combination of hydrolytic enzymes and antibiotics produce synergistic mixtures with strong inhibitory activity on the growth of many pathogenic fungi (Schirmbock *et al.* 1994). Therefore, the filtrates used in this study were heat-treated. Results obtained from boiling the supernatant suggested that proteins in the supernatant were likely to affect spore germination. In addition, spore growth appeared to improve after boiling, likely due to the sugars in the supernatant.

B. cinerea spores were incubated in the fungal filtrates for 72 hours. Inhibition of spore germination was observed after 24 hours compared to the controls. This results agrees with results others have obtained that suggest that enzyme activity in fungal filtrates is seen after 24 hours of exposure of the pathogens to the enzyme solutions (Mischke 1997; Schirmbock *et al.* 1994).

A more quantitative assay was performed using the plant pathogens *F. solani* and *S. sclerotium.*

4.4.2 Inhibition of *F. solani* and *S. sclerotium*

This study used growth inhibition as the measure of putative enzymatic (chitinolytic and glucanolytic) activity. Enzymes from twenty-five isolates were presented to the target fungi either as native (crude) cell free filtrates or boiled. Boiling the supernatant tested whether the enzymes acting directly on fungal mycelia indeed inhibited growth of the pathogens or whether waste metabolites or other growth inhibitory compounds, eg., antibiotics caused nonspecific inhibition of growth. The inhibitory action of the compounds (putative enzymes) in the supernatants was detected by recording fungal growth after 6 days. A combination of hydrolytic enzymes was investigated by Schirmbock *et al* (1994) who found that chitinase, β-1, 3-glucanase and protease activities were present when *T. harzianum* mycelia grown first on glucose and then transferred to fresh medium containing cell walls of *B. cinerea*. Parallel formation of hydrolytic enzymes in *T harzianum* in the presence of cell walls of a potential host suggested that these two classes of proteins may cooperate in mycoparasitism. A concentration of 25 μg/mL of endochitinase resulted in 35% inhibition (Schirmbock *et al.* 1994). In a study by Lorito *et al* (1993), spore germination and tube elongation of *B. cinerea, F. solani, F. graminearum,* and other plant pathogens, were completely

inhibited when the pathogens were grown in an enzyme preparation containing 200-300 μg/mL of endochitinase produced by *T. harzianum*.

4.4.2.1 Effect of temperature

The culture supernatant of nine of the fungi and *S. filum* displayed inhibitory activity on growth of the plant pathogens *S. sclerotium* and *F. solani* (Tables 4.1 and 4.2). All measurements are relative to the control cultures to which no enzyme solution (supernatant) was added. On this basis, all cultures were inhibited. However, it could be argued that inhibition should have been measured relative to the culture to which the boiled supernatant had been added. On this basis, none of the cultures show inhibition. Lima *et al* (1997) carried out an experiment in which enzyme-containing solutions were used to incubate mycelia of *S. rolfsii* and *Rhizoctonia solani*. The hydrolytic effect of the chitinolytic enzyme-containing culture filtrate on *S. rolfsii* mycelium and/or sclerotium cell walls and *R. solani* hyphae was confirmed by scanning electron microscopy. However, unlike evidence from this study, the authors found that heat-denatured enzyme solutions had no effect on the phytopathogens.

Temperature can have a dramatic effect on protein structure by altering the three-dimensional shape of the enzyme and disrupting hydrogen or S-S bonds. However, in some cases, activity can be restored if the modulator, in this case high temperature, is removed and the protein resumes its original shape. In a study by Kang *et al* (1999) a chitinase from the entomopathogenic fungus *Metarhizium anisopliae* was found to be resistant to heat when crude extracts of the enzyme treated at 100º C for 5 minutes in the presence of β-mercaptoethanol. The results obtained in our study also suggest that the dialysed culture filtrates were inhibitory to fungal growth even after boiling. If this is the case, it can be concluded that all the supernatants showed inhibition of pathogen growth. To clarify these results, a preparation of the pure enzyme from the supernatants

could be carried out in future experiments. Purification of the enzyme is achieved by centrifugation of the supernatant, followed by filtration and dialysis. The dialysed enzyme solution is then concentrated using commercial columns followed by fractionation by gel-filtration chromatography (Lima *et al.* 1997; Lorito *et al.* 1994). The purified enzyme preparation is then subjected to boiling and analysed for its ability to inhibit pathogen growth. Previous studies have shown that fungal chitinases have a broad temperature (25-55° C) activity profile (Flach *et al.* 1992), however, none so far have been shown to exceed 55° C, except for the study by Kang *et al* (1999) (Kang *et al.* 1998; Kang *et al.* 1999).

4.4.2.2 Effect of supernatant on fungal inhibition

Evidence seems to suggest that elements in the supernatant caused growth inhibition of the plant pathogens. Microscopic studies were not carried out here where growth inhibition was measured by dry weight of the cultures after being exposed to fungal filtrates. Lorito *et al* (1998) showed that enzymes from *T. harzianum* and *Gliocladium virens* induced morphological abnormalities within hyphae and inhibition of spore germination in *B. cinerea.* Jeffries and Young, (1994) showed that glucanases and chitinases as well as cellulases have been implicated in cell death of *Pythium, Rhizoctonia* and *Sclerotium*. *S. sclerotium* cell-wall contains beta- (1-3)-glucan and chitin (Jeffries and Young 1994). Jones *et al* (1974) observed that an exo-beta- (1-3)-glucanase and an endo-beta- (1-3)-glucanase were needed for the complete degradation of *S. sclerotium* cell-walls (Jones *et al.* 1974).

Lima *et al* (1997), found that filtrate from a *T. harzianum* culture hydrolysed *S. rolfsii* mycelium and sclerotium within 24 hours of exposure to the filtrate and damaged *R. solani* hyphae (Lima *et al.* 1997). The isolated chitinase from *Trichoderma* sp. failed to affect the cell walls of *R. solani* probably because of the high amount (8.5%) of an acid-

insoluble melanin-like material within the *R. solani* cell walls. *R. solani* does not produce significant amounts of spores and it is believed a surface localized polychromatic substance may be the cause of its survival in nature (Lima *et al.* 1997).

4.4.2.3 Effect of carbon sources on inhibition

The effect of different carbon sources on enzymatic activity was studied by growing the test fungi on three different media. In this study, the carbon source used to grow the test fungi did not appear to influence enzyme activity in the filtrates. The enzyme substrates chitin and laminarin, are commonly used to establish simulated mycoparasitic conditions and to induce several enzymes related to mycoparasitism. Although induction of chitinase and glucanase expression was not specifically investigated here, results suggest that media with chitin did not particularly result in higher inhibitory activity. This result agrees with those obtained by Sanz *et al* (2005), where chitin in the medium did not induce expression of a glucanase gene from *T. harzianum* (Sanz *et al.* 2005). Yet, Cohen-Kupiec *et al*, 1999 found that laminarin used as sole carbon source, did induce the production of exoglucanases. De la Cruz *et al* (1993) induced maximum *β*-glucanase specific activity in media supplemented with either, pustulan (β-1, 6-glucan), nigeran (α-1, 3-glucan alternating with α-1, 4-glucan), chitin or *Saccharomyces cerevisiae* or *B. cinerea* cell walls and that highest chitinase specific activity was obtained in medium supplemented with chitin. Interestingly, a study by Takaya *et al* (1998) showed that unlike a chitinase from *T. harzianum* Chit42 induced by chitin in the medium, two chitinases (a class I and a class III) from *Rhizopus oligosporus* were not induced by chitin in the culture medium. This result suggests that gene encoding these two different types of chitinase evolved before these fungi diverged from their common ancestor, and that differing gene-regulating mechanisms have evolved in fungi living in different environments (Takaya *et al.* 1998b). Moreover, chitinase enzymes are

produced during specific stages of fungal development as well as playing a role in growth regulation (Flach *et al.* 1992). In yeast, the enzymes are required for cell separation (Kuranda and Robbins 1991).

In this study *S. sclerotium* were affected by some supernatants from chitin or laminarin grown cultures. However, inhibition, identified as dry weight lower than 55 mg, increased when the isolates were grown on PD Broth with an average of 17.2 mg of dry mycelia in the crude supernatant compared to 18.1 mg for chitin and laminarin.

When comparing performance of media, *F. solani* growth was inhibited most on medium containing laminarin, followed by chitin and PD broth. If, as others have found, chitin induces the production of chitinase, in this case it did not appear to have an effect on the growth of *F. solani*. In the fungal cell wall, chitin molecules appear to be protected by β-glucans, and it may not be readily available to chitinases. It has been speculated that chitinase activity is preceded by glucanase activity (De la Cruz *et al.* 1993).

Accumulation of glucose in the supernatant as a result of degradation of chitin and laminarin can be self-limiting towards enzyme activity (Inglis & Kawchuck, 2002). The supernatants were diluted by the addition of V8 Juice, thus diluting the metabolites accumulated in the filtrates as well as allowing the growth of the pathogenic fungi. The amount of glucose in the V8 juice was 0.32 %. In addition, glucose from carbohydrate catabolism already existed in the supernatant. A concentration of 2% of glucose in the medium represses chitinase and glucanase activities (Cohen-Kupiec *et al.* 1999). Results from an experiment carried out by Vazquez-Garciduenas *et al* (1998) showed 51% reduction in of β-1, 3-glucanase activity levels when the *T. harzianum* was grown with glucose. Moreover, these investigators found that production of β-1,3-glucanase under otherwise inducing conditions was inhibited by the addition of glucose (Vazquez-

Garciduenas *et al.* 1998). Carbon sources other than those used in this study, such as. glycerol or amino acid preparations (peptone) could be trialled in future studies.

4.4.2.4 Time effect on inhibition

The plant pathogens were grown on the filtrates for 6 days (144 hours). This period of time may not be optimal for enzyme effect on fungal growth inhibition according to evidence suggested in this and other studies where inhibitory activity lessened after more than 72 hours. Wang *et al* (2002) reported the results of a study in which they used the supernatant of bacterial cultures of *Bacillus amyloliquefaciens* to inhibit growth of the pathogenic fungus *F. oxysporum*. They found that the culture supernatant displayed inhibitory activity on fungal growth that peaked after 24 hours of incubation and declined afterwards although still retaining significant inhibitory activities at 72 hours (Wang *et al.* 2002). In addition, Schirmbock *et al* (1994) found chitinase and β-1, 3-glucanases activities increased up to 35 hours incubation and then decreased slowly. Furthermore, Lima *et al* (1997) reported production of enzymes in six *Trichoderma* spp grown on chitin-containing medium after 24 hours.

4.5 CONCLUSIONS

The bioassay to assess fungal growth inhibition by fungal enzymes seemed to show evidence that, elements in the supernatants were responsible for the inhibition of pathogenic growth. However, it cannot be categorically concluded that enzymes alone were responsible for growth inhibition of the plant pathogens *F. solani* and *S. sclerotium*. Considering the results, various aspects of the bioassay need to be improved.

The length of time the pathogens were allowed to grow on the filtrates decreased to 24 hours when other investigators have found chitinolytic enzyme activity to peak.

Microscopic studies need to be introduced to obtain evidence of tissue maceration by enzymes present in the supernatants.

Both *F. solani* and *S. sclerotium* are grown in the various media and both boiled and unboiled supernatant are added to fresh cultures of these species to test the effects of adding spent supernatant.

However, more quantitative results were obtained for inhibition of *B. cinerea* spores that showed all isolates tested showed inhibitory effect, in some cases up to 100-fold.

CHAPTER 5

ISOLATION OF FUNGAL CHITINASE AND GLUCANASE GENES

5.1 INTRODUCTION

Chapters 3 and 4 of this manuscript have described the quantification of chitinolytic and glucanolytic enzymes from a number of fungal isolates. This chapter describes experiments to identify the chitinase and glucanase genes that encode the enzymes.

Genes encoding chitinolytic and glucanolytic enzymes from *Trichoderma* have been isolated by PCR using the known amino-acid sequences of the purified chitinase and glucanase to design synthetic oligonucleotides (De la Cruz *et al.* 1995a; De la Cruz *et al.* 1995b; Garcia *et al.* 1994). Where the genomic DNA sequence of interest is unknown but likely to be similar to known homologues, degenerate primers are designed from conserved regions identified by alignment of multiple sequences in the databases. These degenerate primers take into account all possible degeneration of the genetic code in that region.

The aim of the experiments described in this chapter was to isolate novel chitinase and glucanase genes. Two techniques were used to isolate the gene of interest.

- PCR with degenerate primers also known as the genome walking or vectorette PCR method.
- PCR with gene-specific primers.

5.1.1 The vectorette PCR method

To obtain the regions outside the fragment amplified with the degenerate primers, the vectorette method was used (Fig 5.1) (Kilstrup and Kristiansen 2000). Initially, the genomic DNA is digested with restriction enzymes and the resulting chromosomal DNA fragments are ligated to a vectorette or Genome Walking Adaptor. The vectorette consists of a partially double-stranded DNA cassette that is phosphorylated in its 5'-ends. Thus, the library of chromosomal DNA fragments has a known (vectorette) sequence attached to both ends. This library is used as a template for amplification with a gene-specific primer (designed to be complementary within the known portion of the gene) together with a vectorette-specific primer that is complementary to the known end sequences. The inherent background amplification problem that occurs where two vectorette-specific primers amplify fragments not of interest is circumvented by using nested primers. These nested primers bind to the complementary regions present on the product of the first amplification round. This way, the bands obtained are the products of the vectorette and the chitinase specific primers.

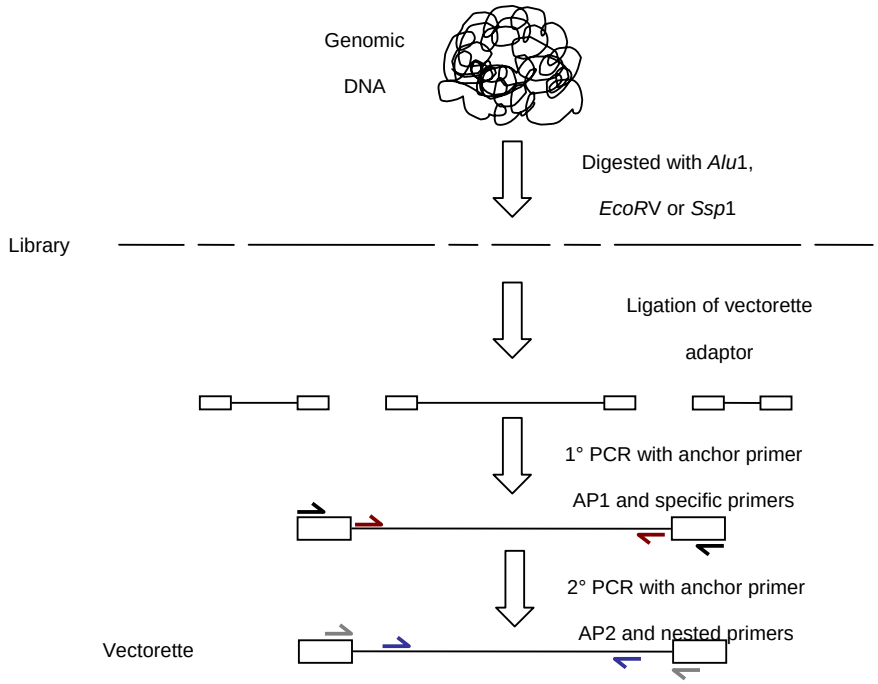


Figure 5.1- Schematic representation of genome walking by the vectorette method. Genomic DNA is digested with one of three blunt-end cutting restriction enzymes, then a vectorette adaptor is ligated to the fragments. An adaptor primer (AP) (indicated in black and grey), is used in combination with a specific primer, (indicated in red and blue), to amplify flanking 5' or 3' regions of the known sequence. A second round of PCR using nested primers is done to obtain specific products.

5.2 MATERIALS AND METHODS

5.2.1 Isolation of fungal DNA

The fungi were grown on liquid SM chitin medium as described in Section 2.1.2, and the mycelia recovered by centrifugation at 8000x g for 10 min. Approximately 500 µg of fresh fungal mycelia was used for each extraction. DNA extraction from the fungal isolates was carried out with a DNeasy Plant Mini Kit (Qiagen) as described in Section 2.3.

5.2.2 PCR conditions

5.2.2.1 For genomic DNA

PCR conditions were as described in Section 2.4 PCR cycle conditions were as described in Table 2.1. Annealing temperatures were estimated by adding together 2 °C for each A or T and 4 °C for each C or G. The reactions were electrophoresed in TAE buffer at 5 V/cm in a 1% agarose gel in a Bio-Rad mini Sub^{TM} cell. Amplification of genomic DNA was carried out using degenerate primers Chi 1 and Chi 2 and Glu 1 and Glu 3 (Table 5.1) designed from conserved regions identified by an alignment of amino acid sequences of known fungal chitinase and glucanase genes by CLUSTAL W (Thompson *et al.* 1994) and deducing the nucleotide sequences using BackTranslate (Accelrys Inc) program (Appendix 1).

Table 5.1-Sequences of degenerate and specific oligonucleotides designed to isolate fungal chitinase and glucanase genes.

Primer name	Sequence[a] (5'>3')
Chi[b] 1	ATCATGRTNTAYTAGGGGNCARAA
Chi 2	KTTTWTKTTKTTTMRAGG
ChitF[d]	CATGACACGCCTTCTTGACG
ChitR[d]	ATTTCTAACCAATGCGAGTAAGC
Glu[c] 1	ACNACNAACYCNGYNNYNCGT
Glu 3	AGCNNCCGAYGGNANCGGNGA

[a]CT=Y GA=R CA=M TG=K CTAG=N
[b]Chi= chitinase
[c]Glu= glucanase
[d]ChitF and ChitR (Viterbo *et al.* 2002)

5.2.2.2 For DNA vectorette library

PCR amplifications were carried out using the specific primers (SPL1 and SPL2), and an adaptor primer (AP1) (Table 5.2) for isolate 04-001, as described in Section 2.4. Similarly, PCR amplifications for isolate 04-013 were carried out using specific primers

(SMS1 and SMS2) and an adaptor primer (AP1) (Table 5.2) using the same method as described for isolate 04-001.

PCR cycle conditions are described in Table 2.2. Separate PCRs were performed using DNA templates digested with *Alu*I, *Eco*RV and *Ssp*I.

5.2.2.3 For nested PCR

The PCR amplicons were diluted 200-fold and used as the template for a second round of PCRs with nested primers. The nested anchor primer AP2 (Table 5.2) was used for all the subsequent amplifications. PCR conditions are described in Section 2.4 and the cycle conditions are listed in Table 2.2.

Table 5.2- Specific and nested primers used in the amplification of the flanking regions of the known sequence.

Primer name	Sequence	Use
NPL 1	5′-GTTATCATCCAGGGCGTTCAG-3′	Forward nested primer- *ChiA*
NPL 2	5′-TGACTCCGCTTCCTCCA-3′	Reverse nested primer- *ChiA*
SPL 1	5′-ATCCAGCGTTGATCTCTAGAT-3′	Forward specific primer- *ChiA*
SPL 2	5′-ATCTAGAGATCAACGCTGGAT-3′	Reverse specific primer- *ChiA*
SMS 1	5′-TCTGCTGTCCGGTTCCCAGT-3′	Forward specific primer- *Glu1*
SMS 2	5′-CCCAGTATTGGGAAGCAGCA-3′	Reverse specific primer- *Glu1*
NPSMS 1	5′-TGCCGACCATCCCGCTGCCG-3′	Nested primer for primary PCR- *Glu1*
NPSMS 2	5′-TAACCCGAACGCTCTGACCC-3′	Nested primer for secondary PCR- *Glu1*
AP1	5′-GTAATACGACTCACTATAGGGC-3′	Anchor primer
AP2	5′-ACTATAGGGCACGCGTGGT-3′	Nested anchor primer

5.2.3 Recovery of DNA from agarose gels

Purification of amplicons from agarose gels was carried out with a QIAquick gel extraction kit (Qiagen) according to the protocol described in 2.4.2.

5.2.4 Cloning of PCR products

The extracted DNA was cloned in pCR®2.1-TOPO® (Invitrogen) vector followed by transformation into *Escherichia coli* One shot TOP 10 chemically competent cells according to the protocol shown in 2.5. Screening of clones was carried out by PCR as described in 2.5.2.

5.2.5 Genome walking

5.2.5.1 Digestion of genomic DNA and ligation of adaptors

Genomic DNA was cut with restriction enzymes; *Ssp*I (Amersham Biosciences), *Alu*I (Amersham Biosciences) and *Eco*RV (Promega) that deliver blunt-end DNA fragments. Restriction digestion was carried out in buffer supplied by the manufacturer. Enzyme was added at 5 U/ μg of DNA. The reactions were incubated at 37° C for 1 hour.

The enzymes used were a 4-bp cutter, (*Alu*I, recognition site AG↓CT); a cutter of AT-rich regions (*Ssp*I, recognition site AAT↓ATT) and a rare 6-bp sequence cutter (*Eco*RV, recognition site GAT↓ATC) (Cato *et al.* 2001).

The vectorette or Genome Walker adaptor was prepared by adding equi-molar amounts (100 pmoles) of Adaptor F (5′ GTAATACGAC TCACTATAGG GCACGCGTGG TCGACGGCCC GGGCAGGT 3′) and Adaptor R (5′- PO_4-ACCTGCCC-NH_2 -3′) (Siebert *et al.* 1995) then incubating at 37 °C for 10 min.

Twenty microlitres of a solution containing 100 pmol of Genome Walker adaptor, 1 U of T4 DNA ligase (Promega), 5mM ATP in 1x OPA buffer (10mM Tris-acetate pH 7.5; 10mM magnesium acetate and 50 mM potassium acetate) (Amersham Biosciences) was added to the digestion reactions from above and incubated at room temperature

overnight. The reaction mixture was diluted ten-fold with 10 mM TRIS-HCl, 0.1 mM EDTA (pH 8.0) and stored at 4 °C.

5.2.6 Sequence analysis

Sequencing was carried out as described in Section 2.6, using primers M13F and M13R that annealed within the vector. Each DNA strand was sequenced twice. The nucleotide sequences were edited using SeqEd^{TM}, version 1.0.3, Applied Biosystems Industries (Foster City, California). Amino acid sequence was deduced by Translate (GCG) program at the Australian National Genome Information Service (ANGIS) Putative function was ascribed to the cloned DNA fragments by comparing them to related sequences using BLASTN (Altschul *et al.* 1997).

Sequence searches were carried out using NCBI (National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD) BLAST (Basic Local Alignment Search Tool) programme and ANGIS.

5.3 RESULTS

5.3.1 Amplification of chitinase genes

The first step in the isolation of chitinase gene from the fungal isolates was the amplification of the genomic DNA with degenerate primers Chi 1 and Chi 2 designed from conserved regions of known fungal genes. The sequences used to deduce these degenerate primers were: *Saccharomyces cerevisiae* (accession number AAB67331; *Rhizopus niveus* (accession number S36931); *Rhizopus oligosporus* (accession number B47022); *Amanita muscaria* (accession number AJ276119); *Cucumis sativus* (accession number A31455); *Beta vulgaris* (accession number AAB28479); *Oryza sativa* (accession number BAC78593); *Grifola umbellata* (accession number AF380832).

Faint PCR products could be observed in most of the lanes, but when the amplification was repeated, no PCR products were obtained from isolates 03-002, 03-003, 03-008, 04-013 and 01-028. Two fragments with an approximate length of 400 bp and 650 bp were amplified from fungal DNA of isolates 04-000, 04-001, 02-023, 04-040 and *S. filum* (Fig 5.2), cloned and sequenced.

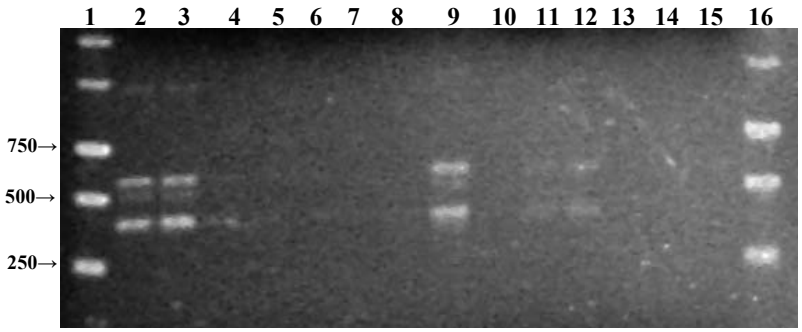


Figure 5.2- Amplified products of chitinase degenerate primers Chi 1 and 2. Lane 1 and 16 Molecular marker 1Kb. Lane 2: isolate 04-000; Lane 3: 04-001; Lane 4: 02-002; Lane 5: 02-003; Lane 6: 04-004; Lane 7: 03-008; Lane 8: 04-013; Lane 9: 02-23; Lane 10: 01-028; Lane 11: 04-040; Lane 12: *S. filum*; Lane 13: 02-016; Lane 14: 05-017; as a negative control; Lane 15: negative control (no template).

The sequence from isolate 04-001 is presented in Fig 5.3.

```
1...ATGGTCTATTGGGGGCAGAACGGTGGTGGTACTATCGAGAACAACGGCCTTTCTGCTCAC 60
61  TGTACTGCTGAAGCCGGTATCGACGTCGTCGTACTTAGTTTTCTTTATCAATATGGTAAT 120
121 GGCGTCGAAATCGCAGCGGGAACAATTGGCCAGAGCTGCTCCATTGATACCTCTGGCAAC 180
181 CCTTCAAACTGTGATGAGCCCAGCGCAGCCATCGCTACCTGCAAGTCCAATGGAGTCAAG 240
241 GTGATCTTATCCCTAGGTGGCGCGGCCGGTGCCTATTTTCTCTCTTCTCAGCAGGAAGCC 300
301 GAGACAATTGGCCAAAATCTCTGGGATGCTTATGGCGCAGGAAATGGTACTGTTCCGAGA 360
361 CCCTTCGGAAGCAATAGTTTGGACGGATGGGATTTCGATGTAGAGGCGAGTAACGGCAAC 420
421 CAGTACTACCAGTACTTGATCGCTAAGCTTCGCTCAAACTTCAACGGCGGCAACTACGTG 480
481 ATTACCGGTGCTCCTCAGTGCCCAATTCCGTCAGTTCTTCTTAGATTTTACAGTTATATG 540
541 GCTGATGTATAGCCTGCTAATAAGGAAAAATAGGGAACCAAATATGCAGCAAATCATTAC 600
601 CACTTCCCAGTTTGACTATCTTTGGGTTCAGTTCTACAA
```

Figure 5.3- Nucleotide sequence of a partial chitinase gene amplified with degenerate primers Chi1 and Chi 2. The nucleotide sequence is 639 nucleotides long not including Chi 2. The primer Chi 1 (boxed) is missing three nucleotides (ATC) at the 5' end.

5.3.1.1 Genome walking to obtain the full chitinase gene sequence

To determine the sequences flanking the conserved region of the putative chitinase gene identified in isolate 04-001 a pair of oligonucleotides ranging in size from 16 to 20 nt was designed (SPL 1 and SPL 2) from the known chitinase sequence (Table 5.2). The internal primers were designed 20-36/40bp from each end so that amplified products would overlap with the known sequence by 20 bp to provide a contiguous sequence. This primary amplification produced fragments up to 2.0 Kbp. Three restriction enzymes were used to provide a subset of fragments that could generate amplicons ranging in size from 100 bp to 1 Kbp. The three enzymes produced approximately the same range of fragment sizes shown as smearing (Fig. 5.4).

5.3.1.2 Secondary amplification

The expected smears were due to non-specific amplification, so a secondary nested amplification was performed using a pair of nested primers (NPL 1 And NPL 2) and a nested adaptor primer (AP2) (Table 5.2). This second amplification was carried out to avoid the possible background problems caused by first strand synthesis by the vectorette primer. Thus, each primary amplification product was used as a template for two secondary amplifications with primer pairs AP2 and NPL1, AP2 and NPL2. Bands of ~ 250bp were seen in Lane 5 and 7 (Fig 5.5 indicated by white arrow). These bands were extracted from the gel and sequenced. The known chitinase sequence was aligned and overlapped with the sequences obtained by the secondary amplification. Thus, the sequence for isolate 04-001, was extended by a further 47 nucleotides.

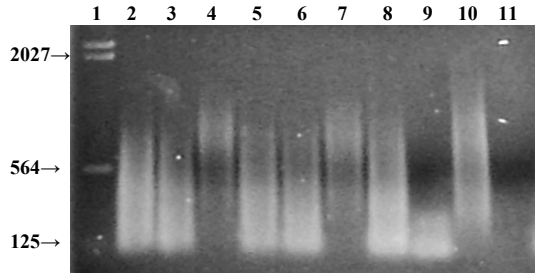


Figure 5.4- Primary amplification of chitinase gene from isolate 04-001. Digestion with *Alu*I (Lanes 2-4); with *Eco*RV (lanes 5-7); with *Ssp*I (lanes 8-10). Amplification was carried out using the anchor primer AP1 and specific primers SPL1 and SPL2. Lane 1: Molecular Marker Lambda/HindIII. Lane 2: with primer SPL1; Lane 3: with primer SPL2; Lane 4: with primer AP1 (control); Lane 5: with primer SPL1; Lane 6: with primer SPL 2; Lane 7: with primer AP1 (control); Lane 8: with primer SPL1; Lane 9: with primer SPL2; Lane 10: with primer AP1 (control). Lane 11: negative control

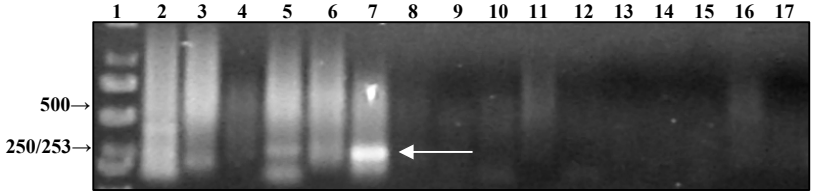


Figure 5.5-Secondary PCR with nested primers NPL1, NPL2, and anchor primer AP2 on amplicons obtained in the primary amplification of 04-001 (above). Amplification was carried out using the anchor primer AP2 and nested primers NPL1 and NPL2. Lane 1: Molecular Marker 1 Kb. Lane 2: *AluI* digest, NPL1, AP2; Lane 3: *AluI* digest, NPL2; AP2. Lane 4: *AluI* digest, NPL1, AP2; Lane 5: *AluI* digest, NPL2; AP2. Lane 6: *AluI* digest, AP2 (control) Lane 7: *EcoRV* digest, AP2, NPL1; Lane 8: *EcoRV* digest, AP2, NPL2; Lane 9: *EcoRV* digest AP2 and NPL1; Lane 10: *EcoRV* digest, AP2, NPL2; Lane 11: *EcoRV* digest, AP2 (control); Lane 12: *SspI* digest, AP2, NPL1; Lane 13: *SspI* digest, AP2, NPL2; Lane 14: *SspI*, AP2, NPL1; Lane 15: *SspI* digest, AP2, NPL2; Lane 16: *SspI* digest, AP2 (control); Lane 17: control (no template)

5.3.1.3 ChiA sequence

A sequence of 639 bp in length, representing part of a putative chitinase gene was obtained (see Fig 5.3). The gene was named *ChiA*. Comparison of the nucleotide sequence showed low homology to other genes in the database, however, six plant chitinase genes show high homology (93%) over 28 bases. The nucleotides showing homology to plants are between nucleotide 611 and 639 of the *ChiA* sequence. Identical nucleotides are found between nucleotide 307 and 335 of the 509 bp endochitinase sequence of *Pyrus pyrifolia,* between 621 and 649 of the 1109 bp basic chitinase

sequence of *Vigna unguiculata,* and between 1146 and 1174 of the 1452 bp acidic chitinase sequence of *Glycine max* (Table 5.3)

Table 5.3- Identity over 28 nucleotides between positions 611 and 639 of the*ChiA* sequence and other chitinases.

Species	Identity (%)	Accession number
Pyrus pyrifolia (sand pear) class III endochitinase mRNA	93% in 28 nt.	AY338248
Vigna unguiculata a basic chitinase class III mRNA	93% in 28 nt.	X88801
Glycine max an acidic chitinase gene	93% in 28 nt.	AB007127
Malus x domestica (cultivated apple) class III acidic chitinase	93% in 28 nt.	AF309514
Psophocarpus tetragonolobus chitinase	93% in 28 nt.	D49953.1
Benincasa hispida class III chitinase	93% in 28 nt	AF 184884

A 191 amino acid protein was deduced from the chitinase nucleotide sequence. The amino acid sequence was compared with other sequences in the database (Table 5.4). *ChiA* was 70% identical to a class III chitinase ChiA2 from *Aspergillus fumigatus* Af293 and 64% identical with *Metarhizium anisopliae*, an entomopathogenic fungus. Genome walking yielded a 250 bp fragment that was not contiguous to the known sequence (Appendix 2). Two stop codons were identified that are investigated further in Chapter 6.

Table 5.4- Identity between the amino acid sequence of the partial putative chitinase *ChiA* and known chitinases using BLASTX.

Species/gene	Identity (%)	Accession number
Aspergillus fumigatus Class III chitinase	70%	EAL85097
M. anisopliae endochitinase CHI2	64%	CAC07216
M. anisopliae chitinase	63%	AAY34347
S. cerevisiae endochitinase	43%	AAT93059
S. cerevisiae endochitinase	43%	NP013388
S. cerevisiae endochitinase precursor	43%	P29029

The known portion of *ChiA* starts 102 bp downstream from the 5' end of *A. fumigatus* chitinase coding region and finishes 586 bp upstream from the 3' end of the *A. fumigatus* chitinase coding region that is 1002 bp long. The sequence starts 100 bp downstream from the 5' end of *M. anisopliae* coding region and finishes approximately 600 bp upstream from the 3' end of the *M. anisopliae* chitinase coding region which is 1200 bp long. In the *ChiA* sequence, there are two stop codons at bases 551 and 572.

5.3.2 Amplification of a chitinase gene using specific primers

Some regions of known fungal chitinase and glucanase genes are highly conserved. For fungal chitinase and glucanase genes, the most highly conserved regions are located within the open reading frame (ORF) therefore the entire length of the genes cannot be isolated using the degenerate primer method. In order to clone the complete open reading frame (ORF) of the chitinase gene from isolate 04-001, that was identified as a *Trichoderma asperellum,* primers designed for *T. asperellum* (*T. harzianum* T-203) in a previous study by Viterbo *et al* (2002) were used.

Bands of the expected size (approximately 1.0 Kb) were obtained from isolates 04-000 and 04-001 (Fig 5.6). Sequences were obtained from bands of <500 bp (Lanes 4-13) but they did not match chitinase genes and were not used further.

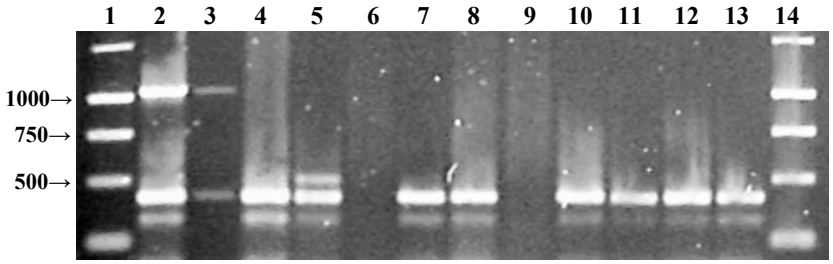


Figure. 5.6-Amplification of fungal genomic DNA using the chitinase specific primers ChitF and ChitR. Lane 1: MW 1Kb; Lane 2: DNA from isolate 04-000; Lane 3: Isolate 04-001; Lane 4: Isolate 03-002; Lane 5: Isolate 03-003; Lane 6: negative control; Lane 7: Isolate 04-004; Lane 8: Isolate 03-008; Lane 9: Isolate 04-013; Lane 10: Isolate 02-023; Lane 11: Isolate 01-028; Lane 12: Isolate 04-040; Lane 13: *S. filum*; Lane 14: MW 1Kb.

The amplification product from isolates 04-000 and 04-001 were cloned and named pSSev2i and pSSev2ii.

5.3.2.1 ChiB sequence

The partial putative chitinase gene was named *ChiB* and its nucleotide sequence is presented in Figure 5.7. The nucleotide sequence was compared to other in the database. High identity was shared between *ChiB* and chitinases from other *Trichoderma* spp. (Table 5.5).

```
1   TTCATGACACGCCTTCTTGACGCCAGCTTTCTGCTGCTGCCTGCTATCGCATCGACGCTA  60
61  TTTGGCACCGCCTCTGCACAGAATGCGACATGCGCACTGAAGGGAAAGCCGGCAGGCAAA 120
121 GTCTTGATGGGATATTGGGAAAATTGGGATGGAGCAGCCAACGGTGTTCACCCTGGATTT 180
181 GGCTGGACACCGATCGAAAACCCCATCATTAAACAGAATGGCTACAATGTGATCAACGCC 240
241 GCCTTCCCCGTTATTCTGTCAGATGGCACAGCATTATGGGAAAACGACATGGCTCCTGAC 300
301 ACTCAAGTCGCAACTCCAGCTGAAATGTGTGAGGCTAAAGCAGCTGGTGCCACAATTCTG 360
361 CTGTCAATTGGAGGTGCTACTGCTGGCATAGATCTCAGCTCCAGTGCAGTCGCTGACAAG 420
421 TTCATCGCCACCATTGTACCAATCTTGAAGCAGTACAATTTTGACGGCATTGATATAGAC 480
481 ATTGAGACGGGGTTGACCAACAGCGGCAATATCAACACACTTTCCACATCCCAGACCAAT 540
541 TTGATTCGCATCATTGATGGTGTTCTTGCTCAGATGCCTTCCAACTTCGGCTTGACTATG 600
601 GCACCTGAGACAGCGTACGTTACAGGCGGTAGCATCACGTATGGCTCTATTTGGGGAGCG 660
661 TACCTACCTATCATCCAGAAATATGTTCAAAACGGCCGGCTGTGGTGGTTAAACATGCAA 720
721 TATTACAACGGCGATATGTACGGTTGCTCTGGCGACTCTTACGCAGCTGGCACCGTCCAA 780
781 GGATTCATCGCTCAGACTGATTGCCTAAATGCAGGACTTACCATCCAAGGCACCACAATC 840
841 AAGGTTCCATACGACATGCAAGTACCAGGCCTACCTGCGCAAT
```

Figure 5.7- Nucleotide sequence of putative chitinase gene, *ChiB* amplified with chitinase specific primers ChitF (boxed) and ChitR (missing).

Table 5.5-Identity between the partial chitinase gene *ChiB* at the nucleotide level with other chitinases in the database.

Species	Identity (%)	Accession number
T. harzianum endochitinase	99% over 874 nt	AF406791
T. harzianum endochitinase	88% over 874 nt	AY028421
T. atroviride chitinase	88% over 874 nt	AY129675
T. inhamatum chitinase	80% over 874 nt	AF525754
T. harzianum chitinase	79% over 874 nt	AF525753

Similarly, the deduced amino acid sequence showed 98% identity with an endochitinase gene from *T. harzianum* Chi36Y (Viterbo and Chet 2001) (Table 5.6).

Table 5.6- Amino acid identity of *ChiB* with other chitinases in the database.

Species	Identity (%)	Accession number
T. harzianum endochitinase chitY36	98%	AAL01372
T. atroviride endochitinase	92%	AAM77132
T. harzianum chitinase	87%	AAM93195
T. inhamatum chitinase	87%	AAM93196
Cordyceps bassiana chitinase	87%	AAN41259

5.3.3 Identification of a glucanase gene

The sequences used to deduce the degenerate primers Glu 1 and Glu 3 were: *Aspergillus oryzae* (accession number BAB92972); *A. phoenicis* (accession number BAB83607); *Trichoderma hamatum* (accession number AAP33112 and AAF80600); *T. harzianum* (accession number CAA05375); *Cochliobolus carbonum* (accession number AAC71062); *Ustilago maydis* (accession number XP757542) and *Hypocrea lixii* (accession number CAA05375). PCR amplification of a putative glucanase gene with degenerate primers, Glu1 and Glu 3 was carried out with all fungal isolates (Fig 5.8).

Fragments of 500 to 1.8 Kb were amplified and sequenced from isolates 04-000, 04-013, 02-023 and *S. filum.* A 1257 nucleotide fragment from isolate 04-013 was obtained (Fig 5.9). Amplicons from isolates 04-000 and 02-023 did not show identity to glucanase genes and were not used further.

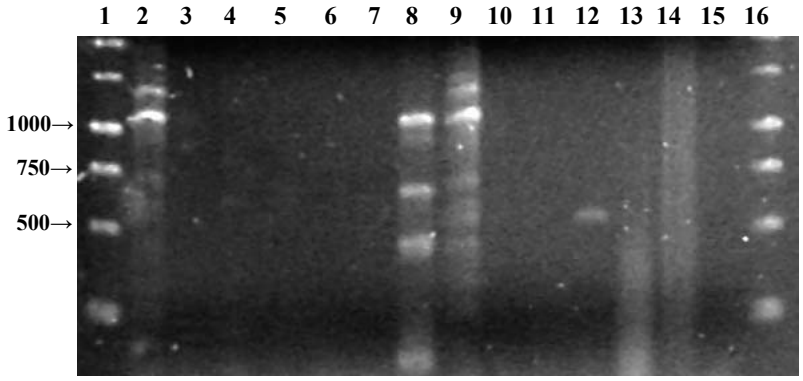


Figure 5.8-Amplified products of glucanase degenerate primers Glu 1 and Glu 3. Lane 1 and 16 Molecular marker 1Kb. Lane 2: isolate 04-000; Lane 3: 04-001; Lane 4: 02-002; Lane 5: 02-003; Lane 6: 04-004; Lane 7: 03-008; Lane 8: 04-013; Lane 9: 02-23; Lane 10: 01-028; Lane 11: 04-040; Lane 12: *S. filum*; Lane 13: 02-016 as a negative control; Lane 14: 05-017 as negative control ; Lane 15: negative control (no template).

```
1     ACCACCAACCTGGCTGCTCGTGCTGCTTCCGAATACTGGGTTGGTACCATCAAACGTCAG
61    GGTGCTGTTGCTTTCGGTAACGGTACCGACTACCAGGTTTACCGTAACGTTAAAGACTTC
121   GGTGCTAAAGGTGACGGTTCCACCGACGACACCGCTGCTATCAACCAGGCTATCTCCTCC
181   GGTAACCGTTGCGGTAAAGGTTGCGACTCCTCCACCGTTACCCCGGCTCTGGTTTACTTC
241   CCGCCGGGTACCTACGTTGTTTCCAAACCGATCGTTCAGTACTACTACACCCAGATCGTT
301   GGTGACGCTGTTAACCTGCCGGTTATCAAAGCTGCTGCTGGTTTCGCTGGTATGGCTGTT
361   ATCGACGCTGACCCGTACGAAGACGACGGTTCCAACTGGTACACCAACCAGAACAACTTC
421   TTCCGTGCTATCCGTAACCTGGTTATCGACCTGACCGCTATGCCGCAGGGTTCCGGTGCT
481   GGTATCCACTGGCAGGTTGGTCAGGCTACCTCCCTGCAGAACATCCGTTTCGAAATGATC
541   AAAGGTGGTGGTGACGCTAACAAACAGCAGGGTATCTTCATGGACAACGGTTCCGGTGGT
601   TTCATGTCCGACCTGACCTTCAACGGTGGTAACTACGGTATGTTCCTGGGTAACCAGCAG
661   TTCACCACCCGTAACCTGACCTTCAACGACTGCAACACCGCTATCTTCATGAACTGGAAC
721   TGGGCTTGGACCTTCAAATCCCTGTCCATCAACAACTGCCAGGTTGGTCTGAACATGTCC
781   AACGCTCCGCAGAACCAGACCGTTGGTTCCGTTCTGATCCTGGACTCCCAGCTGACCAAC
841   ACCCCGACCGGTGTTGTTTCCGCTTTCACCGAAAACTCCATCCCGATCGGTGGTGGTGTT
901   CTGATCCTGGACAACGTTGACTTCTCCGGTTCCAAAGTTGCTGTTGCTGGTATCACCGGT
961   AACACCATCCTGGCTGGTGGTTCCGTTGTTACCAACTGGGTTCAGGGTAACGGTTACCTG
1021  CCGGGTTCCGCTAAACAGAAACGTGAAGCTTCCGTTAAAGTTACCACCCAGACCGTTACC
1081  GAAACCGTTGAAGTTTGCACCGCTGACTACACCGACTCCCCGTCCGCTCCGACCGCTCTG
1141  CCGTCCTCCCTGGGTGAATCCCGTACCGCTGGTCTGCTGCCGACCATCCCGCTGCCGAAC
1201  ATCCCGCTGCTGTCCGGTCTGCTGTCCGGTTCCCAGTCCTCCGCTACCCAGCCGGCT
```

Figure 5.9-Nucleotide sequence of *Glu1* obtained after amplification using degenerate primers Glu 1 and Glu 3 (in bold). Primers designed for genome walking SMS1, SMS2 and NPSMS1 are boxed.

Alignment of the glucanase nucleotide sequence showed identity to other glucanases (Table 5.7).

Table 5.7-Identity of the glucanase gene *Glu1* with other glucanases at the nucleotide level over the entire sequence.

Species	Identity (%)	Accession number
A. fumigatus glucanase	77%	BX649607
A. oryzae exo-1, 3-glucanase	77%	AB074847
A. phoenicis exgS glucanase gene	68%	AB070739
Coniothyrium minitans β-1, 3-glucanase	48%	AF247649
Acremonium sp. β-1, 3-glucanase	43%	AY854012
Blumeria graminis 1, 3-β-glucanase	41%	AF317733
T. harzianum β-1, 3-glucanase	39%	AJ002397

The translated sequence was compared with sequences in the databases using BLASTX (Altschul *et al.* 1997) and showed 99% identity (from residue 22 to residue 550) with part of a 946 amino acid long exo-1, 3-β-glucanase from *Aspergillus oryzae,* an ascomycete, (accession number BAB92972). The top hits are listed in Table 5.8.

Table 5.8-Identity of a putative glucanase *Glu1* with other sequences at the amino acid level.

Species	Identity (%)	Accession number
Exo-1,3 –β-glucanase *A. oryzae*	99%	BAB92972
Exo-1, 3-β-glucanase *A. fumigatus* Af293	77%	CAD29605
Exo-β-1, 3-glucanase *A. phoenicis*	73%	BAB83607
1, 3-β glucanase *B. graminis*	63%	AAL26904
β-1, 3-glucanase *Acremonium* sp.	62%	AAW47927
β- 1, 3-glucanase *C. minitans*	61%	AAL26904
1,3-β-glucosidase *T. harzianum*	43%	CAA05375

5.3.4 Genome walking to identify a glucanase sequence

To determine the sequences flanking the conserved region of the putative glucanase gene identified in isolate 04-013, a pair of oligonucleotides was designed from the sequence obtained from the amplification with degenerate primers. The internal primers were designed 40 bp from each end so that amplified products would overlap with the known sequence by 20 bp to provide a contiguous sequence. The three libraries (*Alu*I, *EcoR*V and *Ssp*I) gave rise to smears and faint bands of between 100 bp and ~1.5Kb (Fig 5.10). Some faint bands could be detected above the background, however dominant products of the same size were produced by amplification of 04-013 chromosomal DNA with primer SMS2 and the anchor template specific primer AP1 of the *EcoR*V library and no background sequences were apparent (indicated with an arrow in Lane 11, Fig 5.10). This fragment of ~400 bp was extracted from the agarose gel and sequenced. Sequencing of this product resulted in 87 bp of sequence corresponding to a fragment upstream from the known sequence that determined the 5' end of the gene (Fig 5.11)

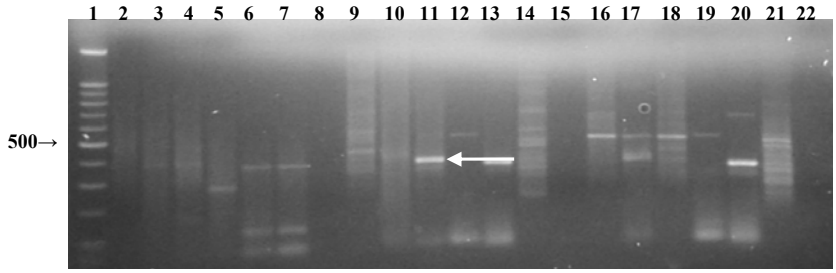


Figure 5.10- Primary genome walking of isolate 04-013 to amplify the flanking regions of the known sequence. Genomic DNA was digested with *Alu*I (Lanes 2 to 7); with *Eco*RV (lanes 9 to 14); with *Ssp*I (lanes 16 to 21). Amplification was carried out using the anchor primer AP1 and specific primers SMS1 and SMS2 at annealing temperatures of 55 and 60 °C respectively. Lane 1: Molecular Marker 100bp; Lane 2: with primer SMS1; Lane 3: with primer SMS1; Lane 4: with primer SMS2; Lane 5: with primer SMS2; Lane 6: with primer AP1 (control); Lane 7: with primer AP1 (control); Lane 8: negative control; Lane 9: with primer SMS1; Lane10: with primer SMS1; Lane 11: with primer SMS2; Lane 12: with primer SMS2; Lane 13: with primer AP1 (control); Lane 14: with primer AP1 (control); Lane 15: negative control; Lane 16: with primer SMS1; Lane 17: with primer SMS1; Lane 18: with primer SMS2; Lane 19: with primer SMS2; Lane 20: with primer AP1; Lane 21: with primer AP1; Lane 22: negative control. Arrow indicates product excised from the gel.

The 87 nt fragment (Fig 5.11) was 96% homologous to an unidentified sequence from the bacterium *Chromobacterium violaceum* (AEO16920) (Vasconcelos *et al.* 2003); 95% homologous to an unidentified sequence from *Oryza sativa* (cv japonica)(Anon 2004) at the nucleotide level.

```
1    ATGCTGTTCCTGGCTCACGTTCTGCTGCTGCTGGGTCTGCCGGCTGGTATGGTTGGTGCT
61   GTTCCGCTGGGTCAGGAAACCGACATC
```

Figure 5.11- Nucleotide sequence of the 5' end of the glucanase gene, obtained after primary genome walking with primers SMS2 and AP1.

5.3.4.1 Secondary amplification

To avoid possible background problems in the primary amplification, a secondary amplification using nested primers NPSMS1 and AP2 was carried out.

This resulted in smears ranging from ~200 bp to 4 Kb (Fig 5.12). A number of amplicons were produced from the amplification of the *Alu*I library. A prominent band in Lane 2 (Fig 5.12) was extracted from the agarose gel and sequenced.

Sequence analysis showed that 1176 bp of the newly sequenced fragment was part of a glucanase gene (Fig 5.13). The fragment obtained was 85% identical to part of a

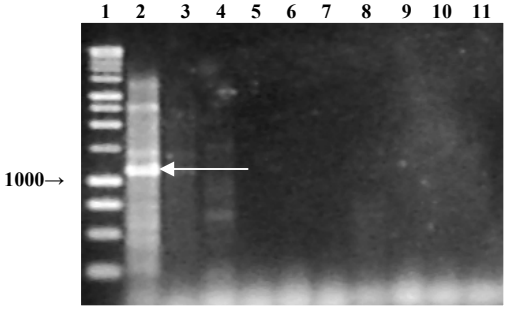


Figure 5.12- Secondary PCR using nested primers NPSMS1, and anchor primer AP2 on amplicons obtained in the primary amplification. Lane 1: Molecular Marker 1Kb. Lane 2: *AluI* digest, NPSMS1, AP2; Lane 3: *AluI* digest, NPSMS1, AP2 ; Lane 4: *AluI* digest AP2 (control) Lane 5: *EcoRV* digest, AP2, NPSMS1; Lane 6:*EcoRV* digest AP2 and NPSMS1; Lane 7: *EcoRV* digest, AP2 (control); Lane 8: *SspI* digest, AP2, NPSMS1; Lane 8: *SspI*, AP2, NPSMS1; Lane 9: *SspI* digest, AP2 (control); Lane 10: control (no template). Amplicon excised from the gel is indicated by white arrow.

glucanase from *A. nidulans* (XM404916), a hypothetical protein (XM653291), a glucanase from *A. fumigatus* (BX649607 and XM747722) and 81% identical with a glucanase from *A. oryzae* (ABO74847).

Using this sequence information one primer was synthesised (NPSMS2) that read from the 3' end of the fragment towards the 5' end of the gene. The *Alu*I library was amplified using this primer and AP2. A single fragment of ~ 600 bp was amplified (Fig 5.14) and sequenced. A sequence of 320 bp downstream from the sequence obtained in the secondary amplification was obtained this way. The sequence is shown in Fig 5.15.

```
   1  GGTGTTCTGTCCTCCGAAGTTCCGGAACCGACCGCTACCCCGTCCACCCCGGAAGAAGCT
  61  GAACCGTCCACCGAAGTTCAGTCCACCCCGCAGCCGTCCGCTCCGGCTCAGTCCCAGCCG
 121  GAAACCCCGGTTGAATCCACCGTTGCTGCTCCGCTGATCCCGTCCCAGCCGTCCCCGACC
 181  GTTCAGGGTTCCTCCTCCGTTGTTACCGGTCCGGCTTCCTCCTCCGTTGCTCACGCTACC
 241  AACCAGTGCTCCGTTAAAACCGTTACCAAAACCCGTCTGCAGACCGCTCTGCCGACCCAC
 301  GCTAAACCGTCCTCCCTGCTGAACGGTGGTAAAGTTTACGAACGTTCCAAACCGCTGTAC
 361  ACCTCCTACGACGCTTCCTCCTTCGTTTCCGTTAAATCCGCTGGTGCTAAAGGTGACGGT
 421  TCCACCGACGACACCGCTGCTATCCAGAAAATCCTGAACTCCGCTAAAGAAGACCAGATC
 481  GTTTACTTCGACCACGGTGCTTACATCATCACCGACACCATCAAAGTTCCGAAAAACGTT
 541  AAAATCACCGGTGAAGTTTGGCCGGTTCTGATGGCTTACGGTCAGAAATTCGGTGACGAA
 601  AAAAACCCGATCCCGATGCTGCAGGTTGGTGAAGTTGGTGAAACCGGTTCCGTTGAAATC
 661  ACCGACATCGCTCTGCAGACCAAAGGTCCGGCTCCGGGTGCTATCCTGATGCAGTGGAAC
 721  CTGGCTGAATCCTCCCAGGGTGCTGCTGGTATGTGGGACACCCACTTCCGTATCGGTGGT
 781  TCCGCTGGTACCGAACTGCAGTCCGACAAATGCGCTAAAACCCCGAAACAGACCACCACC
 841  CCGAACAAAGAATGCATCGCTGCTTTCATGCTGATGCACATCACCGAAAAAGCTTCCGCT
 901  TACATCGAAAACTCCTGGTTCTGGGTTGCTGACCACGAACTGGACCTGCCGGACCACAAC
 961  CAGATCAACGTTTACAACGGTCGTGGTGTTTACATCGAATCCCAGGGTCCGGTTTGGCTG
1021  TACGGTACCGCTTCCGAACACAACCAGCTGTACAACCAGCTGTACAACTACCAGGTTACC
1081  AACGCTAAAAACGTTTTCATGGGTCTGATCCAGACCGAAACCCCGTACTACCAGGCTAAC
1141  CCGAACGCTCTGACCCCGTTCACCCCGCAGACCAAC
```

Figure 5.13 - Nucleotide sequence of a fragment of a glucanase gene obtained after primary genome walking. The primer (NPSMS2) designed to amplify the 3' end of the gene is underlined.

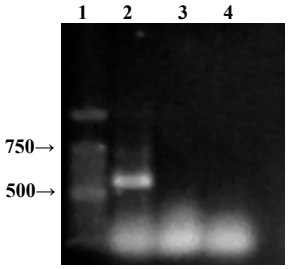


Figure 5.14- Amplification of the *Alu*I library with primer NPSMS2 and AP2. Lane 1. Molecular Marker 100 bp. Lane 2. *Alu*I digest, NPSMS2, AP2. Lane 3 and 4. control (no template).

The secondary PCR with primers NPSMS2 and AP2 yielded a 318 nt fragment (Fig 5.15) with 87% identity to *A. oryzae* (ABO74847), 82% identity to a glucanase from *A. phoenicis* (ABO70739) and 80% identity to the gene ExgO from *A. fumigatus* (XM 747722 and BX649607).

```
  1   TGGAACGACCCGGACTTCTCCTACTGCAAAACCGACGGTTGCCGTAAAGCTTGGGGTCTG
 61   CGTGTTCAGAACACCTCCGACATGTACGTTTACGGTGCTGGTCTGTACTCCTTCTTCGAA
121   AACTACGGTCAGACCTGCCTGGCTACCGAATCCTGCCAGGAAAACATGGTTGAAGTTGAC
181   TGCTCCGACGTTCACATCTACGGTCTGTCCACCAAAGCTTCCACCAACATGATCACCTCC
241   AACTCCGGTGCTGGTCTGGTTCCGCAGGACGAAAACCGTTCCAACTTCTGCTCCACCCTG
301   GCTCTGTTCCAGCAGTCC
```

Figure 5.15- Nucleotide sequence of the glucanase gene obtained after secondary PCR of the genome walking method.

The genome walking method used to amplify *Glu1* is shown in Fig 5.16.

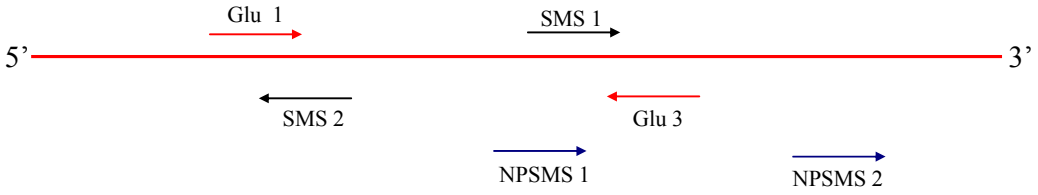


Figure 5.16- The genomic DNA was first amplified with degenerate primers Glu 1 and Glu 3. Primary amplification of the vectorette library was carried out with primers SMS1 and SMS 2. Secondary amplification was carried out with primer NPSMS 1. The resulting sequence was used to design primer NPSMS 2 and obtain the 3' end of the glucanase gene.

A contiguous sequence was constructed from the sequences derived from degenerate primers and genome walking methods. The complete ORF putative glucanase gene is 2844 nucleotides long and was assigned the GenBank accession number DQ312297. This putative glucanase sequence is homologous to glucanase sequences from the ascomycete *Aspergillus* spp (Table 6.5).

5.3.5 Identification of isolates 04-000 and 04-001

Sequencing of the ITS region of isolates 04-000 and 04-001 (results not shown) showed that both sequences were identical to each other, indicating that they were from the same species. The sequence was the same as that from the mycoparasitic fungus *Trichoderma asperellum* (strain Tr 7) 5.8S ribosomal RNA (rRNA) (Fig 5.17).

```
1   gagaccgccactgtatttaggggccggcacccgtgtgaggggtcccgatccccaacgccg 60
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    gagaccgccactgtatttaggggccggcacccgtgtgaggggtcccgatccccaacgccg 416

61  atcccccggaggggttcgagggttgaaatgacgctcggacaggcatgcccgccagaatac 120
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    atcccccggaggggttcgagggttgaaatgacgctcggacaggcatgcccgccagaatac 356

121 tggcgggcgcaatgtgcgttcaaagattcgatgattcactgaattctgcaattcacatta 180
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    tggcgggcgcaatgtgcgttcaaagattcgatgattcactgaattctgcaattcacatta 296

181 cttatcgcatttcgctgcgttcttcatcgatgccagaaccaagagatccgttgttgaaag 240
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    cttatcgcatttcgctgcgttcttcatcgatgccagaaccaagagatccgttgttgaaag 236

241 ttttgattcattttgaatttttgctcagagctgtaaagaaatacgtcc 288
    ||||||||||||||||||||||||||||||||||||||||||||||||
    ttttgattcattttgaatttttgctcagagctgtaaagaaatacgtcc 188
```

Figure 5.17- Alignment between *T. asperellum* (strain Tr7) (AF406791) 5.8S rRNA and partial 18S and 28S rRNA genes and isolate 04-001 (DQ007018) ITS 1 and ITS 4 (indicated in bold).

5.4 DISCUSSION

5.4.1 Chitinase gene

Amplification of a fragment of fungal DNA with homology to a chitinase gene was achieved using PCR techniques with degenerate primers designed from conserved regions of a number of known fungal chitinases. The gene fragment, *ChiA* shows homology to plant, yeast and fungal chitinases. Unfortunately, only one isolate gave an amplicon out of the eleven isolates that showed chitinase activity. It is a fundamental weakness of PCR that a single base mismatch at the 3' end of the primer prevents extension of the strand and the chain reaction is prevented (Cline *et al.* 1996). To overcome this problem, either new primers should be designed or a DNA polymerase with 3'-5' proofreading exonuclease activity (eg. *Pfu* DNA polymerase) could be used to remove 3' mismatches so that strand extension can occur (Cline *et al.* 1996).

5.4.1.1 Genome walking method

The method used to determine the full-length sequence, the genome walking or PCR-vectorette method, did not allow the full-length gene sequence to be determined. The nested PCR used to amplify single-locus products resulted in smearing that may have been caused by the primers annealing to more than one site. A single nested primer, NPL 1 and an adaptor primer AP2 amplified a 250 bp fragment. This fragment proved to be homologous to the known sequence. The failure to amplify and sequence the flanking regions using the genome walking method may be because there were no restriction enzyme recognition sites close to the sequence. When the sequences were searched for restriction enzyme recognition sites, the enzymes used, *Eco*RV and *Ssp*I, were absent (Appendix 1, Fig A2). These restriction enzymes were chosen so that

digestion of the genomic DNA did not produce many small fragments. It is possible that the lengths of the fragments, produced after digestion, were too large for amplification. A restriction map of the *T. asperellum* nucleotide sequence also revealed that there were two *Alu*I sites at positions 156 and 448 downstream from the 5' end. If indeed *Eco*RV and *Ssp*I produced fragments that were too large for amplification, this can be confirmed by "long range" PCR or Southern blotting. Other researchers have amplified segments as large as 10 kbp using the PCR vectorette method (Jeffreys *et al.* 1988). More likely, the anchor template failed to ligate to the ends of the restricted fragment library so that amplification would not occur. The smears produced during the primary PCR may correspond to non-specific DNA amplification likely due to non-specific priming, lack of optimization of the PCR conditions, poor quality genomic DNA, or non specific primers. The nested primers reduced the background. Although in this case the PCR-vectorette technique did not produce additional fragments of the gene, there are ways to improve the technique. Restriction enzyme recognition sites incorporated in the anchor primer and specific primer ought to increase cloning efficiency (Roux and Dhanarajan 1990). Also, the full-length gene can be isolated using inverse PCR and random amplification of cDNA ends (RACE) (Schaeffer *et al.* 1994). The complete sequence could also be obtained by constructing a cDNA library and a genomic library to obtain the sequences of the transcript and the full-length gene respectively.

5.4.2 Isolation of a chitinase gene using specific primers

To overcome the failure to isolate a full-length gene, *Trichoderma* spp. specific primers (Viterbo *et al.* 2002) were used to amplify the fungal genomic DNA of all eleven isolates. The sequence obtained from isolates 04-000 and 04-001 showed 98% homology to an endochitinase gene from *T. harzianum* indicating that the sequence was part of a chitinase gene. There is high homology (98%) of this sequence to the published

sequence by Viterbo *et al* (2002), however they are not identical. This provides evidence on possible genetic diversity between this isolate and that used by the authors. This sequence was named *ChiB*. A sequence analysis will be presented in Chapter 6.

5.4.3 Glucanase gene

Initially a 1257 bp nucleotide sequence corresponding to 400 amino acids was amplified from isolate 04-013 with glucanase degenerate primers. The deduced amino acid sequence showed identity to other known glucanases.

5.4.3.1 Genome walking method

This method was successfully used to obtain a complete glucanase gene that showed homology to a glucanase from *A. oryzae.* A total of 2844 bp were obtained from the putative glucanase gene, named *Glu1*, from the isolate 04-013.

DNA fragments were amplified in a single PCR reaction from an oligonucleotide vectorette library with a single genomic-specific primer in combination with a vectorette specific primer. Except for the sequence corresponding to the 3' end of the gene obtained after primary amplification, no distinct products were produced possibly because the restrictions sites were located too far away from the glucanase gene to be amplified with the genome walking method used in this study. Others have also amplified flanking regions of known sequences in a single PCR reaction from an oligocassette library (Kilstrup and Kristiansen 2000).

To obtain the rest of the putative glucanase gene, libraries were amplified in a secondary PCR. While a few non-specific background products were seen on the gel, the dominant products were extracted and sequenced. As a result, a downstream fragment was obtained that added another 1176 bp towards the end of the gene. Using this nucleotide sequence, a further 320 bp sequence was attained, corresponding to the

3' end of the gene as shown by BLASTX (Altschul *et al.* 1997) alignment with known glucanases.

5.5 CONCLUSIONS

A partial putative chitinase, *ChiA*, 639 nucleotides long, probably from *T. asperellum*, was isolated and its amino acid sequence was predicted. The gene shows homology with a chitinase gene in *A. fumigatus,* a soil-borne filamentous fungus, and a chitinase in *M. anisopliae*, an entomopathogenic fungus. Sequence of the the flanking regions of *ChiA* was not obtained.

A second partial chitinase gene, *ChiB*, 887 nucleotides long, also from *T. asperellum*, showed 98% homology with an endochitinase gene in *T. harzianum*, so it can be concluded that *ChiB* is also the same gene.

A putative glucanase gene, *Glu1*, was obtained using the degenerate primers and genome walking method. The deduced amino acid sequence showed high homology to a known glucanase. This gene can be potentially used to enhance plant resistance to fungal pathogens.

CHAPTER 6

ANALYSIS OF THE CLONED CHITINASE AND GLUCANASE GENES

6.1 INTRODUCTION

In Chapter 5 of this manuscript, two sequences, *ChiA* and *ChiB*, were isolated from one fungal isolate (04-001) that show homology to known chitinase genes. The fungal isolate had been previously identified as *Trichoderma* using morphological and nucleotide analyses.

ChiA, (accession number DQ007018) shows 70% amino acid homology to a class III chitinase from the fungus *Aspergillus fumigatus* and 64% homology to a chitinase from *M. anisopliae*. *ChiB*, (accession number DQ312296) shows 98% amino acid homology to an endochitinase (Chit36Y) from *T. harzianum.*

The glucanase gene, named *Glu1*, (accession number DQ312297) showed 98% homology to an exo-1, 3-glucanase from *Aspergillus oryzae.* This sequence was isolated from fungal isolate 04-013 that had been identified as *Aspergillus* by morphological characteristics.

In this chapter the three sequences will be analysed using bioinformatic tools provided by the available databases such as NCBI and ANGIS. Conducting multiple sequence alignments with the three sequences and related chitinase and glucanase gene sequences in the databases will enable us to characterise the genes predicted from the sequences, thus giving a detailed view of the function of those genes. Knowledge on the gene content of any organism is essential for the study and understanding of its biology and

its ancestry. Therefore, phylogenetic analysis will be conducted to gain information on the species of origin of the sequences named above.

Thus, the aim of this chapter is to use information provided by the genome databases to analyse chitinase and glucanase gene sequences described in Chapter 5.

6.2 MATERIALS AND METHODS

6.2.1 Sequence analysis

Analysis of the DNA sequences and amino acid sequences of a partial putative chitinase gene, *ChiA* and of the complete open reading frame (ORF) of a putative chitinase gene, *ChiB* and a putative glucanase gene *Glu1*, was carried out using software available through the NCBI. The sequences were trimmed to the same size and the primer sequences removed. The accession numbers of the sequences used from the databases, were identified by BLAST (Altschul *et al.* 1997) search of the NCBI protein databases. The top hits were used to create a group of related sequences homologous to our sequences. Protein sequence was deduced from six reading frames of the nucleotide sequence using the BLASTX (Altschul *et al.* 1997).

Prealignment was carried out using T-Coffee multiple sequence alignment program Version_1.37 (Notredame *et al.* 2000) and the results used to "trim" the sequences to a similar length before carrying out a multiple sequence alignment and tree generation.

6.2.2 Catalytic domains

The catalytic domains of the protein sequences were located using BLASTP (Marchler-Bauer and Bryant 2004).

6.2.3 Phylogenetic analysis

Phylogenic trees of the protein sequences were generated using the Phylip software package version 3.63 (Felsenstein 1989; Felsenstein 2004) under the control of perl scripts using BioPerl modules (Stajich *et al.* 2002). Tree distances were calculated using the Phylip "DNAdist" (Kimura two parameter method used) and "Neighbor" (Neighbor-Joining algorithm) modules. Rooted trees were constructed using T-Coffee to align the sequences, Jalview for manual trimming and curation, the Kimura two-parameter method to calculate the distances, and the neighbour-joining method to construct the tree.

Branch reliability figures are based on the generation of 1000 trees using the Phylip Seqboot and Consense modules in addition to those above. Images were generated using a combination of standard unix and Phylip utilities as well as the ATV tree viewer to make the images (Zmasek and Eddy 2001)

Phylogenetic distances for *ChiB* and *Glu1* were calculated with a distance method (Neighbor) using the original data set and 1000 bootstrap data sets generated from the original set by the program Seqboot.

6.3 RESULTS

6.3.1 Analysis of the chitinase sequence *ChiA*

6.3.1.1 Nucleotide sequence comparison with other chitinase genes

ChiA (Fig 6.1) (GenBank accession number DQ007018) has a nucleotide sequence of 639 nt with low identity (5.5%) with other chitinase genes, however, six plant chitinase genes show high homology (93%) over a short region of 28 nucleotides (Fig 6.1).

```
ChiA        TTTGACTATCTTTGGGTTCAGTTCTACAA
AY338248    TTTGACTACGTTTGGGTTCAGTTCTACAA
X88801      TTTGACTTTGTTTGGGTTCAGTTCTACAA
AF184884    TTTGACTACGTTTGGGTTCAGTTCTACAA
AB007127    TTTGACTATGTGTGGGTTCAGTTCTACAA
D49953      TTTGACTATGTGTGGGTTCAGTTCTACAA
AF309514    TTTGACAATGTTTGGGTTCAGTTCTACAA
```

Figure 6.1- Alignment of the nucleotide sequence of *ChiA* with plant chitinases. Conserved nucleotides are indicated by shading. Partial putative chitinase *ChiA* in red; AY338248 *Pyrus pyrifolia* class III endochitinase; X88801 *Vigna unguiculata* basic chitinase class III; AF184884 *Benincasa hispida* class III chitinase; AB007127 *Glycine max* acidic chitinase; D49953 *Psophocarpus tetragonolobus* chitinase; AF309514 *Malus domestica* class III acidic chitinase.

The complete nucleotide sequence with the deduced amino acid sequence of *ChiA* is shown in Fig 6.2. The asterisks at amino acid positions 184 and 191 indicate stop codons.

```
1 atggtctattgggggcagaacggtggtggtactatcgagaacaacggcctttctgctcac   60
1  M  V  Y  W  G  Q  N  G  G  G  T  I  E  N  N  G  L  S  A  H    20
61 tgtactgctgaagccggtatcgacgtcgtcgtacttagttttctttatcaatatggtaat  120
21  C  T  A  E  A  G  I  D  V  V  V  L  S  F  L  Y  Q  Y  G  N   40
121 ggcgtcgaaatcgcagcgggaacaattggccagagctgctccattgatacctctggcaac 180
41   G  V  E  I  A  A  G  T  I  G  Q  S  C  S  I  D  T  S  G  N  60
181 ccttcaaactgtgatgagcccagcgcagccatcgctacctgcaagtccaatggagtcaag 240
61   P  S  N  C  D  E  P  S  A  A  I  A  T  C  K  S  N  G  V  K  80
241 gtgatcttatccctaggtggcgcggccggtgcctattttctctcttctcagcaggaagcc 300
81   V  I  L  S  L  G  G  A  A  G  A  Y  F  L  S  S  Q  Q  E  A  100
301 gagacaattggccaaaatctctgggatgcttatggcgcaggaaatggtactgttccgaga 360
101  E  T  I  G  Q  N  L  W  D  A  Y  G  A  G  N  G  T  V  P  R  120
361 cccttcggaagcaatagtttggacggatgggatttcgatgtagaggcgagtaacggcaac 420
121  P  F  G  S  N  S  L  D  G  W  D  F  D  V  E  A  S  N  G  N  140
421 cagtactaccagtacttgatcgctaagcttcgctcaaacttcaacggcggcaactacgtg 480
141  Q  Y  Y  Q  Y  L  I  A  K  L  R  S  N  F  N  G  G  N  Y  V  160
481 attaccggtgctcctcagtgcccaattccgtcagttcttcttagattttacagttatatg 540
161  I  T  G  A  P  Q  C  P  I  P  S  V  L  L  R  F  Y  S  Y  M  180
541 gctgatgtatagcctgctaataaggaaaaatagggaaccaaatatgcagcaaatcattac 600
181  A  D  V  *  P  A  N  K  E  K  *  G  T  K  Y  A  A  N  H  Y  200
601 cacttcccagtttgactatctttgggttcagttctacaa 639
201  H  Y  H  F  P  V  S  L  G  S  V  L  Q  213
```

Figure 6.2- Nucleotide sequence of *ChiA* and its deduced amino acids sequence. The amino acid sequence is shown in one-letter code under the nucleotide sequence. The stars * at positions 184 and 191 indicate translation stop codons. The nucleotides homologous with plant chitinases are underlined. Amino acids corresponding to the substrate binding (SLGG) and active (DGWDFDVE) sites are indicated in bold.

6.3.1.2 Amino acid sequence comparison with other chitinases and active sites of ChiA

An alignment using BlastP shows the catalytic domains of *ChiA* partial chitinase and known chitinases (Fig. 6.2). Two highly conserved regions, (S-X-G-G and D-G-X-D-X-D-X-E) essential for function (Watanabe *et al.* 1993a), were identified in the catalytic domain of chitinases classified as family 18 glycosyl hydrolases are present in *ChiA* (Fekete *et al.* 1996; Tsujibo *et al.* 2002). This family includes enzymes of fungal, yeast, bacterial and plant origin. These conserved regions represent substrate binding and active sites, respectively, where X represents hydrophobic amino acids (Fekete *et al.* 1996; Tsujibo *et al.* 2002). As expected, *ChiA* possesses a hydrophobic amino acid (leucine) in the substrate binding site and in the catalytic domain it possesses hydrophobic amino acids W (tryptophan), F (phenylalanine) and V (valine).

```
ChiA              QYGNGVEIAAGTIGQSCSIDT--SGNPSNCDEPSAAIATCKSNGVKVILSLGGAAGAYFLSSQQEAETIG
gi|NP013388       QFP----TLGLNFANACSDTF--SDGLLHCTQIAEDIETCQSLGKKVLLSLGGASGSYLFSDDSQAETFA
gi|B41035         QFP----TLGLNFANACSDTF--SDGLLHCTQIAEDIETCQSLGKKVLLSLGGASGSYLFSDDSQAETFA
gi|A41035         QFP----TLGLNFANACSDTF--SDGLLHCTQIAEDIETCQSLGKKVLLSLGGASGSYLFSDDSQAETFA
gi|P36910         TFGNG-QTPALNLAGHCD----PATN---CNSLSSDIKTCQQAGIKVLLSIGGGAGGYSLSSTDDANTFA
gi|P29025         KFNMG-GLPEINLASACEQTFFPNTNLLHCPTVGSDIKTCQSNGVKVLLSLGGAAGSYGFSSDSEGQTFA
gi|CAC07216       QFGNGGNIPSGTIGQSCYIST--SGQGQNCEALTAAIQTCQSAGVKIILSLGGATSSYSLQTQAQAEQIG

ChiA              QNLWDAYG-AGNGTVPRPFGSNSLDGWDFDVEASNGN-QYYQYLIAKLRSNFN---GGNYVITGAPQCPI
gi|NP013388       QTLWDTFG-EGTGASERPFDSAVVDGFDFDIEN--NNEVGYSALATKLRTLFA-EGTKQYYLSAAPQCPY
gi|B41035         QTLWDTFG-EGTGASERPFDSAVVDGFDFDIEN--NNEVGYSALRTKLRTLFA-EGTKQYYLSAAPQCPY
gi|A41035         QTLWDTFG-EGTGASERPFDSAVVDGFDFDIEN--NNEVGYSALRTKLRTLFA-EGTKQYYLSAAPQCPY
gi|P36910         DYLWNTYL-GGQ-SSTRPLGDAVLDGIDFDIES--GDGRFWDDLARALAG-HN-NGQKTVYLSAAPQCPL
gi|P29025         ETIWNLFG-GGT-SDTRPFDDAVIDGIDLDIEG--GSSTGYAAFVTALR------SKGHFLIGAAPQCPF
gi|CAC07216       QYLWDSYGNSGNKTVQRPFGSNFVNGFDFDIEVNGGSSQYYQYMIAKLRSNFGSDEANTYYITGAPQCPI
```

Figure 6.3- Alignment of the amino acid sequences of the catalytic domains of fungal chitinases. Conserved amino acid residues across species are indicated by shading. Partial putative chitinase *ChiA*, DQ007018 (in red); NP013388 *Saccharomyces cerevisiae* chitinase (Johnston *et al.* 1997); B41035 and A41035 *S. cerevisiae* chitinase precursor (Kuranda and Robbins 1991); P36910 acidic endochitinase from sugar beet (Nielsen *et al.* 1993); P29025 chitinase precursor from *Rhizopus niveus* (Yanai 1992), direct submission; CAC07216 putative chitinase from *M. anisopliae* var. acridum (Screen and St Leger 2000), unpublished;. The active site DGWDFDVE and the substrate binding site SLGG are indicated in bold.

6.3.1.3 Phylogeny of ChiA

The relatedness of the *ChiA* to other chitinases was determined. A phylogenetic tree was constructed (Fig 6.3). The root of the tree is a protein from the yeast *Debaryomyces hansenii* CBS767 (Dujon *et al.* 2004). There are two distinct clades. The clade containing *ChiA* is dichotomous. One branch contains the fungal chitinases from *A. fumigatus* (EAL85097) and *M. anisopliae* chitinases (CAC07216 and AAY34347) (Clade 1, Branch A) (Fig 6.3). Branch B contains chitinases from yeasts (Clade 1) (Fig 6.3). This dichotomy indicates that fungal and yeast chitinases may be descendants from a common ancestor. The second clade (Clade 2) (Fig 6.4) comprises class III chitinases from plants such as *Medicago truncatula, Beta vulgaris* and *Cucumis sativus*. The accession number EAC04676 corresponds to a sample of unknown origin.

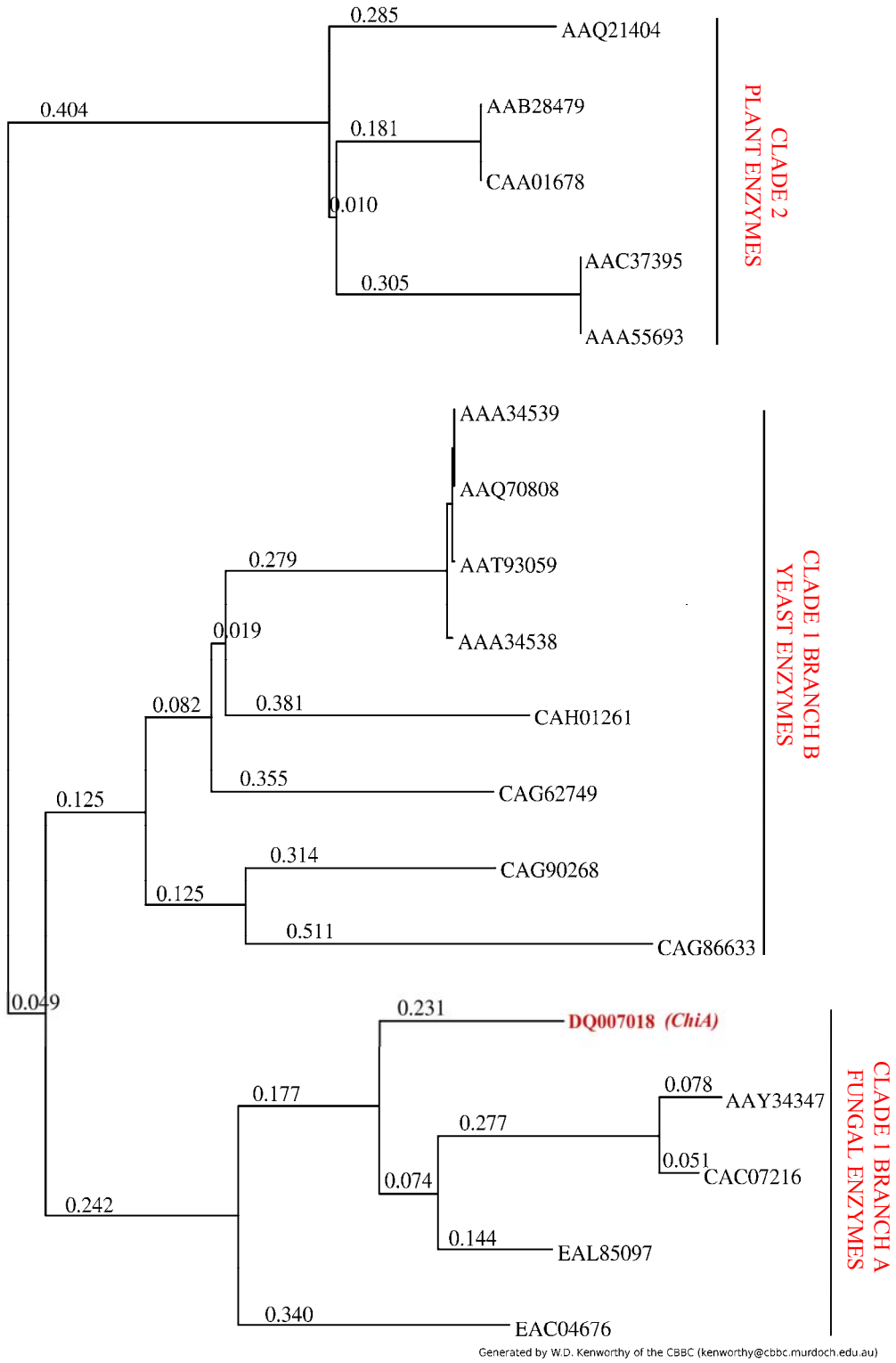


Figure 6.4- Phylogenetic relationship among chitinase genes. The rooted phylogenetic tree was calculated based on an alignment of regions of identical or similar residues from chitinase genes using ClustalW. The numbers on the lines indicate distance, calculated as an estimate from that particular pair of species, of the divergence time between those two species.

6.3.1.4 Branch reliability

A consensus tree was constructed to estimate the reliability of the multiple alignment. This analysis confirmed polytomies like in the phylogenetic tree where at all the branch points there are more than two immediate descendants. The chitinase genes are clustered in three groups, fungal chitinases where *ChiA* is found (Cluster I); a second group comprising yeast chitinases (Cluster II) and a third containing plant chitinases (Cluster III) (Fig 6.5). Yeast chitinase (AAA34539) (in bold, underlined), appears to be an ancestor and may be part of other more inclusive clades of yeasts.

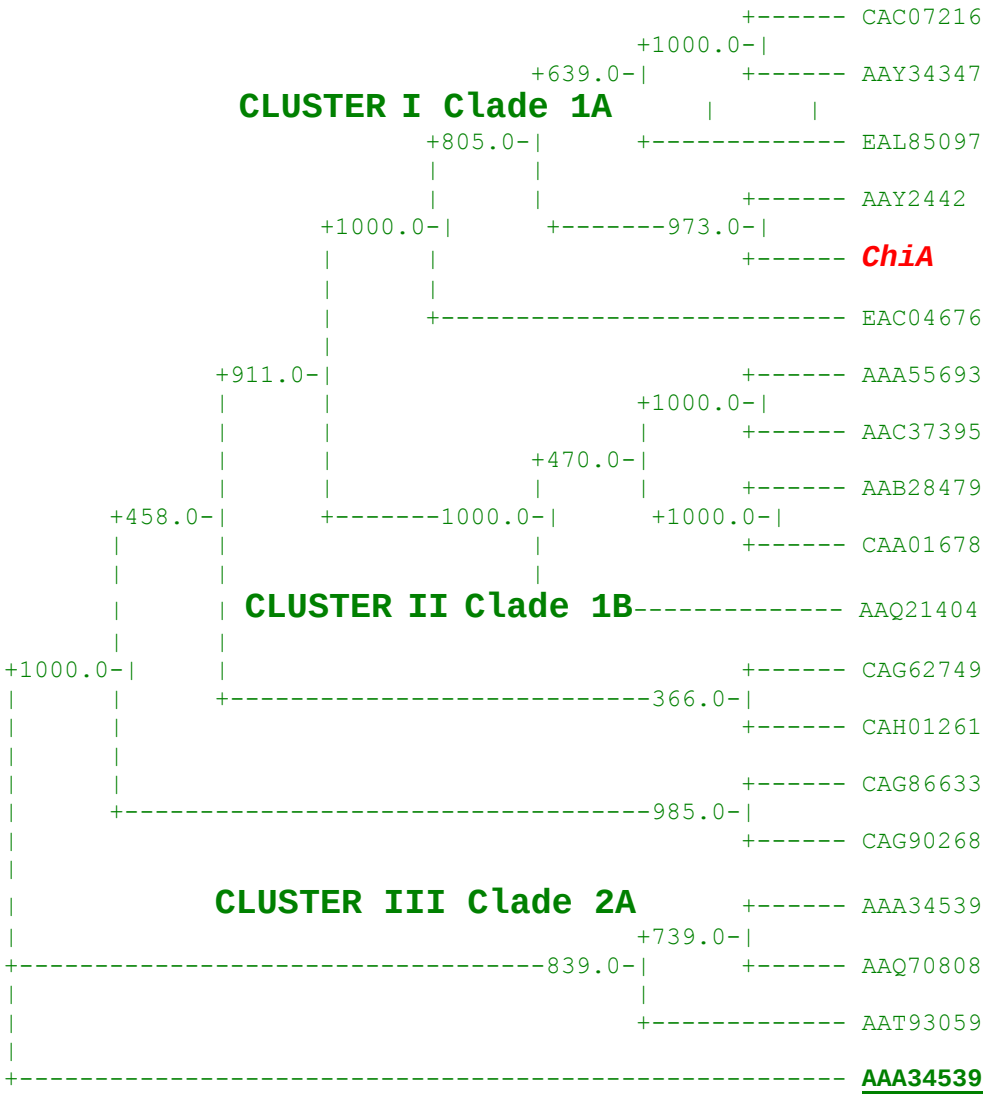


Figure 6.5- Branch reliability of the phylogenetic tree. The numbers on the junctions indicate the number of times the group consisting of the species which are to the right of that junction occurred among the trees, out of 1000. Cluster I comprises fungal enzymes, cluster II comprises yeast enzymes and cluster III comprises plant chitinases.

6.3.2 Analysis of the chitinase sequence *ChiB*

6.3.2.1 Nucleotide sequence comparison with other chitinases

The primer sequences used to amplify *ChiB* were removed from the sequence resulting in a fragment of 871 nucleotides (GenBank accession number DQ312296). *ChiB* was compared with sequences in the database (Fig 6.6). The sequence with the highest identity homology (97%) to *ChiB* was an endochitinase from *T. harzianum* (accession number AF406791) (Viterbo *et al.* 2002). The five genes most closely related were from *Trichoderma* species and are shown below and identity ranged from 81-97%.

```
                1         11        21        31        41        51
ChiB            TGACACGCCTTCTTGACGCCAGCTTTCTGCTGCTGCCTGCTATCGCATCGACGCTATTTG
AY028421        TGACACGCCTTCTTGACGCCAGATTTCTGCTGCTGCCTGCCATCGCATCGACGTTAATTG
AY129675        TGACACGCCTTCTTGACGCCAGATTTCTGCTGCTGCCTGCCATCGCATCGACGTTAATTG
AF406791        TGACACGCCTCCTCGACGCCAGCTTTCTGCTGCTGCCTGCTATCGCATCGACGCTATTCG
AF525754        TGACACGCCTTCTTGACGCAAGCTTTCTGCTTTTGCCTGTCATTGTATCGACATTATTCG
AF525753        TGACACGCCTTCTTGACGCAAGCTTTCTACTTTTGCCTGTCATCGCTTCGACATTATTCG

                61        71        81        91        101       111
ChiB            GCACCGCCTCTGCACAGAATGCGACATGCGCACTGAAGGGAAAGCCGGCAGGCAAAGTCT
AY028421        GCACCGCTTCTGCACAAAATGCAACATGTGCACTCAAGGGAAAACCGGCCGGCAAAGTCC
AY129675        GCACCGCTTCTGCACAAAATGCAACATGTGCACTCAAGGGAAAACCGGCCGGCAAAGTCC
AF406791        GCACCGCCTCTGCACAGAATGCGACATGCGCACTTAAGGGAAAGCCGGCAGGCAAAGTCT
AF525754        GCACTGCATCCGCACAAA--GC-ACATGTGCAACTAAGGGAAAGCCGGCTGGCAAGGTTC
AF525753        GCACTGCATCCGCACAAA--GC-ACATGTGCAACTAAGGGAAAGCCAGCTGGCAAGGTTC

                121       131       141       151       161       171
ChiB            TGATGGGATATTGGGAAAATTGGGATGGAGCAGCCAACGGTGTTCACCCTGGATTTGGCT
AY028421        TGATGGGATATTGGGAGAACTGGGACGGAGCATCCAACGGCGTTCACCCCGGATTCGGCT
AY129675        TGATGGGATATTGGGAGAACTGGGACGGAGCATCCAACGGCGTTCACCCCGGATTCGGCT
AF406791        TGATGGGATATTGGGAAAATTGGGATGGAGCAGCCAACGGTGTTCACCCTGGATTTGGTT
AF525754        TTCAAGGATACTGGGAGAATTGGGATGGATCAGCTAATGGTGTTCACCCCGGATTTGGAT
AF525753        TTCAAGGATACTGGGAGAACTGGGATGGATCAGCTAATGGTGTTCACCCCGGATTTGGAT

                181       191       201       211       221       231
ChiB            GGACACCGATCGAAAACCCCATCATTAAACAGAATGGCTACAATGTGATCAACGCCGCCT
AY028421        GGACACCAATCGAAAACCCTATTATTAAACAGAATGGCTACAATGTGATCAACGCTGCTT
AY129675        GGACACCAATCGAAAACCCTATTATTAAACAGAATGGCTACAATGTGATCAACGCTGCTT
AF406791        GGACACCAATCGAAAACCCCATCATTAAACAGAATGGCTACAATGTGATCAACGCCGCCT
AF525754        GGACGCCTATCGAAAACCCTGTGATCGCACAAAATGGCTACAACGTGATTAATGCCGCTT
AF525753        GGACGCCTATCGAAAACCCTGTGATCGCACAAAATGGCTACAACGTGATTAATGCCGCTT

                241       251       261       271       281       291
ChiB            TCCCCGTTATTCTGTCAGATGGCACAGCATTATGGGAAAACGACATGGCTCCTGACACTC
AY028421        TCCCCGTTATTCTGTCAGATGGCACAGTGCTGTGGGAAAACGACATGGCTCCTGATACTA
AY129675        TCCCCGTTATTCTGTCAGATGGCACAGTGCTGTGGGAAAACGACATGGCTCCTGATACTA
AF406791        TCCCCGTTATTCTGTCAGATGGCACAGCGTTATGGGAAAACGACATGGCTCCTGACACTC
AF525754        TCCCAGTCATCCTCTCAGATGGTACGGCACTGTGGGAAGATGGCATGGATGCTACTGTTA
AF525753        TCCCAGTCATCCTCTCAGATGGTACGGCACTGTGGGAAGATGGCATGGATGCTACTGTTA

                301       311       321       331       341       351
```

```
ChiB        AAGTCGCAACTCCAGCTGAAATGTGTGAGGCTAAAGCAGCTGGTGCCACAATTCTGCTGT
AY028421    CGATTGCAACACCGGCCGAGATGTGTGAGGCTAAAGCAGCTGGAGCAACAATTCTTTTGT
AY129675    CGATTGCAACACCGGCCGAGATGTGTGAGGCTAAAGCAGCTGGAGCAACAATTCTTTTGT
AF406791    AAGTCGCAACTCCAGCTGAAATGTGTGAGGCTAAAGCAGCTGGAGCCACAATTCTGCTGT
AF525754    AAGTTGCGACACCAGCCGAAATGTGCCAGGCCAAAGCAGCTGGTGCCACTATCCTCATGT
AF525753    AAGTTGCGACACCAGCCGAAATGTGCCAGGCCAAAGCAGCTGGTGCCACTATCCTCATGT

                361       371       381       391       401       411
ChiB        CAATTGGAGGTGCTACTGCTGGCATAGATCTCAGCTCCAGTGCAGTCGCTGACAAGTTCA
AY028421    CAATCGGAGGTGCTTCTGCTGGCATAGACCTCAGCTCTAGTACAGTCGCCGACAAGTTTG
AY129675    CAATCGGAGGTGCTTCTGCTGGCATAGACCTCAGCTCTAGTACAGTCGCCGACAAGTTTG
AF406791    CAATTGGAGGTGCTACTGCTGGCATAGATCTCAGCTCCAGTGCAGTCGCTGACAAGTTCA
AF525754    CCATTGGAGGTGCTACTGCTGGCATCGACCTCAGCTCTAGTACTGTCGCCGATAAGTTCA
AF525753    CTATTGGAGGTGCTACTGCTGGCATCGACCTCAGCTCTAGTACTGTCGCCGATAAGTTCA

                421       431       441       451       461       471
ChiB        TCGCCACCATTGTACCAATCTTGAAGCAGTACAATTTTGACGGCATTGATATAGACATTG
AY028421    TCGCGACCATTGTACCAATCTTGAAGCAGTACAACTTTGACGGTATTGATATCGACATTG
AY129675    TCGCGACCATTGTACCAATCTTGAAGCAGTACAACTTTGACGGTATTGATATCGACATTG
AF406791    TCGCCACCATTGTACCAATCTTGAAGCAGTACAATTTTGACGGCATTGATATAGACATTG
AF525754    TCTCGACTATCGTCCCAATCTTGAAGCAGTACAACTTCGACGGTATTGACATCGACATTG
AF525753    TCTCGACTATCGTCCCAATCTTGAAGCAGTACAACTTCGACGGCATTGACATCGATATTG

                481       491       501       511       521       531
ChiB        AGACGGGGTTGACCAACAGCGGCAATATCAACACACTTTCCACATCCCAGACCAATTTGA
AY028421    AGACTGGTTTGACCGGCAGCGGCAATATCAACACGCTTTCCACATCCCAGGCCAACTTGA
AY129675    AGACTGGTTTGACCGGCAGCGGCAATATCAACACGCTTTCCACATCCCAGGCCAACTTGA
AF406791    AGACGGGGTTGACCAACAGCGGTAATATCAACACACTTTCCACATCCCAGACCAACTTGA
AF525754    AGACGGGGTTGGTTGGAAGCGGCAGCATCGGCACCCTGTCCACGTCACAGGCCAACTTGA
AF525753    AGACGGGATTGGTTGGAAGCGGCAGCATCGGCACCCTGTCCACGTCACAGGCCAACTTGA

                541       551       561       571       581       591
ChiB        TTCGCATCATTGATGGTGTTCTTGCTCAGATGCCTTCCAACTTCGGCTTGACTATGGCAC
AY028421    TTCGCATCATTGATGGTGTTCTTGCTGCGATGCCTTCAAACTTTGGCTTGACTATGGCAC
AY129675    TTCGCATCATTGATGGTGTTCTTGCTGCGATGCCTTCAAACTTTGGCTTGACTATGGCAC
AF406791    TTCGCATCATTGATGGTGTTCTTGCTCAGATGCCTTCCAACTTCGGCTTGACTATGGCAC
AF525754    TCCGCATCATTGATGGTGTCCTTGCACAGATGCCTTCCAACTTCGGCTTGACTATGGCAC
AF525753    TCCGCATCATTGATGGTGTCCTTGCACAGATGCCTGCCAACTTCGGCTTGACTATGGCAC

                601       611       621       631       641       651
ChiB        CTGAGACAGCGTACGTTACAGGCGGTAGCATCACGTATGGCTCTATTTGGGGAGCGTACC
AY028421    CGGAGACACCATATGTTACAGGCGGTAGCGTCACGTATGGCTCTATTTGGGGATCATACC
AY129675    CGGAGACACCATATGTTACAGGCGGTAGCGTCACGTATGGCTCTATTTGGGGATCATACC
AF406791    TTGAGACAGCGTACGTTACAGGCGGTAGCATCACGTATGGCTCTATTTGGGGAGCGTACC
AF525754    CAGAGACGGCGTATGTGACAGGTGGCAGTGTTGTGTACGGATCCATCTGGGGGTCTTATC
AF525753    CAGAGACGGCGTATGTGACTGGTGGTAGTGTTGTGTACGGATCCATCTGGGGTTCTTACC

                661       671       681       691       701       711
ChiB        TACCTATCATCCAGAAATATGTTCAAAACGGCCGGCTGTGGTGGTTAAACATGCAATATT
AY028421    TGCCAATCATTCAAAAATATATCCAAAACGGCCGACTGTGGTGGCTCAACATGCAATACT
AY129675    TGCCAATCATTCAAAAATATATCCAAAACGGCCGACTGTGGTGGCTCAACATGCAATACT
AF406791    TACCTATCATCCAGAAATATGTTCAAAACGGCCGGCTGTGGTGGTTAAACATGCAATATT
AF525754    TCCCAATTATCAAGAAGTACGTCGACAACGGCCGGGTGTGGTGGCTCAACATGCAATACT
AF525753    TCCCAATTATCAAGAAGTACGTCGACAACGGCCGGTTGTGGTGGCTCAACATGCAATACT

                721       731       741       751       761       771
ChiB        ACAACGGCGATATGTACGGTTGCTCTGGCGACTCTTACGCAGCTGGCACCGTCCAAGGAT
AY028421    ACAACGACGACTACTACGGTTGCTCAGGCGACTCATACGCAGCCGGCACTGTCGCGGGAT
AY129675    ACAACGACGACTACTACGGTTGCTCAGGCGACTCATACGCAGCCGGCACTGTCGCGGGAT
AF406791    ACAACGGCGACATGTACGGTTGCTCTGGCGACTCTTACGCAGCTGGCACTGTCCAAGGAT
AF525754    ACAACGGCGACATGTATGGTTGCTCCGGCGATTCATATGCGGCCGGCACTGTTCAAGGTT
AF525753    ACAACGGCGACATGTATGGTTGCTCCGGCGATTCATATGCGGCCGGCACTGTTCAAGGTT

                781       791       801       811       821       831
ChiB        TCATCGCTCAGACTGATTGCCTAAATGCAGGACTTACCATCCAAGGCACCACAATCAAGG
AY028421    TTATTGCTCAGACTGATTGCTTGAATGCAGGACTTACTGTCCAAGGCACCACAATCAAGA
AY129675    TTATTGCTCAGACTGATTGCTTGAATGCAGGACTTACTGTCCAAGGCACCACAATCAAGA
AF406791    TCATCGCTCAGACTGACTGCCTAAATGCAGGACTTACCGTCCAAGGCACCACAATCAAGG
```

```
AF525754        TCACCGCACAAACTGACTGCTTAAACAATGGCATCACTATCCAAGGCACCACAATCAAGG
AF525753        TCACCGCACAAACCGACTGCTTAAACAATGGCATCACTATCCAAGGCACCACAATCAAGG

                841         851         861         871
ChiB            TTCCATACGACATGCAAGTACCAGGCCTACCTGC
AY028421        TCCCATACGACATGCAAGTACCCGGCCTACCTGC
AY129675        TCCCATACGACATGCAAGTACCCGGCCTACCTGC
AF406791        TTCCGTACGACATGCAAGTACCAGGTCTACCTGC
AF525754        TTCCCTACAACATGCAAGTTCCTGGATTACCTGC
AF525753        TTCCCTACAACATGCAAGTTCCTGGATTACCTGC
```

Figure 6.6 –Nucleotide alignment of *ChiB* with the five most homologous sequences available. All sequences have been trimmed for accurate alignment. AY028421 *T. harzianum* endochitinase (chit36); AY129675 *T. atroviride* endochitinase (chit36P1); AF406791 *T. harzianum* endochitinase (Chi36Y); AF525754 *T. inhamatum* chitinase (chit37); AF525753 *T. harzianum* (chit37).

Nucleotide 7 of *ChiB* aligns with the first nucleotide of a *T. harzianum* endochitinase (Chit36Y) coding region (1212 bp long) and finishes 332 bp upstream from the 3' end of it. Compared with another *T. harzianum* endochitinase coding region (chit36, 1345 bp long) the *ChiB* sequence starts 102 bp downstream from the 5' end and finishes 367 bp upstream from the 3' end. *ChiB* starts seven nucleotides downstream from the 5' end and finishes betweeen 134 and 166 bp upstream from the 3' end of the other three chitinases (AY129675, AF525753 and AF525754). This indicates that the transcriptional start of *ChiB* and its end have not been obtained.

The deduced amino acid sequence reveals highly conserved regions of chitinases from family 18 (Fig 6.7). These regions correspond to the substrate binding site S-X-G-G and the active site D-G-X-D-X-D-X-E respectively (Tsujibo *et al.* 2002). In addition, an A-X-A sequence commonly associated with signal peptide processing sites of secretory proteins from eucarya and bacteria, (von Heijne 1986) was also found.

```
1   ttcatgacacgccttcttgacgccagctttctgctgctgcctgctatcgcatcgacgcta  60
1    F  M  T  R  L  L  D  A  S  F  L  L  L  P  A  I  A  S  T  L  20
61  tttggcaccgcctctgcacagaatgcgacatgcgcactgaagggaaagccggcaggcaaa.120
21   F  G  T  A  S  A  Q  N  A  T  C  A  L  K  G  K  P  A  G  K  40
121 gtcttgatgggatattgggaaaattgggatggagcagccaacggtgttcaccctggattt.180
41   V  L  M  G  Y  W  E  N  W  D  G  A  A  N  G  V  H  P  G  F  60
181 ggctggacaccgatcgaaaaccccatcattaaacagaatggctacaatgtgatcaacgcc 240
61   G  W  T  P  I  E  N  P  I  I  K  Q  N  G  Y  N  V  I  N  A  80
241 gccttccccgttattctgtcagatggcacagcattatgggaaaacgacatggctcctgac 300
81   A  F  P  V  I  L  S  D  G  T  A  L  W  E  N  D  M  A  P  D  100
301 actcaagtcgcaactccagctgaaatgtgtgaggctaaagcagctggtgccacaattctg 360
101  T  Q  V  A  T  P  A  E  M  C  E  A  K  A  A  G  A  T  I  L 120
361 ctgtcaattggaggtgctactgctggcatagatctcagctccagtgcagtcgctgacaag 420
121  L  S  I  G  G  A  T  A  G  I  D  L  S  S  S  A  V  A  D  K  140
421 ttcatcgccaccattgtaccaatcttgaagcagtacaattttgacggcattgatatagac 480
141  F  I  A  T  I  V  P  I  L  K  Q  Y  N  F  D  G  I  D  I  D  160
481 attgagacggggttgaccaacagcggcaatatcaacacactttccacatcccagaccaat 540
161  I  E  T  G  L  T  N  S  G  N  I  N  T  L  S  T  S  Q  T  N  180
541 ttgattcgcatcattgatggtgttcttgctcagatgccttccaacttcggcttgactatg 600
181  L  I  R  I  I  D  G  V  L  A  Q  M  P  S  N  F  G  L  T  M  200
601 gcacctgagacagcgtacgttacaggcggtagcatcacgtatggctctatttggggagcg 660
201  A  P  E  T  A  Y  V  T  G  G  S  I  T  Y  G  S  I  W  G  A  220
661 tacctacctatcatccagaaatatgttcaaaacggccggctgtggtggttaaacatgcaa 720
221  Y  L  P  I  I  Q  K  Y  V  Q  N  G  R  L  W  W  L  N  M  Q  240
721 tattacaacggcgatatgtacggttgctctggcgactcttacgcagctggcaccgtccaa 780
241  Y  Y  N  G  D  M  Y  G  C  S  G  D  S  Y  A  A  G  T  V  Q  260
781 ggattcatcgctcagactgattgcctaaatgcaggacttaccatccaaggcaccacaatc 840
261  G  F  I  A  Q  T  D  C  L  N  A  G  L  T  I  Q  G  T  T  I  280
841 aaggttccatacgacatgcaagtaccaggcctacctgcgcaat 883
281  K  V  P  Y  D  M  Q  V  P  G  L  P  A  Q   296
```

Figure 6.7- Nucleotide sequence of *ChiB* and its deduced amino acid sequence. The amino acid sequence is shown in one-letter code under the nucleotide sequence. The signal peptide AIA and the highly conserved regions SIGG (substrate binding site) and DGIDIDIE (the active site) are shown in bold.

6.3.2.2 Amino acid comparison with other chitinase genes

As expected from the nucleotide alignments, an alignment of the deduced amino acid sequence of *ChiB* with all available others on GenBank, revealed identity with endochitinases from other *Trichoderma* species. *T. harzianum* Chit36Y is 98% identical over 313 amino acids from a sequence that is 344 amino acids long. Identity was seen with an endochitinase from *T. atroviride* and *T. harzianum* at 92% identity over 292 the aligned portion. *ChiB* shared 87% identity with chitinases from *T. harzianum* and *T. inhamatum*. From this alignment, it is evident that the entire open reading frame of *ChiB* has yet to be obtained (Fig 6.8).

```
                1         11        21        31        41        51
ChiB            TRLLDASFLLLPAIASTLFGTASAQNATCALKGKPAGKVLMGYWENWDGAANGVHPGFGW
AAL01372        TRLLDASFLLLPAIASTLFGTASAQNATCALKGKPAGKVLMGYWENWDGAANGVHPGFGW
AAK54377        TRLLDARFLLLPAIASTLIGTASAQNATCALKGKPAGKVLMGYWENWDGASNGVHPGFGW
AAM77132        TRLLDARFLLLPAIASTLIGTASAQNATCALKGKPAGKVLMGYWENWDGASNGVHPGFGW
AAM93195        TRLLDASFLLLPVIASTLFGTASAQ-STCATKGKPAGKVLQGYWENWDGSANGVHPGFGW
AAM93196        TRLLDASFLLLPVIVSTLFGTASAQ-STCATKGKPAGKVLQGYWENWDGSANGVHPGFGW

                61        71        81        91        101       111
ChiB            TPIENPIIKQNGYNVINAAFPVILSDGTALWENDMAPDTQVATPAEMCEAKAAGATILLS
AAL01372        TPIENPIIKQNGYNVINAAFPVILSDGTALWENDMAPDTQVATPAEMCEAKAAGATILLS
AAK54377        TPIENPIIKQNGYNVINAAFPVILSDGTVLWENDMAPDTTIATPAEMCEAKAAGATILLS
AAM77132        TPIENPIIKQNGYNVINAAFPVILSDGTVLWENDMAPDTTIATPAEMCEAKAAGATILLS
AAM93195        TPIENPVIAQNGYNVINAAFPVILSDGTALWEDGMDATVKVATPAEMCQAKAAGATILMS
AAM93196        TPIENPVIAQNGYNVINAAFPVILSDGTALWEDGMDATVKVATPAEMCQAKAAGATILMS

                121       131       141       151       161       171
ChiB            IGGATAGIDLSSSAVADKFIATIVPILKQYNFDGIDIDIETGLTNSGNINTLSTSQTNLI
AAL01372        IGGATAGIDLSSSAVADKFIATIVPILKQYNFDGIDIDIETGLTNSGNINTLSTSQTNLI
AAK54377        IGGASAGIDLSSSTVADKFVATIVPILKQYNFDGIDIDIETGLTGSGNINTLSTSQANLI
AAM77132        IGGASAGIDLSSSTVADKFVATIVPILKQYNFDGIDIDIETGLTGSGNINTLSTSQANLI
AAM93195        IGGATAGIDLSSSTVADKFISTIVPILKQYNFDGIDIDIETGLVGSGSIGTLSTSQANLI
AAM93196        IGGATAGIDLSSSTVADKFISTIVPILKQYNFDGIDIDIETGLVGSGSIGTLSTSQANLI

                181       191       201       211       221       231
ChiB            RIIDGVLAQMPSNFGLTMAPETAYVTGGSITYGSIWGAYLPIIQKYVQNGRLWWLNMQYY
AAL01372        RIIDGVLAQMPSNFGLTMALETAYVTGGSITYGSIWGAYLPIIQKYVQNGRLWWLNMQYY
AAK54377        RIIDGVLAAMPSNFGLTMAPETPYVTGGSVTYGSIWGSYLPIIQKYIQNGRLWWLNMQYY
AAM77132        RIIDGVLAAMPSNFGLTMAPETPYVTGGSVTYGSIWGSYLPIIQKYIQNGRLWWLNMQYY
AAM93195        RIIDGVLAQMPANFGLTMAPETAYVTGGSVVYGSIWGSYLPIIKKYVDNGRLWWLNMQYY
AAM93196        RIIDGVLAQMPSNFGLTMAPETAYVTGGSVVYGSIWGSYLPIIKKYVDNGRVWWLNMQYY

                241       251       261       271       281
ChiB            NGDMYGCSGDSYAAGTVQGFIAQTDCLNAGLTIQGTTIKVPYDMQVPGLP
AAL01372        NGDMYGCSGDSYAAGTVQGFIAQTDCLNAGLTVQGTTIKVPYDMQVPGLP
AAK54377        NDDYYGCSGDSYAAGTVAGFIAQTDCLNAGLTVQGTTIKIPYDMQVPGLP
AAM77132        NDDYYGCSGDSYAAGTVAGFIAQTDCLNAGLTVQGTTIKIPYDMQVPGLP
AAM93195        NGDMYGCSGDSYAAGTVQGFTAQTDCLNNGITIQGTTIKVPYNMQVPGLP
AAM93196        NGDMYGCSGDSYAAGTVQGFTAQTDCLNNGITIQGTTIKVPYNMQVPGLP
```

Figure 6.8 – Deduced amino acid sequence of *ChiB* (in red) compared with similar sequences. AAL01372 *T. harzianum* endochitinase; AAK54377 *T. harzianum* endochitinase; AAM77132

T. atroviride endochitinase; AAM93195 *T. harzianum* chitinase and AAM93196 *T. inhamatum* chitinase. The colours signify amino acids with similar properties.

6.3.2.3 Active sites of ChiB

ChiB has the highly conserved substrate binding site and catalytic site (S-X-G-G and D-G-X-D-X-D-X-E) shared by chitinases from *Trichoderma* spp., *Hypocrea lixii, Streptomyces coelicolor* and *Bacillus* spp. (Fig 6.9). In *ChiB* the hydrophobic amino acid represented by X is an isoleucine in both sites.

```
ChiB        MCEAKAAGATILLSIGGATAGIDLSSSAVADKFIATIVPILKQYNFDGIDIDIETGLTNS
AAM77132    MCEAKAAGATILLSIGGASAGIDLSSSTVADKFVATIVPILKQYNFDGIDIDIETGLTGS
AAL01372    MCEAKAAGATILLSIGGASAGIDLSSSTVADKFVATIVPILKQYNFDGIDIDIETGLTGS
AAM93195    MCEAKAAGATILLSIGGATAGIDLSSSAVADKFIATIVPILKQYNFDGIDIDIETGLTNS
AAM93196    MCQAKAAGATILMSIGGATAGIDLSSSTVADKFISTIVPILKQYNFDGIDIDIETGLVGS
CAB69724    MCAAKASGQTLLLSIGGATAGIDLNSTAVADRFVDTIVPILKEYNFDGIDIDIETGLTGS
BAC73348    MCQAKASGQTILLSIGGAAAGIDLSSSAVADRFVATIVPLLKKYNFDGIDIDIETGLVGS
JC7996      ISYLKSKGKKVVLSIGGQNGVVLLPDNAAKDRFINSIQSLIDKYGFDGIDIDLESGIYLN
AAP10651    ISYLKSKGKKVVLSIGGQNGVVLLPDNAAKDRFINSIQSLIDKYGFDGIDIDLESGIYLN
```

Figure 6.9-Amino acid sequence alignment of the substrate binding and active sites (shaded) of the chitinase gene *ChiB* (in red) with known chitinases. AAM77132, endochitinase from *T. atroviride* (Viterbo *et al.* 2001). AAL01372, endochitinase from *T. harzianum* (Viterbo *et al.* 2001), AAM93195 chitinase from *T. harzianum* and AAM93196, chitinase from *T. inhamatum* (Viterbo *et al.* 2002), CAB69724, chitinase precursor from *Streptomyces coelicolor* (Schrempf 1999). BAC73348 endochitinase from *S. avermitilis* (Schrempf 1999). JC7996, a chitinase from *Bacillus thuringiensis* (Arora *et al.* 2003). AAP10651, an exochitinase from *B. cereus* (Wang *et al.* 2001). Identities computed with respect to: AAM77132.

6.3.2.4 Phylogeny of ChiB

To visualise the relationship between *ChiB* and other chitinases, a phylogenetic tree was constructed using the amino acid sequences of the amplified fragment and sequences of other chitinases. The tree was rooted using SwissProt sequence P27050, a chitinase from *B. circulans* because proteins from these bacteria are believed to be ancestral to fungal proteins. Note that this is a "pseudo" root as a neighbor-joining tree is un-rooted by definition. The results of the phylogenetic analysis for *ChiB* sequence shows a high degree of identity to that of *Trichoderma* species (Fig 6.10). The tree has two distinct clades that are polytomous. The clade that contains *ChiB* (Clade 1) has two branches. One branch contains *Trichoderma* spp chitinases (AAL01372, AAM77132,

AAM93195, AAM 93196) and a chitinase from the entomopathogenic fungus *Cordyceps bassiana* (syn. *Beauveria bassiana*), accession number AAN41259 (Clade 1, Branch A) (Fig 6.10). A second branch contains bacterial chitinases where a chitinase from *Lactococcus lactis* (accession number NP268107) shares a possible ancestor with *ChiB* (Clade 1, Branch B). Clade 2 also contains bacterial chitinases.

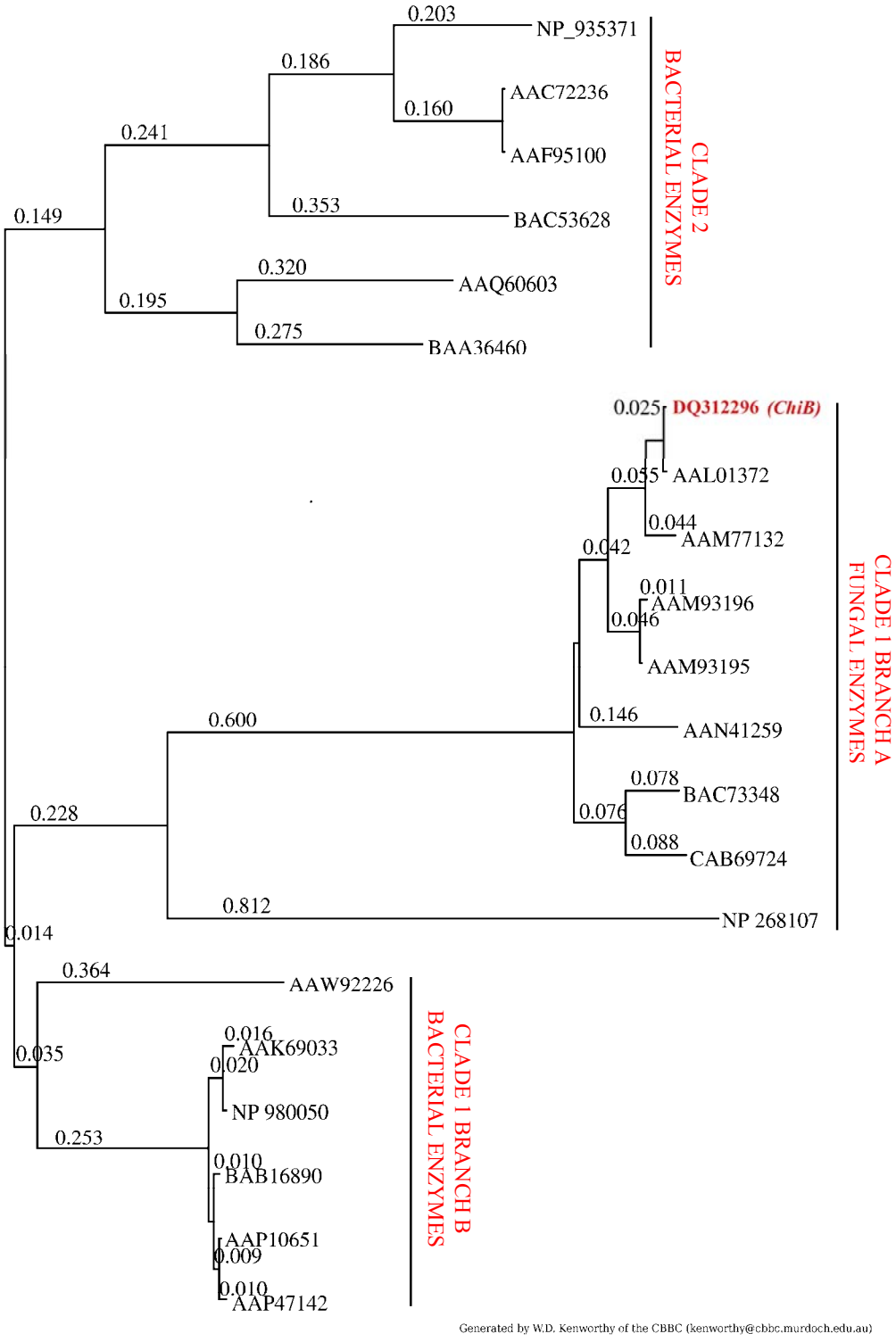


Figure 6.10-Relationship (calculated on genomic differences) between sequences most closely related. The tree is rooted on sequence P27050, a chitinase D precursor from *Bacillus circulans*. The chitinase gene *ChiB* is in a cluster with other chitinases, *T. harzianum* endochitinase Chit36Y (accession number AAL01372); *T. atroviride* chitinase (accession number AAM77132); *T. harzianum* chitinase (accession number AAM93196) and *Cordyceps bassiana* chitinase (accession number AAN41259). The numbers represents distance that is calculated as an estimate of the divergence time between two species.

Multiple sequence alignment of chitinase genes in the database was performed with CLUSTALW (Thompson *et al.* 1994) using the default parameters (Table 6.1). The available sequences were trimmed to equal length before phylogenetic analyses using programs in PHYLIP (Felsenstein 1989). Genetic distances between pairs of nucleotide sequences were calculated using the program DNAdist (maximum likelihood method with transition/transversion ratio of 2.0 and 1 category of substitution rates). Phylogenetic trees were constructed with a distance method (Neighbor) using the original data set and 1000 bootstrap data sets generated from the original set by the program Seqboot. The consensus tree was generated by the program Consense. The percentage nucleotide and amino acid identities were determined using OldDistances (GCG). The sequences included in the matrix shown in Table 6.1 include AY028421, a *T. harzianum* endochitinase (Prot AAM93195); AY129675, a *T. atroviride* endochitinase (Prot AAM77132); AF406791, a *T. harzianum* endochitinase (Prot AAL01372) and AF525753 a *T. harzianum* chitinase (AAM93196).

Table 6.1- Nucleotide and amino acid (in bold) homology (% identity) of partial gene *ChiB* (DQ312296) with chitinase sequences available on GenBank.

Sequence Identity (a)	1	2	3	4	5	6
1	1.0000	1.0000	0.8822	0.8799	0.7933	0.7899
2	**1.0000**	1.0000	0.8822	0.8799	0.7933	0.7899
3	**0.9483**	**0.9483**	1.0000	0.9805	0.7991	0.7945
4	**0.9448**	**0.9448**	**0.9966**	1.0000	0.7979	0.7933
5	**0.8789**	**0.8789**	**0.8997**	**0.8962**	1.0000	0.9805
6	**0.8824**	**1.0000**	**0.9031**	**0.8997**	**0.9896**	**1.0000**

Nucleotide and protein lengths used for the alignment are shown in Table 6.2.

Table 6.2- Data shows the nucleotide and protein lengths of the chitinase sequences used for the CLUSTALW alignment. The letter a, corresponds to the sequence identities in Table 6.1. The letters b and c indicate the length without gaps of the nucleotide and protein sequences, respectively.

(a)	Accession number	Nucleotide length	(b)	Protein length	(c)
1	AY028421	874	874	290	290
2	AY129675	874	874	290	290
3	*ChiB*	874	874	290	290
4	AF406791	874	874	290	290
5	AF525754	874	871	290	289
6	AF525753	874	871	290	289

6.3.2.5 Branch reliability

One thousand bootstrap data sets were generated from the original to indicate reliability figures for the branch structure in Fig 6.11. The numbers on the branches indicate the number of times the partition of the species into the two sets, which are separated by that branch, occurred among the trees, out of 1000.00 trees. A chitinase D precursor from the bacterium *B. circulans*, accession number P27050 (Watanabe *et al.* 1992), could be an ancestor of chitinase *ChiB*. *ChiB* forms a cluster with AAL0137, AAM7713, AAM9319 which, according to the iterations is reliable and are enzymes from *Trichoderma* species (Cluster I) (Fig 6.11). Enzymes from *Streptomyces* spp., accession numbers CAB6972 and BAC7334, form another cluster (Cluster II) (Fig 6.11). Bacterial enzymes from *Bacillus* spp, (AAK6903 and JC79960) form the third cluster (Cluster III), further away from the one containing *ChiB*.

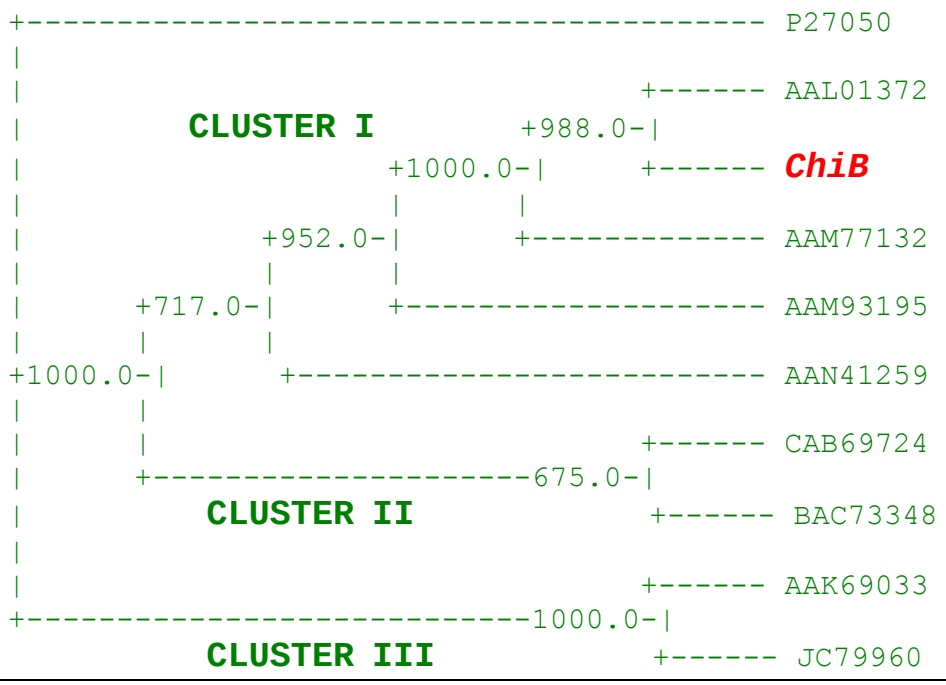


Figure 6.11- Neighbour-joining tree of amino acid sequences of the partial chitinase genes. *ChiB* is shown in red. The clades comprising, fungal, and bacterial chitinases are numbered I and II and III respectively. Bootstrap values are shown at each node.

6.3.3 Comparison between *ChiA* and *ChiB* sequences

6.3.3.1 Nucleotide sequences

There was low identity (18%) between *ChiA* and *ChiB* at the nucleotide level. Homology between the conserved regions (in bold) at the nucleotide level was 58% in the substrate binding region and 25% identical in the active site (Fig 6.12).

```
ChiA  1------------------------------------------------------------   60
Identity
ChiB  1ctagatgcatgctcgagcggccgccagtgtgatggatatctgcagaattcgcccttcatg   60

ChiA 61  ------------------------------------------------------------ 120
Identity
ChiB 61  acacgccttcttgacgccagctttctgctgctgcctgctatcgcatcgacgctatttggc 120

ChiA 121 ----------------------------------------------------atggtctattgg 180
Identity                                                        t g
ChiB 121 accgcctctgcacagaatgcgacatgcgcactgaagggaaagccggcaggcaaagtcttg 180

ChiA 181 gggcagaacggtggtggtactatcgagaacaacggcctttctgctcactgtactgctgaa 240
Identity   g    a  g g    t      g       c  c  t  t   c   g   tg
ChiB 181 atgggatattgggaaaattgggatggagcagccaacggtgttcaccctggatttggctgg 240

ChiA 241 gccggtatcgacgtcgtcgtacttagttttctttatcaatatggtaatggcgtcgaaatc 300
Identity  c    atcga   c  c t  tta       t    a  atg  a    cg cg   tc
ChiB 241 acaccgatcgaaaaccccatcattaaacagaatggctacaatgtgatcaacgccgccttc 300
```

```
ChiA 301 gcagcgggaacaattggccagagctgctccattgatacctctggcaacccttcaaactgt 360
Identity  c g           g     a     t     ga a c         cct   a
ChiB 301 cccgttattctgtcagatggcacagcattatgggaaaacgacatggctcctgacactcaa 360

ChiA 361 gatgagcccagcgcagccatcgctacctgcaagtccaatggagtcaaggtgatctta tcc 420
Identity g  g   c    gc g  at   t      aa  c   tgg g ca   t  t  t tc
ChiB 361 gtcgcaactccagctgaaatgtgtgaggctaaagcagctggtgccacaattctgctg tca 420

ChiA 421 ctaggtggc gcggccggtgcctattttctctcttctcagcaggaagccgagacaattggc 480
Identity  t gg gg gc  c g tg c     tctc   tc       g  gc ga a   t   c
ChiB 421 attggaggt gctactgctggcatagatctcagctccagtgcagtcgctgacaagttcatc 480

ChiA 481 caaaatctc----------------------------tgggatgcttatggcgcaggaaat 540
Identity    a   t                              g    t at   g      a
ChiB 481 gccaccattgtaccaatcttgaagcagtacaatttt gacggcattgatatagacattgag 540

ChiA 541 ggtactgttccgagacccttcggaagcaatagtttggacggatgggatttcgatgtagag 600
Identity       t  c a    c  c   a caa a   t   c  at   a   c at t
ChiB 541 acggggttgaccaacagcggcaatatcaacacactttccacatcccagaccaatttgatt 600

ChiA 601 gcgagtaacggcaaccagtactaccagtacttgatcgctaagcttcgctcaaacttcaac 660
Identity    a  a  g             cag       c            c a
ChiB 601 cgcatcattgatggtgttcttgctcagatgccttccaacttcggcttgactatggcacct 660

ChiA 661 ggcggcaactacgtgattaccggtgctcctcagtgcccaattccgtcagttcttcttaga 720
Identity g        tacgt a    cggt        gt       t   t  g          a
ChiB 661 gagacagcgtacgttacaggcggtagcatcacgtatggctctatttggggagcgtaccta 720

ChiA 721 ttttacagttatatggctgatgtatagcctgctaataaggaaaaatagggaaccaaatat 780
Identity   t  ca   a a    tg t  a a           g     a a        a ta
ChiB 721 cctatcatccagaaatatgttcaaaacggccggctgtggtggttaaacatgcaatattac 780

ChiA 781 gcagcaaatcattaccacttcccagtttgactatctttgggttcagttctacaa------ 840
Identity    g   at   tac   t c c g        t     g t       t caa
ChiB 781 aacggcgatatgtacggttgctctggcgactcttacgcagctggcaccgtccaaggattc 840

ChiA 841 ------------------------------------------------------------ 900
Identity
ChiB 841 atcgctcagactgattgcctaaatgcaggacttaccatccaaggcaccacaatcaaggtt 900

ChiA 901 -------------------------------------
Identity
ChiB 901 ccatacgacatgcaagtaccaggcctacctgcgcaat 937
```

Figure 6.12- Nucleotide comparison between *ChiA* and *ChiB*. Identical nucleotides are shown in between the sequences. The substrate binding and active sites are indicated in bold. The alignment was carried out manually.

6.3.3.2 Amino acid sequences

Comparison of *ChiA* and *ChiB* showed that in the case of the two sites, S-X-G-G and D-G-X-D-X-D-X-E (in bold), representing substrate binding and active sites the residues represented by X (hydrophobic residue) were different in the two sequences. Beyond the conserved regions, the two sequences were only weakly homologous (5%) over 324 amino acids (Fig 6.13).

```
1  ChitA     --------------------------------------------------  50
1  Consensus                                                     50
1  ChitB     LDACSSGRQCDGYLQNSPFMTRLLDASFLLLPAIASTLFGTASAQNATCA  50

51 ChitA     ------MVYWGQNGGGTIENNGLSAHCTAEAGIDVVVLSFLYQYGNGVEI 100
51 Consensus                                i.  ..              100
51 ChitB     LKGKPAGKVLMGYWENWDGAANGVHPGFGWTPIENPIIKQNGYNVINAAF 100

101ChitA     AAGTIGQSCSIDTSGNPSNCDEPSAAIATCKSNGVKVIL**SLGG**AAGAYFL 150
101Consensus       .   .    p       a .   k. g  ..ls.gga     l 150
101ChitB     PVILSDGTALWENDMAPDTQVATPAEMCEAKAAGATILL**SIGG**ATAGIDL 150

151ChitA     SSQQEAETIGQNLWDAYGAGNGTVPRPFGSNSL**DGWDFDVE**ASNGNQYYQ 200
151Consensus ss   a.     .       n            dg d d.e    n     200
151ChitB     SSSAVADKFIATIVPILKQYNF----------**DGIDIDIE**TGLTNSGNI 200

201ChitA     YLIAKLRSNFNGGNYVITGAPQCPIPSVLLRFYSYMADVPANKEKGTKYA 250
201Consensus   ..  ..n       .           .    .y.               250
201ChitB     NTLSTSQTNLIRIIDGVLAQMPSNFGLTMAPETAYVTGGSITYGSIWGAY 250

251ChitA     ANHYHYHFPVSLGSVLQ---------------------------------- 300
251Consensus       .       l                                     300
251ChitB     LPIIQKYVQNGRLWWLNMQYYNGDMYGCSGDSYAAGTVQGFIAQTDCLNA 300

301ChitA     ------------------------ 324
301Consensus
301ChitB     GLTIQGTTIKVPYDMQVPGLPAQX 324
```

Figure 6.13- Comparison of amino acid residues between *ChiA* and *ChiB*. The highly conserved residues typical of family 18 glycosyl hydrolases are indicated in bold.

6.3.4 Analysis of the glucanase gene *Glu1*

6.3.4.1 Nucleotide sequence comparison with other glucanases

Glu1 was submitted to GenBank and given the accession number DQ312297. It is 2844 nucleotides in length and showed highest identity (77%) to the nucleotide sequence of an exo-1, 3-glucanase of 2844 nucleotides from the filamentous fungus *A. fumigatus* (accession number AB074847). The alignment below (Fig 6.14) shows how deletions and insertions in *Glu1* and other homologous glucanases from *Aspergillus* spp. have separated those genes from others more distantly related.

```
            1         11        21        31        41        51
Glu1        ------------------------------------------------------------
AB074847    ------------------------------------------------------------
AB070739    ------------------------------------------------------------
BX649607    ------------------------------------------------------------
AF247649    TTCTCTCATCCGCATTGTGTCGGTTATTCATTCATTCGTTGCCACCTTCACTCCGCTATG
AJ002397    ------------------------------------------------------------
AY854012    ------------------------------------------------------------
AF317733    ------------------------------------------------------------

            61        71        81        91        101       111
Glu1        ------------------------------------------------------------
AB074847    ------------------------------------------------------------
AB070739    ------------------------------------------------------------
BX649607    ------------------------------------------------------------
AF247649    CACGGTGTTTTTATATAGAGCTTCTTTCCTCTTCCACCGTTGCTCCAACAACTTGCATTC
AJ002397    ------------------------------------------------------------
AY854012    ------------------------------------------------------------
AF317733    ------------------------------------------------------------

            121       131       141       151       161       171
Glu1        ------------------------------------------------------------
AB074847    ------------------------------------------------------------
AB070739    ------------------------------------------------------------
BX649607    ------------------------------------------------------------
AF247649    TTTACAAACCAACCCCACCAACGCGTCTCTGTGAGACTAACTTTGATTTCCAAAGTCCCG
AJ002397    ------------------------------------------------------------
AY854012    ------------------------------------------------------------
AF317733    ------------------------------------------------------------

            181       191       201       211       221       231
Glu1        ----------------------------------------------------ATGCTGTTCCT
AB074847    ------------------------------------------------------------
AB070739    ------------------------------------------------------------
BX649607    -----------------------------------ATGGCTTTTCTTCGCACTCCCAT
AF247649    ACAATCCTTCAAACCAACTTTTACATTTTCAAAACTCAAACTAGCTGTCATCATGGTAGC
AJ002397    ---------------------------------------ATGGGGTTTATACGCTC-
AY854012    ---------------------------------------ATGAAGTTCC-ACGCACT
AF317733    ----------------GGCTACTCATAACATCATTCGTTCTCAGCAGCCTTA-TCGAAAT

            241       251       261       271       281       291
Glu1        GGCTCACGTTCTGCTGCTGCTGGGTCTGCCGGCTGGTATGGTTGGTGCTGTTCCGCTGGG
AB074847    ------------------------------------------------------------
AB070739    ------------------------------------------------------------
BX649607    CGTGCTCTTGCTGACGCAGATACTGCTCTTCCTGGGCCTAGTCAATGCAGTTCCTACCCC
AF247649    GTTCGGCGCCACGGCGCTTTTCCTCACGCTGCGCGTGCTTCTTTCGGCAACCCCGTCGTC
AJ002397    CGCCGTGCTTTCGGCCCTCACATTTGCCGCG---GCATGTCGAGGCCTGGCCA--CGC--
AY854012    CATTCAGAAGTCGCTGCTTGCTTTGAGCGCCATCGGCTCAATAGCACTTGCGAGTCCC--
AF317733    GGCACGTTTTACCACACTCCTTTTTGTGTTTCATCAGTTACTTCCATTTGCAACCAGCTC

            301       311       321       331       341       351
Glu1        TCAGGAAACCGACATCACCACCAA---CCTGGCTGCTCGTGCTGCTTCCGAATACTGGGT
AB074847    --AAGAGACGGATATCACAACAAA---TTTAGCTGCCAGGGCGGCGTCAGAATACTGGGT
AB070739    ------------------------------------------------------ACTGGGT
BX649607    GGAATCTCTTGATGTGCATGGAAA---TTTAGCTCGAGATACTGCGTCGAGCTTCTGGGT
AF247649    TGCTGCGCCGGTCGCACAGGCAAACGCTCCTGCACCGGGTGCAGCCAGTGGCTACTGGCT
AJ002397    -CTGGGTCCGAGGCTGAACCTTCTGTT----GA---AAAGCGTGCTTCAAGTTATTGGTA
AY854012    -CTTGCCCCAAGAAATGAGACGCTGGAAGCGGA---AGCCAGAGCCTCAGACTGGTGGTT
AF317733    TCCAGCTCCTATCATTAATCAGGTTGCCCCTCACACAGCCGCTGATTCAAATTTTTGGCT

            361       371       381       391       401       411
Glu1        TGGTACCATC------AAACGTCAGGGTGCTGTTGCTTTCGGTAACGGTAC---CGACT-
AB074847    TGGAACTATC------AAACGGCAGGGTGCTGTTGCTTTTTGGAAATGGCAC---AGATT-
AB070739    TGGAACGATC------AAGCGTCAGGGCACTGTGCCCTTTGGCAACACCACGAGCAGCT-
BX649607    GGCTAATATT------AAGCGTCAAGGCGCCGTGGCTTTCGGCCAGAGCTC---GGGTT-
AF247649    TCCTGAGCTCGCTTCCAAGGGACAAGGAAAGGCTGCATTCAACGCCAACCCCGACACCT-
AJ002397    TGAAAACATT------GCCCATCAAGGAATTGCACCATTCGCCCCCA---G---CAACT-
AY854012    CGGGAATATT------GAGCGCACTGGGCGAGTTCCCTTCGGCAGCAATAG---CAACT-
```

```
AF317733      AGAAAGTATT------GCTAGAAAAGGTAAGGTCGCCTACGGTACAT-------CATCTC

                    421       431       441       451       461       471
Glu1          ACCAGGTTTACCGTAACGTTAAAGACTTCGGTGCTAA----------------------
AB074847      ATCAGGTATATCGTAATGTGAAAGACTTTGGTGCTAAGGGTATGGGCTACCACGTTGCGT
AB070739      ATGCAATCTACCGCAACGTCAAGGATTATGGTGCCAAGGGTGAGTAAGACCTCCTTACAT
BX649607      ATCAGGTTTACCGCAATGTGAAGGACTTTGGCGCAAAG----------------------
AF247649      ACCTGGTCTACCGCAACGTCAAGGACTTCGGAGCCACG----------------------
AJ002397      ACACAGTGTTCCGCAACGTAAAGGACTATGGCGCAAAG----------------------
AY854012      ACCCTGTTTTCCGTAACGTCAAGGACTACGGCGCGCGT----------------------
AF317733      ACCAAGTATTTCGCAACGTCAAGGACTTCGGGGCAAAG----------------------

                    481       491       501       511       521       531
Glu1          --------------------------AGGTGACGGTTCCACCGACGACACCGCTGCTAT
AB074847      GAAGATGACAT-ATGCTAACTGCACTCAGGTGATGGGTCGACGGATGATACTGCTGCCAT
AB070739      GGCTGGTATTTGATGCTGACTACTG-CAGGTGACGGATCTACCGATGATACTGATGCGAT
BX649607      --------------------------GGTGACGGAGTAACCGATGATACTGCGGCGAT
AF247649      --------------------------GGCGACGGCTCGACTGACGATACCGCTGCTAT
AJ002397      --------------------------GGTGATGGTGTTACCGACGACACGGCAGCAAT
AY854012      --------------------------GGAGACGGAAGCACCGACGACACGGCAGCTAT
AF317733      --------------------------GGTGACGGCACTACTGATGACACTGCTGCGAT

                    541       551       561       571       581       591
Glu1          CAACCAGGCTATC------------------TCCTCCGGTAAC------CGTTGCGGTAA
AB074847      TAACCAGGCGATT------------------TCCTCAGGCAAC------CGATGTGGCAA
AB070739      CAACAAGGCCATC------------------TCCTCTGGTGGC------CGCTGTGGTTC
BX649607      CAACCAGGCCATA------------------TCTTCCGGGAAT------CGGTGCGGAAA
AF247649      CAACAAGGCTATCGCTTCCCTGACCAAGGGATCTTCCGACAGCGAAGTGCGTTGCGGTGA
AJ002397      TAACAATGCCATT--------------------CTGTCGGGAGGT-----CGATGTGGCAG
AY854012      CAATGCCGCGATT--------------------ACAGCGGGAGGC-----GGTCGCTGCGG
AF317733      CAATGCTGCTATA--------------------ACTCAAGGATCT-----CGCTGTGCCCA

                    601       611       621       631       641       651
Glu1          AGGT---TGCGACTCCTCCACCGTTACCCCGGCTCTGGTTTACTTCCCGCCGGGTACCTA
AB074847      GGGG---TGTGATTCATCAACCGTCACTCCTGCCCTCGTATACTTCCCTCCTGGTACTTA
AB070739      CGGT---TGTGACTCGTCTACCACTACCCCAGCCCTAGTGTACTTCCCCGCAGGTACCTA
BX649607      AGGA---TGCGATTCCTCCACCACCACTCCCGCTCTGGTTTACTTTCCTCCCGGTACATA
AF247649      GGGA---TGTGACTCTACCACCATCACCCCGGCCGTCGTCTACTTCCCCCCCGGAACCTA
AJ002397      ACT---CTGCACATCGAGCACTCTGACTCCAGCTGTGGTATACTTTCCGGCTGGAACGTA
AY854012      ACTGGGCTGCAAATCAAGCACCAACACACCAGCTCTCGTTTTCTTCCCACCTGGTACCTA
AF317733      AGGG---TGTGACTCGTCTACTATAACTCCTGCATTGGTCTACTTTCCCTCCGGCACCTA

                    661       671       681       691       701       711
Glu1          CGTTGTTTCCAAACCGATCGTTCAGTACTACTACACCCAGATCGTTGGTGACGCTGTTAA
AB074847      TGTCGTCTCCAAACCAATCGTTCAGTATTACTATACCCAAATCGTTGGCGACGCCGTCAA
AB070739      CGTGGTGTCCAAGCCTATTGTGCAGTACTACTACACCCAGATGGTCGGCGATGCCAACGA
BX649607      CGTTGTCTCGAAGCCCATCATCCAGTATTACTACACTCAGATAGTCGGTGATGCCGTTGA
AF247649      CATGATCAGCAAGCCCATTATTCAGTACTACTACACTCAGTTCATCGGTGATGCTAAGCA
AJ002397      CGTGATCTCAACGCCCATCATTGATCAGTACTACACCAACATCATTGGAGATCCAACCAA
AY854012      TCTGGTGTCTCAGCCCATTATTGGCTACTACTACACTCACATTGTCGGGGACTTGAAGGA
AF317733      CAGTATCAGTGAACCTATTGTACAGCTATACTACACCCAATTCGTCGGAAATGCAGTCAC

                    721       731       741       751       761       771
Glu1          CCTGCCGGTTATCAAAGCTGCTGCTGGTTTCGCTGGTATGGCTGTTATCGACGCTGACCC
AB074847      CCTTCCTGTGATCAAGGCGGCGGCGGGCTTTGCTGGTATGGCTGTCATTGATGCCGACCC
AB070739      TTTGCCTGTCCTCAAGGCTGCCTCGAGCTTCAGCGGTATGGCCGTCATTGATGCCGACCC
BX649607      TCTTCCTGTGATCAAAGCATCCGCCGACTTTACAGGCATGGCGGTGATTGACGCCGACCC
AF247649      GCTGCCCACGCTCAAGGCTACCGCTGCTTTCGAGGGCATGGGTCTTATCGACGCTGATCC
AJ002397      CCTGCCAACCATCAAGGCGACCGCAGGCTTCAGCGGCATTGCCCTCATCGATGGTGATAC
AY854012      CATTCCCACCATCAAGGCCACATCCAACTTCCAAGGGTCTGCTGTCATTGACGCGAACCC
AF317733      ACCTCCCACCATCAAGGCTACCTCTGGTTTCAAGGGAATGGCTCTTATCGACTCTAATCC

                    781       791       801       811       821       831
Glu1          GTAC---GAAGACG---ACGGTTCCA--ACTGGT----------------------------
AB074847      TTAT---GAAGATG---ACGGCAGCA--ACTGGT----------------------------
AB070739      CTAC---GACAGCA---ATGGCGACA--ACTGGT----------------------------
BX649607      GTAC---GAGAATG---ACGGCTCCA--ATTGGT----------------------------
AF247649      TTACATTGAGGGTGGCAACGGCGCCA--ACTGGT----------------------------
```

```
AJ002397      TTACTATGG------CGATAATAACCCGAACGA--------------------------
AY854012      CTACGCCAA------CACTGGTAACA--ATTGG--------------------------
AF317733      ATATACAGAAGGAGTCAATTGGTACACTAATCAGGTATGTAGCGAATAATAGTGTGCGAG

                    841       851       861       871       881       891
Glu1          -ACACCAA------CCAGAACAACTTC--TTC---CGTGCTATCCGTAACATCGTTCTGG
AB074847      -ACACCAA------CCAGAACAACTTC--TTC---CGTGCCATTCGTAACCTTGTAATCG
AB070739      -ACACCAA------CCAGAACAACTTC--TTC---CGTGCCGTTCGCAACTTCGTCATTG
BX649607      -ACACCAA------CCAGAACAACTTC--TTC---CGCGCCATTCGCAACCTTGTCATCG
AF247649      -TCACCAA------CCAGAACAACTTC--TAT---CGTTCCATCCGCAACTTCGTCATCG
AJ002397      --CCCCAATTGGATTTCAACCAACGTC--TTCTACCGGCAAGTACGCAACTTTAAGCTTG
AY854012      --TTCCAA----AATCAAAACAACTTC--TTC---CGTCAGATTCGTAACCTCAAGATCG
AF317733      CTTCCTAAATCTAACCAAAACAGAATAATTTCTACAGACAAATTCGCAATTTTGTGATTG

                    901       911       921       931       941       951
Glu1          ACATCACCGCTATGCCGCAGGGTTCCGGTGC------TGGTCTGCACTGGCAGGTTGGTC
AB074847      ATTTGACTGCTATGCCTCAGGGATCTGGTGC------TGGTATCCATTGGCAGGTGGGCC
AB070739      ATCTAACCGCTATGCCCGCTTCCTCGGGTGC------TGGTATCCACTGGCAGGTCGGAC
BX649607      ACCTCAGGGCCATGCCTATGAACAGCGGTGC------TGGTATCCACTGGCAAGTCGGTC
AF247649      ACATCTCGGCA--GCCAAGG----CCGCTGC------CGGTGTGCACTGGCAGGTTTCGC
AJ002397      ACATGACGTCTATTCCTACTTCTGCGCCCAAAATCTATGGTATCCACTGGCCGACAGCCC
AY854012      ATATTCGATCTCAGCCAATGAACACGGGCAC--T----GGAATCCACTGGCAGGTAGCGC
AF317733      ATACGACAGCTATGCCTGCTGAT-CGCGGAG-----CAGGCATTCATTGGCAAACTGCCC

                    961       971       981       991      1001      1011
Glu1          AGGCTACCTCCCTGCAGAACATCCGTTTCGAAATGA--------------------TCAAA
AB074847      AGGCGACCAGTTTGCAGAATATCAGATTCGAGATGA--------------------TCAAG
AB070739      AGGCCACCAGTCTGCAGAACATCCGCTTCGAGATGG--------------------TCAAG
BX649607      AGGCAACCAGCTTGCAGAACATCCGCTTCGAGATGA--------------------TCAGG
AF247649      AAGCCACCAGCATCCAGAATGTCGTCTTCAACATGG--------------------CTGAT
AJ002397      AGGCAACAAGCTTGCAAAACATCCAGATTACTATG-----------------------AG
AY854012      AGGCAACTTCTCTCCAGAACATCGAGTTCTACATGG--------------------TCGAG
AF317733      AAGCTACATCCCTACAGAATATTGTCTTCAATATGAGAACTGATGGAGGCACTAATAATG

                   1021      1031      1041      1051      1061      1071
Glu1          GGTGGTGGT-------------------GACGCTAAC-------AAACAGCAGGGTATCT
AB074847      GGCGGAGGC-------------------GATGCAAAC-------AAGCAACAGGGTATCT
AB070739      GGTGGCACC-------------------AAC---AAC-------AAGCAGCAGGGTATCT
BX649607      GGAGGCGGC-------------------GACGCCAAC-------AAGCAGCAAGGTATCT
AF247649      GCCAGCACCCCG----------------GAAGGTCAG-------GCCCAGAAGGGTATCT
AJ002397      CACAGCG---------------------TCTGGAAAT-------AGCCAAGTCGGTCTTT
AY854012      AACTCCAGT-------------------TCCGAGAAT-------CGGCAGCAGGGTATTT
AF317733      CCCAGCTGGTATGCAAAATCCGTTTCTATCCTAAAATTATTAACAGTATACAGGGTATAT

                   1081      1091      1101      1111      1121      1131
Glu1          TCATGGACAACGGTTCCGGTGGTTTCATGTCCGACCTGACCTTCAACGGTGGTAACTACG
AB074847      TCATGGACAACGGATCTGGTGGTTTCATGTCCGACTTGACTTTCAATGGTGGAAATTACG
AB070739      TCATGGACAACGGATCCGGTGGCTTCATGTCCGACCTGACCTTTAACGGCGGTAACTATG
BX649607      TCATGGACAACGGCTCCGGTGGTTTCATGTCCGACCTGACCTTCAATGGCGGCAACTACG
AF247649      TCCAGGACAACGGCTCCGGTGGTTTCATGACCGACCTCGTCTTCAACGGCGGTGCCATCG
AJ002397      TCATTGAGAACGGATCCGCTGGATTCCTCACCGATATGACCTTCAACGGTGGCTTGATTG
AY854012      TCATGGAGAATGGGTCCGGCGGCTTCATGACCGACCTCAAGTTCTTCGGTGGCAAATACG
AF317733      TTATGGACAATGGATCTGGAGGCTTTATGTCAGATCTAACTTTTAACGGCGGGAAGTATG

                   1141      1151      1161      1171      1181      1191
Glu1          GTATGTTCCTGGGTAACCAGCAGTTCACCACCCGTAACCTGACCTTCAACGACTGCAACA
AB074847      GCATGTTCTTAGGAAACCAGCAATTCACTACACGGAACTTGACCTTCAATGACTGCAACA
AB070739      GTGCCTTCTTCGGTAACCAGCAGTTCACCACCCGCAACCTGACCTTCAACGACTGTAACA
BX649607      GCATGTTCGTTGGCAACCAGCAGTTCACCACCCGCAACCTGAGCTTCAACGACTGCAACA
AF247649      GTGCCTTCCTCGGTAGCCAGCAGTTCACTACCCGCAACATGACCTTCAACAAGTGCGGCA
AJ002397      GTGCTGCTATTGGCAATCAGCAGTACACGATGCGCAACTTGGTATTCAACAACTGCGC-A
AY854012      GCGCTGTTTTCGGCTCGCAGCAGTTCACAGCTCGAAACCTTGAGTTTAACAACGCCCAGA
AF317733      GCGCTTTTTTTGGAAGCCAACAATTTACAACTCGCAACTTGATATTCAACAATTGTCAAA

                   1201      1211      1221      1231      1241      1251
Glu1          CCGCTATCTTCATGAACTGGAACTGGGCTTGGA--CCTTCAAATCCCTGTCCATCAACAA
AB074847      CAGCCATTTTCATGAACTGGAACTGGGCCTGGA--CCTTCAAGTCACTTTCCATCAACAA
AB070739      CCGCCATCTACATGAACTGGAACTGGGCCTGGA--CCTTCAAGTCGGTGTCTATCAACAA
```

```
BX649607      CGGCTGTTTTCATGAACTGGAACTGGGCCTGGA--CCTTCAAGTCCATCTCCATCAACAA
AF247649      CTGGTATCTACATGAACTGGAACTGGCTCTGGA--CCATGAAGTCGAACACCTTCAACGG
AJ002397      CAGCCATTGTCAGCGGCTTCGATTGGGAGTGGG-TTTACCAGGGCCATCAGCATCAACAA
AY854012      CGGGCATCTTGCTGATCTGGAACTGGATCTGGA-ACTTCCATGGCTTGAAGG-TCAATAA
AF317733      CTGCCATATTTTTTGAACTGGAACTGGGCCTGGACTCTTTCAGGTGTTGCA--ATCAACAA

                1261      1271      1281      1291      1301      1311
Glu1          CTGCCAGGTTGGTCTGAACATGTCCAACTTCCCG------CAGAACCAGACCGTTGGTTC
AB074847      CTGCCAGGTCGGTCTGAATATGTCCAATGCCCCG------CAGAATCAGACTGTCGGTTC
AB070739      CTGCCAGGTCGGCCTCAACATGTCCGCCTCTCCG------TCCAACCAGACCGTGGGGTC
BX649607      CTGCCAGGTCGGTCTCAATATGTCCAACGCGCCT------CAGAATCAGACAGTTGGCTC
AF247649      CTGCAAGGTCGGACTCGACATGGCCAACGCTCCT------GACAACCAGACTGTCGGCTC
AJ002397      CTGTGGATTGGGAATTGACATGAC----GGCCGC--------CGAGTCC------------
AY854012      CTGCGGCATTGGCATTGATCTGACAACTGGCGGTAGTGGTGCGAGTCAGAGTGTTGGTTC
AF317733      TGCAGGTATCGGCATTGATATGTCAAACGGAGGTTCTGGTGCACAAAC---TGTTGGATC

                1321      1331      1341      1351      1361      1371
Glu1          CGTTCTGATCCTGGACTCCCAGCTGACCAACACCCCGACCGGTGTTGTTTCCTTCGCTAC
AB074847      TGTCCTGATTCTGGACAGTCAACTTACGAACACACCCACTGGTGTTGTTTCTGCATTCAC
AB070739      TGTCCTGATGCTGGACAGCACGTTCACTAGCACTCCTACTGGTGTCGTGACTGCCTTCAG
BX649607      CGTTCTTCTGCTCGACAGCTCGGTAACCAACACTCCCACGGGCGTTGTCACCGCGTTCAC
AF247649      TGTGCTTCTCTTGGACAGCAAGTACATCTCCACACCTGTCGGTGTCAACACTTCTTTCTC
AJ002397      -ATCACGCTGATCGACAGTTCCATCAGCGGGACGCCAGTTGGAATCAAGACCAGCTTCAG
AY854012      TACCATTGTGCAGGACGCTACCTTCACTGGCACTCGCGTTGGTATTACCACGACGTACGA
AF317733      CGTTCTACTGGTAGACAGTAAAATTTCCAATACACCTATAGGTGTCTCTACAGCGTACTC

                1381      1391      1401      1411      1421      1431
Glu1          CGAAAACTCCATCCCGATCGGTGGTGGTGTTCTGATCCTGGACAACGTTGACTTCTCCGG
AB074847      CGAGAACAGCATTCCCATTGGCGGAGGTGTCCTGATTCTTGACAACGTCGACTTCAGCGG
AB070739      CAGCGACAGCATCCCCACCGGTGGCGGTGCCCTGGTCCTCGAAAACGTCGACTTCTCCGG
BX649607      GCAGGACAGTATTCCCGTGGGCGGCGGGGTGTTGATCCTTGAGAATGTCGATTTTACCGG
AF247649      CGAGACCAGCATCCCCATCGGTGGTGGCACGCTCATCATCGATAATGTTGACTTCTCTGG
AJ002397      GCGAAACCAGTCGCCCGCCACGTCCAACAGTCTCATCGTTGAGAACCTTTCTCTCAACAA
AY854012      CCGTAACCAAAAGGGTACCTTTGGCACGCTTGTCATCGACAACACCGACTTTGTCAACAC
AF317733      TACTTCACAAAGCGTCACTAATGGTACTCTTATATTGGACAACGTCGACTTTTCTGAAAA

                1441      1451      1461      1471      1481      1491
Glu1          TTCCAAAGTTGCTGTTGCTGGTATCACCGGTAA----CACCATCCTGGCTGGTGGTTCCG
AB074847      TTCTAAGGTTGCAGTTGCGGGTATTACTGGTAA----TACTATTCTGGCAGGCGGCTCAG
AB070739      CTCAACAGTTGCCATTGCAAGTGTGAACGGCGA----CACCATCCTCAAGGGTGGGTCCG
BX649607      CTCCAATGTGGCCGTGGCCGGTATCAGTGGGAA----CACCATCCTCAAAGGCGGATCTG
AF247649      CACCGAGACGGCTGTTCAGGACTTCCAAGGCAA----GAAGCTCCTCGCTGGTGGTTCGG
AJ002397      TGTCCCAGTGGCCAT-TCAGTCGTCGAGTGGAAGCACCATTCTGGCCGGAGGAACCACAA
AY854012      TGGTATTGCGG---T-TCAGAACCCGAGCACAGGGGCTACGATTCTTGGAGGCAATAGGA
AF317733      TGTCCCTGTTGCGATCTCTGATGCGGCAAGCAAAAA-TACGATCCTGGCTGGTAACGCAA

                1501      1511      1521      1531      1541      1551
Glu1          TTGTTACCAACTGGGTTCAGGGTAACGGTTACCTGCCGGGTTCCGCTA--AACAGAAACG
AB074847      TCGTGACGAACTGGGTCCAGGGCAATGGTTACCTCCCTGGCAGCGCGA--AGCAGAAGCG
AB070739      TTGTCTCGAACTACGTGCAGGGCAATGTCTACACCCCGGGCAGCAGCAGCAACAGCAACA
BX649607      TTGTGGCTAGCTGGATTCAGGGTAACACCTACAGTCCAGCAAACTCTCTCAACAAGCGGG
AF247649      TCGTCAAGACCTGGGCTCAGGGTAACGCTCTTGCCTCTGGCGGCACCC---AAGGCCGCG
AJ002397      CAATTGCTGCGTGGGGCCAGGGTCACCAGTATAC--GC-CGAATGGACC-GACGA-CATT
AY854012      AGGTCGGGGTCATTCGTCCAGGGTCGCTCATACACCGGCTCAAATGGTC---AAGT-CGT-
AF317733      AAATATCTTCTTGGGCACAAGGACATCAGTATGAA-GCTGGTACACAAG-GAAGAGCAGT

                1561      1571      1581      1591      1601      1611
Glu1          TGA---AGCTT-CCGTTAAAGTTACCACCCAGACCGT---TACCGAAACCGTTGAAGT--
AB074847      TGA---GGCTT-CCGTGAAGGTGACTACCCAAACTGT---GACAGAGACAGTGGAGGT--
AB070739      ACA---ACAGCACCGTCACCAAGCGCTCCCCTGCCGTTCAGGCCGAGGATGTTCAGGTGA
BX649607      CCACTCAGGGCAAGGTTCGCGTGGTGACAGAGACGGT---GGTCGAGACCGTCCTTGC--
AF247649      TCCAAGGCGATGTTCAGAACGGACCTACCAAGCCCCA--GAGTCTTCTTGGTAACGACGG
AJ002397      CCA---AGGC--AGCATTACGC------CAAACTCTC--GCCCCTCGTCCCTTTTGAGCG
AY854012      CCA---AGGCG-AGCGCCCAGCGCCGTCCAAGCCCGC--TGGCCTCCTGGCCTCC-AACG
AF317733      TCA---AGAAA-ATCTCGCTGCAACTTCTAAGCCAGC--ATCTCTTTTAGATGCC-TCTG

                1621      1631      1641      1651      1661      1671
Glu1          TTGCACCGCTGACTACACCGAC--TCCCCGTCCGCTCCGACCTTCCTGCCGTCCTCCCTG
```

```
AB074847      CTGCACTGCCGATTATACAGAC--TCTCCGTCCGCACCAACAGCGCTTCCTTCGTCTTTG
AB070739      TTACCCAGACTATCACCGAGACCATCACAGCCTGCGGTGCCGACTACGCCACTGAAACTG
BX649607      TTGCCCTGCGCAGCACACA-AC----TGCGGCAGCGTCGACAGAGGCTCG--CCAATCGA
AF247649      TGGGCTCTTTGAGCGCAGCAAG----CCTCTTTACGCCGACGTTGCTGCCGGCCAGTTCG
AJ002397      GATCCAACTATTACACGCGCTCAAAGCCCCAGTATGAAACTCTGCCAGTTTCTTCATTCA
AY854012      GTCACATC---TTCACGCGATCCAAGCCTCAGTATGAGAATGTTCCCGCAAACAAGTTCA
AF317733      GAAACGTT---TTTACTCGCTCTAGACCGCAGTATACTGAAGTACCTTTAACTTCCTTCA

                    1681      1691      1701      1711      1721      1731
Glu1          GGTGAATCCCGTACCGC-TGGTCT---GCTGCCGACCCTGCCGCT-GCCGAACATC--CC
AB074847      GGAGAGAGTCGTACTGC-AGGGCT---TTTGCCGACTCTTCCGTT-GCCTAACATC--CC
AB070739      CTGCGGGTTCGTTCGGA-ACTGCC---TCTGACGTCGGAGCGGAGAGTTCGACTACGACC
BX649607      ATGCAGATGCTTACGAG-TCGACTCAGGTAGCAGGCCAGCCCAGTGCAGCCAAGACTACC
AF247649      TCAGCCTGAAGACTGCT-GGTGCCAAGGGTGACGGTAAGACCGATGACACCAAGGCTGTG
AJ002397      GGAGCGTCC-GATCTGCTGGTGCCACCGGTAACGCGGTCACCGATGACACGGCCGCTCTG
AY854012      TCTCTGTGAAGAAATTC-GGAGCTCGCGGCGATGGCGTCGCTGACGATACAGCCGCTATC
AF317733      AAAGTGTAAAGGAC-GCTGGCGCGAAAGGTGATGGTGTCACAGATGACACTGCGGCGATT

                    1741      1751      1761      1771      1781      1791
Glu1          GCTGCTGTCCGGTCTGCTG--TCCGGTTCCCAG-TCCTCCGC--TACCCAGC-CGGCTGG
AB074847      ACTCTTGAGCGGTTTGTTG--TCCGGCTCCCAG-TCATCTGC--CACCCAGC-CCGCTGG
AB070739      GCCGCTG-GTGGTGTGGTGCCTCTGTCGTCGAT-GTCTGTGCAGTACCCCGC-C-GCTGG
BX649607      CCTGTGGTGTACCCCACTCTGTCTGGTGGCCTT-TCCAAGCCTGGTTTTAGTTCTGGTTC
AF247649      CAAGCTGCCATCGATGGTCTCACCGATGGCCAGGTCCTCTGG---ATCGACCAC-GGTGC
AJ002397      CAAAGTGTCATCAACAGTGCCACTGCTTGCGGCCAAATCGTGTACTTTGATGCC-GGTAT
AY854012      CAAGCCGCGGTCAAC---GCCGCAGCCGCCGATGAGGTGGTCTTCTTTCCCCAC-GGCGC
AF317733      CAAGCAGCTTTCAATTCAGCGA-AGCCATC--TGATATAGACTATTTTGACCAC-GGCGC

                    1801      1811      1821      1831      1841      1851
Glu1          TGTTCTGTCCTCCGAAGTTCCGGAACCGACCGCTACCCCGTCCACCCCGGAAGAATTCGA
AB074847      GGTACTGAGCAGCGAGGTCCCCGAGCCCACTGCCACTCCGTCAACTCCAGAGGAGGCTGA
AB070739      AAGCTACAGCTGGGAAGGTTTTGACAGCGAAACCTCTCAGGCCACCACGGCCACCAACGA
BX649607      GACCATCAGCTCTCAAGGGCCATCGAGTGGAAACATGCCTGCCCCACCTGCGGAAACTGT
AF247649      CTACATCCTCTCTGACACCATCGTCATCCCTGCCGAGAAGAACGTCAAGATCGTCGGTGA
AJ002397      CTACCGCATCACCAGCACGCTCAGTATCCCCCCTGGCGCGAAGATTGTTGGAGAGGAATA
AY854012      TTACCTCATCCACGACACCGTAAACATTCCCGCCAACATTCGCATCACGGGAGAGATCTG
AF317733      ATATGTTATCACTAATACTGTTAAAGTACCAAAGGATATTAAGATTACCGGCGAAATATG

                    1861      1871      1881      1891      1901      1911
Glu1          ACCGTCCACCGAAGTTCAGTCCACCCC-GCAGCCGTCCGCTCC--GGCTCAGTCCCAGCC
AB074847      ACCATCTACTGAAGTACAGTCGACGCC-TCAGCCCTCAGCCCC--TGCACAGTCACAGCC
AB070739      GGCTTCTGTCCCTGCTGCCGGAACTTC-TGCTGGCTCGACTCC--TGCTGAGCCTACTTC
BX649607      CGCGGCCGGAAAGCCTCCAGCAGCTCCATCTGGTGGCGGCTCCACTGGAGAGACGCCTGT
AF247649      GACCTACCCTCTCCTCATGGCCACTGGTGCCAACTTCGAGGACA-TGGAGAACCCCAAGC
AJ002397      CCCCATCATCATGTCTTCCGGAA------GCTTCTTCAATGACCAAAGCAACCCGAAGCC
AY854012      GCCCCTGATCATGGCGGCTGGAAA-CA--GCAACTTCCAGGATCAGCGCAACCCGAAGCC
AF317733      GCCGATGATCATGGCTGGCGGTA--CC--GCTTTTTCA--GACGCTTCAAAACCAACACC

                    1921      1931      1941      1951      1961      1971
Glu1          GGAAACCCCGGTTGAATCCACCGTTGCTGCTCCGCTGATCCCGTCCCAGCCGTCCCCGAC
AB074847      AGAGACTCCAGTTGAGTCCACCGTGGCCGCGCCCCTCATTCCCTCCCAGCCATCCCCAAC
AB070739      CCAGTCGGCCGGTGCTTCTATCGT-----CGCCCAGGGCTCCGGCTCTGCCT----CGGC
BX649607      CGTCGTTCCAAATGC-TCCCCAGA-------CCACGAGCACCAAGCCAAATATTC-CCAC
AF247649      CGCTCTTCCAGGTCGGCAAGCAGA----------CAGGTGATAAGGGCGCCTT---CGAG
AJ002397      CGTGGTTCAAGTTGG-----CACA---------CCGGGCCAGACTGGACAGGT---GGAG
AY854012      CATGCTGCGAGTTGG-----CCGA---------GCTGGAGATGTTGGCAATGT---TGAG
AF317733      CGTATTTCAAGTCGG-----CCAA---------GATGGTGATGTAGGATCGGT---TGAG

                    1981      1991      2001      2011      2021      2031
Glu1          CGTTCAGGGTTCCTCCTCCGTTGTTACCGGTCCGGCTTCCTCCTCCGTTGCTCACGCTAC
AB074847      TGTTCAGGGGTCTTCCTCGGTGGTTACTGGTCCAGCTTCGTCTTCGGTCGCTCATGCAAC
AB070739      TGCTACCAGCACCTCGTC-----TTCCGGATCCTACTTCGG-TACATCCAGCAACTCCAC
BX649607      TGCGCAGGCCTCCGCCTCCTCGGCCACGG---CCGCTTCGATAGCCGTGTCCCACAGTGC
AF247649      ATGTCCGACTCCATCATCACCA-CCAAGGGTCCCACTCCCGGTGCTATC-CTCATGGAGT
AJ002397      TGGTCGGACATGATTGTG-TCCACGCAGGGCACCCAAGCTGGAGCTGTT-CTCATTGAGT
AY854012      ATTTCAGACCTGATGCTC-GAGACCAAAGGCGCGCAGCCTGGCGCTATT-CTCCTCGAGT
AF317733      ATATCTGATATGATATTT-GAAACTCAGGGTCCACAGCCAGGTGCTATC-ATGATACAAT
```

```
                2041        2051        2061        2071        2081        2091
Glu1            CAACCAGTGCTCCGTTAAAACCGTTACCAAAACCCGTCTG-CAGACCGCTCTGCCGACCC
AB074847        AAATCAATGCTCCGTCAAAACGGTCACCAAGACTCGTCTC-CAAACTGCTCTCCCAACTC
AB070739        CGGCCAGTGCTCAACCAAGACCGTCACCAAGACCCGTATC-GAGACCACCATTCCCTCCC
BX649607        CGCTGCATGCGCGAGCAAGGCAATCACCAAGACCCGTGTT-CAGACCGTCATGTCTCAAG
AF247649        GGAACA--TCAACGCCGAGAACGGCGCC---GCTGGTCTCTGGGACGTCCACTTCCGCAT
AJ002397        GGAACTTGGCCACCTCTGGAACGC---C--CAGCGGCATGTGGGATGTGCACACGAGAAT
AY854012        GGAACGTCGCAGCAGCAAGCCCGGGATC--TGCCGGACTCTGGGATGTGCATGCACGAGT
AF317733        GGAATGTCGCGCAATCTTCCCCTGGTTC--GGTTGGAATGTGGGATGTTCATGCACGTAT

                2101        2111        2121        2131        2141        2151
Glu1            ACGCTAAACCGTCCTCCCTGCTGAACGG---TGGTAAAGTTTACGAACGTTCCAAACCGC
AB074847        ATGCTAAGCCCAGCTCCCTGCTCAATGG---TGGAAAGGTTTACGAGCGCTCTAAGCCTC
AB070739        AGGCTATGGCCAGCTCTCTTCTTACCAGCTCTGGCAGTGTCTTCGAGAAGTCCAAGCCTT
BX649607        CGACCAAGCCAAGCTCGTTATTGAATGG---CCCGAAGGTGTTTGAGCGATCCAAGCCGC
AF247649        TGGTGGTTTCCAGGGTACTGAGCTCCAA-TCCGACACTTGCAGCAAGCAGAACGGCACCG
AJ002397        CGGCGGCTTCAAGGGATCGAACCTCCAA-GTTGCGCAATGCCCTGTA-ACTGCGAGCAGC
AY854012        TGGCGGTAGCGCTGGTACGGAGCTACA--GTCAAACAAATGCCTGAAGACCCCGAACGTG
AF317733        TGGGGGTTCTGCAGGGTCTAAACTACAA-ACTGAT-ACCTGCTCGAAAAGTCCAACTAGT

                2161        2171        2181        2191        2201        2211
Glu1            TGTACACCTCC-TACGAC----GCTTCC--TCCTTCGT-TTCCGTTAAATCCGCTGGTG-
AB074847        TGTATACCAGC-TACGAT----GCCTCT--TCGTTCGT-CAGCGTCAAGTCTGCGGGCG-
AB070739        TGTACGGTTCG-TATGCC----GCTTCG--TCATTCGT-CAGCGTCAAGTCGGCCGGTG-
BX649607        AGTACGCGGAA-TACGCC----GCTTCG--TCCTTTGT-GAGCGTCAAGTCGGCCGGTG-
AF247649        CGCACGACGCC-AACCCCAAATGCATCGGTTCCTTCATGCAGCTCCACATCACCAAGAAA
AJ002397        ACGACTGTT---AACACGGCTTGTATTGGAGCTTACATGTCCATGCATATCACTGCTAGC
AY854012        AATACACAGCCCAACAAGGACTGCATTGGTGCTTGGATGCTCATGCACATCACCCCTTCT
AF317733        GCGTCAGTA---AACCCAGCATGCGAAGGTGCATTCTTACTTCTGCATGTCACCCCGAAA

                2221        2231        2241        2251        2261        2271
Glu1            -CTA---AAGGTGACGGTTCCACCGACGACACCGCTGCTATCCAGAAAATCCTGAACTCC
AB074847        -CAA---AGGGAGACGGATCGACCGACGATACAGCAGCCATCCAGAAGATTCTGAACAGC
AB070739        -CCA---AGGGTGACGGTACCACCGATGACACGGCTATCATCCAGAGCGTCCTGAACAAC
BX649607        -CCA---AGGGTGATGGCCAGACCGATGACACCGCCGCCATCCAAAAAGTGCTGAATAGC
AF247649        TCTA---GCGGCTACTTCGAGAACGTCTGGCTTTGGACCGCCGACCACGAGCTCGACCTC
AJ002397        GCAAGCAACCTTTACATGGAGAATAACTGGCTCTGGACCGCAGACCACGATATTGA-CGA
AY854012        GCGT-CTGCCTATC--TCGAAAATACTTGGCTGTGGGTGTCTGATCATGAGCTTGATCTC
AF317733        GCTT---CCCTTTACCTAGAGAACAATTGGTTTTGGGTCGCGGATCATCAACTTGATGGA

                2281        2291        2301        2311        2321        2331
Glu1            GCTAAAGAAGAC-CAGATCGTTTACTTCGACCACGGTGCTTACATCATCACCGACACCAT
AB074847        GCCAAGGAGGAC-CAGATAGTTTACTTCGACCACGGAGCCTACATCATCACCGATACCAT
AB070739        GCCACCGAGGAC-CAGATCGTGTACTTCGACCACGGTGCCTACATCATCACCGACACCAT
BX649607        GTGACGGAAGAC-CAGATCGTCTACTTTGATCACGGTGCCTACATCATCACCGACACTAT
AF247649        AAGGACCACAAC-CAGATTG---ACATTTACAACGGACGCGGCATGCTCGTCGAGTCG--
AJ002397        CTCGAGCAACACGCAAATCA---CCATTTTCAGCGGCAGAGGTCTGTACGTTGAAAGCAC
AY854012        CCCGATGAGGGC-CAGATCA---ATATCTATAATGGCCGAGGCTTGCTCGTGGAATCCAC
AF317733        CCTAATTTCAAT-CAACTAT---CTATATACAATGGACGAGGTATACTCATCGAATCGGC

                2341        2351        2361        2371        2381        2391
Glu1            C-AAAGTTCCGAAAAAACGTTAAAATCACCGGTGAAGTTTGGCCGGTTCTGATGGCTTACG
AB074847        C-AAGGTGCCCAAGAACGTCAAGATCACGGGTGAAGTATGGCCCGTCCTGATGGCCTACG
AB070739        C-AAGGTTCCCAAGAACATCAAGATCACTGGTGAGGTGTGGCCCATGCTCATGGCGTATG
BX649607        C-AAAGTGCCCAAGAACATCAAAATCACCGGTGAGGTCTGGCCCCTTCTCATGGCTCACG
AF247649        --CAGGGGCCCGTCTGGCTCTACGGCACCGCTTCGGAGCACTCTGTCCTCTCGCAGTACC
AJ002397        AGCAGGAACGTTCTGGTTTGTTGGG-ACAGCGGTTGAGCATCATACCCTCTACCAGTATC
AY854012        --CAAG--CCTACTTGGCTCTGGGGCACAGCATCGGAACACAACGTGCTATACAACTATC
AF317733        A-AAGGGACCTGTATGGATGTACGGAACTAGTTCGGAGCATAATGTCTTGTACAACTATC

                2401        2411        2421        2431        2441        2451
Glu1            GTCAGAAATTCGGTGACGAAAAAAACCC---GATCCCGATGCTGCAGGTTGG---TGAAG
AB074847        GCCAGAAGTTCGGCGATGAGAAGAACCC---TATCCCCATGCTTCAGGTCGG---AGAAG
AB070739        GCGACAAGTTCTCCGACCAGAAGAACCC---CATCCCTATGCTCCAGGTCGG---TGAGG
BX649607        GCGAGAAATTTGCCGACGAAAAGAACCC---AATTCCCCTACTTCAGATTGG---TCAGC
AF247649        ------ACTTCCAGGGCGCAAAGAAC------AT--C--TACCTCGGCGCCA---TCCAG
AJ002397        ------AATTTGCCAACACGCAGAATATCTATGCCGGAGTTATCCAGACAGAGACACCCT
AY854012        ------AGTTCCAAAACGCGCGCAATGTCTACATGACGGTAATCCAAACCGAGACTGCCT
```

```
AF317733      ------AGATATCGAACGCTAACAATGTGTATATGGCCCTTATCCAGACCGAGACTCCTT

                    2461       2471       2481       2491       2501       2511
Glu1          TTGGTGAAACCGGTTCCGTTGAAATCACCGACATCGCTCTGCAGACC------AAAGGTC
AB074847      TAGGTGAGACCGGCTCCGTCGAAATCACCGACATCGCGCTGCAGACC------AAGGGCC
AB070739      CCGGTGAGTCTGGCTCCGTTGAGATCAGTGACCTCGTCCTGGAGACC------AAGGGCC
BX649607      CCGGCGAAAAGGGCTCTGTGGAGATCACCGACCTCGCCATCCAGACC------AAGGGCC
AF247649      ACCGAGACGCCG--TACTACCAGCCCAACCCCAACGCGCTGCAGCCCT--TCAAGAAGAA
AJ002397      ACTACCAGCCAAATCCCGATGCCCCAACGCCCTTCAATGTCAACACTGCTCTCAACGATC
AY854012      ACTTCCAGGCCAATCCCGATGCCAGAGTTCCTTTTGAGACCCTGTCAGCGTGGAATGACC
AF317733      ATTTCCAAGGTAATCCAGATGCTAGCAAGCCATTTGCTCCTATGGCACAATATCATGATC

                    2521       2531       2541       2551       2561       2571
Glu1          CGGCTCCGGGTGCTATCCTGA--TGCAGTGGAACCTGGCTGAATCCTCCCAGGGTGCTGC
AB074847      CCGCCCCTGGCGCAATCCTAA--TGCAGTGGAACCTCGCGGAGTCCTCCCAGGGCGCCGC
AB070739      CCGCTCCTGGTGCCATCCTGG--TGCAGTGGAACCTCCAAGCTGCTTCCCAAGGCTCTGT
BX649607      CGGCACCTGGGGCAATCCTGA--TGGAATGGAATGTTGCCGAAGCCAGCCAGGGATCTGT
AF247649      CGACAAGTACTTCGACCCGGACATGAACACCAAGAGCGAC-AAGACGGCCTGGGCGGTGC
AJ002397      CCAACTTTGCCACCTCCTGTTCCGGCAGTTCTGGCCGCTGTGCCGAGGCTTGGGGCTTGC
AY854012      CTGACTGGTCTAGCTGCA---CGAGCAA------CAACTGCGCCAAGACCTGGGGTCTTC
AF317733      CAGTCTTTAAT-GGGCCAACTAATGCAA---------A-----CAAGGCCTGGGGCCTTC

                    2581       2591       2601       2611       2621       2631
Glu1          TGGTATGTGGGA-CACCCACTTCCGTATCGGTGGTTCCGCTGGTACCGAACTGCAGTCCG
AB074847      CGGTATGTGGGA-CACCCACTTCCGCATCGGTGGATCAGCAGGCACCGAGCTGCAGAGCG
AB070739      TGGTATGTGGGA-TGTCCATGCCCGCATCGGTGGCTCTGCCGGCACTCAGCTTCAGTCCG
BX649607      GGGTATGTGGGA-TGTGCACTTCCGCATTGGCGGCTCCGCGGGCACGGAATTGCAGAGCG
AF247649      GCATCATCGAGAGCGACAACATCTGGGTCTACGGC---GCGGGCACGTACAGCTTCTTCC
AJ002397      GAATCGTTAGCTCTCAGAACATTCTCATTTATGCT---GCCGGCTTGTATTCGTTCTTCG
AY854012      GGGTTCACGACTCTGACGATATCCTTTTCTACGGC---GGAGGCATGTACTCCTTCTTCG
AF317733      GTGTAACAGATTCCTCTGATGTTTTCGTATATGGA---GCTGGTTTATACAGTTTTTTG

                    2641       2651       2661       2671       2681       2691
Glu1          ACAAATGC-------GCTAAAACCCCG---------AAACAGACCACCACCCCGAACAAA
AB074847      ACAAGTGT-------GCCAAGACGCCG---------AAGCAAACCACCACG---------
AB070739      ACACCTGC-------GCGAAGACCCCC---------AGCCAGACCACCACACCCAACGAG
BX649607      ACAAGTGC-------TCCAAGACGCCC---------AAGATGACCACAACCCCCAACCCG
AF247649      AGGACTACA------GCCAGGACTGCG---------TCGCGACCAACGACTGCCAGGAGC
AJ002397      AGAACAACGATGGAAACACTGGTTGTGACGTGGCTCTTGGGCCGGAAAATTGCCAGAACA
AY854012      AGAATTAC-------ACGCAGGAT-TG-------TCTCGTGACGGAGGACTGTCAGCAGA
AF317733      AAAATTACG------ATCAAACTTGTG---------TAACGGCGGGTAATTGCCAGCCCA

                    2701       2711       2721       2731       2741       2751
Glu1          GAATGCATCGC--TGCTTTCATGCTGATGCACATCACCGAAAAAGCTTCCGCTTACATCG
AB074847      ------------------------------------------------------------
AB070739      AAGTGCATTGG--CGCCTTCCTCCTCTTCCACGCCACCCAGAACGCCAGTGTCTACCTGG
BX649607      CAGTGCATCGG--CGCGTTCATGTTGCTGCATCTTACAGAGCAGGCAAGCGCCTACATCG
AF247649      ACATGAACGAGATCGACGCCAAGTCGACCAACATCAACGTGTTTGGGCTGTCCTCCAAGG
AJ002397      ACATTTTCGACCTTGAAGGCACGCTGACCAACATTAACGTCTACAACTTGGGAACCGTGG
AY854012      ACATGGTTGACATT--ACGAACTCGGAC----ATTGTGCTATATGGTGTAAACACCAAGG
AF317733      ATATGGTCTCACTTGAA--AATTCTTCC-AAAATATACTTCTACGGCTTATCAACAAAGG

                    2761       2771       2781       2791       2801       2811
Glu1          AAAAC---TCCTGGTTCTGGGTTGCTGACCACGAACTGGACCTGCCGGACCACAACCAGA
AB074847      ------------------------------------------------------------
AB070739      AGAAC---ACCTGGTTCTGGACTGCTGACCACGACCTTGACGCTTCCGGCCACGGACAGA
BX649607      AGAAC---GCTTGGTTCTGGACGGCGGACCACGAGCTCGATCTGGCGGACCACAACCAGA
AF247649      CATCCGTCAACATGATCACGCAGGACGGCAAGGGCCTTGCGCTCGACAGCGACAACAGGT
AJ002397      GCGTCGTCAACCAGATCACGCAGAACGGAAACGTGCTTGCGA--CCTCTTCTTC-CAACG
AY854012      CCTCATCCAATGTGATTACACAGAACGGCGCAGGAGTTGTGAAGCATCTGCCTGACAACC
AF317733      CTTCTTCCGCCATGGTTACTATCAACGGAGCCCAAACCGCAATGGACAAAGAT---AATA

                    2821       2831       2841       2851       2861       2871
Glu1          TCAACGTTTACAACGGTCGTGGTGTTTACATCGAATCCCAGGGTCCGGTTTGGCTGTACG
AB074847      ------------------------------------------------------------
AB070739      TTAACATTTACAACGGCCGCGGTATCCTCCTCGAGAGTCAGGGTCCAGCTTGGCTGTATG
BX649607      TCAACATCTACAACGGTCGTGGTGTCCTGATAGAGAGCAATGGACCCGTCTGGTTGTATG
AF247649      CCAACTTCTGCGCCACGCTGGGCATCTTTGCCCAGGCGTAGTTGTGTCCGCGCCTGCTCC
```

```
AJ002397      TCAATGCCTTTGCTGATGTTATTGCTCTCTTTC-GACTCGCTTCCGGAAGCGGAGGCGTG
AY854012      TCAATACCTTCTGCGCTGCAATTGCGGAATACC-GTCCATCATGG--------------
AF317733      GAAACAACTTTTGTGCTACTATTGCTAGGTT-----CTCTATTTAAGATTAGGCGGTATC

                    2881      2891      2901      2911      2921      2931
Glu1          GTACCGCTTCCGAACACAACCAGCTGTACAACCAGCTGTACAACTACCAGGTTACCAACG
AB074847      ------------------------------------------------------------
AB070739      GCACGGCTGCCGAACACAACCAGCTGTACAAC------------TACCAGGTCTCTAACG
BX649607      GCACGGCCTCGGAACACCACCAGCTGTACAAC------------TACCAGGTTGCGAACG
AF247649      TCAGGCTGACGAGTCTCCTATTTCTCTTCTTC----TT------TTCGCCGCGAGATACC
AJ002397      ACTCCTCCTCCTTCTAGCACCACCAAAGCACAGT---------CCACAACATTTTCCACA
AY854012      ------------------------------------------------------------
AF317733      TTGCTGGCTGGTTCCCAATTTACACCTGCTTCGTT--T-----CCATTATACTGTGAATA

                    2941      2951      2961      2971      2981      2991
Glu1          CTAAAAACGTTTTCATGGGTCTGATCCAGACCGAAACCCCGTACTACCAGGCTAACCCGA
AB074847      ------------------------------------------------------------
AB070739      CCAAGAACGTCTTCATGGGCTTGATCCAGACCGAGACTCCTTACTACCAGGCCAACCCCA
BX649607      CCCAAAACGTGTTCATGGCCTTGATCCAGACCGAGACGCCATACTACCAGTCCAACCCAG
AF247649      TCCTCGATTTAGACTTTGATTCTTTCCCTCTTGCGGGGCCCTATTGGTTAGCATAGCACA
AJ002397      ATCATCACCAGCTCGCCG--CCGAAGCAAACTGGATGGAATTTCTTGGGATGCTACTCCG
AY854012      ------------------------------------------------------------
AF317733      TTTTTAATGTATTTAAAT--TACTCGGGGATTGATTTTAGTAAGAAGG--CGTTATAGAA

                    3001      3011      3021      3031      3041      3051
Glu1          ACGCTCTGACCCCGTTCACCCCGCAGAC-CAACTGGAACGACCCGGACT--TCTCCTACT
AB074847      ------------------------------------------------------------
AB070739      ACGCTCTGACTCCCTTCACTCCGCAGAC-CTCCTGGTCCGACCCCGACT--TCTCGTACT
BX649607      ACGCACTGACACCCTTCACGCCCCAGAC-CACATGGAACGATCCTACCT--TCTCCCACT
AF247649      AAGCA-TGTA----TTTACCTTTCTTACTCTCGTTCAAGAACCTTGACTG-TCGCGCAAA
AJ002397      ATAACGTCAACGGTCGAACTTTGGCCAACCAGGTCCAGGTCGCAGGAGGTGCATCTGCCA
AY854012      ------------------------------------------------------------
AF317733      ATGACCGTATGCATTGAA------------------------------------------

                    3061      3071      3081      3091      3101      3111
Glu1          GCAAAACCGACGGTTGCCGTAAAGCTTGGGGTCTGCGTGTTCAGAACACCTCCGACATGT
AB074847      ------------------------------------------------------------
AB070739      GCACGACCGACTCCTGCCGCAAGGCCTGGGGTCTCCGTATCATCAACAGCTCCGAGACCT
BX649607      GCACAACCGCCTCGTGCCGCAAGGCCTGGGGTCTGCGCGTCATGAACACCTCAGATCTGT
AF247649      GCACAGCACCAGCTAAGCATATGTTATA---CCCGAGTTGCTGGAAGTGATGGGATATGG
AJ002397      TGTCGATCGAGGCTTGTGAAACTGCTTCGGAATCCGCCGGATACACCATTGCTGGCGTCG
AY854012      ------------------------------------------------------------
AF317733      ------------------------------------------------------------

                    3121      3131      3141      3151      3161      3171
Glu1          ACGTTTACGGTGCTGGTCTGTACTCCTTCTTCGAAAACTACGGTCAGACCTGCCTGGCTA
AB074847      ------------------------------------------------------------
AB070739      ACATCTACGGTGCCGGTCTC----------------------------------------
BX649607      TCGTGTACGGGGCGGGTCTGTACAGCTTCTTCGAGAACTATGGCCAGACATGCTTGGATA
AF247649      TGGTAAACTGAAGTGAAGGGGTTGCATAGGCGGAGGATGAAGGACGCGCATACA-GTATG
AJ002397      AATACTCTGGAGAATGCTGGTGTGATACCAAGTTCCAGAACGGCGGCGGCCCAGCCTCTG
AY854012      ------------------------------------------------------------
AF317733      ------------------------------------------------------------

                    3181      3191      3201      3211      3221      3231
Glu1          CCGAATCCTGCCAGGAAAACATGGTTGAAGTTGACTGCTCCGACGTTCACATCTACGGTC
AB074847      ------------------------------------------------------------
AB070739      ------------------------------------------------------------
BX649607      CCGAGTCGTGCCAGGAAAACATGGTGGAGGTGGACTGCTCGAATGTCCATTTGTACGGAC
AF247649      TAGCAACCGAACGAAATGCAATGAAAGATGAACACATCCAAAAAAAAAAAAAAAAA----
AJ002397      ATGGAAGTGCTCAATGCACCATGACTTGCAGCGGTGCGCCACAGGAAACCTGCGGAGGCC
AY854012      ------------------------------------------------------------
AF317733      ------------------------------------------------------------

                    3241      3251      3261      3271      3281      3291
Glu1          TGTCCACCAAAGCTTCCACCAACATGATCACCTCCAACTCCGGTGCTGGTCTGGTTCCGC
AB074847      ------------------------------------------------------------
AB070739      ------------------------------------------------------------
```

```
BX649607      TAAGCACCAAGGCCAGCGTCAACATGATCACGTCGTCCAACGGCGCGGGGGATTGTGCCAC
AF247649      ------------------------------------------------------------
AJ002397      CGAACCGCCTCGATGTTTACAGCTTGGCGACCGCAACTGGGAGCGCCTCGCCTCCCGCGG
AY854012      ------------------------------------------------------------
AF317733      ------------------------------------------------------------

                 3301      3311      3321      3331      3341      3351
Glu1          AGGACGAAAACCGTTCCAACTTCTGCTCCACCCTGGCTCTGTTCCAGCAGTCCGTTACA-
AB074847      ------------------------------------------------------------
AB070739      ------------------------------------------------------------
BX649607      AGGACGAGAACGAGAGTAACTTTTGCTCTACTATTGCTCTCTTTGAACAATCGTGA----
AF247649      ------------------------------------------------------------
AJ002397      CCACCGGATGGAACTTTAGAGGATGCTATACCGACAGTGTCAATGCTCGAGCGTTGATTG
AY854012      ------------------------------------------------------------
AF317733      ------------------------------------------------------------

                 3361      3371      3381      3391      3401      3411
Glu1          ------------------------------------------------------------
AB074847      ------------------------------------------------------------
AB070739      ------------------------------------------------------------
BX649607      ------------------------------------------------------------
AF247649      ------------------------------------------------------------
AJ002397      CCGAGTCGGTCCCCAACGGCCCCTCGTCGATGACTATAGAGGCGTGTCAAAGTGTGTGCA
AY854012      ------------------------------------------------------------
AF317733      ------------------------------------------------------------

                 3421      3431      3441      3451      3461      3471
Glu1          ------------------------------------------------------------
AB074847      ------------------------------------------------------------
AB070739      ------------------------------------------------------------
BX649607      ------------------------------------------------------------
AF247649      ------------------------------------------------------------
AJ002397      AGGGCCTGGGCTACACTCTTGCGGGCCTTGAGTATGCCGACGAATGCTACTGTGGTAACA
AY854012      ------------------------------------------------------------
AF317733      ------------------------------------------------------------

                 3481      3491      3501      3511      3521      3531
Glu1          ------------------------------------------------------------
AB074847      ------------------------------------------------------------
AB070739      ------------------------------------------------------------
BX649607      ------------------------------------------------------------
AF247649      ------------------------------------------------------------
AJ002397      GCCTCGCCAATGGCGCAACCATTGCCCCAGATGGAAATGCCGGTTGCAACATGAATTGCG
AY854012      ------------------------------------------------------------
AF317733      ------------------------------------------------------------

                 3541      3551      3561      3571      3581      3591
Glu1          ------------------------------------------------------------
AB074847      ------------------------------------------------------------
AB070739      ------------------------------------------------------------
BX649607      ------------------------------------------------------------
AF247649      ------------------------------------------------------------
AJ002397      CGGGCAACGCTGCTGAAACATGCGGCGGTCCTAACAGGTTGGACATTTACAGTTACGGAC
AY854012      ------------------------------------------------------------
AF317733      ------------------------------------------------------------

                 3601      3611      3621
Glu1          -------------------------
AB074847      -------------------------
AB070739      -------------------------
BX649607      -------------------------
AF247649      -------------------------
AJ002397      AGGCCAACGGCACCCAGCCACTTTAA
AY854012      -------------------------
AF317733      -------------------------
```

Figure 6.14- Nucleotide alignment of *Glu1* and other glucanases in the database. AB074847 *A. oryzae* exgO gene exo 1,3-glucanase; AB070739 *A. phoenicis* exgS glucanase; BX649607

A.fumigatus glucanase; AF247649 *C. minitans* β-1, 3-glucanase; AJ002397 *T. harzianum* β-1, 3-glucanase; AY854012 *Acremonium* sp. β-1, 3-glucanase; AF317733 *B. graminis* 1, 3-β-glucanase.

The nucleotide sequence and its translation product are shown in Fig. 6.15. Two highly conserved regions were found in *Glu1*. These highly conserved regions of the exo-glucanases are reported to include putative substrate binding regions (Nikolskaya *et al.* 1998). A signal peptide cleavage site was also identified near the 5' end of the gene followed by the N-terminal that showed 100% identity to the N-terminal sequence of the exo-glucanase of *A. oryzae* (accession number BAB92972). Seven putative asparagine-linked glycosylation sites (Asn-X-Thr/Ser) were also found within the sequence (Fig 6.15, underlined)

```
1   atgctgttcctggctcacgttctgctgctgctgggtctgccggctggtatggttggtgct...60
1    M  L  F  L  A  H  V  L  L  L  L  G  L  P  A  G  M  V  G  A    20
61  gttccgctgggtcaggaaaccgacatcaccaccaacctggctgctcgtgctgcttccgaa..120
21   V  P  L  G  Q  E  T  D  I  T  T  N  L  A  A  R ↓A  A  S  E    40
121 tactgggttggtaccatcaaacgtcagggtgctgttgctttcggtaacggtaccgactac  180
41   Y  W  V  G  T  I  K  R  Q  G  A  V  A  F  G  N  G  T  D  Y    60
181 caggtttaccgtaacgttaaagacttcggtgctaaaggtgacggttccaccgacgacacc  240
61   Q  V  Y  R  N  V  K  D  F  G  A  K  G  D  G  S  T  D  D  T    80
241 gctgctatcaaccaggctatctcctccggtaaccgttgcggtaaaggttgcgactcctcc  300
81   A  A  I  N  Q  A  I  S  S  G  N  R  C  G  K  G  C  D  S  S   100
301 accgttaccccggctctggtttacttcccgccgggtacctacgttgtttccaaaccgatc  360
101  T  V  T  P  A  L  V  Y  F  P  P  G  T  Y  V  V  S  K  P  I   120
361 gttcagtactactacacccagatcgttggtgacgctgttaacctgccggttatcaaagct  420
121  V  Q  Y  Y  Y  T  Q  I  V  G  D  A  V  N  L  P  V  I  K  A   140
421 gctgctggtttcgctggtatggctgttatcgacgctgacccgtacgaagacgacggttcc  480
141  A  A  G  F  A  G  M  A  V  I  D  A  D  P  Y  E  D  D  G  S   160
481 aactggtacaccaaccagaacaacttcttccgtgctatccgtaacatcgttctggacatc  540
161  N  W  Y  T  N  Q  N  N  F  F  R  A  I  R  N  I  V  L  D  I   180
541 accgctatgccgcagggttccggtgctggtctgcactggcaggttggtcaggctacctcc  600
181  T  A  M  P  Q  G  S  G  A  G  L  H  W  Q  V  G  Q  A  T  S   200
601 ctgcagaacatccgtttcgaaatgatcaaaggtggtggtgacgctaacaaacagcagggt  660
201  L  Q  N  I  R  F  E  M  I  K  G  G  G  D  A  N  K  Q  Q  G   220
661 atcttcatggacaacggttccggtggtttcatgtccgacctgaccttcaacggtggtaac  720
221  I  F  M  D  N  G  S  G  G  F  M  S  D  L  T  F  N  G  G  N   240
721 tacggtatgttcctgggtaaccagcagttcaccacccgtaacctgaccttcaacgactgc  780
241  Y  G  M  F  L  G  N  Q  Q  F  T  T  R  N  L  T  F  N  D  C   260
781 aacaccgctatcttcatgaactggaactgggcttggaccttcaaatccctgtccatcaac  840
261  N  T  A  I  F  M  N  W  N  W  A  W  T  F  K  S  L  S  I  N   280
841 aactgccaggttggtctgaacatgtccaacttcccgcagaaccagaccgttggttccgtt  900
```

```
281  N  C  Q  V  G  L  N  M  S  N  F  P  Q  N  Q  T  V  G  S  V   300
901 ctgatcctggactcccagctgaccaacaccccgaccggtgttgtttccttcgctaccgaa  960
301  L  I  L  D  S  Q  L  T  N  T  P  T  G  V  V  S  F  A  T  E   320
961 aactccatcccgatcggtggtggtgttctgatcctggacaacgttgacttctccggttcc 1020
321  N  S  I  P  I  G  G  G  V  L  I  L  D  N  V  D  F  S  G  S   340
1021aaagttgctgttgctggtatcaccggtaacaccatcctggctggtggttccgttgttacc 1080
341  K  V  A  V  A  G  I  T  G  N  T  I  L  A  G  G  S  V  V  T   360
1081aactgggttcagggtaacggttacctgccgggttccgctaaacagaaacgtgaagcttcc 1140
361  N  W  V  Q  G  N  G  Y  L  P  G  S  A  K  Q  K  R  E  A  S   380
1141gttaaagttaccacccagaccgttaccgaaaccgttgaagtttgcaccgctgactacacc 1200
381  V  K  V  T  T  Q  T  V  T  E  T  V  E  V  C  T  A  D  Y  T   400
1201gactccccgtccgctccgaccttcctgccgtcctccctgggtgaatcccgtaccgctggt 1260
401  D  S  P  S  A  P  T  F  L  P  S  S  L  G  E  S  R  T  A  G   420
1261ctgctgccgaccctgccgctgccgaacatcccgctgctgtccggtctgctgtccggttcc 1320
421  L  L  P  T  L  P  L  P  N  I  P  L  L  S  G  L  L  S  G  S   440
1321cagtcctccgctacccagccggctggtgttctgtcctccgaagttccggaaccgaccgct 1380
441  Q  S  S  A  T  Q  P  A  G  V  L  S  S  E  V  P  E  P  T  A   460
1381accccgtccacccccggaagaattcgaaccgtccaccgaagttcagtccaccccgcagccg 1440
461  T  P  S  T  P  E  E  F  E  P  S  T  E  V  Q  S  T  P  Q  P   480
1441tccgctccggctcagtcccagccggaaaccccggttgaatccaccgttgctgctccgctg 1500
481  S  A  P  A  Q  S  Q  P  E  T  P  V  E  S  T  V  A  A  P  L   500
1501atcccgtcccagccgtccccgaccgttcagggttcctcctccgttgttaccggtccggct 1560
501  I  P  S  Q  P  S  P  T  V  Q  G  S  S  S  V  V  T  G  P  A   520
1561tcctcctccgttgctcacgctaccaaccagtgctccgttaaaaccgttaccaaaacccgt 1620
521  S  S  S  V  A  H  A  T  N  Q  C  S  V  K  T  V  T  K  T  R   540
1621ctgcagaccgctctgccgacccacgctaaaccgtcctccctgctgaacggtggtaaagtt 1680
541  L  Q  T  A  L  P  T  H  A  K  P  S  S  L  L  N  G  G  K  V   560
1681tacgaacgttccaaaccgctgtacacctcctacgacgcttcctccttcgtttccgttaaa 1740
561  Y  E  R  S  K  P  L  Y  T  S  Y  D  A  S  S  F  V  S  V  K   580
1741tccgctggtgctaaaggtgacggttccaccgacgacaccgctgctatccagaaaatcctg 1800
581  S  A  G  A  K  G  D  G  S  T  D  D  T  A  A  I  Q  K  I  L   600
1801aactccgctaaagaagaccagatcgtttacttcgaccacggtgcttacatcatcaccgac 1860
601  N  S  A  K  E  D  Q  I  V  Y  F  D  H  G  A  Y  I  I  T  D   620
1861accatcaaagttccgaaaaacgttaaaatcaccggtgaagtttggccggttctgatggct 1920
621  T  I  K  V  P  K  N  V  K  I  T  G  E  V  W  P  V  L  M  A   640
1921tacggtcagaaattcggtgacgaaaaaaacccgatcccgatgctgcaggttggtgaagtt 1980
641  Y  G  Q  K  F  G  D  E  K  N  P  I  P  M  L  Q  V  G  E  V   660
1981ggtgaaaccggttccgttgaaatcaccgacatcgctctgcagaccaaaggtccggctccg 2040
661  G  E  T  G  S  V  E  I  T  D  I  A  L  Q  T  K  G  P  A  P   680
2041ggtgctatcctgatgcagtggaacctggctgaatcctcccagggtgctgctggtatgtgg 2100
681  G  A  I  L  M  Q  W  N  L  A  E  S  S  Q  G  A  A  G  M  W   700
2101gacacccacttccgtatcggtggttccgctggtaccgaactgcagtccgacaaatgcgct 2160
701  D  T  H  F  R  I  G  G  S  A  G  T  E  L  Q  S  D  K  C  A   720
2161aaaaccccgaaacagaccaccaccccgaacaaagaatgcatcgctgctttcatgctgatg 2220
721  K  T  P  K  Q  T  T  T  P  N  K  E  C  I  A  A  F  M  L  M   740
2221cacatcaccgaaaaagcttccgcttacatcgaaaactcctggttctgggttgctgaccac 2280
741  H  I  T  E  K  A  S  A  Y  I  E  N  S  W  F  W  V  A  D  H   760
2881gaactggacctgccggaccacaaccagatcaacgtttacaacggtcgtggtgtttacatc 2340
761  E  L  D  L  P  D  H  N  Q  I  N  V  Y  N  G  R  G  V  Y  I   780
2341gaatcccagggtccggtttggctgtacggtaccgcttccgaacacaaccagctgtacaac 2400
781  E  S  Q  G  P  V  W  L  Y  G  T  A  S  E  H  N  Q  L  Y  N   800
2401cagctgtacaactaccaggttaccaacgctaaaaacgttttcatgggtctgatccagacc 2460
801  Q  L  Y  N  Y  Q  V  T  N  A  K  N  V  F  M  G  L  I  Q  T   820
2461gaaaccccgtactaccaggctaacccgaacgctctgaccccgttcaccccgcagaccaac 2520
821  E  T  P  Y  Y  Q  A  N  P  N  A  L  T  P  F  T  P  Q  T  N   840
2521tggaacgacccggacttctcctactgcaaaaccgacggttgccgtaaagcttggggtctg 2580
841  W  N  D  P  D  F  S  Y  C  K  T  D  G  C  R  K  A  W  G  L   860
2581cgtgttcagaacacctccgacatgtacgtttacggtgctggtctgtactccttcttcgaa 2640
861  R  V  Q  N  T  S  D  M  Y  V  Y  G  A  G  L  Y  S  F  F  E   880
2641aactacggtcagacctgcctggctaccgaatcctgccaggaaaacatggttgaagttgac 2700
881  N  Y  G  Q  T  C  L  A  T  E  S  C  Q  E  N  M  V  E  V  D   900
```

```
2701tgctccgacgttcacatctacggtctgtccaccaaagcttccaccaacatgatcacctcc 2760
901  C  S  D  V  H  I  Y  G  L  S  T  K  A  S..T..N..M..I..T..S.  920
2761aactccggtgctggtctggttccgcaggacgaaaaccgttccaacttctgctccaccctg 2820
921  N  S  G  A  G  L  V  P  Q  D  E  N  R  S  N  F  C  S  T  L  940
2821gctctgttccagcagtccgttaca 2844
941  A  L  F  Q  Q  S  V  T.. 948
```

Figure 6.15-Nucleotide sequence of *Glu1* and its deduced amino acid sequence. The arrow indicates the signal peptide cleavage site at residue 36. Two highly conserved regions are indicated in bold. The N-terminal sequences are indicated in bold and underlined. Seven putative asparagine-linked glycosylation sites are underlined.

6.3.4.2 Amino acid comparison with other glucanases

An alignment of the deduced amino acid sequence of *Glu1* revealed a 949 amino acid sequence homologous to an exo-1, 3-glucanase from *A. oryzae* (99% identity) (accession number BAB92972) which is a 946 amino acid protein (Oda *et al.* 2001), followed by an exo-1, 3-glucanase ExgO from *A. fumigatus* Af293 (77% identity)(accession number CAD29605) (Nierman *et al.* 2005) (Fig 6.16). Comparison of the amino acid sequences revealed that *Glu1* shares high homology with other glucanases from *Aspergillus* spp. but shares low homology with the other glucanases in the database. There is a region between amino acids at position ~407 and at position ~600 where little homology is observed between *Glu1* and glucanases from *B. graminis*, *Acremonium* sp., *T. harzianum* and *C. minitans* (Fig 6.16)

```
                1         11        21        31        41        51
Glu1            ----MLFLAHVLLLLG----LPAGMVGAVPLGQETDITTNLAARAASEYWVGTIKR--QG
BAB92972        ----MLFLAHVLLLLG----LPAGMVGAVPLGQETDITTNLAARAASEYWVGTIKR--QG
CAD29605        ----MAFLRTPIVLLLTQILLFLGLVNAVPTPESLDVHGNLARDTASSFWVANIKR--QG
BAB83607        ----MLFVSYVCLLLS----VPVALVNAVPTAGSVDVTSHLAVR-ASDYWVGTIKR--QG
AAF63758        ----MLAFSAGAFLLT----LRVFLTATPSAAAPVAQAVEVPQAGASGYWFGNIKR--QG
AAL26904        ----MARFTTLLFVFH----Q--LLPFATSSPAPIINQVAPHTAADSNFWLESIAR--KG
AAW47927        -MKFHALIQKSLLALS----AIGSIALASPL-APRNETLEAEAR-ASDWWFGNIER--TG
CAA05375        ----MGFIRSAVLSAL------TFAAACRGLATPGSEAEPSVEKRASSYWYENIAH--QG

                61        71        81        91        101       111
Glu1            AVAFGNGTD-YQVYRNVKDFGAKGDGSTDDTAAINQAISSGN--------RCGKGCDSST
BAB92972        AVAFGNGTD-YQVYRNVKDFGAKGDGSTDDTAAINQAISSGN--------RCGKGCDSST
CAD29605        AVAFGQSSG-YQVYRNVKDFGAKGDGVTDDTAAINQAISSGN--------RCGKGCDSST
BAB83607        TVPFGNTTSSYAIYRNVKDYGAKGDGSTDDTDAINKAISSGG--------RCGSGCDSST
AAF63758        IAPYNENPAAYKVFRNVKLLGAKGDGVTDDTAAINAAIADGN--------RCGQGCDSTT
AAL26904        KVAYGTSSH--QVFRNVKDFGAKGDGTTDDTAAINAAITQGS--------RCAQGCDSST
AAW47927        RVPFGSNSN-YPVFRNVKDYGARGDGSTDDTAAINAAITAGG-------GRCGLGCKSST
CAA05375        IAPFAPSNY--TVFRNVKDYGAKGDGVTDDTAAINNAILSGG--------RCGRLCTSST

                121       131       141       151       161       171
Glu1            VTPALVYFPPGTYVVSKPIVQYYYTQIVGDAVNLPVIKAAAGFAGMAVIDADPYE--DDG
BAB92972        VTPALVYFPPGTYVVSKPIVQYYYTQIVGDAVNLPVIKAAAGFAGMAVIDADPYE--DDG
```

```
CAD29605        TTPALVYFPPGTYVVSKPIIQYYYTQIVGDAVDLPVIKASADFTGMAVIDADPYE--NDG
BAB83607        TTPALVYFPAGTYVVSKPIVQYYYTQMVGDANDLPVLKAASSFSGMAVIDADPYD--SNG
AAF63758        TSPAIIYFPAGTYLISEPIIQYYYTQFVGDATNPPTLKAKDTFEGMGLIDADPYIPGGDG
AAL26904        ITPALVYFPSGTYSISEPIVQLYYTQFVGNAVTPPTIKATSGFKGMALIDSNPYT-----
AAW47927        NTPALVFFPPGTYLVSQPIIGYYYTHIVGDLKDIPTIKATSNFQGSAVIDANPYANTGN-
CAA05375        LTPAVVYFPAGTYVISTPIIDQYYTNIIGDPTNLPTIKATAGFSGIALIDGDTYYGDNNP

                181       191       201       211       221       231
Glu1            S--NWYTNQNNFFRAIRNIVLDITAMPQ--GSGAGLHWQVGQATSLQNIRFEMIKGG--G
BAB92972        S--NWYTNQNNFFRAIRNLVIDLTAMPQ--GSGAGIHWQVGQATSLQNIRFEMIKGG--G
CAD29605        S--NWYTNQNNFFRAIRNLVIDLRAMPM--NSGAGIHWQVGQATSLQNIRFEMIRGG--G
BAB83607        D--NWYTNQNNFFRAVRNFVIDLTAMPA--SSGAGIHWQVGQATSLQNIRFEMVKGG---
AAF63758        A--NWYTNQNNFYRQIRNFVIDIKDTK----AAAGIHWQVSQATSLQNIRFEMATG--E-
AAL26904        -------EGNNFYRQIRNFVIDTTAMPA--DRGAGIHWQTAQATSLQNIVFNMRTDG--G
AAW47927        ---NWFQNQNNFFRQIRNLKIDIRSQPM--NTGTGIHWQVAQATSLQNIEFYMVENS--S
CAA05375        NDPNWIS-TNVFYRQVRNFKLDMTSIPTSAPKIYGIHWPTAQATSLQNIQITMSTAS---

                241       251       261       271       281       291
Glu1            DANKQQGIFMDNGSGGFMSDLTFNGGNYGMFLGNQQFTTRNLTFNDCNTAIFMNWNWAWT
BAB92972        DANKQQGIFMDNGSGGFMSDLTFNGGNYGMFLGNQQFTTRNLTFNDCNTAIFMNWNWAWT
CAD29605        DANKQQGIFMDNGSGGFMSDLTFNGGNYGMFVGNQQFTTRNLSFNDCNTAVFMNWNWAWT
BAB83607        TNNKQQGIFMDNGSGGFMSDLTFNGGNYGAFFGNQQFTTRNLTFNDCNTAIYMNWNWAWT
AAF63758        AGANQKGIFQDNGSGGFMSDLVFNGGAIGAFLGSQQFTTRNMTFNNCGTAIFMNWNWLWT
AAL26904        TNNAQLGIFMDNGSGGFMSDLTFNGGKYGAFFGSQQFTTRNLIFNNCQTAIFLNWNWAWT
AAW47927        SENRQQGIFMENGSGGFMTDLKFFGGKYGAVFGSQQFTARNLEFNNAQTGILLIWNWIWN
CAA05375        -GNSQVGLFIENGSAGFLTDMTFNGGLIGAAIGNQQYTMRNLVFNNCAQPLSAASIGSGF

                301       311       321       331       341       351
Glu1            FKSLSINNCQVGLNMSNFPQ--NQTVGSVLILDSQLTNTPTGVVSFATENSIPIGGGVLI
BAB92972        FKSLSINNCQVGLNMSNAPQ--NQTVGSVLILDSQLTNTPTGVVSAFTENSIPIGGGVLI
CAD29605        FKSISINNCQVGLNMSNAPQ--NQTVGSVLLLDSSVTNTPTGVVTAFTQDSIPVGGGVLI
BAB83607        FKSVSINNCQVGLNMSASPS--NQTVGSVLMLDSTFTSTPTGVVTAFSSDSIPTGGGALV
AAF63758        LKSIFINDCKLGLDMANSPD--NQTVGSVLLLDSKFTNTPIGINSSFTQDSVPHTGGTLI
AAL26904        LSGVAINNAGIGIDMSNGGSG-AQTVGSVLLVDSKISNTPIGVSTAYS-TSQSVTNGTLI
AAW47927        FHGLKVNNCGIGIDLTTGGSGASQSVGSTIVQDATFTGTRVGITTTYD-RNQKGTFGTLV
CAA05375        TRAISINNCGLGIDMTAAES--------ITLIDSSISGTPVGIKTSFRRNQSPATSNSLI

                361       371       381       391       401       411
Glu1            LDNVDFSG-SKVAVAGITGN-TILAGGSV-VTNWVQGNGYLPGSAKQKREAS--------
BAB92972        LDNVDFSG-SKVAVAGITGN-TILAGGSV-VTNWVQGNGYLPGSAKQKREAS--------
CAD29605        LENVDFTG-SNVAVAGISGN-TILKGGSV-VASWIQGNTYSPANSLNKRATQGK------
BAB83607        LENVDFSG-STVAIASVNGD-TILKGGSV-VSNYVQGNVYTPGSSSNSNNNSTVTKRSPA
AAF63758        IDNVDFEG-SNVAVQNVAGE-TLLAGKSK-VATWAQGNAMAAGQAQAG------------
AAL26904        LDNVDFSENVPVAISDAASKNTILAGNAK-ISSWAQGHQYEAGTQGR-------------
AAW47927        IDNTDFVN-TGIAVQNPSTGATILGGNRK-VGSFVQGRSYTG-SNGQ-------------
CAA05375        VENLSLNN-VPVAIQSSSGS-TILAGGTTTIAAWGQGHQYTPNGPTT-------------

                421       431       441       451       461       471
Glu1            -----VKVTTQTVTETVEVCTADYTDSPSAPTFLPSSLGESRTAGLLPTLP--LPNIPLL
BAB92972        -----VKVTTQTVTETVEVCTADYTDSPSAPTALPSSLGESRTAGLLPTLP--LPNIPLL
CAD29605        -----VRVVTETVVETVLACPAQHT-TAAASTEARQSNADAYESTQVAGQPSAAKTTPVV
BAB83607        VQAEDVQVITQTITETITACGADYATETAAGSFGTASDVGAESSTTTAAGG----VVPLS
AAF63758        ------------------------R--VQG-------------------------------
AAL26904        ----------------------------------------------------------------
AAW47927        ----------------------------------------------------------------
CAA05375        ----------------------------------------------------------------

                481       491       501       511       521       531
Glu1            SGLLSGSQSSATQPAG-VLSSEVPEPTATPSTPEEFEPSTEVQSTPQPSAPAQSQPETPV
BAB92972        SGLLSGSQSSATQPAG-VLSSEVPEPTATPSTPEEAEPSTEVQSTPQPSAPAQSQPETPV
CAD29605        YPTLSGGLSKPGFSSGSTISSQGPSSGNMPAPPAETVAAGKPPAAPSGGG---STGETPV
BAB83607        S------MSVQYPAAG-SYSWEGFDSETSQATTATNEAS--VPAAGTSAG---STPAEPT
AAF63758        ------------------------------------DVNNP---------------------
AAL26904        -----------------------------------AVQEN---------------------
AAW47927        ----------------------------------VVQGE---------------------
CAA05375        ----------------------------------FQGS---------------------

                541       551       561       571       581       591
Glu1            ESTVAAPLIPSQP-SPTVQGSSSVVTGPASSSVAHATNQCSVKTVTKTRLQTALPTHAKP
BAB92972        ESTVAAPLIPSQP-SPTVQGSSSVVTGPASSSVAHATNQCSVKTVTKTRLQTALPTHAKP
CAD29605        VVPNAPQTTSTKPNIPTAQASASSAT-AASIAVSHSAAACASKAITKTRVQTVMSQATKP
BAB83607        SQSAGASIVAQGSGSASAATSTSSSG-SYFGTSSNSTGQCSTKTVTKTRIETTIPSQAMA
AAF63758        -----------P-------------------------------------------TKP
AAL26904        ----------------------------------------------------LAATSKP
AAW47927        ----------------------------------------------------RPAPSKP
CAA05375        ----------------------------------------------------ITPNSRP
```

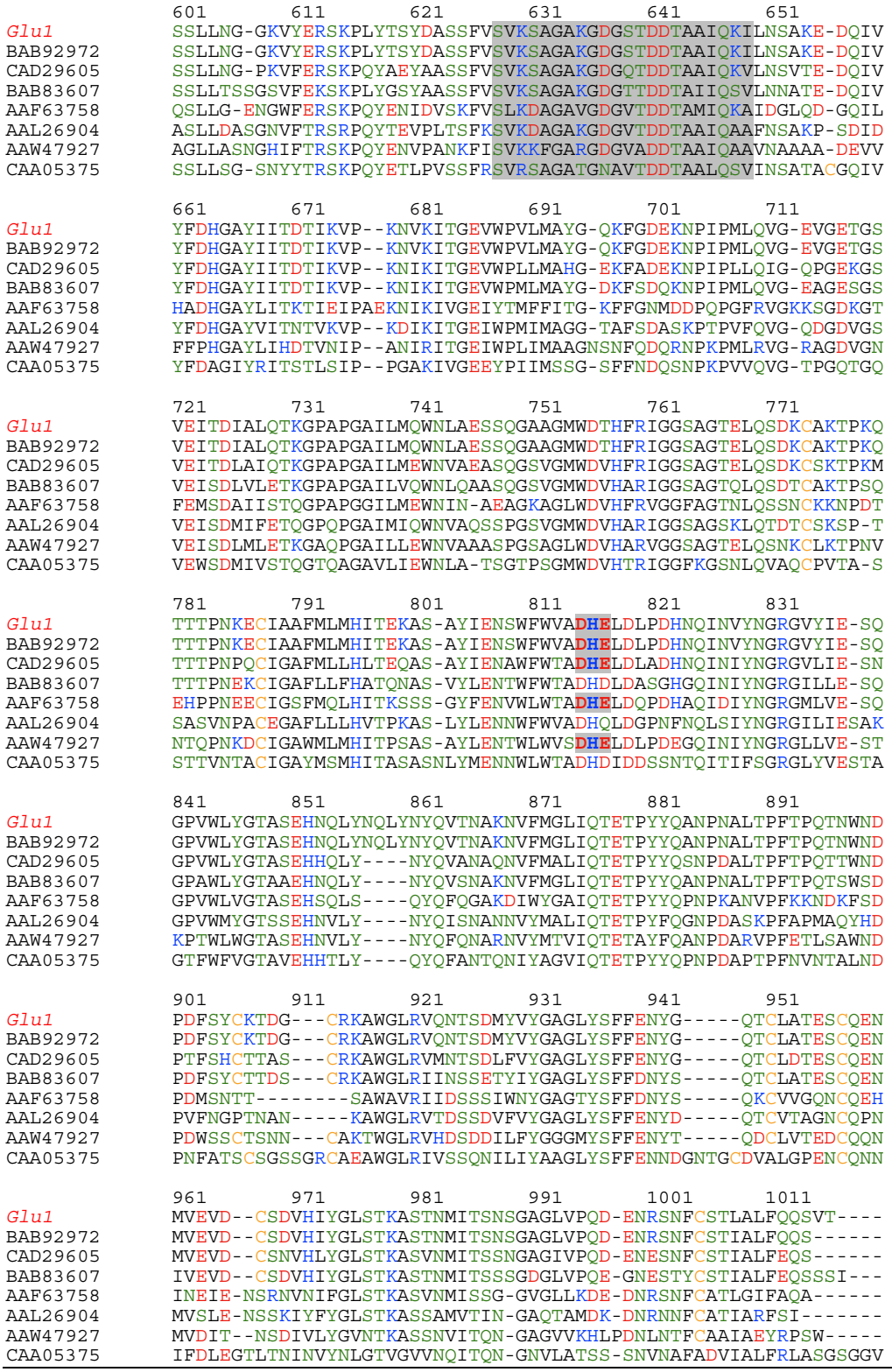


Figure 6.16- Colours indicate amino acid with similar properties. Alignment of amino acid sequence of *Glu1* (in red) with related glucanases in the database. BAB92972, exo-1, 3-glucanase from *A. oryzae* . CAD29605, exo-1, 3-glucanase from *A. fumigatus* . BAB83607, a glucanase from *A. phoenicis.* AAF63758, a β-1, 3-glucanase from *Coniothyrium minitans.* AAL26904, a β-1, 3-glucanase from *Blumeria graminis.* AAW47927, a β-1, 3- glucanase from

Acremonium sp. and CAA05375, a β-1, 3-exoglucanase from *Hypocrea lixii.* The N-terminal amino acid sequence that characterise these enzymes is indicated in bold and underlined. The two sequences involved in substrate binding are shaded. The amino acid residues indicated in bold and shaded (DHE) are believed to be important in the catalytic reaction of these enzymes.

6.3.4.3 Active sites of Glu1

An alignment of the amino acid sequence of *Glu1* showed two consensus sequences NVKDFGAKGDGSTDDTAAINQAI and SVKSAGAKGDGSTDDTAAIQKI (in bold) that indicate substrate binding regions (Nikolskaya *et al.* 1998; Oda *et al.* 2002). These sequences are typical of glucanase enzymes and always appear in tandem (Donzelli *et al.* 2001). The N-terminal (bold and underlined) has also been identified previously as one of the characteristic features of these enzymes (Oda *et al.* 2002) (Fig 6.16).

6.3.4.4 N-terminal amino acid sequence comparison

The N-terminal amino acid sequence of *Glu1* was 100% homologous to the N-terminal of *A. oryzae* but was not significantly homologous to other glucanases from fungal origin (Table 6.3).

Table 6.3-N-terminal sequences of *Glu1* and other fungal glucanases.

Gene	N-terminal amino acid sequence	Accession number
Aspergillus spp. *Glu1*	AASEYWVGTIKRQGAVAF	DQ312297
A. oryzae exo-1, 3-glucanase	AASEYWVGTIKRQGAVAF	BAB92972
A. satoi exo-β-D-1, 3-glucanase gene exgS	ASDYWVGTIKRQTGTVPF	ABO70739
A. fumigatus exo-1, 3-glucanase exgO	TASSFWVANIKRQGAVAF	EAL90777
T. atroviride glucan 1, 3-glucosidase GLUC78	AATSWWLPNIARNGNVPF	AAF80600

C. carbonum exo-beta 1, 3-glucosidase precursor	IVDGYWLNDLSGKGRAPF	AAC71062

6.3.4.5 Phylogeny of Glu1

To visualise the relationship between *Glu1* and other glucanases including those more distantly related than the ones shown above (Fig 6.16), a phylogenetic tree was constructed using the amino acid sequences of the amplified fragment and sequences of other glucanases. A rooted tree using SwissProt sequence AAP33112, a glucanase from *T. harzianum* (Donzelli *et al.* 2001) as the root, showed the high degree of identity relationship of this sequence to that of *Aspergillus* species. The tree has two clades one is polytomous (Clade 1) and one is monotomous (Clade 2). The clade that contains *Glu1* (Clade 1) has two branches. One branch contains *Aspergillus* spp. glucanases (BAB92972, EAL90777 syn CAD29605 and BAB83607) (Clade 1, Branch A) (Fig 6.17). A second branch contains fungal proteins including a hypothetical protein from *Neurospora crassa* (XP327809) (Birren 2003b) a glucanase from *Giberella zeae* (accession number EAA76089) (Birren *et al.* 2004) and a glucanase from *Blumeria graminis* (accession number AF317733) (Zhang and Gurr 2003) (Clade 1, Branch B). Clade 2 contains a glucanase from *Ampelomyces quisqualis* (accession number AAC09172) (Rotem *et al.* 1998) that may share an ancestor.

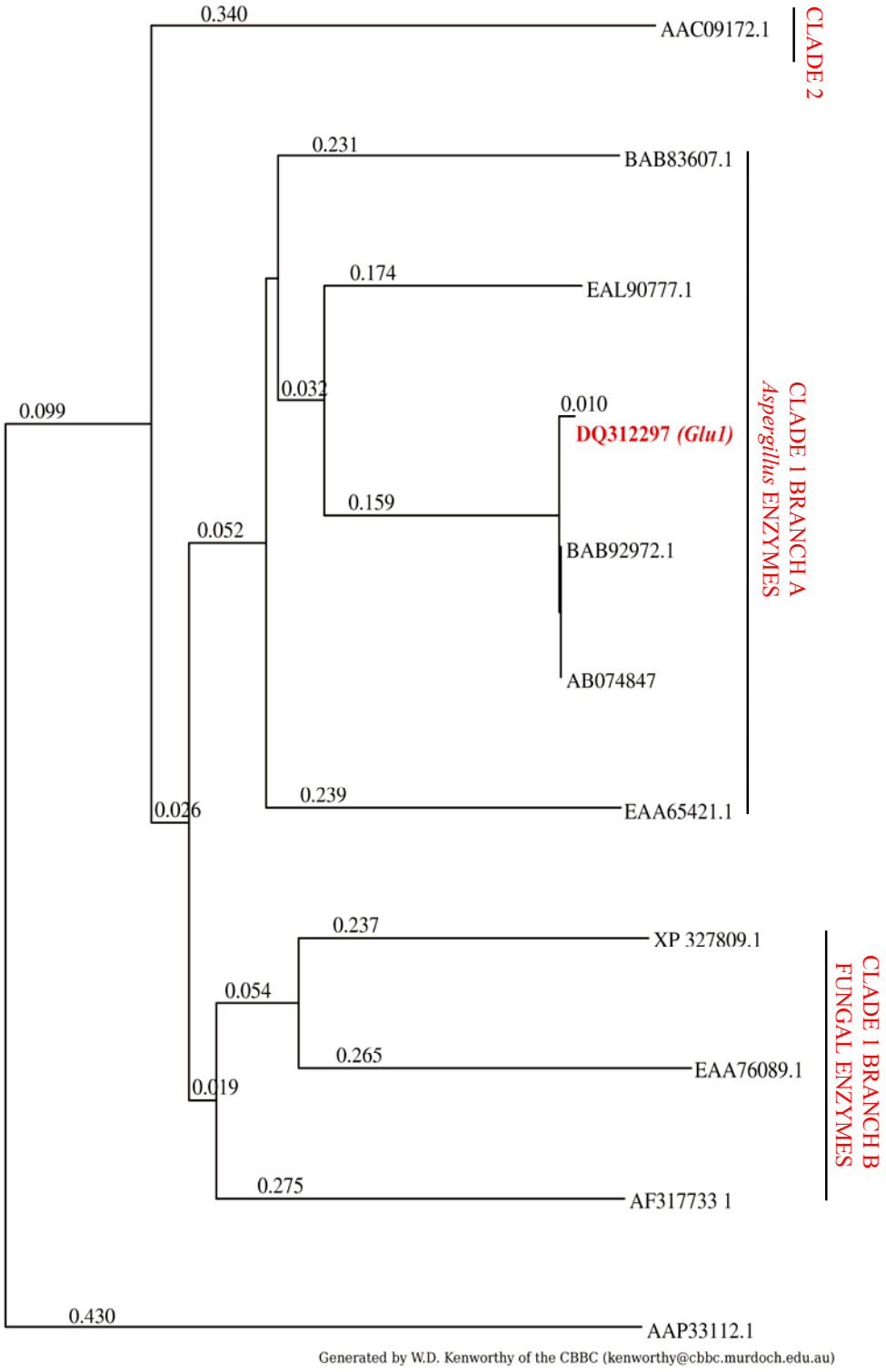


Figure 6.18-Relationship between amino acid sequences (calculated on genomic differences) between sequences most closely related. The tree is rooted on sequence AAP33112, a glucanase from *T. harzianum.* The numbers represents distance that is calculated as an estimate of the divergence time between two species. Accession numbers correspond to the SwissProt numbers.

Multiple sequence alignment was performed with CLUSTALW (Thompson *et al.* 1994) using the default parameters. The available sequences were trimmed to equal length before phylogenetic analyses using programs in PHYLIP (Felsenstein 1989). Genetic distances between pairs of nucleotide sequences were calculated using the program DNAdist (maximum likelihood method with transition/transversion ratio of 2.0 and 1 category of substitution rates). The percentage nucleotide and amino acid identities were determined using OldDistances (GCG) (Table 6.4). The sequences used for the alignment were AB074847, an *Aspergillus oryzae* exgO gene exo 1, 3- glucanase (Prot BAB92972); AB070739, an *A. phoenicis* exgS glucanase (Prot BAB83607); BX649607, an *A. fumigatus* glucanase (Prot CAD29605); AF247649, a *Coniothyrium minitans* β-1, 3-glucanase (Prot AAF63758); AJ002397, a *T. harzianum (Hypocrea lixii)* β-1, 3-exoglucanase (Prot CAA05375); AY854012, an *Acremonium* sp. B-1, 3-glucanase gene (Prot AAW47927) and AF317733 a *Blumeria graminis* 1, 3-β-glucanase (Prot AAL26904). The relationship shows that *Glu1* is closely related to a gene that encodes for an exo-glucanase from *A. oryzae.*

Nucleotide and protein length used for the alignment are shown in Table 6.5.

Table 6.4-Nucleotide and amino acid (in bold) homology (% identity) of the glucanase gene (DQ312297) with glucanase sequences available on GenBank.

Sequence Identity (a)	1	2	3	4	5	6	7	8
1	1.0000	0.7657	0.6808	0.6748	0.4824	0.3945	0.4281	0.4072
2	**0.9947**	1.0000	0.6634	0.6602	0.5125	0.4037	0.4227	0.4306
3	**0.7344**	**0.7204**	1.0000	0.6639	0.5031	0.4080	0.4184	0.4025
4	**0.7698**	**0.7738**	**0.7206**	1.0000	0.4740	0.4030	0.4328	0.3960
5	**0.6139**	**0.6152**	**0.6088**	**0.6178**	1.0000	0.4174	0.5077	0.4702
6	**0.4304**	**0.4334**	**0.4381**	**0.4424**	**0.5109**	1.0000	0.5723	0.5044
7	**0.6199**	**0.6212**	**0.6199**	**0.6161**	**0.5547**	**0.5191**	1.0000	0.5616
8	**0.6336**	**0.6362**	**0.6258**	**0.6428**	**0.5750**	**0.5332**	**0.6102**	1.0000

Table 6.5- Data shows the nucleotide and protein lengths of the glucanase sequences used for the CLUSTALW alignment. The letter a, corresponds to the sequence identities in Table 6.4. The letters b and c indicate the length without gaps of the nucleotide and protein sequences, respectively.

(a)	Accession number	Nucleotide length	(b)	Protein length	(c)
1	*Glu1*	3626	2844	1264	948
2	AB074847	3626	2160	1264	946
3	AB070739	3626	2544	1264	947
4	BX649607	3626	2844	1264	945
5	AF247649	3626	2966	1264	777
6	AJ002397	3626	3099	1264	767
7	AY854012	3626	2352	1264	784
8	AF317733	3626	2601	1264	1032

6.3.4.6 Branch reliability

The consensus tree confirmed polytomies shown in the phylogenetic tree where at all the branch points there are more than two immediate descendants. The glucanase genes are clustered in two groups, *Aspergillus* spp. glucanases where *Glu1* is found (Cluster I) and second group comprising other fungal glucanases (Cluster II) and includes two *Aspergillus* proteins, a hypothetical protein from *A. nidulans* (EAA65421) and an exo-β-1, 3-glucanase from *A. fumigatus* (CAD29605), more distantly related to the others (Fig.6.19).

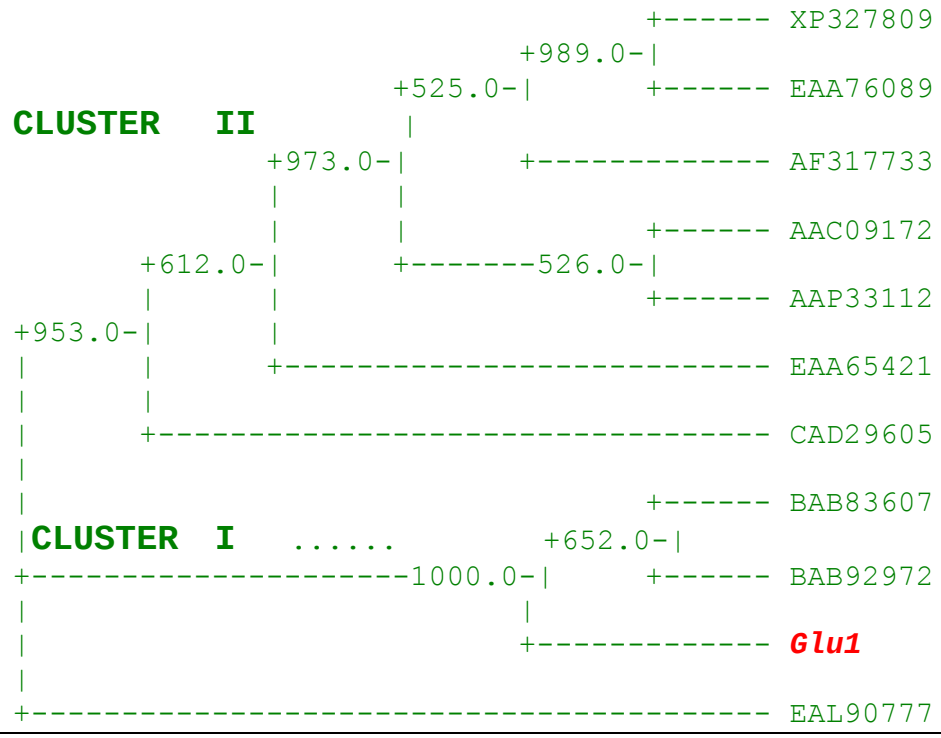


Figure 6.19- Neighbour-joining tree from the amino acid sequences of the partial chitinase genes. *Glu1* is shown in red. The clades comprising, fungal glucanases are numbered I and II. Bootstrap values are shown at each node.

6.4 DISCUSSION

Two chitinase sequences were determined from *T. asperellum*. One of these, *ChiA*, contained two stop codons and is therefore not a functional chitinase gene. *ChiB* did not contain stop codons, and although the complete gene sequence was not determined, it is

likely to be a functional gene, though this was not proven experimentally. A glucanase gene (*Glu1*) from an *Aspergillus* sp.. is a complete gene that is likely to be functional. All three sequences show homology to genes that encode for glycosyl hydrolase enzymes. *ChiA* and *ChiB* belong to family 18 of these enzymes whilst *Glu1* belongs to family 17.

Glycoside hydrolase-like family 18 chitinases are characterised by several conserved sequence motifs, the most prominent being the D-G-X-D-X-D-X-E (Aspartic acid-X-Aspartic acid-X-Glutamic acid, where X is a hydrophobic amino acid) motif that spans strand 4 of the $(\beta\alpha)_8$ (TIM barrel) fold and includes the glutamic acid (E) that acts as the catalytic acid. All family 18 chitinases hold a TIM barrel structure as a scaffold bearing the active site. The active site grooves of these chitinases are lined with aromatic amino acids that contribute to substrate binding (Synstad *et al.* 2004; Tsujibo *et al.* 2002; Watanabe *et al.* 1993b). The importance of the glutamic acid for catalysis was demonstrated by Synstad *et al* (2004) who observed a large reduction (1×10^4 –1×10^5 –fold) in enzyme activity upon mutation to glutamine. Furthermore mutation of glutamic acid to aspartate in other family 18 chitinase also reduced activity (1×10^3-fold) dramatically (Watanabe *et al.* 1993b). In addition to site directed mutagenesis experiments, crystallographic data have also shown that a conserved glutamate is involved in the catalytic mechanism and probably acts as a proton donor. Another highly conserved motif is the consensus sequence S-X-G-G (Serine-X-Glycine-Glycine, where X is a hydrophobic amino acid) which corresponds to the substrate binding site in family 18 glycosyl hydrolases (Watanabe *et al.* 1993b).

Families 17 and 18 include fungal, yeast, bacterial and some plant genes (Henrissat *et al.* 1995). Table 6.6 shows family 18 conserved motifs.

Table 6.6-Signature sequences found in the active sites of chitinases for family 18 glycosyl hydrolases. The glutamic acid (E) residue is crucial to the catalytic mechanism.

Source of gene	Substrate binding site	Catalytic site
Coccidioides immitis	L**SIGGWT**Y**S**PN**F**	**FDG**I**DIDWE**Y**P**ED
T. harzianum	L**SIGGWT**W**S**TN**F**	**FDG**I**DIDWE**Y**P**AD
Aphanocladium album	L**SIGGWT**W**S**TN**F**	**FDG**I**DIDWE**Y**P**AD
Serratia marcescens	P**SIGGWT**L**S**DP**F**	**FDG**V**DIDWE**F**P**GG
T. asperellum ChiA	L**SLGGAA**G**A**YFL	**LDG**W**DFDVE**A**S**NG
T. asperellum ChiB	L**SIGGAT**A**G**IDL	**FDG**I**DIDIE**TGLT

Structural groups of chitinases are referred to as classes (Gooday 1999). For example, class I, II and IV chitinases belong to family 19 whereas class III and V to family 18 (Henrissat 1999). One exception are the chitinases I, II and III of *Rhizopus oligosporus*, a fungus, belong to family 18. Table 6.7 shows chitinases listed by their functions and the classes and families they belong to. The enzyme encoded by *ChiA* showed similarity to a class III chitinase from *Aspergillus fumigatus* and the enzyme encoded by *ChiB* showed high similarity to endochitinases from *Trichoderma* strains that also belong to class III, family 18.

Table 6.7-Chitinase gene from different families and classes and their functions.

Source of chitinase genes	Family	Class	Function	Author and Accession numbers
Saccharomyces cerevisiae	18	III	Cell separation	(Johnston *et al.* 1997) NP013388
Trichosanthes kirilowii	18	III	Defensive and ribosome-inactivating activity	(Shih *et al.* 1997) N/A*
Trichoderma asperellum chit36Y	18	III	Mycoparasitic activity	(Viterbo and Chet 2002) AAL01372
T. asperellum ChiA	18	III	Mycoparasitic activity	(Severgnini *et al.* 2005) DQ007018
T. asperellum ChiB	18	III	Mycoparasitic activity	(Severgnini *et al.* 2005) DQ312296
Rhizopus oligosporus	18	III	Hyphal growth	(Takaya *et al.* 1998b) BAA13489
Glycine max	18	III	Seed development	(Yeboah *et al.* 1998) BAA77677
Oryza sativa	19	I	Defence	(Nishizawa *et al.* 1999) CAA40107
Hordeum vulgare	19	II	Defence	(Jach *et al.* 1995) CAA02316
Picea abies	19	I	Defence	(Salzer *et al.* 1997) N/A
Citrus sinensis	19	II	Defence	(Nairn *et al.* 1997) CAA93847
Streptomyces coelicolor	19	II	Parasitic activity	(Bentley 2002) CAB69724

*N/A-accession number not available.

Some members of family 17 glycosyl hydrolases include laminarinases (glucanases) and exo-β-1, 3-glucanases (Table 6.8).

Table 6.6-Members of glycosyl hydrolases belonging to family 17.

Source of glucanase genes	Family	Author and accession number
Hordeum vulgare (plant) laminarinase	17	(Xu *et al.* 1992) Q02437
Nicotiana plumbaginifolia (plant) laminarinase	17	(Gheysen *et al.* 1990)CAA38540
Aspergillus saitoi (fungus) exo-beta-D-1, 3-glucanase gene exgS	17	(Oda *et al.* 2002) AB070739
T. atroviride (fungus) 1,3-beta-glucosidase	17	(Donzelli *et al.* 2001)AF253421
Glu1	17	Severgnini, 2005 DQ312297

6.4.1 Sequence analysis of *ChiA*

A comparison of the deduced amino acid sequence of *ChiA* with others revealed that it was related to the class III from *A. fumigatus* and a chitinase from *M. anisopliae* var. acridum.

Two stop codons were found at positions 184 and 191. These were shown not to be within typical intronic splice sequences (5' GT and 3' AG). There was a possibility that the observed stop codons were artifacts of PCR and/or sequencing but they were seen in multiple sequencing reactions, a good indication that the stop codons were genuine and therefore *ChiA* is most probably a pseudogene. A cDNA library was constructed to compare mature RNA with the DNA sequence however efforts to amplify it were not successful. To ascertain that these were not *Taq* DNA polymerase-induced mismatches, another amplification of the *ChiA* fragment was done from genomic DNA and sequenced. This confirmed that the stop codons were real, and therefore that *ChiA* is a pseudogene.

Chitinase pseudogenes have been described in other species. A chitinase-like pseudogene in *Pinus strobus, Pschi4*, had a complete coding sequence with a premature stop codon in the first exon (Wu *et al.* 1997). Comparison with the functional gene *Pschi1*, revealed 90% identity through the putative coding region excluding introns, and 83% identity through the 5' flanking sequence (Wu *et al.* 1997). Glycosyl hydrolases commonly acquire new substrate specificities through time (Henrissat 1991). This phenomenon is not unexpected given the stereochemical resemblance between their substrates. New carbohydrate metabolites emerge over time leading to selection for catalysis of different substrates and genetic divergence to acquire new specificity. Thus, gene families have been observed elsewhere among glycosyl hydrolases (Henrissat 1991). Henrissat (1991) speculated that copies of genes that do not produce functional enzymes could serve as templates for encoding enzymes with altered properties such as new specificity directed to a stereochemically similar substrate (Henrissat 1991). If one substrate is not available over time, the enzyme is not produced and the gene becomes non-functional because it is not selected for, which may be the case with *ChiA*.

A further indication that *ChiA* is part of a chitinase gene is the identification of two conserved regions typical of the family 18 chitinases. A glutamic acid residue was found in the catalytic region of *ChiA* with a D-G-W-D-F-D-V-E (Aspartic acid-Glycine-Tryptophan- Aspartic acid-Phenylalanine-Aspartic acid-Valine-Glutamic acid) motif. *ChiA*, also contains the conserved motif S-L-G-G (Serine-Leucine-Glycine-Glycine) corresponding to the substrate binding site of the gene.

As expected *ChiA* grouped with chitinase-like genes closely related to other fungi and more distantly related to bacterial chitinases. Unlike *ChiB* and *Glu, ChiA* was too distantly related at the nucleotide level to other chitinases to construct a meaningful matrix.

6.4.2 Sequence analysis of *ChiB*

The partial gene sequence, *ChiB*, was 98% identical to the nucleotide sequence of an endochitinase from *T. harzianum* (accession number AF406791), giving a strong indication that the gene isolated from *T. asperellum* encodes a chitinase.

The 293 amino acid sequence of *ChiB* from *T. asperellum*, showed 98% amino acid similarity with the 344 amino acid protein from *T. harzianum* chit36Y, accession number AAL01372 (Fekete *et al.* 1996; Viterbo and Chet 2001). In view of these results it can be said that *ChiB* is likely to be the same chitinase gene as chit36Y. The sequence of genomic DNA revealed no introns in the coding region of *ChiB* or in chit36Y. Chit36Y from *T. harzianum* has been shown to be an endochitinase with apparent molecular mass of 33 kDa, heat-resistant, with a p*I* value of 4.8. In other *Trichoderma* species, homologous genes occur as a single copy. Numerous overlapping ORFs were encoded on the same strand that correlate with the amino acid sequences closest in identity to the partial chitinase gene.

In the case of *ChiB* the active site is composed of D-G-I-D-I-D-I-E (Aspartic acid-Glycine-Isoleucine-Aspartic acid-Isoleucine-Aspartic acid-Isoleucine-Glutamic acid) and its substrate binding site is Serine-Isoleucine-Glycine-Glycine. Site-directed mutagenesis studies indicated that E-204 and D-200 were directly involved in the chitin catalysis (Watanabe *et al.* 1993a). This glutamate (E) is the last residue of this active site signature (Watanabe *et al.* 1993a). In general, these two residues (D and E) have been reported to be important in chitinase from class III of which *ChiB* is one. Another motif, the Ala-X-Ala sequence is frequently observed at signal peptide processing sites of secretory proteins from eucarya and bacteria (Takana *et al.* 1999). This signal peptide is represented by A-I-A in *ChiB* (Alanine-Isoleucine-Alanine).

Phylogenetic analysis showed *ChiB* in a cluster with other fungal chitinases, and closest to chit36Y (Viterbo *et al.* 2002) from *T. asperellum*. The alignment contained members of fungal, yeast and bacterial chitinases. The clade that contains *ChiB*, possesses a hanging branch with a protein from *Lactococcus lactis*, a bacterium, which may share an ancestor with *ChiB* and/or may indicate that a horizontal gene tranfer occurred at some point in the evolutionary history of these genes (Viterbo *et al.* 2002)

The fact that chitinase genes of similar organisms isolated from different environments exhibit homology and phylogenetic relationship like *ChiB* and chit36Y suggests that these organisms may have a close evolutionary relationship. Furthermore, a cluster of bacterial chitinases appear in the tree which agrees with earlier reports that *Trichoderma* species chitinases are similar to bacterial chitinases (Takaya *et al.* 1998b).

Despite efforts, the full-length gene was not amplified in this study. It is possible that base changes prevented amplification especially at the 3' end of the primer site. Attempts to amplify the full gene should be made in future investigations. This is already discussed in Chapter 5.

6.4.3 Comparison of *ChiA* and *ChiB*

The two sequences, *ChiA* and *ChiB*, amplified in this study showed little homology. This result is in agreement with those from other studies where different chitinases from the same organism are not the result of mutation of a single gene but instead are the end products of different genes of little or no homology (Felse and Panda 1999; Garcia *et al.* 1994; Irene *et al.* 1994). However, both partial genes, *ChiA* and *ChiB* possess the typical consensus sequences corresponding to substrate binding and catalytic sites. A complex system of more than six enzymes with the ability to degrade chitin has been described in *Trichoderma* strains (Haran *et al.* 1995). Some of these proteins with both endo- and exo-hydrolytic activities have been fully characterized, their genes cloned and evidence

of their participation in the mycoparasitic process described (Carsolio *et al.* 1999; Peterbauer *et al.* 1996). Additionally, the protein coded by chit36Y inhibited spore germination of three phytopathogens, *Alternaria alternata, Fusarium oxusporum* and *B. cinerea* demonstrating the direct involvement of the chit36Y endochitinase in the mycoparasitic process (Viterbo and Chet 2002). This points to a possible function to *ChiB* although these properties are yet to be determined.

6.4.4 Sequence analysis of *Glu1*

A glucanase gene from a putative *Aspergillus* species, *Glu1* showed high identity to glucanases of other *Aspergillus* species including *A. oryzae*, *A. fumigatus*, *A. nidulans* and *A. phoenicis*, a strong indication that *Glu1* is indeed a glucanase gene. *Glu1* is 77% homologous at the nucleotide level and 99% homologus at the amino acid level to an exo-1, 3-glucanase from *A. oryzae* and it is likely they have the same function. The difference in degree of identity between nucleotides and amino acids indicates codon preference in these fungi and that *Glu1* is a novel gene. Amplification of the ITS region of the isolate was not successful therefore it cannot be concluded that isolate 04-013 is *A. oryzae.* However, *Aspergillus* is a ubiquitous fungus found in nature and commonly isolated from soil, plant debris, and indoor air environment (Agrios 2005), thus certain to be represented in the collection of soil isolates in this study. For example, *A. fumigatus* is the most encountered species causing infection (Agrios 2005), it is not surprising that an *Aspergillus* (isolate 04-013) was isolated from chitin-amended soil that possesses the gene for this enzyme (De la Cruz *et al.* 1993).

There are a number of reports providing amino acid sequence data for fungal and yeast exo-1, 3-glucanases such as *T. harzianum*, *C. carbonum, S. cerevisiae* and *C. albicans*

among others but none of these display significant homology in their N-terminal amino acid sequence to *Glu1*.

There are many structural elements (motifs) that are conserved among these proteins that were found in *Glu1*. For example carbohydrates can be attached to the amino acid asparagine in proteins through *N*-glycosylation sites which are indicated by the consensus sequence N-Xaa-S/T. The first amino acid is Asparagine (N), the second amino acid can be any of the 20 amino acids (Xaa) except P (Donzelli *et al.* 2001), and the third amino acid is either Serine (S) or Threonine (T). In *Glu1*, seven glycosylation sites were found whereas an exo-1, 3-glucanase from *A. saitoi exgS* gene possesses eleven such sites (Oda *et al.* 2002). Aspartic acid and glutamic acid (residues D and E, respectively) are part of the catalytic sites in glycosyl hydrolases, and *Glu1* shows high conservation of these residues, however, experimental work beyond the scope of this project needs to be carried out to predict with certainty the catalytic residues in *Glu1*. Nonetheless, a motif of key residues; in particular, the Asp-His-Glu that has been found to play an important role in the catalytic reaction of the enzyme (White *et al.* 1994), was found in *Glu1* at posititon 759. Additionally, there are two imperfect copies of a 23-amino acid sequence (NVKDFGAKGDGSTDDTAAINQAI and SVKSAGAKGDGSTDDTAAIQKIL), starting at amino acid 65 and 625 respectively. The two copies are ~70% identical overall. These amino acid sequences were found in related proteins, including a novel exo-β-1, 3-glucanase (EXG1p) from the fungus *Cochliobolus carbonum* (Nikolskaya *et al.* 1998), an exo-β-1, 3-glucanase gene from *A. saitoi* (Oda *et al.* 2002), a viral endoneuroaminidase (sialidase) (Ertesvag *et al.* 1995), a viral neck appendage protein (Gerardy-Schahn *et al.* 1995) and several plant (Kalaitzis *et al.* 1995) and bacterial polygalacturonases (He and Collmer 1990). Although the proteins sharing this conserved 23-amino acid motif have different enzymatic activities,

they all interact with polysaccharides. β-1, 3-glucanase, *N*-acetylneuraminidase and polygalacturonase catalyse the structural modification of polysaccharides, whereas the viral neck appendage protein is involved in binding to cell surface carbohydrates (Nikolskaya *et al.* 1998; Villanueva and Salas 1981).

6.5 CONCLUSIONS

A partial chitinase gene *ChiA* (accession number DQ007018) and a chitinase gene *ChiB* (accession number DQ312296) with 98% similarity to the chit36Y gene of *T. harzianum* were characterized. Both genes were amplified from genomic DNA of fungal isolate 04-001 from a chitin-amended soil. ITS analysis revealed it to be a *T. asperellum*.

The partial chitinase gene, *ChiA*, was, at first, believed to be a good candidate for a novel gene with 70% identity homology to a chitinase gene from *A. fumigatus*. However, after analysis, *ChiA* was shown to have no potential use to protect plants against plant pathogens. On the other hand, analysis of *ChiB*, revealed that this gene could be used as a source of protection for plants against fungal pathogens.

The full length glucanase gene, *Glu1* (accession number DQ312297) from an *Aspergillus* species is a novel, functional gene that can be used for further studies as a good candidate for enhancing glucanase production in other organisms.

CHAPTER 7

GENERAL DISCUSSION

Chitinases and glucanases are produced by plants, fungi, bacteria and viruses for roles in protection induction and normal development. Chitinase and glucanase are potentially useful for engineering plants to resist attack by fungal pathogens. Such genes have been isolated from plants and soil organisms such as bacteria and fungi and expressed in plants (Patil *et al.* 2000). However, not all chitinase genes function equally well. Chitinases from fungi have shown a broader spectrum of activity against fungal pathogens (Lorito *et al.* 1998). Furthermore, chitinase and glucanase enzymes act in synergism to degrade the cell wall of the pathogens (Bolar *et al.* 2001; Lorito *et al.* 1994).

7.1 PROJECT AIM

The aim of this project was to isolate chitinolytic and glucanolytic fungi from soil. The hypothesis being tested was that, because of the high diversity of fungi in Western Australian soils (Bougher 1994; Bougher and Syme 1998; Glen 2001; Tommerup and Bougher 1999), we would find novel genes that would be useful for engineering plants against fungal pathogens. Novel enzymes have been found previously in other parts of the world (De la Cruz *et al.* 1995b; Kang *et al.* 1999; LeCleir *et al.* 2004) but this is the first search here in W.A.

To achieve this aim, five steps were undertaken:

i) To conduct an assay to identify soil fungi in W.A. from a range of ecosystems.

ii) To assay the hydrolytic activity of enzymes from identified fungi against chitin and laminarin

iii) To assay the hydrolytic activity of those enzymes against the main components of fungal cell walls from pathogenic fungi *Rhizoctonia solani, Sclerotinia sclerotium, Fusarium solani, Botrytis cinerea, Ascochyta faba* and *Leptosphaeria maculans.*

iv) To assess their ability to inhibit the growth of fungal plant pathogens economically important such as *F. solani*, *S. sclerotium* and *Botrytis cinerea.*

v) To isolate and characterise genes that could be used to enhance plant defence against fungal pathogens using two methods:

-chitinase and glucanase degenerate primers and genome walking and

-chitinase specific primers

Chitinolytic fungi were isolated from various sites in WA (natural forest, mine rehabilitation site, agricultural land, compost heap and chitin-baited soil) so that we would recover and test a range of fungal species. Isolations were made on chitin agar to select for chitinolytic fungi. Other studies have shown that the fungal species in a given environment identified by culture techniques represents a small fraction of the total species detected by molecular methods (Magnuson and Lasure 2002) and that 80-90% of soil fungi cannot be cultured *in vitro* (Kaeberlein *et al.* 2002), It is likely that many more species existed in the soil samples than was seen. Moreover, results presented by Seki (1966) and Okutani (1975) who studied marine environments, suggested that marine bacteria capable of degrading chitin represented 0.4 to 19% of total cultured bacteria (Okutani 1975; Seki 1966).

The isolates with the best chitinase production were *Trichoderma* species and two unrelated partial chitinase sequences (*ChiA* and *ChiB*) were cloned from one isolate.

Comparison of the two sequences revealed low identity over the entire sequence. Other studies have also revealed that different chitinase genes from the same organism share little or no homology to each other (De la Cruz *et al.* 1993; Felse and Panda 1999). Sequencing data revealed that *ChiA* and *ChiB* belong to family 18 glycosyl hydrolases exhibiting the typical active and substrate binding sites that identify members of this family. We also cloned a novel gene from an *Aspergillus* that was homologous to a glucanase gene from *A. oryzae.*

In this study, chitinolytic and glucanolytic activity was detected when the fungi were grown on colloidal chitin or laminarin as their sole carbon source. The activity of chitinolytic and glucanolytic enzymes was quantified with assays with chromogenic *p*-nitrophenyl analogs of disaccharides of *N*-acetylglucosamine and dinitrosalicylic acid and by measuring the increase in reducing sugars produced from hydrolysis of pathogenic fungi cell wall preparations. The antagonistic ability of the chitinolytic and glucanolytic fungi was demonstrated by using chitinolytic fungi filtrates to suppress the growth of three economically important plant pathogens; *Fusarium solani*, *Sclerotinia sclerotium* and *Botritys cinerea.* Eleven isolates showed significant activity against these pathogens.

The cloning of chitinase and glucanase sequences was carried out using degenerate primers. The chitinase primer sequences were designed from conserved sequences in chitinase genes from fungi, yeast and plants while the glucanase primers were designed from fungal glucanase genes. Since chitinases are classified as either family 18 from bacteria, fungi and some plants or 19 from plants glycosyl hydrolases based on amino acid sequence similarity (Henrissat and Bairoch 1993), the degenerate primers designed for this project targeted family 18. Similarly, glucanases belong to family 17 enzymes and the degenerate primers targeted that particular family.

We must consider whether the use of degenerate primers is an appropriate way to look for novel enzymes. By their nature, degenerate primers are targeted to regions conserved amongst known enzymes so completely novel enzymes that do not possess similar conserved areas will be missed. LeCleir *et al* (2004) used degenerate primers to isolate chitinase genes from diversed aquatic habitats and identified 108 potential chitinase sequences. All chitinase sequences were novel compared to previously identified sequences. The authors did not characterise the enzymes but concluded that unique signature sequences found in some of the sequences could translate into fundamental differences in enzyme properties (LeCleir *et al.* 2004).

An alternative approach that has been used to isolate novel enzymes is to purify enzymes with novel substrate activity and use the protein sequence to pull out the corresponding gene (De la Cruz *et al.* 1992; De la Cruz *et al.* 1995b; Kang *et al.* 1999). Another approach that has considerable potential, but as yet is largely unexplored is the use of phage display. In phage display, proteins synthesized from transgenes cloned within the phage genome are displayed on the surface of the phage particle (Rosenberg *et al.* 1996). Enzymes with novel substrate binding characteristics and their corresponding genes can be easily isolated by affinity chromatography (Rosenberg *et al.* 1996). Alternatively, cDNA and genomic DNA libraries can be used to retrieve chitinase genes from environmental DNA, without the need to rely on conserved nucleotide sequences, by screening the clones within the library for activity using a fluorogenic analogue of chitin to identify those genes. Dragborg *et al* (1995) reported the cloning of chitinase genes from *T. harzianum* by constructing a cDNA library in yeast. The library was screened for genes encoding chitinases that hydrolyse a fluorogenic analogue of chitin, 4-methylumbelliferyl-β-D-acetylglucosamine (MUF-1), 4-methylumbelliferyl-β-D-*N, N'*-diacetylchitobioside (MUF-2) and 4-

methylumbelliferyl-β-D-*N, N', N''* triacetylchitobioside (MUF-3) and the release of 4-MU was visualised under UV light. The authors reported the isolation of an endochitinase with 76% identity to an endochitinase from the fungus *Aphanocladium album* and two exochitinases 56% identical to each other and 50% identical to mammalian and fungal hexoseaminidases (Dragborg and Christgau 1995). Similarly, Cotrell *et al* (1999) constructed genomic DNA libraries from organisms taken from marine and estuarine water in a λ phage cloning vector and screened for chitinases using MUF-2. Plaque assays revealed 5.5% and 0.12% of MUF-2-positive clones for the estuarine and coastal sample respectively (Cottrell *et al.* 1999).

7.2 FUTURE WORK

Based on previous findings that fungal enzymes display higher activity against fungal pathogens and also possess a broader spectrum of activity (Lorito *et al.* 1998), we set out to find fungal genes. However, a study carried out by Metcalfe *et al* (2002) who, like us, baited soil with chitin, revealed that most chitinase sequences isolated from such soils belonged to actinobacteria and that these play an important role in the soil chitinolytic community (Metcalfe *et al.* 2002). Therefore, in future studies we recommend the focus be not only on fungal but also bacterial enzymes to investigate their potential to enhance plant defense against pathogenic fungi.

Our aim to find novel genes was accomplished, however we do not know if their spectra of activity are novel. Therefore, we propose that future studies use an alternative approach for generating chitinases and glucanases with novel properties by the use of the techniques for protein engineering. Site specific mutagenesis has been used to demonstrate the importance of amino acid residues such as aspartic and glutamic acids in the catalytic properties of chitinases (Thomas *et al.* 2000; Walton 1994; Watanabe *et al.* 1993a). Thomas *et al* (2000) studied the effects of mutagenesis of these two residues

in *Autographa californica* nucleopolyhedrovirus *chiA* gene. Chitinase protein production was unaffected by the mutation of these residues, however, chitinase assays revealed that altering the glutamate at position 315 of the protein led to decreased exochitinase activity. Watanabe *et al* (1993) demonstrated that mutagenesis of the glutamate of *Bacillus circulans* chitinase caused a significant reduction in the amount of chitinase activity detected (Watanabe *et al.* 1993b). These results suggest that glutamate is a major determinant of chitinase activity. Also, mutagenesis of the aspartate residue resulted in a reduction of the exochitinase detected and an increase in endochitinolytic activity (Thomas *et al.* 2000). The properties of enzymes such as substrate affinity, thermostability and specific activity can be markedly altered by the use of site directed mutagenesis, and/or exo shuffling (Bittker *et al.* 2002; Ghadessy *et al.* 2004; Zhao *et al.* 2004).

Changes to less crucial and flanking residues that alter conformation of the enzyme may be more likely to modulate useful changes to substrate specificities but screening of such large numbers of randomly-generated mutants would require a great deal of work. For practical purposes, further screening to identify members of hydrolase gene families generated during the evolution of individual soil-inhabiting and parasitic microbes, such as those isolated and characterised in this thesis, is much more likely to be useful.

BIBLIOGRAPHY

Agrios GN (2005) 'Plant Pathology.' (Elsevier Academic Press: Burlington/USA)

Alcoa (2004) Alcoa World Alumina Australia Bauxite Mine Rehabilitation. In. (www.doir.wa.gov.au/investment/BauxiteMineRehab: Perth)

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389-3402.

ANGIS Bb http://www.angis.org.au. In.

Anon (2004) *Oryza sativa* (japonica cultivar group).

Anuratha CS, Zen KC, Cole K, Mew T, Muthukrishnan S (1996) Induction of chitinases and β-1,3-glucanases in *Rhizoctonia solani* infected rice plants. Isolation of an infection-related chitinase cDNA clone. *Physiologia plantarum* **97**.

Arora N, Ahmad T, Rajagopal R, Bhatnagar RK (2003) A constitutively expressed 36kDa exochitinase from *Bacillus thuringiensis* HD-1. *Biochem. Biophys. Res. Commun.* **307**, 620-625.

Baek JM, Howell CR, Kenerly CM (1999) The role of an extracellular chitinase from *Trichoderma virens* Gv29-8 in the biocontrol of *Rhizoctonia solani. Curr. Genet* **35**, 41-50.

Barnett HL, Hunter BB (1998) 'Illustrated Genera of Imperfect Fungi.' (The American Phytopathological Society: St. Paul, Minnesota)

Bartnicki-Garcia S (1968) Cell wall chemistry, morphogenesis and taxonomy of fungi. *Annual Review of Microbiology* **22**, 87-105.

Bartnicki-Garcia S, Nickerson WS (1962) Isolation, composition and structure of cell walls of filamentous and yeast like forms of *Mucor rouxii. Biochimica et Biophysica Acta* **58**, 102-119.

Benhamou N, Broglie K, Broglie R, Chet I (1993) Antifungal effect of bean endochitinase on *Rhizoctonia solani*: ultra structural changes and cytochemical aspects of chitin breakdown. *Canadian Journal of Microbiology* **39**, 318-328.

Bentley SD (2002) Complete genome sequence of the model actinomycete *Streptomyces coelicolor. Nature* **417**, 141-147.

Bieri S, Potrykus I, Flutterer J (2000) Expression of active barley seed ribosome-inactivating protein in transgenic wheat. *TAG Theoretical and Applied Genetics* **100**, 755-763.

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**, 1513.

Birren B (2003a) The genome sequence of *Ustilago maydis* 521. *direct submission*.

Birren B (2003b) The glucanase sequence of the filamentous fungus *Neurospora crassa*. *Nature* **422**, 859-868.

Birren B, Nusbaum C, Abonelleil Aea (2004) *Giberella zeae* (anamorph: *Fusarium graminearum*). *direct submission*.

Bittker J, Le B, Liu D (2002) Nucleic acid evolution and minimization by nonhomologous random recombination. *Nature biotechnology* **20**, 1024-1029.

Bliffeld M, Mundy J, Potrykus I, Flutterer J (1999) Genetic engineering of wheat for increased resistance to powdery mildew disease. *TAG Theoretical and Applied Genetics* **98**, 1079-1086.

Blount JW, Korth KL, Masoud S, Rasmussen S, Lamb C, Dixon RA (2000) Altering expression of cinnamic acid- 4 hydroxylase in transgenic plants provides evidence for a feedback loop at the entry point into the phenylpropanoid pathway. *Plant Physiology* **122**, 107-116.

Bolar JP, Norelli JL, Harman G, Brown SK, Aldwinckle HS (2001) Synergistic activity of endochitinase and exochitinase from *Trichoderma atroviride* (*T. harzianum*) against the fungus *Venturia inaequalis* in transgenic apple plants. *Transgenic Research* **10**, 533-543.

Bolar JP, Norelli JL, Wong KW, Hayes CK, Harman G, Aldwinckle HS (2000) Expression of endochitinase from *Trichoderma harzianum* in transgenic apple increases resistance to apple scab and reduces vigor. *Phytopathology* **90**, 72-77.

Bougher N (1994) Diversity of ectomycorrhizal fungi associated with eucalypts in Australia. In 'International symposium and workshop'. Kaiping Guangdong Province, PR of China. (Ed. D Brundett L., B., Malajczuk, L., Mingpin, G.). (Aranwang Information Bureau Pty. Ltd. Canberra)

Bougher N, Syme K (1998) 'Fungi of Southern Australia.' (University of Western Austrtalia Press: Perth)

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry* **72**, 248-254.

Bridge PD, Singh T, Arora DK (2004) The application of molecular markers in the epidemiology of plant pathogenic fungi. In 'Fungal Biotechnology in Agricultural, Food, and Environmental applications.' (Ed. DK Arora). (Marcel Dekker, Inc: New York)

Broekaert W, Terras F, Cammue B, Vanderleyden J (1990) An automated quantitative assay for fungal growth inhibition. *FEMS Microbiology letters* **69**, 55-60.

Broglie K, Chet I, Holliday M, Cressman R, Biddle P, Knowlon S, Mauvais C, Broglie R (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. *Science* **254**, 1194-1197.

Brown AG, Turnbull JW, Booth TH (1997) The Australian Environment. In 'Australian Trees and Shrubs: Species for land Rehabilitation and Farm Planting in the Tropics.' (Ed. JC Doran, Turnbull, J.W.) pp. 9-20. (Australian Centre for Agricultural Research: Canberra, Australia)

Buchner P, Rochat C, Wuilleme S, Boutin JP (2002) Characterisation of a tissue-specific and developmentally regulated β−1, 3−glucanase gene in pea. *Plant Molecular Biology* **49**, 171-186.

Busam G, Kassemeyer H-H, Matern U (1997) Differential expression of chitinases in *Vitis vinifera* L. responding to systemic acquired resistance activators or fungal challenge. *Plant Physiology* **115**, 1029-1038.

Carsolio C, Benhamou N, Haran S, Cortes C, Gutierrez A, Chet I, Herrera-Estrella A (1999) Role of the *Trichoderma harzianum* endochitinase *ech42*, in mycoparasitism. *Appl. and Env. Microbiol.* **65**, 929-935.

Cato SA, Gradner RC, Kent J, Richardson TE (2001) A rapid PCR-based method for genetically mapping ESTs. *Theor. Appl. Genet.* **102**, 296-306.

Chen WP, Chen PD, Liu DJ, Kynast R, Friebe B, Velazhahan R, Muthukrishnan S, Gill BS (1999) Development of wheat scab symptoms is delayed in transgenic wheat plants that constitutively express a rice thaumatin-like protein gene. *TAG Theoretical and Applied Genetics* **99**, 755-760.

Cherif M, Benhamou N (1990) Cytochemical aspects of chitin breakdown during the parasitic action of a *Trichoderma* sp on *Fusarium oxysporum* f. sp. *radicis-lycopersici. Phytopathology* **80**, 1406-1414.

Chernin L, Ismailov Z, Haran S, Chet I (1995) Chitinolytic *Enterobacter agglomerans* Antagonistic to fungal plant pathogens. *Appl. and Env. Microbiol.* **61**, 1720-1726.

Chye L, Zhao KJ, He ZM, Ramalingam S, Fung KL (2005) An agglutimating chitinase with two chitin-binding domains confers fungal protection in transgenic potato. *Planta* **220**, 717-730.

Cline J, Braman JC, Hogrefe HH (1996) PCR fidelity of *Pfu* DNA polymerase and other thermostable DNA polymerases. *Nucleic Acids Res.* **24**, 3546-3551.

Cohen-Kupiec R, Broglie K, Friesem D, Broglie R, Chet I (1999) Molecular characterisation of a novel β-1, 3-exoglucanase related to mycoparasitism of *Trichoderma harzianum*. *Gene* **226**, 147-154.

Cottrell MT, Moore JA, Kirchman DI (1999) Chitinases from uncultured marine microorganisms. *Appl. and Env. Microbiol.* **65**, 2553-2557.

Datta K, Muthukrishnan S, Datta SK (1999) Expression and function of PR-protein genes in Transgenic Plants. In 'Pathogenesis-related proteins in Plants'. (Ed. S Muthukrishnan, Datta, S.K) pp. 261-277. (CRC Press: Boca Raton)

De la Cruz J, Hidalgo-Gallego A, Lora JM, Benitez T, Pintor-Toro JA, Llobell A (1992) Isolation and characterisation of three chitinases from *Trichoderma harzianum*. *Eur. J. Biochem.* **206**, 859-867.

De la Cruz J, Llobell A (1999) Purification and properties of a basic endo-β-1, 6-glucanase from the antagonistic fungus *Trichoderma harzianum*. *Eur. J. Biochem.* **265**, 145-151.

De la Cruz J, Pintor-Toro JA, Benitez T, Llobell A (1995a) Purification and characterisation of an endo-β-1, 6-glucanase from *Trichoderma harzianum* that is related to its mycoparasitism. *Journal of Bacteriology* **177**, 1864-71.

De la Cruz J, Pintor-Toro JA, Benitez T, Llobell A, Romero L (1995b) A novel endo-β-1, 3-glucanase BGN13.1, involved in the mycoparasitism of *Trichoderma harzianum. Journal of Bacteriology* **177**, 6937-6945.

De la Cruz J, Rey M, Lora JM, Hidalgo-Gallego A, Dominguez F, Pintor-Toro JA, Llobell A, Benitez T (1993) Carbon source control on β−glucanases, chitobiase and chitinase from *Trichoderma harzianum. Arch. Microbiology* **159**, 316-322.

Di Pietro A, Lorito M, Hayes CK, Broadway RM, Harman G (1993) Endochitinase from *Gliocladium virens:* Isolation, characterisation and synergistic antifungal activity in combination with Giotoxin. *Phytopathology* **83**, 308-313.

Domsch KH, Gams W, Anderson T-H (1993) 'Compedium of soil fungi.' (IHW-Verlag: Eching)

Donzelli B, Lorito M, Scala F, Harman G (2001) Cloning, sequence and structure of a gene encoding an antifungal glucan 1, 3-beta-glucosidase from *Trichoderma atroviride* (*T. harzianum*). *Gene* **277**, 199-208.

Dragborg H, Christgau S (1995) Molecular cloning and expression in yeast of an endochitinase and two exochitinases from *Trichoderma harzianum*. In 'Advances in chitin science'. (Ed. A Dormand, Jeuniaux, C., Muzzarelli, R., Roberts, G.) pp. 48-58. (Jacques Andre: Lyon)

Dujon B, Sherman D, Fischer G, Durrens P (2004) Genomic evolution of yeast. *Nature* **430**, 35-44.

Ellis MB (1971) 'Dematiaceous Hyphomycetes.' (Commonwealth Mycological Institute: Kew, England)

Ellis MB (Ed.) (1976) 'More Dematiaceous Hyphomycetes.' (Commonwealth Mycological Institute: Kew, England)

Ertesvag H, Hoidal HK, Hals IK, Rian A, Doseth B, Valla S (1995) A family of modular type mannuronan C-5-epimerase genes controls alginate structure in *Azotobacter vinelandii*. *Mol. Microbiol.* **16**, 719-731.

Faize M, Faize L, Koike N, Ishii H (2003) Acibenzolar-S-methyl-induced resistance to Japanese pear scab is associated with potentiation of multiple defense responses. In. (Ed. GenBank).

Fekete C, Weszely T, Hornok L (1996) Assignment of a PCR-amplified chitinase sequence cloned from *Trichoderma hamatum* to resolved chromosomes of potential biocontrol species of *Trichoderma. FEMS Microbiology letters* **145**, 385-391.

Felse PA, Panda T (1999) Regulation and cloning of microbial chitinase genes. *Appl. Microbiol Biotechnol* **51**, 141-151.

Felsenstein J (1989) PHYLIP: Phylogeny Inference Package (Version 3.2). In 'Cladistics'. pp. 164-166. (Seattle)

Felsenstein J (2004) PHYLIP: Phylogeny Inference Package (Version 3.6). In. (Department of Genome Science. University of Washington.: Seattle)

Flach J, Pilet P-E, Jolles P (1992) What's new in chitinase research? *Experientia* **48**, 701-716.

Fuglsang C, Berka R, Wahleithner J, Kauppinen S, Shuster J, Rasmussen G, Halkier T, Dalboge H, Henrissat B (2000) Biochemical analysis of recombinant fungal mutanases. A new family of alpha- 1, 3-glucanases with novel carbohydrate-binding domains. *J. Biol. Chem.* **275**, 2009-2018.

Gao AG, Hakimi SM, Mittanck CA, Wu Y, Woerner BM, Stark DM, Shah DM, Liang J, Rommens C (2000) Fungal pathogen protection in potato by expression of a plant defensin peptide. *Nature Biotechnology* **18**, 1307-1310.

Garcia I, Lora JM, de la Cruz J, Benitez T, Llobell A, Pintor-Toro JA (1994) Cloning and characterization of a chitinase (chit42) cDNA from the mycoparasitic fungus *Trichoderma harzianum*. *Current Genetics* **27**, 83-89.

Gerardy-Schahn R, Bethe A, Brennecke T, Muhlenhoff M, Eckhardt M, Ziesing S, Lottspeich F, Frosch M (1995) Molecular cloning and functional expression of bacteriophage PKIE-encoded endoneuraminidase Endo NE. *Mol. Microbiol.* **16**, 441-450.

Geremia R, Jacobs D, Goldman GH, Van Montagu M, Herrera-Estrella A (1991) 'Induction and Secretion of hydrolytic enzymes by the biocontrol agent *Trichoderma harzianum*.' (Elsevier: Amsterdam)

Ghadessy FJ, Ramsay N, Bousdsocq F, Loakes D, Brow n, A, Iwai S, Vaisman A, Woodgate R, Holliger P (2004) Generic expansion of the substrate spectrum of a DNA polymerase by directed evolution. *Nature biotechnology* **22**, 755-759.

Gheysen G, Inze D, Soetaert P, Van Montagu M, Castresana C (1990) Sequence of a *Nicotiana plumbaginifolia* beta (1,3)-glucanase gene encoding a vacuolar isoform. *Nucleic Acids Res.* **18**, 6685.

Glen M (2001) Genetic variation in ectomycorrhizal fungi and its exploitation in ecological investigations of eucalypt forests. PhD thesis, Murdoch University.

Goettel MS, St. Leger RJ, Rizzo NW, Staples RC, Roberts DW (1989) Ultrastructural Localisation of a Cuticle Degrading Protease produced by the Entomopathogenic fungus *Metarhizium anisopliae* during Penetration of Host (*Manduca sexta)* cuticle. *J. Gen. Microbiol.* **135**, 2223-2239.

Gooday GW (1999) 'Aggressive and defensive roles of chitinases.' (Birkhauser, Verlag: Basel/ Switzerland)

Graham GC, Mayers P, J HR (1994) A simplified method for the preparation of fungal genomic DNA for PCR and RAPD analysis. *BioTechniques* **16**, 48-50.

Haran S, Schickler H, Oppenheim A, Chet I (1995) New components of the chitinolytic system of *Trichoderma harzianum. Mycol. Res.* **99**.

Harman GE, Hayes CK, Broadway RM, Di Pietro A, Peterbauer C, Tronsmo A (1993) Chitinolytic enzymes of *Trichoderma harzianum*: Purification of chitobiosidase and endochitinase. *Phytopathology* **83**, 313-318.

He SY, Collmer A (1990) Molecular cloning, nucleotide sequence and marker exchange mutagenesis of the exo-poly-a-D-galacturonosidase encoding pehX gene of *Erwinia chrysanthemi* EC16. *J. Bacteriol.* **172**, 4988-4995.

Henrissat B (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem J* **280**, 309-316.

Henrissat B (1999) 'Classification of chitinases modules.' (Birkhauser Verlag: Basel/Switzerland)

Henrissat B, Bairoch A (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem J* **293**, 781-788.

Henrissat B, Callebaut I, Fabrega S, Lehn P, Mornon JP, Davies G (1995) Conserved catalytic machinery and the prediction of a common fold for several families of glycosyl hydrolases. *Proc. Natl. Acad. Sci. USA* **92**, 7090-7094.

Herrera-Estrella A, Chet I (1999) 'Chitinases in biological control.' (Birkhauser Verlag: Basel/Switzerland)

Hird DL, Worrall D, Hodge R, Smartt S, Paul W, Scott R (1993) The anther-specific protein encoded by the *Brassica napus* and *Arabidopsis thaliana* genes display similarity to β-1, 3-glucanases. *Plant J.* **4**, 1023-1033.

Hoitink HAJ, Boehm MJ (1999) Biocontrol within the context of soil microbial communities: a substrate-dependent phenomenon. *Annu. Rev. Phytopathol.* **37**, 427-446.

Howie W, Joe L, Newbigin E, Suslow T, Dunsmuir P (1994) Transgenic tobacco plants which express the *chiA* gene from *Serratia marcescens* have enhanced tolerance to *Rhizoctonia solani. Transgenic Research* **3**, 90-98.

Hunsley D, Burnett JH (1970) The ultrastructural architecture of the walls of some hyphal fungi. *Journal of General Microbiology* **62**, 203-218.

Inglis GD, Kawchuck LM (2002) Comparative degradation of oomycete, ascomycete, and basidiomycete cell walls by mycoparasitic and biocontrol fungi. *Canadian Journal of Microbiology* **48**, 60-70.

Irene G, L JM, de la Cruz J, Tahia B, Pintor JA (1994) Cloning and characterisation of chitinase (CHIT42) cDNA from mycoparasitic fungus *Trichoderma harzianum. Curr. Genet* **27**, 83-89.

Irie K, Hosoyam H, Takeuchi T, Iwabuchi K (1996) Trangenic rice established to express corn cystatin exhibit strong inhibitory activity against insect gut proteinases. *Plant Molecular Biology* **30**, 149.

Ish-Horowicz D, Burke JF (1981) Rapid and efficient cosmid cloning. *Nucleic Acids Res.* **9**, 2989.

Jach G, Gornhardt B, Mundy J, Logemann J, Pinsdorf E, Leah R, Schell J, Maas C (1995) Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. *The Plant Journal* **8**, 97-109.

Jeffreys AJ, Wilson V, Neumann R, Keyte J (1988) Amplification of human minisatellites by the polymerase chain reacion-amplified DNA. *Nucleic Acids Res.* **16**, 10953-10971.

Jeffries P, Young TWK (1994) Interfungal parasite relationships. In 'Interfungal parasite relationships'. (Ed. D Krupa S.V., Y.R.) pp. 296. (CAB International: Wallingford)

Jensen LG, Olsen O, Kops O, Wolf N, Thomsen KK, von Wettstein D (1996) Transgenic barley expressing a protein-engineered, thermostable B-1, 3-, B-1, 4-glucanase during germination. *Proc. Natl. Acad. Sci. USA* **93**, 3487.

Jin W, Horner H, Palmer R, Shoemaker R (1999) Analysis and mapping of gene families encoding β-1, 3-glucanases of soybean. *Genetics* **153**, 445-452.

Johnson R (1992) 'Past, present and future opportunities in breeding for disease resistance with examples from wheat.' (Kluwer Academic Publishers: Dordecht/The Netherlands)

Johnston M, Hillier L, Riles L, Albermann K, Andre B, Ansorge W, Benes V, Bruckner M, Delius H, Dubois E, Dusterhoft A, Entian KD, Floeth M, Goffeau A, Hebling U, Heumann K, Heuss-Neitzel D, Hilbert H, Hilger F, Kleine K, Kotter P, Louis EJ, Messenguy F, Mewes HW, Hoheisel JD (1997) The nucleotide sequence of Saccharomyces cerevisiae chromosome XII. *Nature* **387**, 87-90.

Jones D, Gordon A, Bacon J (1974) Co-operative action by endo- and exo- β-glucanases from parasitic fungi in the degradation of cell-wall glucans of *Sclerotinia sclerotium. Biochem. J.* **140**, 47-55.

Kaeberlein T, Lewis K, Epstein SS (2002) Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. *Science* **296**, 1127-1129.

Kalaitzis P, Koehler SM, Tucker ML (1995) Cloning of a tomato polygalacturonase expressed in abscission. *Plant Molecular Biology* **28**, 647-656.

Kang SC, Park S, Lee DG (1998) Isolation and characterisation of a chitinase cDNA from the entomopathogenic fungus *Metarhizium anisopliae*. *FEMS Microbiology letters* **165**, 267-271.

Kang SC, Park S, Lee DG (1999) Purification and characterisation of a novel chitinase from the entomopathogenic fungus, *Metarhizium anisopliae. J. Invertebr. Pathol* **73**, 276-281.

Keen NT, Bent A, Staskawicz B (1992) Plant disease resistance genes: Interaction with pathogens and their improved utilisation to control plant diseases. In 'Biotechnology in Plant Disease Control'. (Ed. I Chet). (John Wiley and Sons Inc: New York)

Keen NT, Yoshikawa M (1983) β-1, 3-endoglucanase from soybean releases elicitor-active carbohydrates from fungal cell walls. *Plant Physiology* **71**, 460-465.

Kellmann J-W, Kleinov T, Engelhardt K, Phillipp C, Wegener D, Schell J, Schreier PH (1996) Characterisation of two class II Chitinase genes from peanut and expression studies in transgenic tobbaco plants. *Plant Molecular Biology* **30**, 351-358.

Kikkert JR, Ali GS, Wallace PG, Reisch B, Reustle GM (2000) Expression of a fungal chitinase in *Vitis vinifera* L. Merlot and Chardonnay plants produced by biolistic transformation. *Acta hortic.* **528**, 297-303.

Kilstrup M, Kristiansen KN (2000) Rapid genome walking: a simplified oligo-casette mediated polymerase chain reaction using a single genome-specific primer. *Nucleic Acids Res.* **28**, e55.

Kim J-K, Duan X, Wu R, Seok SJ, Boston RS, Jang IC, Eun MY, Nahm BH (1999) Molecular and genetic analysis of transgenic rice plants expressing the maize ribosome-

inactivating protein b-32 gene and the herbicide resistance bar gene. *Molecular Breeding* **5**, 85-94.

Knight SC, Anthony VM, Brady AM, Greenland AJ, Heaney SP, Murray DC, Powell KA, Schulz MA, Sinks CA, Worthington PA, Youle D (1997) Rationale and perspectives on the development of fungicides. *Annual Review Phytopathology* **35**, 349-372.

Kobayashi DY, Keen NT (1985) Cloning of a factor from *Pseudomonas syringae* pv. *tomato* responsible for a hypersensitive response on soybean. *Phytopathology* **75**, 1355.

Kobayashi DY, Tamaki SJ, Keen NT (1989) Cloned avirulence genes from the tomato pathogen *Pseudonomas syringae* pv. *tomato* confer cultivar specificity on soybean. *Proc. Natl. Acad. Sci. USA* **88**, 2336-2340.

Koga D, Mitsutomi M, Kono M, Matsumiya M (1999) 'Biochemistry of chitinases.' (Birkhauser Verlag: Basel/Switzerland)

Kong LR, Tzeng DD, Yang CH (2001) Generation of PCR-based DNA fragments for specific detection of *Streptomyces saraceticus* N45. *Proc. Natl.Sci. Counc. Rep. China* **25**, 119-127.

Kotake T, Nakagawa N, Takeda K, Sakurai M (1997) Purification and characterisation of a wall-bound exo-1, 3-β-glucanase from barley seedlings. *Plant Cell Physiol.* **38**, 194-200.

Krsek M, Wellington EMH (2001) Assessment of chitin decomposer diversity within an upland grassland. *Antoine Leeuwenhoek* **79**, 261-267.

Kuranda MJ, Robbins PW (1991) Chitinase is required for cell separation during growth of Saccharomyces cerevisiae. *J. Biol. Chem.* **226**, 19758-19767.

LeCleir GR, Buchan A, Hollibaugh JT (2004) Chitinase gene sequences retrieved from diverse aquatic habitats reveal environment-specific distributions. *Appl. and Env. Microbiol.* **70**, 6977-6983.

Leong SA (2004) Biotechnological Approaches in Plant Protection: Achievements, New Initiatives and Prospects. In 'Fungal Biotechnology in Agricultural, Food and Environmental Applications.' (Ed. DK Arora) pp. 1-18. (Marcel Dekker Inc.: New York)

Letcher PM, McGee PA, Powell MJ (2004) Distribution and diversity of zoosporic fungi from soils of four vegetation types in New South Wales, Australia. *Canadian Journal of Botany* **82**, 1490-1500.

Lewis K, Whipps JM, Cooke RC (1989) Biotechnology of Fungi for Improving Plant Growth. In. (Ed. JM Whipps, Lumsden, R.D.). (Cambridge University Press: Cambridge)

Lima LHC, Ulhoa CJ, Fernandes AP, Felix CR (1997) Purification of a chitinase gene from *Trichoderma* sp and its action on *Sclerotinia rolfsii* and *Rhizoctonia solani* cell walls. *J. Gen. Appl. Microbiol.* **43**, 31-37.

Limon MC, Lora JM, Irene G, De la Cruz J, Llobell A, Benitez T, Pintor JA (1995) Primary structure and expression pattern of the 33-kDa chitinase gene from the mycoparasitic fungus *Trichoderma harzianum. Current Genetics* **28**, 478-483.

Lin W, Anuratha CS, Datta K, Potrykus I, Muthukrishnan S, Datta SK (1995) Genetic engineering of rice for resistance to sheath blight. *BioTechniques* **13**, 686.

Lindhorst HJM, van Loon LC, van Rossum CAM, Mayer A, Bol JF, van Roekel JSC, Meulenhoff EJ, Cornelissen BJC (1990) Analysis of acidic and basic chitinases from tobacco and petunia and their constitutive expression in transgenic tobacco. *Molecular Plant-Microbe Interactions* **3**, 252-258.

Liu D, Rhodes D, D-Urzo MP, Xu Y, Narasimhan ML, Hasegawa PM, Bressan RA, Abad L (1994) *In vivo* and *in vitro* activity of truncated osmotin that is secreted into the extracellular matrix. *Plant Science* **121**, 123.

Liu M, Sun Z-X, Zhu J, Xu T, Harman GE, Lorito M (2004) Enhancing rice resistance to fungal pathogens by transformation with cell wall degrading enzyme genes from *Trichoderma atroviride. Journal of Zhejiang University SCIENCE* **5**, 133-136.

Logemann J, Schell J (1993) The impact of biotechnology on plant breeding, or how to combine increases in agricultural productivity with an improved protection of the environment. In 'Biotechnololgy in plant disease control'. (Ed. I Chet) pp. 2-14. (John Wiley and sons Inc.: New York)

Lora J, de la Cruz J, Llobel A, Benitez T, Pintor-Toro J (1995) Molecular characterisation and heterologous expression of an endo-β-1, 6-glucanase gene from the mycoparasitic fungus *Trichoderma harzianum. Mol. Gen. Genet.* **247**, 639-645.

Lorito M, Farkas V, Rebuffat S, Bodo B, Kubicek CP (1996) Cell wall synthesis is a major target of mycoparasitic antagonism by *Trichoderma harzianum. Journal of Bacteriology* **178**, 6382-6385.

Lorito M, Harman GE, Hayes CK, Broadway RM, Tronsmo A, Woo SL, Di Pietro A (1993) Chitinolytic enzymes produced by *Trichoderma harzianum*: Antifungal activity of purified endochitinase and chitobiosidase. *Phytopathology* **83**, 302-307.

Lorito M, Peterbauer C, Hayes CK, Harman GE (1994) Synergistic interaction between fungal cell wall degrading enzymes and different antifungal compounds enhances inhibition of spore germination. *Microbiology* **140**, 623-629.

Lorito M, Woo SL, Garcia Fernandez I, Colucci G, Harman GE, Pintor-Toro JA, Filippone E, Muccifora S, Lawrence CB, Zoina A, Tuzun S, Scala F (1998) Genes from mycoparasitic fungi as a source for improving resistance to fungal pathogens. *Proc. Natl. Acad. Sci. USA* **95**, 7860-7865.

Lucas GB, Campbell CL, Lucas LT (1992) 'Introduction to Plant Diseases: Identification and Management.' (Van Nostrand Reinhold: New York/USA)

Magnuson JK, Lasure LL (2002) Fungal diversity in soils as assessed by direct culture and molecular techniques. In. (Pacific Northwest National Laboratory: Richland, WA)

Manjula K, Podile AR (2001) Chitin-supplemented formulations improve biocontrol and plant growth promoting efficiency of *Bacillus subtilis* AF1. *Canadian Journal of Microbiology* **47**, 618-625.

Marchant R, Davey MR, Lucas JA, Lamb C, Dixon RA, Power JB (1998) Expression of a chitinase transgene in rose (*Rosa hybrida* L) reduces development of black spot disease (*Diplocarpon rosae* Wolf). *Molecular Breeding* **4**, 187-194.

Marchler-Bauer A, Bryant SH (2004) CD-Search: protein domain annotations on the fly. *Nucleic Acids Res.* **32**, 327-331.

Martin JP (1950) Use of acid, rose bengal, and streptomycin in the plate method for estimating soil fungi. *Soil Science* **69**, 215-232.

Masoud SA, Zhu Q, Lamb C, Dixon RA (1996) Constitutive expression of an inducible B-1, 3-glucanase in alfalfa reduced disease severity caused by the oomycete pathogen *Phytophthora megasperma* f. sp *medicaginis*, but does not reduce severity of chitin-containing fungi. *Transgenic Research* **5**, 313.

Mauch F, Mauch-Mani B, Boller T (1988) Antifungal hydrolases in pea tissue. Inhibition of fungal growth by chitinases and β-1, 3-glucanases. *Plant Physiology* **88**, 936-942.

Metcalfe AC, Krsek M, Gooday GW, Prosser JI, Wellington EMH (2002) Molecular analysis of a bacterial chitinolytic community in an upland pasture. *Appl. and Env. Microbiol.* **68**, 5042-5050.

Metraux JP, Bukhart W, Moyer M, Dincher S, Middlesteadt W, Williams S, Payne G, Carnes N, Ryals J (1989) Isolation of a cDNA encoding a chitinase with structural homology to a bifunctional chitinase. *Proc. Natl. Acad. Sci. USA* **86**, 896-904.

Miller GL (1959) Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry* **31**, 426-428.

Mischke S (1997) A quantitative bioassay for extracellular metabolites that antagonize growth of filamentous fungi, and its use with biocontrol fungi. *Mycopathologia* **137**, 45-52.

Muhammad S, Amusa NA (2003) In-vitro inhibition of growth of some seedling blight inducing pathogens by compost-inhabiting microbes. *African Journal of Biotechnology* **2**, 161-164.

Nairn CJ, Niedz RP, Hearn CJ, Osswald WF, Mayer RT (1997) cDNA cloning and expression of a class II acidic chitinase from sweet orange. *Biochimica et Biophysica Acta* **1351**, 22-26.

Nehls U, Bock A, Hampp R (2000) A chitinase gene from the ectomycorrhizal basiodiomycete *Amanita muscaria. direct submission*.

Nelson EC (1981) 'Phytogeography of southern Australia.' (The Hague)

Newsham KK, Watkinson AR, Fitter AH (1995) Rhizosphere and root-infecting fungi and the design of ecological field experiments. *Oecologia* **102**, 230-237.

Nielsen KK, Mikkelsen JD, Kragh KM, Bojsen K (1993) An acidic class III chitinase in sugar beet: induction by Cercospora beticola, characterization, and expression in transgenic tobacco plants. *Mol. Plant Microbe Interact.* **6**, 495-506.

Nierman W, Wortman J, Pain A, Anderson MJ, Arroya J, Hall N, Barrell B, Fraser C, Denning DW (2005) Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus. Unpublished- Direct submission*.

Nikolskaya AN, Pitkin JW, Schaeffer HJ, Ahn JH, Walton JD (1998) EXG1p, a novel exo-beta-1, 3-glucanase from the fungus *Cochliobolus carbonum*, contains a repeated motif present in other proteins that interact with polysaccharides. *Biochimica et Biophysica Acta* **1425**, 632-636.

Nishizawa Y, Nishio Z, Nakazono K, Soma M, Nakajima E, Ugaki M, Hibi T (1999) Enhance resistance to blast (*Magnaporthe grisea*) in transgenic Japonica rice by constitutive expression of rice chitinase. *Theor. Appl. Genet.* **99**.

Noronha EF, Kipnis A, Junqueira-Kipnis AP, Ulhoa CJ (2000) Regulation of 36-kDa β-1, 3-glucanase synthesis in *Trichoderma harzianum. FEMS Microbiology letters* **188**, 19-22.

Northcote KH, Hubble GD, Isbell RF, Thompson CH, Bettenay E (1975) 'Yellow and Yellow-grey Duplex Soils, The Dy Soils.' (Wilke and Co. Ltd.: Clayton, VIC)

Notredame C, Higgins D, Heringa J (2000) T-Coffee: A novel method for multiple sequence alignments. *Jounal of Molecular Biology* **302**, 205-217.

Oda K, Abe K, Akita O, Nakajima T (2001) Cloning and nucleotide sequencing of the exo-beta-1, 3-glucanase gene (exog0) from *Aspergillus oryzae. direct submission*.

Oda K, Kasahara S, Yamagata Y, Abe K, Nakajima T (2002) Cloning and expression of the exo-beta-1, 3-glucanase gene exgS from *Aspergillus saitoi. Biosci. Biotechnol. Biochem.* **66**, 1587-1590.

Ohtakara A (1988) Chitinase activity. In 'Methods in Enzymology'. (Ed. WA Wood, Kellogg, S.T.) pp. 462-470. (Academic Press: San Diego)

Okutani K (1975) 'Microorganisms related to mineralisation of chitin in aquatic environments.' (University of Tokyo Press: Tokyo)

Oldach KH, Becker D, Lorz H (2001) Heterologous expression of genes mediating enhanced fungal resistance in transgenic wheat. *Molecular Plant-Microbe Interactions* **14**, 832-838.

Pappinen A, Degefu Y, Syrjala L, Keinonen K (2002) Transgenic silver birch expressing sugarbeet chitinase 4 shows enhanced resistance to *Pyrenopeziza betulicola. Plant Cell Rep.* **20**, 1046-1051.

Patil RS, Ghormade V, Deshpande MV (2000) Chitinolytic enzymes: an exploration. *Enzyme and Microbial Technology* **26**, 473-483.

Peterbauer C, Lorito M, Hayes CK, Harman G, Kubicek CP (1996) Molecular cloning and expression of the *nagI* gene from *Trichoderma harzianum* PI. *Curr. Genet* **30**, 325-331.

Pinto AS, Barreto CC, Schrank A, Ulhoa CJ, Vainstein MH (1997) Purification and characterisation of an extracellular chitinase from the entomopahogen *Metarhizium anisopliae. Canadian Journal of Microbiology* **43**.

Punja ZK, Raharjo SH (1996) Response of transgenic cucumber and carrot plants expressing different chitinase enzymes to inoculation with fungal pathogens. *Plant Disease* **80**, 999-1005.

Ridout C, Coley-Smith J, Lynch J (1986) Enzyme activity and electrophoretic profile of extracellular protein induced in *Trichoderma* spp. by cell walls of *Rhizoctonia solani. J. of Gen. Microbiol.* **132**, 2345-2352.

Roberts WK, Selitrennikoff CP (1986) Isolation and partial characterisation of two antifungal proteins from barley. *Biochimica et Biophysica Acta* **880**, 161-170.

Rodriguez-Kabana R, Godoy G, Morgan-Jones G, Shelby RA (1983) The determination of soil chitinase activity: conditions for assay and ecological studies. *Plant Soil* **75**, 95-106.

Rohini VK, Sankjara Rao K (2001) Transformation of peanut (*Arachis hypogaea* L.) with tobacco chitinase gene, variable response of transformants to leaf spot disease. *Plant Science* **160**, 889-898.

Rommens C, Kishore G (2000) Exploiting the full potential of disease-resistance genes for agricultural use. *Curr. Opin. Biotechnol.* **11**, 120-125.

Rosenberg A, Griffin K, Studier FW, McCormick M, Berg J, Novy R, Mierendorf R (1996) T7Select Phage display system: a powerful new protein display system based on bacteriophage T7. *Advanced products and protocols for molecular biology research* **6**, 1-6.

Rosenberger RF (1976) The Cell Wall. In 'Filamentous fungi'. (Ed. BDR Smith J.E.) pp. 328-344. (Edward Arnold: London)

Rotem Y, Yarden O, Sztejnberg A (1998) The mycoparasite *Ampelomyces quisqualis* expresses several exo-beta-1, 3-glucanases in culture and during mycoparasitism. *unpublished*.

Roux KH, Dhanarajan P (1990) A strategy for single site PCR amplification of dsDNA: Priming digested cloned or genomic DNA from an anchor-modified restriction site and a short internal sequence. *BioTechniques* **8**, 48-57.

Sahai AS, Monacha MS (1993) Chitinases of fungi and plants: their involvement in host parasite interaction. *FEMS Microbiology Rev* **11**, 317-338.

Salzer P, Hubner B, Sirrenberg A, Hager A (1997) Differential effect of purified spruce chitinases and β-1, 3-glucanases on activity of elicitors from ectomycorrhizal fungi. *Plant Physiology* **114**, 957-968.

Sambrook J, Fritsch EF, Maniatis T (1989) '"Molecular cloning: A laboratory manual".' (Cold Spring Harbor Laboratory Press: New York)

Sanz L, Montero M, Redondo J, Llobell A, Monte E (2005) Expression of an alpha-1,3-glucanase during mycoparasitic interaction of *Trichoderma asperellum*. *FEBS Journal* **272**, 493-499.

Sasaki T, Matsumoto T, Katayore Y (2002) *Oryza sativa* nipponbare (GA3) genomic DNA. *direct submission*.

Schaeffer HJ, Leykam J, Walton JD (1994) Cloning and targeted gene disruption of EXG1, encoding exo-beta 1, 3-glucanase, in the phytopathogenic fungus *Cochliobolus carbonum. Appl. and Env. Microbiol.* **60**, 594-598.

Schaffrath U, Mauch F, Freydl E, Schweizer P, Dudler R (2000) Constitutive expression of the defense-related Rir 1b gene in transgenic rice plants confers enhanced resistance to the rice blast fungus *Magnaporthe grisea. Plant Molecular Biology* **43**, 59-66.

Schirmbock M, Lorito M, Wang Y-L, Hayes CK, Arisan-Atac I, Scala F, Harman GE, Kubicek CP (1994) Parallel Formation and Synergism of Hydrolytic Enzymes and Peptaibol Antibiotics, Molecular Mechanisms involved in the Antagonistic Action of *Trichoderma harzianum* against Phytopathogenic Fungi. *Appl. and Env. Microbiol.* **60**, 4364-4370.

Schrempf H (1999) 'Characteristics of chitin-binding proteins from Streptomycetes.' (Birkhauser Verlag: Basel/Switzerland)

Screen SE, St Leger RJ (2000) Cloning, expression and analysis of chitinase genes from the entomopathogenic fungus Metarhizium anisopliae. In. (Ed. GenBank).

Seki H (1966) Seasonal fluctuation of heterotrophic bacteria in the sea of Aburatsubo Inlet. *J. Oceanogr. Soc. Jpn.* **22**, 15-26.

Severgnini SME, Wylie SJ, O'Brien P (2005) Chitinase ChitA from *Trichoderma asperellum*. In. (GenBank: Perth)

Shih NR, McDonald KA, Jackman AP, Girbes T, Iglesias R (1997) Bifunctional plant defence enzymes with chitinase and ribosome inactivating activities from *Trichosanthes kirilowii* cell cultures. *Plant Science* **130**, 145-150.

Siebert PD, Chenchik A, Kellogg DE, Lukyanov KA, Lukyanov SA (1995) An improved PCR method for walking in uncloned genomic DNA. *Nucleic Acids Res.* **23**, 1087-1088.

St Leger RJ, Charnley AK, Cooper RM (1986) Cuticle degrading enzymes of entomopathogenic fungi: Synthesis in culture on cuticle. *J. Invertebr. Pathol* **48**, 85-95.

Stajich, JE, Block D, Boulez K, Brenner SE, Chervitz SA, Dagdigian C, Fuellen G, Gilbert JGR, Korf I, Lapp H, Lehvaslaiho H, Matsalla C, Mungall CJ, Osborne B, Pocock MR, Schattner P, Senger M, Strein LD, Stupka ED, Wilkingson M, Birney E (2002) The Bioperl Toolkit: Perl Model for the Life Sciences. *Genome Research* **12**, 1611-1618.

Steyaert JM, Stewart A, Ridgway HJ (2003) Co-expression of two genes, a chitinase (chit42) and a proteinase (prb1) implicated in mycoparasitism by *Trichoderma hamatum*. *direct submission*.

Stirling M, Stirling G (1997) Disease management: Biological control. In 'Plant pathogens and plant diseases'. (Eds JFaO Brown, H J and .). (Rockvale Publications: Armidale)

Sutton BC (1980) 'The Coelomycete: Fungi Imperfecti with Pycnidia, Acervuli and Stromata.' (Commonwealth Mycological Institute: Kew, England)

Synstad B, Gaseidnes S, van Aalten DMF, Vriend G, Nielsen JE, Eijsink VGH (2004) Mutational and computational analysis of the role of conserved residues in the active site of a family 18 chitinase. *Eur. J. Biochem.* **271**, 253-262.

Tabei Y, Kitade S, Nishizawa Y, Kikuchi N, Kayano T, Hibi T, Akutsu K (1998) Transgenic cucumber plants harboring aq rice chitinase gene exhibit enhanced resistance to gray mold *(Botrytis cinerea)*. *Plant Cell Reports.* **17**, 159.

Takana T, Fujiwara S, Nishikori S, Fukui T, Takagi M, Imanaka T (1999) A unique chitinase with dual active sites and triple substrate b inding from the hyperthermophilic archaen *Pyrococcus kodakaraensis* KOD1. *Appl. and Env. Microbiol.* **65**, 5338-5344.

Takaya N, Yamazaki D, Horiuchi H, Ohta A, Takagi M (1998a) Cloning and characterisation of a chitinase-encoding gene (chiA) from *Aspergillus nidulans*, disruption of which decreases germination frequency and hyphal growth. *Biosci. Biotechnol. Biochem.* **62**, 60-65.

Takaya N, Yamazaki D, Horiuchi H, Ohta A, Takagi M (1998b) Intracellular chitinase gene from *Rhizopus oligosporus*: molecular cloning and characterisation. *Microbiology* **144**, 2647-2654.

Terakawa T, Takaya N, Horiuchi H, Koike M, Takagi M (1997) A fungal chitinase gene from *Rhizopus oligosporus* confers antifungal activity to transgenic tobacco. *Plant Cell Reports.* **16**, 439.

Thomas CJ, Gooday GW, King LA, Possee RD (2000) Mutagenesis of the active site coding region of the *Autographa californica* nucleopolyhedrovirus *chiA* gene. *Journal of General Virology* **81**, 1403-1411.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W; improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalty and weight matrix choice. *Nucleic Acids Res.* **22**, 4673-4680.

Tommerup IC, Bougher NL (1999) The role of ectomycorrhizal fungi in nutrient cycling in temperate Australian woodlands. In 'Temperate Eucalypt Woodlands in Australia: Biology, Conservation, Management and Restoration.' (Ed. RJ Hobbs, Yates, C.) pp. 190-224. (Surrey Beatty and sons: Chipping Norton, Australia)

Tronsmo A, Harman GE (1993) Detection and quantification of *N*-acetyl-B-D-glucosaminidase, chitobiosidase and endochitinase in solutions and on gels. *Analytical biochemistry* **208**, 74-79.

Tsujibo H, Orikoshi H, Baba N, Miyahara M, Miyamoto K, Yasuda M, Inamori Y (2002) Identification and characterisation of the gene cluster involved in chitin degradation in a marine bacterium, *Alteromonas* sp. Strain O-7. *Appl. and Env. Microbiol.* **68**, 263-270.

Ulhoa CJ, Peberdy JF (1991) Regulation of chitinase synthesis in *Trichoderma harzianum*. *J. Gen. Microbiol.* **137**, 2163-2169.

Usui T, Matsui H, Isobe K (1990) Enzymic synthesis of useful chito-oligosaccharides utilizing transglycosylation by chitinolytic enzymes in a buffer containing ammonium sulfate. *Carbohydr. Res.* **203**, 65-77.

Valadaris-Inglis V, Cheria M, Peter IW, Peberdy JF (1997) Sequence analysis of the catalytic domain of *Metarhizium anisopliae* chitinase. *Braz J Genet* **20**, 161-164.

Vasconcelos AT, de Almeida DF, Almeida FC (2003) The complete genome sequence of *Chromobacterium violaceum* reveals remarkable and exploitable bacterial adaptability. *Proc. Natl. Acad. Sci. USA* **100**, 11660-11665.

Vasseur V, M.V M, Goldman GH (1995) *Trichoderma harzianum* genes induced during growth on *Rhizoctonia solani* cell walls. *Microbiology* **141**, 767-774.

Vazquez-Garciduenas S, Leal-Morales C, Herrera-Estrella A (1998) Analysis of the β-1, 3-glucanolytic system of the biocontrol agent *Trichoderma harzianum. Appl. and Env. Microbiol.* **64**, 1442-1446.

Villanueva N, Salas M (1981) Adsorption of bacteriophage phi29 to *Bacillus subtilis* through the neck appendage of the viral particle. *J. Virol.* **38**, 15-19.

Viterbo A, Chet I (2001) Characterisation of chitinases from *Trichoderma. Unpublished- Direct submission*.

Viterbo A, Chet I (2002) Expression regulation of chit36 in *Trichoderma asperellum*. *unpublished-direct submision*.

Viterbo A, Haran S, Friesem D, Ramot O, Chet I (2001) Antifungal activity of a novel endochitinase gene (chit36) from *Trichoderma harzianum Rifai* TM. *FEMS Microbiology letters* **200**, 169-174.

Viterbo A, Montero M, Ramot O, Friesem D, Monte E, Llobell A, Chet I (2002) Expression regulation of the endochitinase chit36 from *Trichoderma asperellum* (*T. harzianum* T-203). *Curr. Genet* **42**.

von Heijne G (1986) A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* **14**, 4683-4690.

Walton JD (1994) Deconstructing the Cell wall. *Plant Physiology* **104**, 1113-1118.

Wang S-L, Shih I, Liang T, Wang C (2002) Purification and characterisation of two antifungal chitinases extracellularly produced by *Bacillus amyloliquefaciens* V656 in a shrimp and crab shell powder medium. *J. Agric. Food Chem.* **50**, 2241-2248.

Wang S-Y, Wu S-J, Thottapilly G, Locy RD, Singh NK (2001) Purification and characterisation of a *Bacillus cereus* exochitinase. *Enzyme and Microbial Technology* **28**, 492-498.

Watanabe A, Kobori K, Yamada T, Ito Y, Uchida M, Tanaka H (1993a) Domain structures and functions of bacterial chitinases. In 'Chitin Enzymology'. (Ed. RAA Muzzarelli) pp. 329-336. (Eur Chitin Society: Ancona)

Watanabe T, Oyanagi W, Suzuki K, Ohnishi K, Tanaka H (1992) Structure of the gene encoding chitinase D of Bacillus circulans WL-12 and possible homology of the enzyme to other prokaryotic chitinases and class III plant chitinases. *J.Bacteriol* **174**, 408-414.

Watanabe TK, Kobori K, Miyashita T, Fujii H, Sakai M, Uchida M, Tanaka H (1993b) Identification of glutamic acid 104 and aspartic acid 200in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. *J. Biol. Chem.* **268**, 18567-18572.

Wei H, Scherer M, Singh A, Liese R, Fischer R (2001) *Aspergillus nidulans* alpha-1, 3-glucanase (mutanase) mutA, is expressed during sexual development and mobilises mutan. *Fungal Genetics and Biology* **34**, 217-227.

Wessels JGH (1965) Morphogenesis and biochemical processes in *Schizophyllum commune*. *Wentia* **13**, 1-113.

Wessels JGH, Kreger DR, Marchant R, Regensburg BA, De Vrie OMH (1972) Chemical and morphological characterisation of the hyphal wall surface of the Basidiomycete *Schizophyllum commune. Biochimica et Biophysica Acta* **273**, 346-358.

White A, Withers SG, Gilkes NR, Rose DR (1994) Crystal structure of the catalytic domain of the beta-1, 4-glycanase cex from *Cellumonas fimi. Biochemistry* **33**, 12546-12552.

Woo SL, Donzelli B, Scala F, Mach RL, Harman G, Kubicek CP, Del Sorbo G, Lorito M (1999) Disruption of the *ech42* (endochitinase-encoding) gene affects biocontrol activity in *Trichoderma harzianum* P1. *Mol. Plant Microbe Interact.* **12**, 419-429.

Wu G, Shart BJ, Lawrence EB, Lovine EB, Fitzsimmons KC, Shah DM (1995) Disease resistance conferred by expression of a gene encoding H2O2--generating glucose oxidase in transgenic potato plants. *Plant Cell* **7**, 1357.

Wu H, Echt CS, Popp MP, Davis JM (1997) Molecular cloning, structure and expression of an elicitor-inducible chitinase gene from pine trees. *Plant Molecular Biology* **33**, 979-987.

Xia H, Guo S (2001) Cloning and identification of a chitinase cDNA from *Grifola umbellata. direct submission*.

Xu D, Xue QZ, McElroy D (1996a) Constitutive expression of a cowpea trypsin inhibitor gene, *CpTi*, in transgenic rice plants confers resistance to two major rice insect pests. *Mol. Breed.* **2**, 167.

Xu P, Wang J, Fincher GB (1992) Evolution and differential expression of the 1-3-beta-glucan-endohydrolase encoding gene family in barley *Hordeum vulgare. Gene* **120**, 157-165.

Xu Y, Zhu Q, Panbangred W, Shirasu K, Lamb C (1996b) Regulation expression and function of a new basic chitinase gene in rice (*Oryza sativa* L.). *Plant Molecular Biology* **30**, 387-401.

Yamamoto T, Iketani H, Ieki H, Nishizawa Y, Notsuka K, Hibi T, Hayashi T, Matsuta N (2000) Transgenic gravine plants expressing a rice chitinase with enhanced resistance to fungal pathogens. *Plant Cell Reports.* **19**, 639-646.

Yanai K (1992) Random hydrolysis of N-acetyl-beta-D-glucosaminide 1,4-beta-linkages in chitin and chitodextrins. Belongs to the glycosyl hydrolase 18 family. Chitinase class II subfamily. In. (Ed. GenBank).

Yanai K, Takaya N, Kojima N, Horiuchi H, Ohta A, Takagi M (1992) Purification of two chitinases from *Rhizopus oligosporus* and isolation and sequencing of the encoding gene. *J. Bacteriol.* **174**, 7398-7406.

Yeboah NA, Arahira M, Nong VH, Zhang D, Kadokura K, Watanabe A, Fukazawa C (1998) A class III acidic endochitinase is specifically expressed in the developing seeds of soybean (*Glyine max* [L.] Merr.). *Plant Molecular Biology* **36**, 407-415.

Zhang Z, Gurr SJ (2003) A 1, 3-beta-glucanase from *Blumeria graminis* related to the fungal cell wall. *unpublished*.

Zhao HM, Woodyer R, Johannes T (2004) Protein engineering of phosphite dehydrogenase for the development of a novel NAD(P)H regeneration system. *Abstract of Papers of the American Chemical Society* **227**, U130-U130.

Zhu B, Chen THH, Li PH (1996) Analysis of late-blight disease resistance and freezing tolerance in transgenic potato plants expressing sense and antisense gene for an osmotin-like protein. *Planta* **198**, 70-77.

Zhu Q, Maher EA, Masoud S, Dixon RA, Lamb CJ (1994) Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. *Biotechnology* **12**, 807-812.

Zmasek CM, Eddy SR (2001) ATV: Display and manipulation of annotated phylogenetic trees. *Bioinformatics* **17**, 383-384.

APPENDIX 1

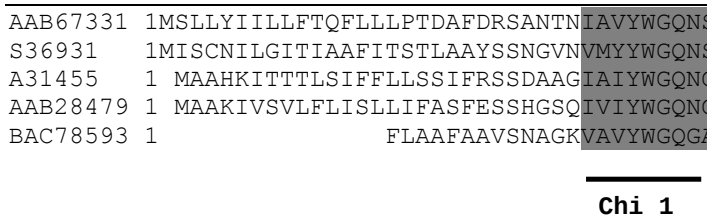


Figure A1.1-Amino acid sequence alignment of known chitinase genes used to design degenerate primer Chi 1 (N→C terminus). *S. cerevisiae*, AAB67331 (Johnston *et al.* 1997); *R. niveus*, S36931 (Yanai 1992); *Cucumis sativus*, A31455 (Metraux *et al.* 1989); *Beta vulgaris*, AAB2847 (Nielsen *et al.* 1993); *Oryza sativa*, BAC78593 (Sasaki *et al.* 2002)

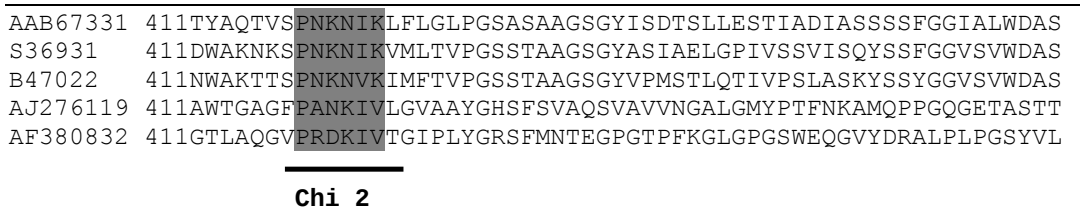


Figure A1.2-Amino acid sequence alignment of known chitinase genes used to design degenerate primer Chi 2 (N→C terminus). *S. cerevisiae* AAB67331(Johnston *et al.* 1997) *R. niveus* S36931(Yanai 1992) *R. oligosporus* B47022(Yanai *et al.* 1992) *Amanita muscaria* AJ276119(Nehls *et al.* 2000) *Grifola umbellata* AF380832(Xia and Guo 2001)

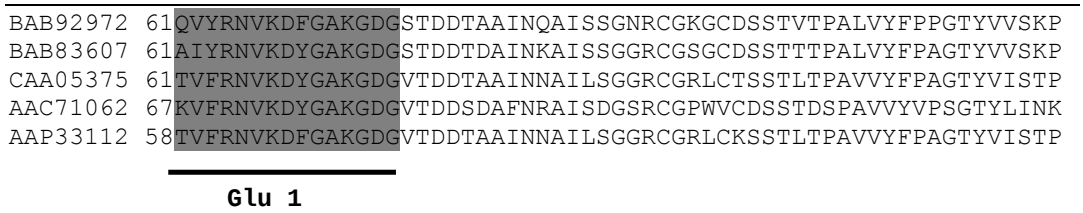


Figure A1.3-Amino acid sequence alignment of known glucanase genes used to design degenerate primer Glu 1 (N→C terminus). *A. oryzae*, BAB92972 (Oda *et al.* 2001)*; A. phoenicis* BAB83607 (Oda *et al.* 2002); *Trichoderma harzianum*, CAA05375 (Donzelli *et al.* 2001); *Cochliobolus carbonum*, AAC71062 (Nikolskaya *et al.* 1998); *T. hamatum*, AAP33112 (Steyaert *et al.* 2003).

```
BAB92972 ...LLPTLPLPNIPLLSGLLSGSQSSATQPAGVLSSEVPEPTATPSTPEEAEPSTEVQSTP
AAP33112 ...TLPLSSFKSVRDDTTALQNIINSATAAGQVVYFDAGIYRITKTLTIPPGAKIVGEEYP
AAF80600 ...KAHGAKGDGSTDDTAAIQAIFNSATSGQVVYFDHGAYVITDTIKVPANIKIVGEIWPL
XP757542 ...FPPGKYLVSSPITSYYYTQLVGSATDRPTLLAAPSFQGIAVIDEDPYASDGSNWYINQ
CAA05375 ...RSAGATGNAVTDDTAALQSVINSATACGQIVYFDAGIYRITSTLSIPPGAKIVGEEYP
```

Glu 3

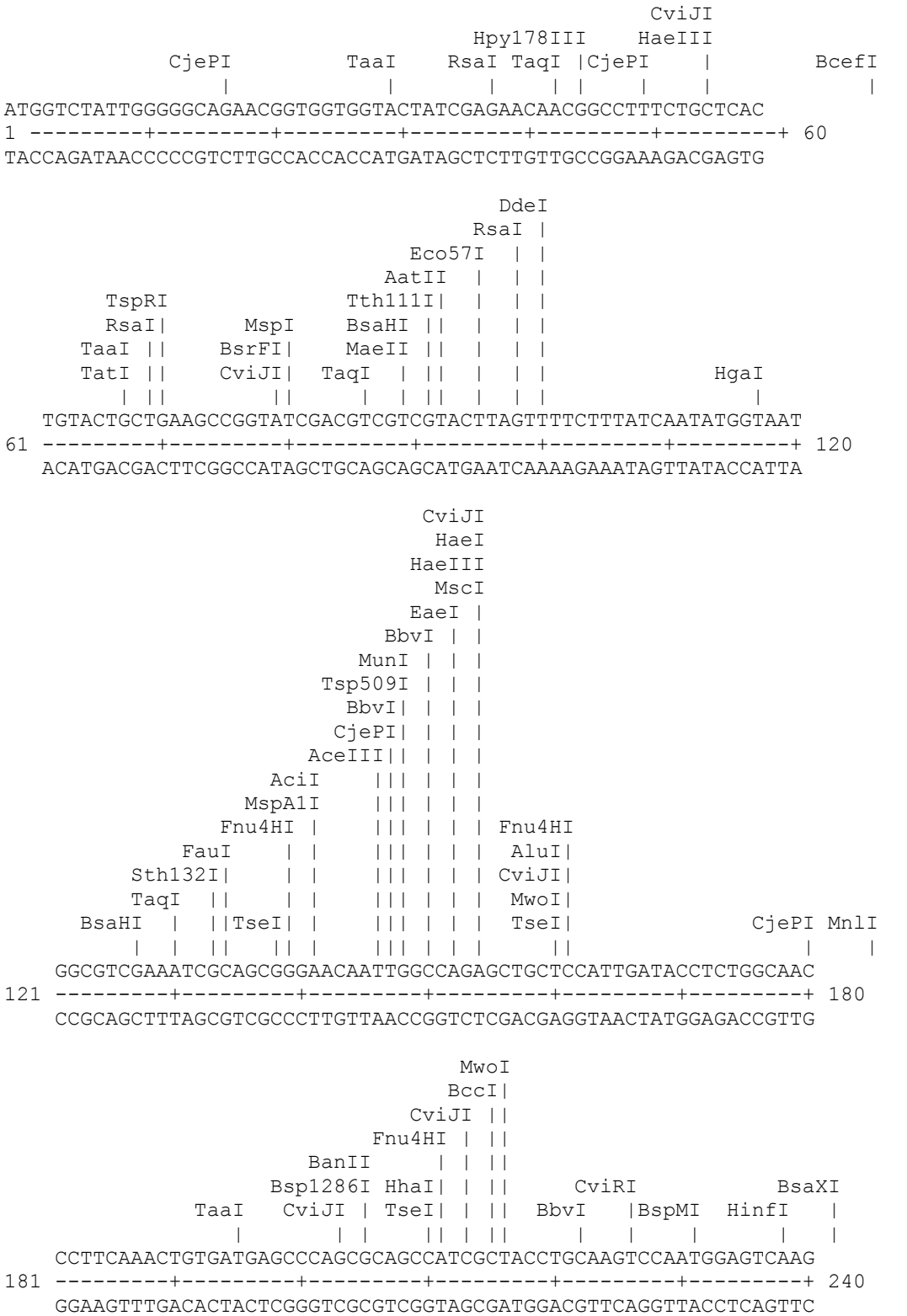
Figure A1.4-Amino acid sequence alignment of known glucanase genes used to design degenerate primer Glu 3 (N→C terminus). *A. oryzae*,BAB92972 (Oda *et al.* 2001); *T. hamatum*, AAP33112 (Steyaert *et al.* 2003)*; T. harzianum*, AAF80600 (Donzelli *et al.* 2001)*; Ustilago maydis*, XP_757542 (Birren 2003a)*; Hypocrea lixii*, CAA05375 (Cohen-Kupiec *et al.* 1999).

APPENDIX 2

```
Query: 177 tgagcaatagtagttgttgtagaactgaatccagaggtagtcaaactg 224
           ||||||||||||||||||||||||||| ||| || ||||||||||| 
Sbjct: 655 tgagcaatagtagttgttgtagaactgaacccaaagatagtcaaactg 60
```

Figure A2- Overlapping sequences obtained from the genome walking method using 04-001 nucleotide sequence. Forty seven bases of the original sequence overlap with the newly amplified sequence that was homologous to the known sequence.

APPENDIX 3


CviJI
Hpy178III HaeIII
CjePI TaaI RsaI TaqI |CjePI BcefI
ATGGTCTATTGGGGGCAGAACGGTGGTGGTACTATCGAGAACAACGGCCTTTCTGCTCAC
1 60
TACCAGATAACCCCCGTCTTGCCACCACCATGATAGCTCTTGTTGCCGGAAAGACGAGTG
DdeI
RsaI
Eco57I
AatII
TspRI Tth111I
RsaI| MspI BsaHI
TaaI || BsrFI| MaeII
TatI || CviJI| TaqI HgaI
TGTACTGCTGAAGCCGGTATCGACGTCGTCGTACTTAGTTTTCTTTATCAATATGGTAAT
61 120
ACATGACGACTTCGGCCATAGCTGCAGCAGCATGAATCAAAAGAAATAGTTATACCATTA
CviJI
HaeI
HaeIII
MscI
EaeI
BbvI
MunI
Tsp509I
BbvI|
CjePI|
AceIII||
AciI |||
MspA1I |||
Fnu4HI | ||| Fnu4HI
FauI | ||| AluI|
Sth132I| | ||| CviJI|
TaqI || | ||| MwoI|
BsaHI | ||TseI| | ||| TseI| CjePI MnlI
GGCGTCGAAATCGCAGCGGGAACAATTGGCCAGAGCTGCTCCATTGATACCTCTGGCAAC
121 180
CCGCAGCTTTAGCGTCGCCCTTGTTAACCGGTCTCGACGAGGTAACTATGGAGACCGTTG
MwoI
BccI|
CviJI ||
Fnu4HI | ||
BanII | | ||
Bsp1286I HhaI| | || CviRI BsaXI
TaaI CviJI | TseI| | || BbvI |BspMI HinfI
CCTTCAAACTGTGATGAGCCCAGCGCAGCCATCGCTACCTGCAAGTCCAATGGAGTCAAG
181 240
GGAAGTTTGACACTACTCGGGTCGCGTCGGTAGCGATGGACGTTCAGGTTACCTCAGTTC

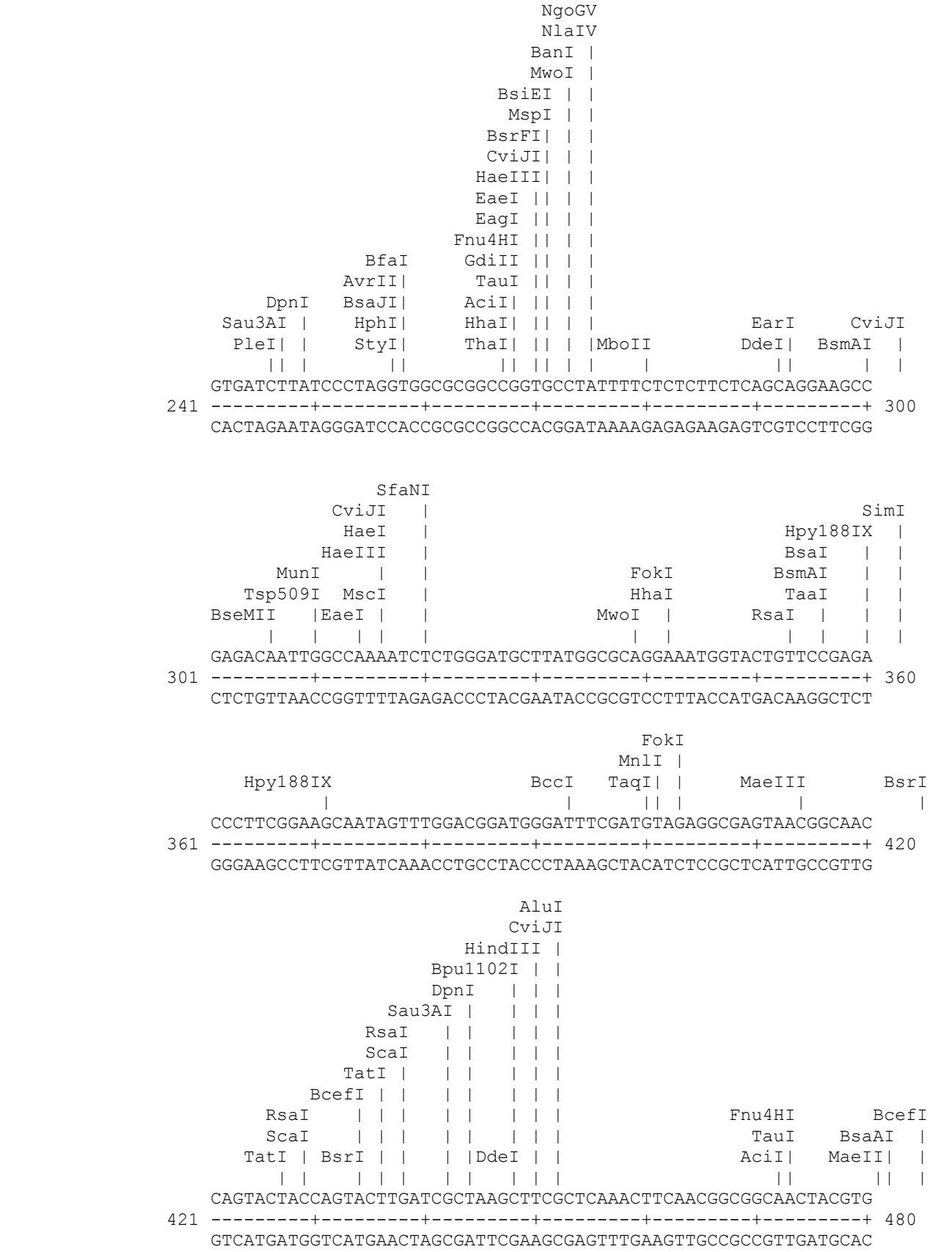

NgoGV
NlaIV
BanI
MwoI
BsiEI
MspI
BsrFI
CviJI
HaeIII
EaeI
EagI
Fnu4HI
BfaI
GdiII
AvrII
TauI
DpnI
BsaJI
AciI
Sau3AI
HphI
HhaI
PleI
StyI
ThaI
MboII
EarI
CviJI
DdeI
BsmAI
GTGATCTTATCCCTAGGTGGCGCGGCCGGTGCCTATTTTCTCTCTTCTCAGCAGGAAGCC
241
CACTAGAATAGGGATCCACCGCGCCGGCCACGGATAAAAGAGAGAAGAGTCGTCCTTCGG
300
SfaNI
CviJI
HaeI
HaeIII
MunI
Tsp509I
MscI
BseMII
EaeI
SimI
Hpy188IX
BsaI
FokI
BsmAI
HhaI
TaaI
MwoI
RsaI
GAGACAATTGGCCAAAATCTCTGGGATGCTTATGGCGCAGGAAATGGTACTGTTCCGAGA
301
CTCTGTTAACCGGTTTTAGAGACCCTACGAATACCGCGTCCTTTACCATGACAAGGCTCT
360
FokI
MnlI
Hpy188IX
BccI
TaqI
MaeIII
BsrI
CCCTTCGGAAGCAATAGTTTGGACGGATGGGATTTCGATGTAGAGGCGAGTAACGGCAAC
361
GGGAAGCCTTCGTTATCAAACCTGCCTACCCTAAAGCTACATCTCCGCTCATTGCCGTTG
420
AluI
CviJI
HindIII
Bpu1102I
DpnI
Sau3AI
RsaI
ScaI
TatI
BcefI
RsaI
ScaI
TatI
BsrI
DdeI
Fnu4HI
TauI
BsaAI
AciI
MaeII
BcefI
CAGTACTACCAGTACTTGATCGCTAAGCTTCGCTCAAACTTCAACGGCGGCAACTACGTG
421
GTCATGATGGTCATGAACTAGCGATTCGAAGCGAGTTTGAAGTTGCCGCCGTTGATGCAC
480

Figure A3- Restriction map of the putative chitinase sequence, *ChiA* {Accelrys Inc)