CELL WALL DEGRADING ENZYMES AND INTERACTION BETWEEN *TRICHODERMA AGGRESSIVUM* AND *AGARICUS BISPORUS*

BY

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ABSTRACT

Agaricus bisporus is the most commonly cultivated mushroom in North America and has a great economic value. Green mould is a serious disease of *A. bisporus* and causes major reductions in mushroom crop production. The causative agent of green mould disease in North America was identified as *Trichoderma aggressivum* f. *aggressivum*. Variations in the disease resistance have been shown in the different commercial mushroom strains.

The purpose of this study is to continue investigations of the interactions between *T. aggressivum* and *A. bisporus* during the development of green mould disease. The main focus of the research was to study the roles of cell wall degrading enzymes in green mould disease resistance and pathogenesis.

First, we tried to isolate and sequence the N-acetylglucosaminidase from *A. bisporus* to understand the defensive mechanism of mushroom against the disease. However, the lack of genomic and proteomic information of *A. bisporus* limited our efforts. Next, *T. aggressivum* cell wall degrading enzymes that are thought to attack *Agaricus* and mediate the disease development were examined. The three cell wall degrading enzymes genes, encoding endochitinase (*ech*42), glucanase (*β-1,3 glucanase*) and protease (*prb1*), were isolated and sequenced from *T. aggressivum* f. *aggressivum*. The sequence data showed significant homology with the corresponding genes from other fungi including *Trichoderma* species. The transcription levels of the three *T. aggressivum* cell wall degrading enzymes were studied during the *in vitro* co-cultivation with *A. bisporus* using RT-qPCR. The transcription levels of the three genes were significantly upregulated compared to the solitary culture levels but were upregulated to

a lesser extent in co-cultivation with a resistant strain of *A. bisporus* than with a sensitive strain.

An *Agrobacterium tumefaciens* transformation system was developed for *T. aggressivum* and was used to transform three silencing plasmids to construct three new *T. aggressivum* phenotypes, each with a silenced cell wall degrading enzyme. The silencing efficiency was determined by RT-qPCR during the individual *in vitro* co-cultivation of each of the new phenotypes with *A. bisporus*. The results showed that the expression of the three enzymes was significantly decreased during the *in vitro* co-cultivation when compared with the wild type. The phenotypes were co-cultivated with *A. bisporus* on compost with monitoring the green mould disease progression. The data indicated that *prb1* and *ech*42 genes is more important in disease progression than the *β-1,3 glucanase* gene. Finally, the present study emphasises the role of the three cell wall degrading enzymes in green mould disease infection and may provide a promising tool for disease management.

ACKNOWLEDGMENT

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LIST OF ABBREVIATIONS

6PAP	:	6-n-Pentyl-2-pyrone
Abs	:	Absorbance
BSA	:	Bovine serum albumin
cDNA	:	Complementary DNA
DEPC	:	Diethylpyrocarbonate
DMSO	:	Dimethyl sulfoxide
dNTP	:	Deoxynucleotide triphosphate
dsRNA	:	Double-stranded RNA
DTT	:	Dithiothreitol
EDTA	:	Ethylene diamine tetra-acetic acid
EtOH	:	Ethanol
GFP	:	Green fluorescence protein
GlcNAc	:	N-acetyl-β-glucosamine
GlcNAcases	:	N-acetylglucosaminidases
GPD	:	Glyceraldehyde-3-phosphate dehydrogenase
IEF	:	Isoelectric focusing
LB	:	Luria-Bertani broth
LB	:	Left border
MCS	:	Multi-cloning site
MEA	:	Malt Extract agar
miRNA	:	Micro RNA
ORF	:	Open reading frame
PCR	:	Polymerase chain reaction
pI	:	Isoelectric point
ppm	:	Parts per million
qPCR	:	Quantitative PCR
RB	:	Right border
RISC	:	RNA-induced silencing complex
RNAi	:	RNA interference
RT	:	Reverse transcription
SCM	:	*Schizophyllum* complete yeast medium

SDS	:	Sodium dodecyl sulfate
SDS-PAGE	:	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
shRNA	:	Short hairpin RNA
siRNA	:	Small interfering RNA
UV	:	Ultraviolet
vir	:	Virulence genes
X-gal	:	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
IMAS		Induction medium with acetosyringone

INTRODUCTION

Agaricus bisporus is an edible mushroom and the most commonly cultivated mushroom in North America (Rinker and Chalmers, 1998). Cultivation of this species has great economic value; in fact *A. bisporus* is the most valuable Canadian vegetable crop, generating an average of over $200 million dollars in revenue annually (adapted from source: Statistics Canada).

Commercial crops of this mushroom have suffered from epidemics of a green mould disease, caused by the soil filamentous fungus initially identified as *Trichoderma harzianum*. The epidemic started in Europe and was later found in North America (Williams *et al.*, 2003). In 1995, Chester County, Pennsylvania, mushroom farms posted record crop losses (30-100%) worth millions of dollars (Wuest *et al.*, 1996).

Green mould disease is characterized by starting with white mycelium and developing to green sporulation over the mushroom area (Ospina *et al.*, 1999). The green mould inhibited the formation of *Agaricus* fruiting bodies and mushroom yields by up to 100% (Anderson *et al.*, 2001). *A. bisporus* contamination by green mould can happen by several sources such as dust particles, workers' clothing, machinery and air (Seaby, 1996b).

Rifai (1969) isolates *T. harzianum* were initially recovered on mushroom farms with green mould and were classified into four biotypes Th1, Th2, Th3 and Th4 based on morphological differences ((Rinker *et al.*, 1997; Seaby, 1987)). Of these forms, Th2 was identified as the European biotype causing a green mould disease while Th4 is the North American biotype (Chen *et al.*, 1999). They were later renamed *Trichoderma*

aggressivum f. *europaeum* and *Trichoderma aggressivum* f. *aggressivum*, respectively (Samuels *et al.*, 2002). These two biotypes are responsible for the major crop losses in Europe and North America.

Based upon the antagonism of fungi by other *Trichoderma* species, it is possible that *T. aggressivum* may exploit a variety of antagonistic mechanisms in the presence of other fungi. It was suggested that *T. aggressivum* could attack *A. bisporus* through antibiotic secretion. Krupke *et al.* (2003) found that secretion of volatile metabolites by *T. aggressivum* Th4 inhibits *A. bisporus* fungal growth. These metabolites were not present in non-aggressive *Trichoderma* Th1 and Th3.

Another important mechanism that has been implicated in *Trichoderma* pathogenicity is the production of hydrolytic enzymes such as chitinases, glucanases, and proteases. These hydrolytic enzymes are thought to be involved in degradation of fungal cell walls (Woo *et al.*, 1999). It has been suggested that the interaction between *T. harzianum* and a host may play a role in the induction of these specific enzymes (Haran *et al.*, 1996).

T. harzianum has been extensively used in the biocontrol of a wide range of phytopathogenic fungi. Cortes (1998) reported that hydrolytic enzymes secreted by the parasite have been directly implicated in host cell wall lysis. Chitinase and glucanase activities were found in soil when *T. harzianum* attacked *Sclerotium rolfsii* or *Rhizoctonia solani* (Elad *et al.*, 1982). It was found that the *T. harzianum* strain that was capable of attacking the pathogen secreted more β-1,3-glucanase and chitinase than the strains that were incapable of attack. The authors also investigated the ability of an antagonistic strain of *T. harzianum* to attack *S. rolfsii* sclerotia. They found that the

invading *T. harzianum* made holes in their surfaces. These sclerotia eventually lost their regular shape and their cytoplasm (Elad *et al.*, 1984).

Considering the complexity of the *in vivo* interaction between a mycoparasitic fungus and its host (in the plant rhizosphere or in the soil) dual-interaction assays provide the closest approximation and the most feasible methodological approach for studying the biochemical events and molecular biology of mycoparasitism. Flores *et al.* (1997) used this method to investigate the expression of a *T. harzianum* alkaline protease gene (*prb*1) during the antagonistic interaction between *T. harzianum* and *R. solani*. They found that the mRNA levels of *prb*1 increased as the mycoparasitic interaction progressed. Protease activity seemed to be crucial for lysing fungal cells.

The strict mechanism of mycoparasitism implicated in the green mould disease is unknown. In order to develop methods to better control the disease, the mechanism of disease production during mycoparasitic interaction needs to be well understood. Therefore, in this study we will investigate the roles of the cell wall degrading enzymes during the interaction between *T. aggressivum* and *A. bisporus*.

CHAPTER I

LITERATURE REVIEW

1.1- *AGARICUS BISPORUS*

A. bisporus is commonly referred to as the common button mushroom and accounts for up to 90% of all commercial mushroom production in North America (Rinker and Chalmers, 1998). In general, there are several different strains of commercial mushrooms such as white strains including Horst U1 and off-white hybrid, and Sylvan 130 and brown strains including Sylvan SB65 and Amycel 2400. The content of mushroom in general is made up of 90% water and 10% dry matter. Of the dry matter, the mushroom contains between 27% and 48% protein content, 60% or less carbohydrates, and 2-8% lipids (Crisan and Sands 1978; Ranzani and Sturion 1998; Morais *et al.*, 2000).

1.1.1- Classification

A. bisporus is a member of the Kingdom Fungi and belongs to Division Basidiomycota. The members of this division are characterized by the formation of basidiospores on an external basidium (Moore-Landecker, 1996). *A. bisporus* is classified in the class Basidiomycetes which is further subdivided into 17 orders, where *A. bisporus* belongs to the order Agaricales. The shape of *A. bisporus* is characterized by its gills that carry basidia over its surface (Moore-Landecker, 1996). The genus *Agaricus* contains many species and all of them are edible. The *A. bisporus* species reflects the presence of two spores on each basidium. This fungus is widespread in different habitats such as soil, wood and dung (Moore-Landecker, 1996). This group includes many of the

most familiar commercial mushrooms such as the button mushroom, which will be included in this study.

1.1.2- Economic significance of *A. bisporus*

The most important commercial mushroom worldwide is *A. bisporus* (Lange) Imbach (Button mushroom), with a widespread growth area including North America, Europe, Far East, and Australasia (Chang, 1999). Cultivation of this species has great economic value; in fact, *A. bisporus* is the most valuable Canadian vegetable crop. The annual quantity of produced Canadian mushroom in 2008 is shown in Table 1.1. Thirty three states in the US cultivate this species with a value of production worth $963 million in 2006 (National Agricultural Statistics Service, 2009) (Figure 1.1).

Table 1.1: Annual commercial mushroom revenue by region with nation's total ($million). Adapted from Source: Statistics Canada, July 27/2009, ht tp://www.statcan.gc.ca/pub/22-003-x/22-003-x2009001-eng.pdf

2008	Canada	Maritime provinces and Quebec	Ontario	Prairie provinces	British Columbia
Area \.000 sq ft					
Under cultivation	5.380	133	2.727	248	2.272
Harvested	39.468	759	23.668	1.712	13.329
Production\.000 lb					
Sold fresh	173.522	3.450	95.532	8.952	65.882
Sold to processors	18.162	0	13.750	0	4.412
Total production	191.684	3.450	109.282	8.952	70.000
Sales\ .000 dollars					
Fresh market sales	277.272	4.733	173.431	16.856	82.253
Sales to processors	13.938	0	9.526	0	4.412
Total sales	291.210	4.733	182.957	16.856	86.665
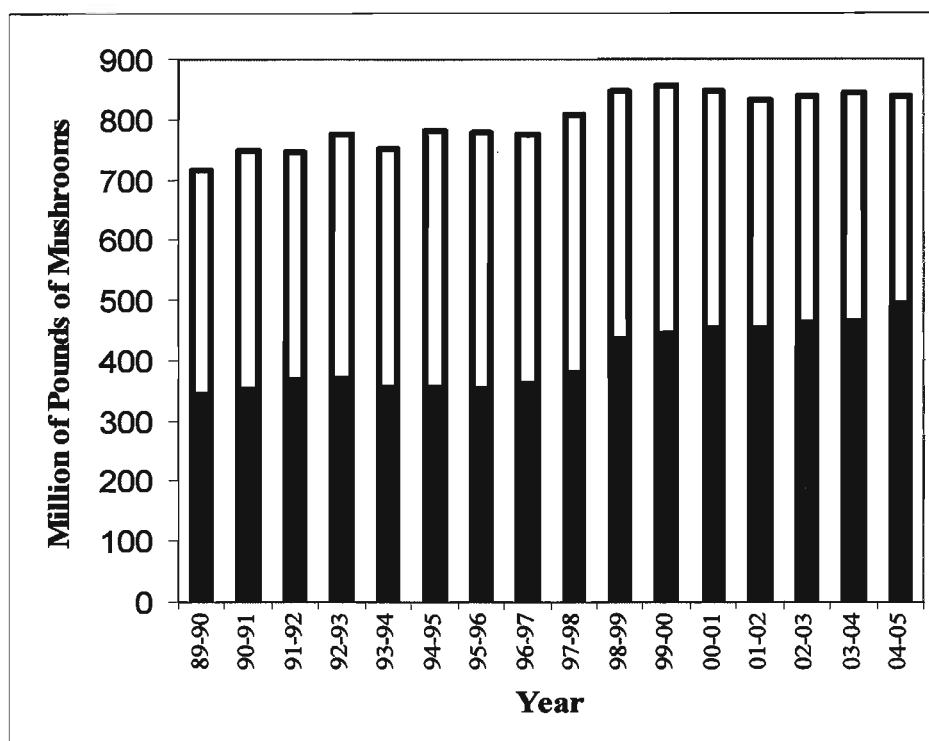
Figure 1.1: The quantity of the mushrooms production, in lbs, in Pennsylvania (black) and the USA (black and white) from 1989 to 2005 (National Agricultural Statistics Service 2009).

1.1.3- Commercial mushroom production

Several cultivation methods are used to grow mushrooms. Commercial mushroom cultivation methods may vary between operations; however mushroom production can generally be outlined in six principal stages (Wuest *et al.*, 1985).

1.1.3.1- Phase 1: Composting

The first phase in mushroom cultivation is preparing suitable compost. The compost is an assemblage of organic materials (wheat straw), which provides a carbon source for fungal growth. The substrate is readily colonized by microbes which actively degrade organic materials including sugars, proteins and polysaccharides. The compost is

a selective substrate for *A. bisporus*. After 10-14 days, the compost is broken up and is moved into the shelf beds of the mushroom house. Furthermore, the temperature of the mushroom house is raised to 55-60 °C for 4-8 days (Ingold, and Hudson, 1993). At this stage the compost has a high concentration of lignin, a neutral pH, and is a source of insoluble nitrogen (protein). The compost is then pasteurized at 70 °C for 1 to 2 hours in order to kill any remaining organisms. After that, compost cools to 25 °C and is inoculated with a spawn.

1.1.3.2- Phase 2: Spawning

The spawning phase lasts for two weeks and is important to colonize the compost completely by the *Agaricus* mycelium. The spawn itself is the hydrated and autoclaved cereal grain that has been buffered and inoculated by *A. bisporus*. After the complete colonization of the grain by the mycelium, the spawn is inoculated into the compost. This process requires the adjustment of the CO_2 concentration [20,000 to 30,000 parts per million (ppm)], growth temperature (24 to 27°C) and relative humidity (85%). After about two weeks, the *A. bisporus* mycelia should cover about 90% of the compost.

1.1.3.3- Phase 3: Casing

The fully colonized compost is then covered with a casing soil layer 3-5 cm thick. The most common casing soil is a mixture of loam and peat moss. This step requires the readjustment of the CO_2 concentration at 3000 ppm and relative humidity is raised to over 95% with no change in the temperature and hydration. Within a week, *A. bisporus* mycelium will colonize completely the casing layer.

1.1.3.4- Phase 4: Pinning and cropping

When the *A. bisporus* mycelium reaches the surface of the casing layer, it starts the pinning step where the pin refers to the small initiated mushroom fruit body. The process continues until the formation of the complete fruiting bodies takes place. This process requires the adjustment of growth conditions, the CO_2 concentrations drop to 1000 ppm, relative humidity drop to 85% and temperature lowered to 20°C, to facilitate the formation of the fruiting bodies. Almost one week is required under the optimum growth conditions. In the cropping stage, the grown mushrooms are picked by hand to reduce damage. Mushrooms are formed on about three successive flushes with one week intervals in between. The three week cropping stage yields up to 30-35 kg of mushrooms per square meter. To eliminate any fungal or bacterial contamination of the mushroom growing room, a steam treatment that raise room temperature to 65°C for 40 hours is applied after final crop removal.

1.1.4- Mushroom pathogens

Mushroom production is hampered by different pathogens that significantly reduce yield or increases the production and disease control costs. The first reported mushroom pathogen was the La France Virus isolated in 1948 in USA (Sinden and Hauser 1950). Since that time, other mushroom pathogens have been identified (Table 1.2) included *T. aggressivum*, the causative agent of the green mould disease.

Table 1.2: Reported mushroom pathogens (Grogan, 2009).

Pathogen	Year reported	Country	Reference
La France Virus	1948	USA	Sinden and Hauser (1950)
Pythium artotrogus	1960	USA	Fergus *et al.* (1963)
Pythium oligandrum	1986	UK	Fletcher *et al.* (1990)
Trichoderma aggressivum f. *europium*	1984	Ireland	Seabe (1987)
Trichoderma aggressivum f. *aggressivum*	1990	Canada	Muthumeenakshi *et al.* (1994) and Castle *et al.* (1998)
Cladobotryum mycophilum Type II	1992	Ireland	McKay *et al.* (1999)
Mushroom Virus X	1996	UK	Gaze *et al.* (2000)

1.2- *TRICHODERMA*

The genus *Trichoderma* is described as filamentous fungi that are distributed ubiquitously in soil, plant material, decaying vegetation, and wood (Thornton *et al.*, 2002). This genus includes a large variety of species, several of which are commonly present at mushroom farms: *T. viride, T. koningii, T. atroviride, T. longibrachiatum T. hamatum* and *T. harzianum* (Castle *et al.*, 1998). Most species of the *Trichoderma* genus do not pose a threat to mushroom crops, however, *Trichoderma aggressivum* is implicated in major crop devastation of *A. bisporus* worldwide (Samuels *et al.*, 2002). This problem in commercial mushroom crops is called green mould disease.

1.2.1- Geographic distribution of *Trichoderma* species

The different *Trichoderma* species vary in their geographical distribution, as some are widely distributed and others are limited. Species such as *T. aureoviride* is limited to the UK and northern Europe (Lieckfeldt *et al.*, 2001) and *T. koningii* was found in different areas of the world (Turner *et al.*, 1997). *T. reesei*, which is used commonly in cellulose production, was isolated from the Solomon Islands of the Pacific area. Other species are very restricted. For example, *T. stromaticum* which is present only in association with cacao trees (*Theobroma cacao*) in tropical America (Samuels *et al.*, 2000) and *T. longibrachiatum* is widely distributed in North and South America, Europe, Africa, and India, while Southeast Asia or Australasia do not have this species (Turner *et al.*, 1997). On the other hand, *T. citrinoviride* which is closely related to *T. longibrachiatum* is absent from Africa and India and is found in other area such as North and South America, Europe, Southeast Asia, and Australasia. Finally, some *Trichoderma* species such as *T. harzianum* is widely distributed internationally (Chaverri *et al.*, 2003).

1.2.2- *Trichoderma* as an antagonist

Biological control measures are unlikely to eradicate a pathogen or its damage, however, it can reduce the pathogen's ability to produce and maintain high levels of inocula. For example, *Trichoderma* spp. are able to suppress disease caused by pathogens such as *Botrytis* and *Sclerotinia* by reducing the pathogen's sporulation in the primary infection cycle as well as by preventing accumulation of sclerotia, through a combination of nutrient competition and mycoparasitism (Dubos, 1987). *Trichoderma* species are potent agents for biocontrol of plant pathogens. Chet (1987) states that among the action

mechanisms proposed for them is mycoparasitism with concomitant production of enzymes that degrade fungal cell walls.

There are two possible mechanisms for the antagonistic effect mediated by *Trichoderma*. The first is based on the nutrients and space competition and the second is mediated through the ability of *Trichoderma* to resist the toxic metabolites of the pathogen as well as producing its own toxic metabolites. The metabolites produced by *Trichoderma* can inhibit the germination of spores and kill the cells of the pathogenic fungus. The physical interaction between *Trichoderma* and the pathogen involves the synergistic effect mediated by the production of hydrolytic enzymes and antibiotics.

Trichoderma harzianum acts as an antagonist toward several fungal hosts (de la Cruz and Llobell 1999). A principal mechanism of parasitism employed by *T. harzianum* is the secretion of lytic enzymes: proteases, chitinases and glucanases (Haran *et al.*, 1996). These enzymes degrade fungal cell walls, causing cellular lysis, and the resulting cellular matter is absorbed and incorporated into the parasite cells (de la Cruz and Llobell 1999). I t is plausible that *T. aggressivum* uses enzymatic mechanisms similar to *T. harzianum* during its interaction with *A. bisporus*.

1.3- GREEN MOULD DISEASE

1.3.1- History of disease

Problems caused by *Trichoderma* species in *A. bisporus* production was first described by Sinden and Hauser (1953). The disease was a minor problem because it periodically infected small areas of the compost and casing materials. However, in 1985, some Irish mushroom farms detected a new severe problem that they termed as green

mould disease and that caused a very large reduction in crop yield. Some mushroom houses completely lost their crops. Initial investigations on this disease suggested that it was due to a strain of *Trichoderma harzianum* and was designated Th2 (Seaby, 1987). The disease symptoms were marked with a green sporulation grown on the casing of the infected areas. These symptoms appeared after two to five weeks after the spawning phase. This infection spread in different UK mushroom farms and caused a major economic problem. The production loss by the end of 1986 was estimated to be 10 million English pounds (Seaby, 1987). To identify the causative agent, *Trichoderma* strains were isolated from different farms and were identified as *T. harzianum* Rifai. This was confirmed by the inoculation of *A. bisporus* compost with this *Trichoderma* strain and observation of the same symptoms. T his *Trichoderma* biotype was named Th2 (Seaby, 1987).

It wasn't until 1990 that the first North America report of the disease was in British Columbia. Later, in 1992, it was reported that the disease had spread to Ontario (Rinker, 1993). The symptoms of this disease were identical to that observed in Ireland and by 1994 it had spread to Pennsylvania (Muthumeenakshi *et al.*, 1998; Rinker *et al.*, 1997). The effect of the disease spread in North America was dramatic with massive production losses especially in California, Pennsylvania, British Colombia and Ontario. However, further investigations revealed that the disease is caused by another *Trichoderma* biotype other than the one discovered in Ireland. This new biotype was designated Th4 (Seaby, 1996a).

1.3.2- History of names

Many fungal species, such as *Penicillium* spp., *Aspergillus* spp. and *Cladosporium* spp. as well as many other *Trichoderma* spp. infect mushroom farms and produce green color symptoms (Rinker, 1993; Rinker *et al.*, 1997). However, only *Trichoderma* biotypes Th2 and Th4 were found to cause loss in the crop production (Castle *et al.*, 1998).

Different species of *Trichoderma* are associated with green mould disease and cause a significant damage to mushrooms. However, not all *Trichoderma* species colonize *A. bisporus* crops (Castle *et al.*, 1998). *T. harzianum* had been classified into four biotypes Th1, Th2, Th3, and Th4 according to morphology. Th2 is the European biotype while Th4 is the North American biotype (Chen *et al.*, 1999). They were later renamed *Trichoderma aggressivum* f. *europaeum* and *Trichoderma aggressivum* f. *aggressivum*, respectively (Samuels *et al.*, 2002). Muthumeenakshi and Mills (1995); Castle *et al.* (1998) and Ospina *et al.* (1999) showed that the causative agent Th2 in Europe and Th4 in North America were genetically distinct from *T. harzianum* Th1 (Castle *et al.*, 1998) and Th3 biotype is now classified as *Trichoderma atroviride* not *T. harzianum*.

1.3.2.1- Th1 biotype

The name *T. harzianum* was initially used for the Th1 strain, which is the most common type of *T. harzianum*. It can grow on the compost regardless of the presence of *A. bisporus* (Muthumeenakshi *et al.*, 1998; Seaby, 1987 and Samuels *et al.*, 2002). This strain grows rapidly at 27-30°C, and within two days, produces dark green spores which

are small and spherical. Cultivating this strain with *A. bisporus* on the same compost results in the complete inhibition of growth both of them in areas that were occupied by the other (Mumpuni *et al.*, 1998). The secondary metabolites of this strain are very toxic to *A. bisporus*, however, they do not reduce the production yield significantly (Mumpuni *et al.*, 1998).

1.3.2.2- Th2 biotype

This strain was first identified in Northern Ireland (Seaby, 1996). Unlike the Th1 strain, the growth of Th2 is completely dependent on the presence of *A. bisporus* in the compost. Th2 can completely infect the *A. bisporus* spawn, however, in absence of *A. bisporus*, no Th2 growth could be detected (Muthumeenakshi *et al.*, 1998; Seaby, 1987). This strain has an optimal growth temperature of 22-30°C and within four days it forms tufts of green spores (Muthumeenakshi *et al.*, 1998; Seaby, 1987; 1989). Comparison of the growth rate, spore size, color and amount of aerial mycelia between Th1 and Th2 strains suggested that Th2 originally evolved from Th1 (Seaby, 1987). However, based on molecular biology tools, both strains were proven to be genetically different (Muthumeenakshi *et al.*, 1994) and therefore, Th2 was named as *Trichoderma aggressivum* f. *europaeum* (Samuels *et al.*, 2002). Th2 can grow in areas occupied by *A. bisporus* and not be affected by its metabolites. The growth is widespread across the compost wherever *A. bisporus* is growing (Mumpuni *et al.*, 1998; Williams *et al.*, 2003). The aggressive nature of the Th2 against *A. bisporus* reduces the mushroom quality and production yield by up to 80% (Williams *et al.*, 2003).

1.3.2.3- Th3 biotype

This is another biotype of *Trichoderma* also found in Northern Ireland and can grow on the compost independently from *A. bisporus* (Seaby, 1989). The genetic analysis of the Th3 strain revealed that it is *T. atroviride* not *T. harzianum* (Castle *et al.*, 1998; Muthumeenakshi *et al.*, 1998). *T. atroviride* is similar to Th1 in its non-aggressive nature towards *A. bisporus* growth (Muthumeenakshi *el al.*, 1998).

1.3.2.4- Th4 biotype

The identification of the fourth *Trichoderma* strain (Th4) took place in North America after the first outbreak of green mould disease. Genetic analysis proved that this strain is different from the previously identified Th2 strain (Muthumeenakshi *et al.*, 1998). Comparison of the four *Trichoderma* strains on the molecular levels proved that they originated from different ancestors (Castle *et al.*, 1998). This last strain was named *Trichoderma aggressivum* f. *aggressivum* since it has aggressive nature towards *A. bisporus* and has the ability to lower the mushroom production yield by up to 100%, therefore reflecting a more aggressive interaction than the Th2 strain (Samuels *et al.*, 2002; Williams *et al.*, 2003).

1.3.3- Epidemiology

Green mould disease (Figure 1.2) is characterized by starting with white mycelium and developing to green sporulation over the mushroom area (Ospina *et al.*, 1999). Green mould disease was found in different areas involved in the mushroom production including the steamed rooms prepared for phase II compost material (Seaby, 1996b and Rinker *et al.*, 1997). Poor sanitation techniques are responsible for the high transmission

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of the disease between the different production areas and phases. The key factors involved in the spread of the green mould disease are the lack of cleanup and proper removal of compost waste in addition to the movement of workers and equipments contaminated with the disease (Rinker *et al.*, 1997). Other factors include animals such as Red-pepper mites, mice and sciarid flies that can carry and spread the spores all over the mushroom farm.
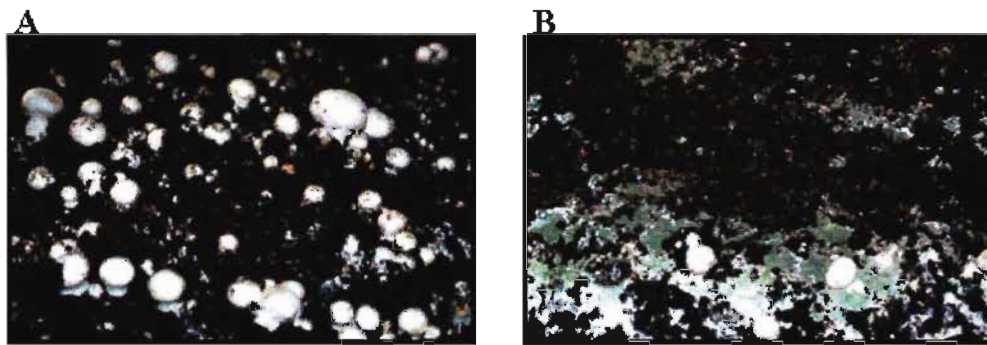
Figure 1.2: Compost infected with green mould disease at the mushroom facility of the Vineland station, Ontario, Canada. (A): Intact mushroom and (B): Infected mushroom.

1.3.4- Disease management

In the past few years, the large growth in mushroom production has influenced the development of mushroom disease management strategies to increase the production efficiency. Unsuccessful disease management, especially in the early stages of outbreak, can lead to dramatic and costly consequences. This includes the spread of the disease to all areas of the mushroom farm. In turn, this will severely reduce crop yield and add more cost for treatment and labour (Grogan, 2009).

The use of the rigorous sanitation programs can help in controlling the infection of the newly spawned compost (Kurpke *et al.*, 2003). The introduction of strict hygiene and

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sanitation methods in the mushroom farms can reduce the risk of infection, regardless of its high cost. In addition, control of the insects and mites can be used to reduce disease spread (Rinker, 1993).

Many pesticides have been developed to protect and treat farms for a variety of problems. However concerns about health or environmental damage have reduced the pesticide use and led to more dependence on few chemicals or on disease prevention methods. The use of fewer chemicals magnifies another problem which is resistance by the pathogen to chemical treatment. Many fungal pathogens tend to develop resistance against the fungicides. This can lead to the complete inefficiency of the applied fungicide (Romaine *et al.*, 2005).

The chemical reagent Benlate 50WP (DuPont Canada Inc., Mississauga, Ontario) has been used in the chemical control of green mould disease however; it is no longer being produced. Benzimidazole formulations such as Benlate inhibit microtubule formation in fungi. The dry powder of the chemical was used to cover the spawn grains prior to spawning (Rinker and Alm, 1998). The chemical was highly efficient in preventing the crop loss when Th4 was used to infect the chemically treated spawn. However, the yield loss was 100% in absence of the chemical treatment (Rinker and Alm, 1998).

Although the use of the chemical treatment is much cheaper than the sanitation methods, the main drawback of the chemical compound is the quick development of fungicide resistance. Many fungal pathogens have developed resistance to the benzimidazole fungicides, among these pathogens are *Verticillium fungicola*, *Cladobotryum mycophilum* and *T. aggressivum* (Fletcher and Yarham, 1976; Grogan and

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Gaze, 2000 and Romaine *et al*, 2005). In many pathogens, such resistance to the benzimidazole fungicides originates from mutations in the β-tubulin gene (Koenraadt *et al.*, 1992).

Fungicide resistance is not the only challenge that faces green mould disease control, since the chemical inefficiency can occur for reasons other than the resistance mechanism. The fungicide is applied to the farm soil which contains different microorganisms. Some of these organisms can degrade fungicides such as benomyl and carbendazim rendering them inactive and reduce or eliminate their effect on the pathogen (Fletcher *et al.*, 1980 and Yarden *et al*, 1990). The biodegradation is advantageous from the environmental point of view, since the degradation of the chemical will reduce the toxicity and accumulation. But, the fast degradation can also limit the effectiveness of the treatment.

1.3.5- Fungal antagonism by *Trichoderma* species

Both Th1 and Th3 are non-aggressive and they permit mushroom growth within or along side the *Trichoderma*-infected areas. The two aggressive biotypes (Th2 and Th4) completely inhibit the mushroom growth. The mechanisms of interaction between these biotypes and mushrooms are not understood (Rinker and Castle, 2005). It is hypothesized that the physical interaction, volatile antifungal compounds (Claydon *et al.*, 1987; Ghisalberti and Rowland, 1993 and Faull *et al.*, 1994) and cell wall degrading enzymes (Elad *et al.*, 1982; de la Cruz *et al.*, 1995; Noronha and Ulhoa, 1996) could be main players in the interaction.

1.3.5.1- Attachment and penetration

Mycoparasitism can be detected through the microscopic examination of the parasite fungus and its host fungus. Evidence of such interaction is the coiling of parasite hyphae around the host hyphae (Jeffries and Young, 1994). In addition, the mycoparasite can penetrate the hyphae of the host fungi by partial degradation of its cell wall (Chet *et al.*, 1981). There is evidence that *Trichoderma* interacts with some fungal species through hyphal attachment and penetration (Elad *et al.*, 1983 and Benhamou and Chet, 1993), however, no such evidence was found between the *T. aggressivum* and *A. bisporus* (Castle, unpublished data).

1.3.5.2- Antifungal compounds

Trichoderma species are well known to produce a variety of antibiotics. For example, *T. harzianum* has been shown to produce 6-pentyl-2-pyrone (6PAP) (Claydon *et al.*, 1987). It has been found that liquid cultures of *T. aggressivum* and *T. harzianum* have compounds that inhibit the growth of *A. bisporus*. The metabolites produced by Th2 are less toxic to *Agaricus* growth than the metabolites produced by Th1 allowing simultaneous growth of both fungi in the compost (Mumpuni *et al.*, 1998). However, the inhibition caused by the presence of Th2 is enough to prevent the formation of *Agaricus* fruiting bodies. Large growth inhibition zones of *A. bisporus* on agar plates were obtained when crude extracts of *T. aggressivum* or *T. harzianum* were used. However, the inhibition was greater with the *T. harzianum* crude extract (Krupke *et al.*, 2003). To identify the specific compounds responsible for this effect, silica column chromatography was used to purify the crude extract of both *T. aggressivum* and *T. harzianum*. Two compounds were obtained from *T. aggressivum* and both showed an

inhibitory effect. Neither of these two compounds was purified from *T. harzianum*, only 6PAP (Krupke *et al.*, 2003). One *T. aggressivum* compound was identified as 3,4-dihydro-8-hydroxy-3-methyl isocoumarin (Krupke *et al.*, 2003). The other compound has yet to be identified.

1.3.5.3- Cell-wall-degrading enzymes

A defining characteristic of the fungal kingdom is the rigid cell wall (Kendrick, 2000). The function of the fungal cell wall is to protect the cell from unfavorable physical, chemical and biological conditions (Adams, 2004). The cell wall is an amorphous matrix composed of different materials which vary between taxonomic groups (Adams, 2004). In general, the structural components of fungal cell walls contain mannan, proteins, chitin and various types of β-glucans as structural components (Adams, 2004). Glucans are polysaccharides that are composed of glucose units that are held together by glycosidic linkages (Pitson *et al.*, 1993). β-glucans are homogeneous polymers, made up of D-glucose molecules, in a β configuration (Pitson *et al.*, 1993). Structurally, β-glucans are classified according to their chain configuration and principal linkage type. When these chains appear in a triple helical structure conformation, they are classified as β-1,3-glucans. In fungal cell walls, β-glucans occur predominantly in β-1,3-linkage configurations, as well as in β-1,6-glycosidic linkages (Pitson *et al.*, 1993). These β-glucans reinforce chitin microfibrils forming the fungal wall structure (Figure 1.3).
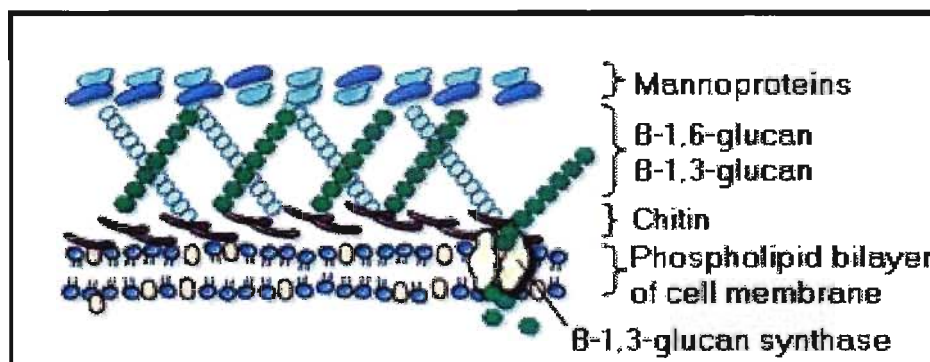
Figure 1.3: Major fungal cell wall components (Adapted from Source: Dr. Fungus) http://www.doctorfungus.org/thedrugs/images/tn/glucan_target.jpg

Although variations in fungal cell wall composition exist, the collective phylum Basidiomycota shares two common fibrous structural elements, chitin and glucan (Kendrick, 2000). Specifically, the cell walls of the commercially produced mushroom, *A. bisporus* are composed of 43% chitin, 41% glucans, 16% protein and 1.5% lipid (Michalenko *et al.,* 1976).

Hydrolytic enzymes are highly associated with mycoparasitic events in plants and fungi (Adams, 2004). During a parasitic event, hydrolytic enzymes such as proteases, chitinases and glucanases are secreted by the parasitic organism, and once secreted they begin to degrade the host organism's cell wall materials, thus disrupting cell wall integrity and synthesis. The hydrolysis of the polymers causes the cell walls to lyse, and consequently the host organism is unable to persist (Kendrick, 2000).

The production of cell wall degrading enzymes such as chitinases, glucanases, and proteases is an important mechanism that has been implicated in *Trichoderma* pathogenicity. These hydrolytic enzymes are thought to be involved in the degradation of *A. bisporus* cell wall (Zeilinger *et al.*, 1999). The penetration of the host mycelium is reported to occur through the partial digestion of the cell wall, a process that is facilitated

by the mycolytic enzymes that are produced and secreted from the parasitic fungus. Among these enzymes, chitinases and glucanases play an essential role. These enzymes have different roles which are complementary for the final antagonistic action (Lorito *et al.*, 1993; 1994a). This allows these parasites to have a maximum antifungal effect on their host. A list of *Trichoderma* cell wall degrading enzymes thought to have roles in fungal antagonism is given in Table 1.3.

Table 1.3: Summary of lytic enzymes from *T. harzianum* that have potential biocontrol capability (Viterbo *et al.*, 2002).

Gene	App. MW, (kDa)	Activity	Strain	Reference
Chitinases				
–	102	*N*-acetylglucosaminidase	TM, 39.1	Haran et al. (1995)
			T25-1	
–	73	*N*-acetylglucosaminidase	TM	Haran et al. (1995)
exc2	73		T25-1	Draborg et al. (1995)
exc1/nag1	64–69		T25-1, PI[a]	Peterbauer et al. (1996)
–	28	*N*-acetylglucosaminidase	T189	Deane et al. (1998)
–	52	endochitinase	TM, TY	Haran et al. (1996)
ech42	42	endochitinase	IMI206040[a]	Carsolio et al. (1994)
chit42	44		CECT 2413	Garcia et al. (1994)
chit42	42		Gv2908	Baek et al. (1999)
ThEn42	42		O1[a]	Lorito et al. (1998)
–	40	chitobiosidase	PI[a]	Harman et al. (1993)
–	37	endochitinase	CECT 2413, 109	de la Cruz et al. (1992)
chit36	36	endochitinase	TM	Viterbo et al. (2001)
chit33	33	endochitinase	CECT 2413	de la Cruz et al. (1992)
–	31	endochitinase	TM,TY	Haran et al. (1996)
Glucanases				
bgn13.1	78	β-1,3-endoglucanase	PI[a], CECT 2413	de la Cruz et al. (1995)
	74		T24	El-Katatny et al. (2001)
–	36	β-1,3-endoglucanase	39.1	
–	17	β-1,3-endoglucanase	CECT 2413	Lorito et al. (1994)
b16-2	43	β-1,6-endoglucanase	CECT 2413	Lora et al. (1995)
lam1.3	110	β-1,3-exo glucanase	T-Y	Cohen-Kupiec et al. (1999)
–	75	β-1,3-exo glucanase		Ramot et al. (2000)
egl1	50	β-1,4-endoglucanase	RUT C30[b]	Migheli et al. (1998)
Proteases				
prb1	31	alkaline protease	IMI 206040[a]	Flores et al. (1997)

[a] = *T. atroviride*, [b] = *T. longibrachiatum.*

The interaction between *T. harzianum* and the host may play a role in the induction of these specific enzymes. Several *T. harzianum* chitinases were induced by the host fungus *Botrytis cinerea, S. rolfsii* or *R. solani* (De La Cruz *et al.*, 1993; Carsolio *et at.*, 1994; Haran *et al.*, 1996).

1.3.5.3.1- Chitinases

Chitin polymers are an essential structural component in the fungi kingdom, as well as in some members of the animal kingdom (Kendrick, 2000). In fact, chitin is one of the most abundant polysaccharides found in nature, second to cellulose. The degradation of chitin is catalyzed by chitinolytic enzymes, called chitinases (Sahai and Manocha, 1993). Chitinases are ubiquitous in nature, being found in eukaryotes, prokaryotes, archaea, and viruses (Kollar *et al.*, 1995). They consist of a group of hydrolytic enzymes that are able to break down polymeric chitin into simple monomers of N-acetyleglucosamine (Sahai and Manocha, 1993). Fungal chitinases are involved in different stages in fungal growth, including hyphal elongation, cell separation, branching, spore swelling, germination, sporangium formation and response to mechanical injuries (Sahai and Manocha, 1993).

Chitinases cleave the bond between C1 and C4 of N-acetyl-β-glucosamine (GlcNAc). Haran *et al.* (1996) divided these enzymes into three classes. The first class contains the enzymes that work in exo-type to produce GlcNAc monomers. This class of enzyme, known as 1,4-ß- N-acetylglucosaminidases (EC 3.2.1.30) [also known as hexosaminidase (EC 3.2.1.52)], is responsible for cleaving the terminal chain of chitin to release N-acetylglucosamine. The second class contains chitinases that cleave inside the chitin chain in internal sites and these enzymes are called endochitinases (EC 3.2.1.14). The last class is known as exochitinases or chitobiosidases and responsible for cleaving

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the chitin to produce dimers of GlcNAc units but no monomers or oligomers (Harman *et al.*, 1993). Analysis of the amino acid sequences of these enzymes has used to group them in families 18-20 of the glycosyl hydrolases (Viterbo, 2002). Slight variations in the gene sequence, amino acid sequence and molecular weights of these enzymes are present between the different *Trichoderma* species (Viterbo, 2002).

1.3.5.3.1.1- N-acetylglucosaminidases

Numerous studies were conducted to identify N-acetylglucosaminidases (GlcNAcases) from different *Trichoderma* species (Koga *et al.*, 1991; Ulhoa and Peberdy 1991; Lorito *et al.*, 1994a and Haran *et al.*, 1995). Three enzymes have been isolated from different strains and have been found to have slightly different molecular masses 102 and 73 kDa, as revealed by the SDS-PAGE. These enzymes were expressed during the interaction with *S. rolfsii*, where 102 kDa appeared first during the interaction (Haran *et al.*, 1996). In addition, two different GlcNAcases genes (*exc1* and *exc2*) were isolated from the same *Trichoderma* spp. (Draborg *et al.*, 1995 and Peterbauer *et al.*, 1996). The production of GlcNAcases appears to happen during the parasitic interactions of *Trichoderma* spp. as a result of different stimuli, such as the stimulation conducted by the enzyme itself.

1.3.5.3.1.2- Endochitinases

The *Trichoderma* chitinolytic enzyme system is both complicated and not fully understood. Differences between these enzymes might occur between the different fungal strains that vary in their habitats, nutritional and antagonistic behaviour. Several endochitinases have been isolated from *T. harzianum* such as the endochitinases of molecular weight 37 and 33 kDa (de la Cruz *et al.*, 1992) or 33 and 31 kDa (Haran *et al.*, 1996). Also, the 42 kDa endochitinase (*ech*42) has been isolated from various *Trichoderma* species included *T. harzianum* (Carsolio *et al.*, 1994; Garcia *et al.*, 1994 and Lorito *et al.*, 1998).

Ech42 is involved in the mycoparasitic action of *Trichoderma* on three levels, including cell wall hydrolysis, spore germination inhibition and the inhibition of the elongation of germ tubes of several filamentous fungi (Lorito *et al.*, 1993 and 1994a,b and Schirmbock *et al.*, 1994). The expression of this enzyme is highly induced during the interaction between the mycoparasite and its host (Carsolio *et al.*, 1994 and Garcia *et al.*, 1994), suggesting that the *ech*42 is a key enzyme during the parasitic interaction. It has been reported that the *ech*42 enzyme is over expressed during the interaction between *T. atroviride* and *R. solani* (Kulling *et al.*, 2000). Despite their important role in the biocontrol activities of *Trichoderma* spp., neither the chitinolytic enzymes nor their genes have been used commercially.

1.3.5.3.2- Glucanases

The second group of enzymes that is important for the mycoparasitic activities of *Trichoderma* spp. is the glucanases. The fungal cell wall is composed mainly, along with

chitin, of ß- 1,3-glucan (laminarin) and 1,4-β-D-glucan (cellulose). Glucan is a major component in fungal cell walls and its primary role is to provide structure, rigidity and protection (Kollar *et al.,* 1995). The synthesis of glucan-degrading enzymes is a shared characteristic between many organisms, although fungi are the principal producers (de la Cruz and Llobell 1999).

Glucanases are classified according to the type and location of glycosidic linkages that they cleave (Pitson *et al.,* 1993). A glucanase that cleaves β-1,3-glucan-type bonded glucans is a *β-1,3-glucanase*, and a glucanase that cleaves bonds located within the chain are endo-glucanases (Figure 1.4). Thus, endo-β-1,3-glucanase cleaves covalent bonds in the triple helical structure of D-glucose molecules in a β-configuration, or β-1,3-glucans (Pitson *et al.,* 1993).
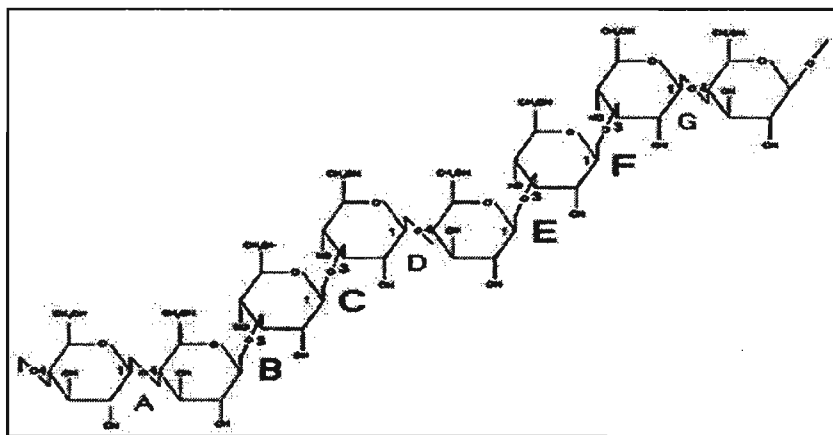
Figure 1.4: Action of ß-1,3glucanase on a 1,3 or 1,4 ß-glucan, where ß-1,3-glucanase cleaves linkages B, C, E, and F (Pitson *et al.,* 1993).

In fungi, β-1,3 glucan degrading enzymes are prevalent, due to the vast availability of their substrates in fungal cell walls (de la Cruz and Llobell 1999). *β-1,3-glucanases* that hydrolyse laminarin have been subdivided into two classes, endo and exo-ß-glucanases based on the activity against the substrate. Endo-ß-glucanases hydrolyse laminarin into oligosaccharides whereas exo-ß-glucanases produces monosaccharides, since they cleave the terminal units. Both the endo-ß-glucanases and the exo-ß-glucanases have multiple roles in fungi and are involved in the differentiation and metabolism of β-glucans (Peberdy, 1990). The formation of *β-1,3-glucanases* is inhibited by glucose, a feature that glucanases share with chitinases (Ramot *et al.*, 2000).

A second class of glucanase enzymes that is also produced by *Trichoderma* is the ß-1,6-glucanases. These enzymes work on the minor structural polymers that are present in the fungal cell wall. Various β-glucanase activity levels were induced against cell-wall preparations from several pathogens (Markovich and Kononova, 2003). Moreover, these enzymes play a role in the *Trichoderma* antagonism of *Pythium* (Thrane *et al.*, 1997). *Pythium*, an oomycete contains a cell wall that is a mixture from ß-1,3-glucans and 1,6-glucans, in addition to cellulose, but not chitin.

Several glucanases have been purified from *T. harzianum*, however the most important are the β-1,3-glucanases with up to seven enzymes identified from this fungus, with differences in their molecular mass and isoelectric point (p*I*) (de La Cruz *et al.*, 1993, Lorito *et al.*, 1994a, Noronha and Ulhoa, 1996 and Vazquez-Garciduenas *et al.*, 1998). Laminarin and purified cell walls were found to induce the highest activity levels of *T. harzianum* β-1,3- glucanases (Elad *et al.*, 1982; de La Cruz *et al.*, 1993 and Noronha and Ulhoa, 2000). It is believed that these enzymes are important for the lysis of

the host fungi cell walls during the mycoparasitic interaction (Vazquez-Garciduenas *et al.*, 1998).

1.3.5.3.3- Proteases

Fungal cell wall structure is sustained by various proteins and glycoproteins (Kendrick, 2000). Consequently, proteases are considered to be factors in fungal cell wall degradation (Pitson *et al.*, 1993). Therefore, the presence of protease enzymes in concert with chitinases and glucanases has been shown to increase subjectivity of host cell walls lysis (Sivan and Chet, 1989). *Trichoderma* spp. secretes proteases in the presence of a host fungus and proteases have been isolated from several species of *Trichoderma* (Viterbo *et al.*, 2004). The penetration into the host fungus is facilitated by the effect of protease that breaks down the peptide bonds in the host cell walls (Pozo *et al.*, 2004).

Prb1 is a 31 kDa serine protease with a p*I* of 9.2 and is one of the hydrolytic enzymes produced by *T. harzianum*. To study the activity of Prb1, a system was developed with cloned multiple copies (2-10 copies) of the *prb*1 gene and were transformed to construct multicopy *Trichoderma* spp. strains. The *prb*1 gene was found to be active when the fungus is grown on media containing *R. solani* cell walls. It is also repressed by glucose (Geremia *et al.*, 1993). Over expression of the *prb*1 gene in *T. harzianum* was also used to improve the biological control of soil-borne plant pathogens (Flores *et al.*, 1997).

The induction of two hydrolytic enzymes prb1 and ech42 in *T. harzianum* has been reported by Cortes and co-workers (1998). Both enzymes were expressed in dual cultures of the fungus and its host, even under the limited fungal contact. Furthermore, the

investigation of such induction revealed that it is contact-independent and is mediated by a diffusible compound produced by the host fungus.

Another serine protease (*tvsp*1) has been identified from *T. virens*. This *Trichoderma* species is used as a biocontrol agent, since the over expression of the extracellular serine protease gene *tvsp*1 can protect against *R. solani* (Pozo *et al.*, 2004). In addition, it is suggested that the inhibition of the enzymatic activity of the phytopathogenic fungus *B. cinerea* by *T. harzianum* is due to the proteolytic enzymes that are secreted and degrade the *B. cinerea*'s hydrolytic enzymes (Elad and Kapat, 1999).

1.3.6- Interaction of *T. aggressivum* with *A. bisporus*

Resistance against green mould disease has gained the attention of mushroom scientists. Studies have reported that commercial brown mushroom strains are more tolerant against green mould disease than the white and off-white mushroom strains (Rinker and Alm, 1996). The comparison between the loss in yield due to green mould was found to be 96% for the white strains, 56% to 73% for the off-white strains and 8% to 14% for the brown strains (Anderson *et al.*, 2001).

Research was performed to explain the increased resistance in the commercial brown strains. This included the investigation of the molecular differences between the difference strains during their interactions with the green mould disease. Gutherie and Castle (2006) measured the activity of different chitinases during the dual cultivation of the white and brown strains of *A. bisporus* and *T. aggressivum*. They found increased activity of the 96 kDa N-acetylglucosaminidase in the brown strain relative to the white

strain. Therefore, it is possible that this enzyme plays a role in the resistance of *A. bisporus* brown strain to *T. aggressivum*. In addition, the inhibition zone of *A. bisporus* resulting from the secondary metabolites of *T. aggressivum* was lower for the brown strain SB65 compared to off-white U1 (Krupke *et al.*, 2003). However, the mechanism of this resistance is not fully understood.

Dual cultures of *Trichoderma* and *A. bisporus* (brown, white or off-white strains) were used as means to study the chitinases production of *T. aggressivum*. (Gutherie and Castle, 2006). *A. bisporus* 111, 105, and 96 kDa N-acetylglucosaminidases have been detected in the dual culture during the interaction. The levels of the 96 kDa protein were produced in all of the *A. bisporus* strains however; the activity levels were higher in the brown strain than the white or off-white strains. Since the brown strain is known to be more resistant to the green mould disease, it is therefore suggested that the elevated levels of the 96 kDa N-acetylglucosaminidase are responsible for such resistance (Gutherie and Castle, 2006).

On the other hand, several hydrolytic enzymes were detected from *T. aggressivum* during the dual cultivation with *A. bisporus*. This includes 131, 125, and 122 kDa N-acetylglucosaminidases, 40 kDa chitobiosidase and 36 kDa endochitinase. Elevated activities of the 122 kDa N-acetylglucosaminidase were obtained, which points towards its involvement in the antifungal effect of *T. aggressivum* (Gutherie and Castle, 2006). One interesting finding was that the early production of *A. bisporus* 111 kDa chitinase enzyme and *T. aggressivum* 131 kDa chitinase enzyme only in the first four days of the dual culture (Gutherie and Castle, 2006).

AIM OF THE STUDY

The aim of this study is to investigate the role of *T. aggressivum* cell wall degrading enzyme genes in the development of the green mould disease and to understand the parasitism resistance mechanism of the commercial brown *A. bisporus* strain (SB65) compared to the off-white *Agaricus* strain (U1). The study tasks included:

1- Isolation and purifying of 96 kDa N-acetylglucosaminidase enzyme and the corresponding gene produced by brown strains of *A. bisporus*.

2- Isolation and characterization of several cell wall degrading enzyme genes from *T. aggressivum*.

3- Studying the genes expression regulation of *T. aggressivum* cell wall degrading enzyme

4- Utilizing RNAi technology to study the roles of these enzymes in green mould disease severity.

CHAPTER II

MATERIALS AND METHODS

2.1- BACTERIAL MANIPULATION

2.1.1-Bacterial strains and maintenance

Strains of bacteria used as hosts in various cloning and transformation experiments described in this study include *Escherichia coli* DH5α, *Agrobacterium tumefaciens* AGL-1 and LBA-4404. Luria-Bertani broth (LB: 1 % (w/v) bacto-tryptone, 0.5 % (w/v) bacto-yeast extract, 1 % (w/v) NaCl, sterilized by autoclaving) was used to grow these bacteria. LB-agar was prepared by the addition of 2 % (w/v) agar to LB and autoclaved. The sterilized LB-agar was cooled to 50-55°C before the addition of the appropriate antibiotic for bacterial selection. The LB-agar was poured into Petri dishes (10 cm) and stored at 4°C after solidification. DH5α was grown at 37°C and AGL-1 and LBA-4404 were grown at 28°C.

2.1.2- DH5α competent cells

A stock cell population (New England Biolabs) was used to prepare competent cells according to Inoue *et al.* (1990). Cells were obtained from a single colony grown on LB-agar, inoculated into 2 mL of antibiotic-free LB and incubated with shaking for 16 h at 37°C. The culture was transferred into a 2 L flask containing 250 mL SOB medium [2% (w/v) bacto-tryptone, 0.5 % (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl$_2$, 10 mM MgSO$_4$, 20 mM glucose] and incubated at 18°C with vigorous shaking

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(250 rpm). When the culture reached an OD_{600} value of 0.6, it was divided into 50 mL tubes, chilled on ice for 10 min and centrifuged at 3000 x g in an Eppendorf 5415D Centrifuge at 4°C. After discarding the supernatant, the cell pellet of each tube was resuspended in 16 mL of ice-cold transformation buffer (10 mM PIPES, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, pH 6.7) and chilled on ice for 10 min. Cells were centrifuged again at 4°C for 10 min at 3000 x g followed by removal of the supernatant. The cell pellet of each tube was resuspended in 2.5-3 mL of ice-cold transformation buffer followed by the stepwise addition of dimethyl sulfoxide (DMSO) with gentle swirling on ice to a final concentration of 7% and kept on ice for 10 min. The cells were aliquoted, frozen in liquid nitrogen and transferred immediately to a -80°C freezer.

2.1.3- *Agrobacterium* competent cells

Stock cell populations from *A. tumefaciens* AGL-1 strain (Dr. Vincenzo De Luca, Brock University) and LBA-4404 strain (Dr. Mike Bidochka, Brock University) were used to prepare competent cells of both strains. For each strain, cells were obtained from a single colony and inoculated into 5 mL of medium containing both ampicillin (100 μg/ml) and carbenicillin (50 μg/ml), with incubation at 28°C for 2 days with vigorous shaking. Then, 1 mL from the grown culture was used to inoculate 25 mL of fresh LB medium containing ampicillin (100 μg/ml) and carbenicillin (50 μg/ml) in a 100 mL flask with vigorous shaking at 28°C for 36 h to 48 h. When the culture OD_{600} reached 0.5 to 0.8, the culture was chilled on ice for 30 min, and then centrifuged at 3000 x g in an Eppendorf 5415D Centrifuge for 5 min at 4°C. After discarding the supernatant, the cell pellet was resuspended in ice-cold $CaCl_2$ (20 mM) and aliquoted 100 μL in 1.5 ml tubes. The cells were frozen in liquid nitrogen and stored immediately in -80°C.

2.1.4- DH5α transformation

For transformation into competent cells, water or TE buffer [1mM ethylene diamine tetra-acetic acid (EDTA), 10mM TrisHCl pH 8.0] was used to resuspend plasmid DNA to a total volume of 20 μL. Competent cells (100 μL) were thawed on ice before their mixing with plasmid DNA and incubation on ice for 30 min. The cells were heat shocked by incubation for 45 second at 42°C followed with the immediate chilling of the cells at 4°C for 2 min. Antibiotic-free LB (700 μL) was added to the cells and incubated at 37°C with shaking for 45 min. Then, the transformed bacteria were spread on LB-agar plates with the appropriate antibiotic (ampicillin 50 μg/ml, carbenicillin 50 μg/ml and kanamycin 50 μg/ml) and incubated at 37°C for 16 h.

2.1.5- *Agrobacterium* transformation

One microgram (1μg) of plasmid DNA in a total volume of 5 μL was added into the frozen *Agrobacterium* competent cells and incubated immediately in a water bath at 37°C for 20 min. The cells were chilled on ice for 30 min, followed by the addition of 900 μL of antibiotic-free LB medium and incubated at 28°C for 2 h with gentle shaking. Afterwards, the cells were spun down at 3000 x g for 5 min at room temperature, and 600 μL from the supernatant was discarded carefully and the remaining supernatant (400 μL) was used to resuspend the cell pellet. The resuspended cells were divided into two pre-warmed (28°C) antibiotic-containing LB plated and incubated for 2 days at 28°C.

2.1.6- *Agrobacterium*- mediated transformation

The *Agrobacterium* mediated transformation of *T. aggressivum* using *Agrobacterium tumefaciens* AGL-1 strain harboring the binary vector pFBENGFP, pFGC1008-GPD, pGlu -Silencer, pEch-Silencer and pPrb1-Silencer was based on the protocol previously described by Fang *et al.* (2006), with some modifications. Each AGL-1 carrying different vectors from 2 days old plates were inoculated into 10 ml of LB broth containing the proper antibiotic, for example: carbenicillin and kanamycin (50 µg/ml) were used for AGL-1-pFBENGFP and for AGL1- with pFGC1008-GPD, pGlu-Silencer, pEch-Silencer and pPrb1-Silencer chloramphenicol (100µg/mL) and carbenicillin (50 µg/ml) were used, followed by incubation at 27°C for 16-20 hrs under constant agitation (250 rpm). At the OD_{600} achieved 0.6- 0.8, the liquid culture was diluted to an OD_{600} value of 0.15 with induction medium containing 200µM acetosyringone (IMAS) and was reincubated at 27°C fro 4 hrs (OD_{600} = 0.4-0.8) under steady agitation (250 rpm). *T. aggressivum* conidia from 7 day old plate were harvested using 5 ml autoclaved H_2O, the conidial suspension was filtered through sterile glass wool to eliminate the remaining mycelial mass. The conidia concentration was calculated with haemocytometer (2.2.2) and was readjusted to final concentration of 1×10^7 conidia/ ml with IMAS. 100µl of conidia was mixed, by gentle inversion, with an equal volume of diluted AGL-1, and the mixture was plated on steriled black filter paper (Ahlstrom, 0.18mm thickness). After 2 days of co-cultivation at 27°C, the black filter papers were transferred onto freshly M-100 plates containing cefotaxime 400 µg/ml and benomyl 0.6 µg/ml for AGL-1-pFBENGFP with *T. aggressivum* plates and hygromycin (200µg/ml) for pFGC1008-GPD, pGlu-Silencer, pEch-Silencer and pPrb1-Silencer with *T. aggressivum.*

After day 1 of incubation, the plateswere overlaid with freshly of M-100 agar containing benomyl 0.6 µg/ml and the plates were reincubated again at 27°C.

2.1.7- Colony selection

Different antibiotics were used for selection including ampicillin (100µg/mL), carbenicillin (50µg/mL), chloramphenicol (100µg/mL) and kanamycin (50µg/ml). The selection antibiotic(s) was added into the LB-agar plates as described in (2.1.1). The transformed bacteria were spread over the plate and incubated overnight at 37°C for *E.coli* DH5α and 2-3 days at 27°C for *Agrobacterium*. The next day, *E.coli* colonies were picked from the plates and inoculated into 2 mL of LB containing the same antibiotic(s) and grown overnight at 37°C with vigorous shaking. X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) was used when cloning inside the β-galactosidase gene. 0.8 mg of X-gal (40µL from a stock of 20mg/mL) was applied on the plate prior to spreading the transformed bacteria. Positive colonies that carry the inserted fragment were selected by their white color due to disruption of the β- galactosidase gene, which differentiates them from the blue color generated by `cells with a formational β-galactosidase enzyme.

2.1.8- Small-scale plasmid DNA preparation

Two different procedures were used for small scale plasmid DNA preparation. The first is the alkaline lysis (Sambrook *et al.*, 1989) and the second is the preparation by using Plasmid DNA MiniPrep Kit (Norgen Biotek Corp.), according to the manufacturer's instructions. The isolated plasmid(s) were stored at -20°C for short term storage or -80°C for long-term storage.

2.1.9- Medium-scale plasmid DNA preparation

Medium-scale plasmid DNA was prepared by using Plasmid Midi Kit (Qiagen), according to the manufacturer's instructions. The isolated plasmid (s) were stored at -20°C for short term storage or -80°C for long-term storage.

2.2- FUNGAL MANIPULATION

2.2.1- Fungal strains and growth conditions

T. aggressivum f. *aggressivum* strain T586 (Brock University) was maintained on malt extract agar (MEA: 0.06 % malt extract, 0.018 % maltose, 0.06 % dextrose, 0.012 % yeast extract and 2 % agar) medium. The *Trichoderma* fungus was subcultured by transferring a loop of conidia onto a new MEA plate. These cultures were incubated at 25°C with minimal light exposure.

Liquid cultures were grown in malt extract broth (MEB: 0.06 % malt extract, 0.018 % maltose, 0.06 % dextrose and 0.012 % yeast extract). A loopful of conidia was obtained from mature plate culture and placed in the medium. The medium was poured into Petri dishes grown at 28°C for 4-6 days, and transferred to 4°C for storage.

A. bisporus commercial strains Sylvan SB65 large brown and Horst U1 off-white hybrid were maintained on *Schizophyllum* complete yeast medium (SCM: 2% agar, 2 % dextrose, 0.2 % yeast extract, 0.2 % peptone, 0.1 % K_2HPO_4, 0.05 % $MgSO_4 \cdot 7H_2O$ and 0.046 % KH_2PO_4) (Raper *et al.*, 1972). The *Agaricus* cultures were subcultured by transferring an inoculum plug (5 mm x 5 mm) from actively growing culture onto the center of a new SCM Petri dish (100 mm). These cultures were incubated at 25°C with minimal light exposure.

Liquid cultures were grown in SCM without agar. Sections were excised from the mature culture, blended in a small amount of medium, and placed in the remaining medium. The medium was poured into Petri dishes grown at 28°C for three weeks, and transferred to 4°C for storage.

2.2.2- Spore counting

T. aggressivum mature culture plate was washed with 5 ml sterile water and, the fungal conidia were carefully transferred from the plate to 15 cc Falcon tube and a serial dilution was prepared. 10 µl of spores were quantified using a haemocytometer covered with cover glass. A light microscope was used to visualize and count the spores and the starting number was calculated.

2.2.3- Fungal co-cultivation and harvesting

A. bisporus culture was grown in SCM broth for 3 weeks followed by inoculation with 1 ml of *T. aggressivum* conidia suspension (10^7 conidia/ml) to form a dual culture of both fungi. At the desired collection time point, samples were harvested by filtering the dual culture through a Whatman filter paper No.4. The harvested samples were frozen in liquid nitrogen and ground with a mortar and pestle into a fine powder. The ground samples were collected in 15 cc tubes and stored at -80°C.

2.2.4- Compost-spawn mixture preparation and conidia inoculation

Mushroom compost (Mushroom Producers' Co-op Inc, Harley, Ontario) was used in the field co-cultivation study. Plastic trays (30 x 30 x 10 cm) were used to prepare the compost-spawn mixture. Nine-hundred grams of compost per tray was mixed with 1.16 g of the *A. bisporus* A15 spawn (Sylvan Inc.). *T. aggressivum* spore inoculation was

performed immediately after preparing the mixture. *T. aggressivum* conidia from 7 day old plate were harvested using 5 ml autoclaved H_2O per plate, the conidial suspension was filtered through sterile glass wool to eliminate the remaining mycelial mass. The conidia concentration was calculated with haemocytometer (2.2.2) and was readjusted to final concentration of 1×10^7 conidia/ ml. The inoculation was done by using 1×10^7 spores in the center of the trays at 2-3 cm deep and the trays were covered tightly with a plastic lid to avoid compost dryness and incubated in the growth room at 24°C.

2.3- DNA MANIPULATION

2.3.1- Fungal DNA extraction

T. aggressivum genomic DNA was extracted from liquid cultures according to Castle *et al.* (1998). The culture was filtered by using Whatman No.4 paper, collected into 15 cc tubes and washed three times with 25 mM EDTA. The samples were frozen by liquid nitrogen and lyophilized overnight in a MicroModulyo® freeze dryer.

Approximately 50 μg of lyophilized culture was placed into a microcentrifuge tube with 500 μL of 10 mM EDTA-10 mM Tris-(pH 8.0). The sample was mixed gently by inversion followed by the addition of 25 μL of sodium dodecyl sulphate (SDS-10 %) and 0.26 mg pronase. The sample was vortexed and incubated at 37°C for 30 min. 500 μL of phenol-chloroform was added, vortexed and centrifuged at 12,500 x g for 15 min.

The aqueous phase of nucleic acids was transferred to a fresh microcentrifuge and precipitated by adding 0.25 volume of 7.5 M ammonium acetate (NH4OAc) and the tube was filled with 95% ice-cold ethanol (EtOH). The sample was incubated at -20°C for 30

min and centrifuged (12,500 x g) for 15 min. EtOH was discarded and the sample was air dried at room temperature in a fume hood for 10 min. The DNA was re-suspended in 300 µL of TE buffer. Following resuspension, µL of 20mg/mL RNase I (Invitrogen) was added and incubated at 37°C for 15 min. NH4OAc addition and EtOH precipitation steps were repeated as above. The DNA was resuspended in 50µL of TE and stored at -20°C.

2.3.2- Extraction of DNA from agarose gel

After running the sample on agarose gel electrophoresis, the desired DNA fragment was excised from the gel and cleaned by using the DNA Gel Extraction Kit (Norgen Biotek Corp.), according to the manufacturer's instructions.

2.3.3- DNA quantification

Spectrophotometer was used to quantify the concentration of DNA samples according to Sambrook et al, (1989). Samples were diluted with water and the absorbance was then measured at wavelengths of 260 nm (Abs_{260}) and 280 nm (Abs_{280}). The ratio between Abs_{260}/Abs_{280} was used to check the purity of the sample. A sample with a ratio of about 1.8 was considered pure. The following equation was used to determine the concentration:

$$\text{[DNA] (µg/ µL)} = \frac{(Abs_{260}) \times (\text{dilution factor}) \times (50\ \text{ng/ µL})}{1000}$$

2.3.4- DNA cleaning

Cleaning of the DNA samples was performed by using the CleanAll Kit (Norgen Biotek Corp.), according to the manufacturer's instructions.

2.3.5- PCR product cleaning

DNA from polymerase chain reaction (PCR) amplification was cleaned by using the PCR Purification Kit (Norgen Biotek Corp.), according to the manufacturer's instructions.

2.3.6- Restriction enzyme digestion

All the restriction enzymes used were purchased from New England Biolabs (NEB). Restriction enzyme digestion and subsequent heat inactivation were performed according to the manufacturer's instructions. The digestion of DNA was carried out by using 1 to 5 μg of DNA with two to ten units of the restriction enzyme in a total reaction volume of 20 μL. The samples were incubated for two hours at 37°C. Greater amounts of DNA in larger volumes and for prolonged digestion periods were used when needed for other applications.

When using multiple restriction enzymes in one reaction, the reaction buffer in which the different enzymes shows high activities according to manufacture's specificities was used with equal units of the restriction enzymes. The success of the digestion was visualized by using agarose gel electrophoresis. Enzyme units and digestion time were increased when incomplete digestion was encountered. When co-digestion with different enzymes was not suitable, sequential digestion was applied. The inactivation of the restriction enzymes was carried out by heating for 20 min at 70°C. Different inactivation temperatures were used when recommended by the manufacturer.

2.3.7- Ligation of DNA

T4 DNA ligase (New England Biolabs) was used to ligate DNA fragments with blunt ends or with compatible cohesive ends, according to the manufacturer's instructions. When ligating cohesive ends, the reaction was performed at room temperature for 2 h however; ligation was performed for 8 h at room temperature when ligating blunt ends. Heat inactivation of the T4 ligase was performed, if needed, at 65°C for 20 min.

Restriction fragments used in molecular cloning were usually gel-purified to enhance the efficiency of isolation of the desired clone. In a typical reaction of 20μL, two fragments were added in a 10:1 molar ratio of insert to vector ensuring successful ligation.

2.3.8- Sequencing of DNA

DNA sequencing was carried out at York University Core Molecular Biology and DNA Sequencing Facility. Bigdye Terminator chemistry on the Applied Biosystems 337 DNA Sequencer was used for obtaining the sequence with the appropriate primers.

2.3.9- PCR

PCR (Mullis *et al.*, 1986) was performed according to the polymerase manufacturer's instructions, using forward and reverse primer sets obtained from Sigma Genosys Canada (Table 2.1). A working concentration of 5 μM was prepared from each primer and stored at -20°C. Power Block System-Model Ericomp was used to carry out the reactions in a 20 μL reaction volume contains the following components: about 0.1 to 1 ng DNA template, 1.2 μL of each primer (5 μM stock), 0.5 μL dNTPs (10 mM), 2 μL

of the 10X PCR buffer (Buffer composition for *Taq*: 100 mM Tris-HCl (pH 8.8 at 25°C), 500 mM KCl, 0.8% (v/v) Nonidet P40 and for native *Pfu*: 200 mM Tris-HCl (pH 8.8 at 25°C), 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 1% (v/v) Triton X-100, 1 mg/ml BSA) and 0.5 µL (5 units/µL) *Taq* DNA polymerase (Fermentas) or 0.5 µL (2.5 unit/µL) native *Pfu* DNA polymerase (Fermentas), with bringing the total reaction volume to 20 µL using dH_2O.

The reaction was started with melting the DNA template using initial step at 94°C for three minutes. Then, 30 to 35 amplification cycles were performed by the successive heating to 94°C for 30 seconds, then cooling to allow primers annealing at a temperature less than the lowest primer melting temperature by 5°C and the subsequent incubation of the reaction at 72°C to allow the extension and formation of the new DNA strands. The used extension time was 1 min/kbp when using *Taq* DNA polymerase and 2 min/kbp with *Pfu* DNA polymerase. Finally, the

reaction was held at 72°C for 5 min and incubated at 4°C afterwards.

Table: 2.1: Names and sequences of the different PCR primers. Melting temperatures can be obtained by using Sigma Genosys's online DNA calculator (http://www.sigma-genosys.com/calc/DNACalc.asp).

Name	Sequence	Tm °C
ALAN-1	5'CAGCACCCAGAAGAATAAC3'	57
ALAN-2	5'CAGATCATCTTGATGAGACG3'	58
AP1	5'GTAATACGACTCACTATAGGGC3'	56
AP2	5'ACTATAGGGCACGCGTGGT3'	64
BGlu-WKF	5'GACGGACGACACCGTAGC3'	64
BGlu-WKR	5'CCGGGAGGGATCTTGAGT3'	64
BTub-WKF	5'GGCGATCTGAACCACCTG3'	64
BTub-WKR	5'GGGAACGGCACCATGTTA3'	64
ChF1	5'TGGTCCACCAACTTCCCTT3'	64
ChR1	5'AAAGAGATGAGCTCCTTG3'	54
Ech42-F1S	5'GCTGGTATCTGGGATTACAAG3'	59
Ech42-R1S	5'CAATCCTTCATGAAGGTAATG3'	58
Ech-F3Sc	5'GGAAGCCGGATATCG3'	57
Ech-R3S	5'TCGGCAGGCTGGAAG3'	62
ED-WKF	5'ATGCCCATCTACGGACGA3'	64
ED-WKR	5' ATGCCCATCTACGGACGA'	64

Name	Sequence	Tm °C
End (F-Fu)	5'GTTGAGCTTCCTCGGAAAATC3'	63
F-eGFP	5'ACGGATCCAGCCATGGTGAGCAA3'	75
Glu walker F1	5'CACACGCTCAAAGCCTCAATATGAAACC3'	70
Glu walker F2	5'CCGCTGCAGGCCAAATTGTGTACTTCG3'	77
Glu walker R1	5'GGGAAGATTGGTCGCATCTCCGATAATG3'	75
Glu walker R2	5'GGAAGTACACGATGGCCGGAGTCAAAG3'	74
Glu(F-Fu)	5'CTTTGGAAAAGCGTGCTTCGT'3	67
Glu-Cter	5'ATGTTGCTCAGCGTGCC3'	63
Glu-Nter	5'GTTACCGACGACACGGC3'	62
GPD-F	5'CCTGAGGTCCGCAAGTAGATTG3'	66
GPD-R	5'CCTAGGTCGACGCTTGTTGTGTGTAGATGGGAGA3'	78
MAS-F	5'CCTACACTTTTGACTAGCGAGGCTTG3'	68
MAS-R	5'CAGACGTCGCGGTGAGTTC3'	67
Prb1 (F-Fu)	5'TTGCAGTCTACCTTGGAGCTT3'	62
Prb1-Cter	5'GCTGATTGTGTTGGTAGC3'	56
Prb1-F1S	5'CGCACCCTCCTTGAGAGTAAC3'	64
Prb1-Nter	5'TGCTCCCGGCTGTCCTC3'	68
Prb1-R1S	5'TTGTTGATTCCGGTATCAACA3'	62
Prb-WKF	5'GCGCTTTGACCACACAGA3'	63
Prb-WKR	5'TGCCAGCCGAGGTATCAT3'	64
R-eGFP	5'GATCTAGACTTGTACAGCTCGTCCA3'	64
siRNA- Prb1 (F)	5'AGACTAGTGGCGCGCCGTTTCAGGAACTATTGGTGG3'	82
siRNA- Prb1 (R)	5'CTGGATCCATTTAAATGAGCGCAGCAACAGTAATAGC3'	77
siRNA-End (F)	5'AGACTAGTGGCGCGCCGTCATCTACTCCTTCATGAAC3'	81
siRNA-End (R)	5'CTGGATCCATTTAAATCTTGAGCAGAAGAACCATGTT3'	75
siRNA-GLU (F)	5'AGACTAGTGGCGCGCCTATTGGTATGAGAATATTGC3'	76
siRNA-GLU (R)	5'CTGGATCCATTTAAATCATATCAAGCTTAAAGTTGCG3'	73

2.3.10- Agarose gel electrophoresis

Agarose gel electrophoresis was performed according to Sambrook *et al.* (1989). Appropriate amounts of agarose (Bioshop Biotechnology Grade) were mixed with 1X electrophoresis buffer containing ethidium bromide (0.1 μg/mL w/v) and boiled until completely dissolved. The solution was allowed to cool to 45°C before pouring. Generally, the voltage applied across the electrodes ranged from 10V/cm to 20V/cm, depending on the application. Upon completion, as indicated by the position of the loading dye. The gels were visualized by ultraviolet (UV) light exposure by using the Bio-Rad Gel Doc 1000.

2.3.11- Quantitative PCR

Quantitative PCR (qPCR) was carried out on equal volumes of DNA or cDNA templates using Mx4000® multiplex quantitative PCR system (Stratagene). Mx4000 v4.20 software was used to design the gene specific primers. The reaction mixture contains 10 µL of 2X SYBR GREEN mastermix (Qiagen), 1.2 µL of each primer (5 µM stock). The total reaction volume was completed to 20 µL with dH_2O. The hotstart enzyme was activated by an initial incubation at 95°C for 15 min followed with 45 amplification cycles carried out as follow: incubation at 95°C for 15 seconds, annealing at 60°C for 30 seconds and elongation at 72°C for 1 min/kbp. Then an 80 cycles dissociation curve was performed by the sequential temperature increment by 0.5°C every 10 seconds, starting at 57°C.

2.4- RNA MANIPULATION

2.4.1- Total RNA isolation

Mycelia from liquid cultures were harvested by filtration through Whatman filter paper No.4 and washed; mycelia then applied to the liquid nitrogen and ground it. Extraction of fungal total RNA was performed by using the Total RNA Purification Kit (Norgen Biotek Corp.), according to the manufacturer's instructions.

2.4.2- DNase treatment of RNA

DNA background in the isolated RNA samples was digested with DNase I (Ambion). The digestion was performed in a total volume of 50 µL containing the RNA sample, the supplied buffer (100 mM Tris, pH 7.5, 25 mM $MgCl_2$, 5 mM $CaCl_2$) and 1

μL (2 units) of DNase I. RNase-free water was used to bring up the total reaction volume. The reaction was incubated at 37°C for 1 h.

2.4.3- RNA cleaning

RNA samples were cleaned, after DNase I treatment, by using the RNA CleanUp and Concentration Kit (Norgen Biotek Corp.), according to the manufacturer's instructions.

2.4.4- RNA quantification

A spectrophotometer was used to quantify the concentration of RNA samples according to Sambrook *et al.*, (1989). Samples were diluted with diethylpyrocarbonate (DEPC)-treated water and the absorbance was then measured at wavelengths of 260 nm (Abs_{260}) and 280 nm (Abs_{280}).

$$[RNA]\ (\mu g/\,\mu L) = \frac{(A_{260}) \times (\text{dilution factor}) \times (40\ ng/\,\mu L)}{1000}$$

2.4.5- Formaldehyde agarose gel electrophoresis

Formaldehyde agarose gel electrophoresis was used to separate RNAs and visualize the integrity of the isolated RNA samples. The gel was prepared according to Sambrook *et al.* (1989). RNA was verified by visualizing 18S and 28S ribosomal RNA on a denaturing agarose gel. To prepare the RNA samples for electrophoresis, 20μL of formamide, 7μL of formaldehyde, 4μL of 10X MOPS buffer (200 mM MOPS, pH 7.0, 80 mM Sodium Acetate and 10 mM EDTA, pH 8.0 in DEPC treated distilled water) and

5μL of RNA loading buffer were added to 15μL of each RNA sample. The sample was then heated to 65°C for 5 minutes and immediately chilled on ice until electrophoresis. A 1.5% agarose gel was prepared by dissolving 1.5g of agarose in 72.5mL DEPC treated distilled water. Ten mL of 10X MOPS buffer was also added. The mixture was then heated until the agarose was dissolved and cooled to approximately 45°C. 17.5 mL of formaldehyde and 5μL of 20mg/mL ethidium bromide were added to the cooled gel solution and poured into casting tray. Chilled RNA samples were loaded onto the solidified gel and run in 1X MOPS buffer usually at 10V/cm, the picture of the processed gel was then captured under UV light by using the Bio Rad Gel Doc 1000.

2.4.6- Reverse transcription

Reverse transcription was performed on RNA to synthesize the first strand cDNA molecules from mRNA species. Five ug of total RNA were used in 50 μL reaction volume. Initially, RNA was mixed with 2 μL of 50 μM oligo (dT)$_{18}$ primer (Invitrogen) and 1 μL of 0.1 M dithiothreitol (DTT) and completing the reaction volume to 31 μL using DEPC-treated water. This mixture was incubated for 5 min at 70°C and chilled on ice immediately afterwards. Meanwhile, another solution was prepared containing 10 μL of the 5X First Strand Buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl and 15 mM MgCl$_2$), 4μL DTT, 2 μL of 10 mM dNTPs, 1 μL (40 units) of RNase out (Invitrogen) and 2 μL Superscript III reverse transcriptase (Invitrogen). Ninteen μL from this solution was mixed with the on ice chilled mixture and the reaction was continued as follow: 10 min at room temperature, 3 h at 50°C followed with 3 h at 46°C and a final 10 h at 42°C.

2.5- PROTEIN MANIPULATION

2.5.1- Isolation and purifications of fungal proteins

A. bisporus total protein was extracted based on Guthrie *et al.* (2005) method. The cultures were harvested for protein isolation using a modified procedure by Haran *et al.* (1995). Ten plates of liquid cultures were filtered through Whatman No.4 filter paper to separate mycelia which was used to extract intracellular protein. Mycelia were then ground using a mortar and pestle, and were suspended in 120 ml of protein extraction buffer (10 mM Tris-HCl, [pH 7.4], 1mM EDTA, 1μM PMSF). The suspension was then homogenized using a Sorvall Omni-Mixer for 5 min at 4°C, sonicated for 5 min at 4°C, and homogenized again 5 min at 4°C. Homogenate was centrifuged at 14000 x g for 30 minutes at 4°C, after that the protein containing supernatant was collected.

2.5.2- Quantification of protein concentration

The BioRad protein assay, based on the Bradford assay (Bradford, 1976), was used to determine the concentrations of the *A. bisporus* total proteins. In this assay, the standard curve (Figure 2.1) was constructed using known amounts of bovine serum albumin (BSA) (NEB, Pickering, ON, Canada). The concentrations of protein samples were determined based on this standard curve of known protein concentration. The absorbance (Abs) readings were taken at 595 nm.
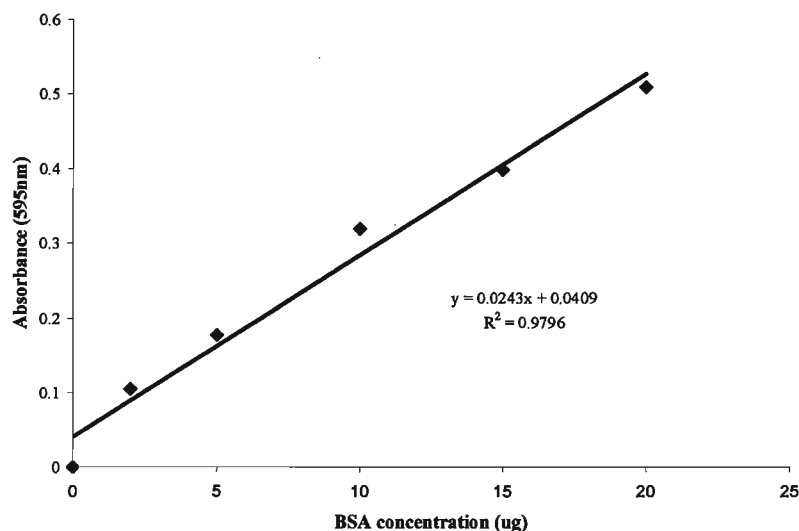
Figure 2.1: BSA standard curve.

2.5.3- Ammonium sulphate precipitation

A. bisporus protein was precipitated from the supernatant by the addition of different amount of ammonium sulphate [$(NH4)_2SO_4$] concentrations of 0.0%, 55%, 65%, and 75% w/v. Once the $(NH4)_2SO_4$ had dissolved, the solution was kept at 4 °C for 1 h. The proteins were collected by centrifuging at 12000 x g for 20 min. The pellet was resuspended and following by dialyzing with tubing cut off (MWCO 12000-14000) overnight against the appropriate extraction buffer at 4 °C. Samples in dialysis bags were placed on a dry bed of polyethylene glycol and concentrated to an approximate volume of 1mL. Proteins were stored at -20 °C until needed.

2.5.4- Gel filtration chromatography

The dialyzed crude *A. bisporus* protein sample obtained from the ammonium sulphate precipitation was fractionated by gel filtration column chromatography with Sephadex G-100 in 30 × 1.7 cm colum equilibrated with 0.25M phosphate buffer (pH

5.6). The elution step was performed with the same buffer and 1 mL fractions were collected by gravity. The void volume of the column was measured by using dextran blue. 96 kDa N-acetylglucosaminidase activities were tested in each fraction. The fractions containing the major enzyme activity were collected for SDS-PAGE.

2.5.5- Rotofor preparative isoelectrofocusing cell

Preparative isoelectric focusing (IEF) was performed in a Rotofor (BioRad) column according to the manufacturer's instructions and based on the method described previously by Small *et al.* (2004). Fifty five mL of a 1% solution of ampholyte (BioRad, pH 3–10) was mixed with 200 mg of the protein sample. The mixture was applied into the chamber of the BioRad Rotofor cell and separated at 4°C with a constant power of 15 W for about 3 h; twenty fractions were simultaneously aspirated into harvesting box through vacuum action. Fractions were assayed for the enzymeatic activities and protein levels. The pH was determined in each fraction. The fractions were concentrated and cleaned from the ampholytes by using the Millipore Centriplus Concentrators according to manufacturer's direction.

2.5.6- Enzymatic activity

For detection of specific chitinolytic activity, proteins were separated using sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) according to the procedure of Guthire *et al.* (2005). Samples were mixed with sample buffer (125mM Tris-Cl [pH 6.8], 20% glycerol (v/v), 4% SDS (w/v), and 0.2% bromophenol blue. Samples were heated for 5 min at 37 °C. The samples were applied onto 1.0 mm gels with a 4% acrylamide bis-acrylamide stacking gel, and separated an 11% acrylamide

resolving gel in a Bio-Rad Mini-Protean® 3 cell. Gels were run in the presence of running buffer (25 mM Tris, 250 mM glycine, 0.1% SDS) for 2 h at 100 V. Following electrophoresis, gels were washed with isopropanol to remove SDS and gels were then washed with dH$_2$O for 5 minutes. The substrate solution was made by adding the substrate 4-methylumbelliferyl N-acetyl-P-D-glucosaminide [4-MU (GlcNAc] (Sigma) to 20 ml of 100 mM sodium acetate [pH4.8] to obtain a final concentration of 0.025mg/ml. The [4-MU (GlcNAc] produces the fluorescent product 4-MU which is detected under UV light.

CHAPTER III

RESULTS

3.1- PURIFICATION AND ANALYSIS OF N-ACETYLGLUCOSAMINIDASE FROM AGARICUS BISPORUS AND SEQUENCING

The resistance of the commercial *A. bisporus* brown strain SB65 to the green mould disease may be linked to the high expression level of a 96 kDa N-acetylglucosaminidase (Guthrie *et al.*, 2005), since both solitary and dual cultures of the brown strain with *T. aggressivum* contained elevated activity of this enzyme. Therefore, the molecular characterization of this enzyme may be important to understanding disease resistance mechanisms. In this section, we isolated, and characterized the 96 kDa N-acetylglucosaminidase from *A. bisporus* SB65 using various methods. The ultimate goal was to get sequence information in order to transfer the gene copy from brown strain into non-resistant various commercial mushrooms such as the white and off- white strain of *A. bisporus* and to assess disease resistance of the transformants.

3.1.1- Ammonium sulphate precipitation

A. bisporus protein was fractionated by using sequential concentrations of $(NH_4)_2SO_4$, 55%, 65% and 75%, respectively. The supernatant of the 55% precipitation was adjusted by adding extra $(NH_4)_2SO_4$ to reach 65% concentration. Similarly, the supernatant of the latter $(NH_4)_2SO_4$ concentration was readjusted to 75%. The isolated protein was purified in triplicate and qualitatively analyzed on 11% SDS-PAGE gels (Figure 3.1). The highest protein concentration was obtained at 65% ammonium

sulphate, as quantified by Bradford assay (Figure 3.2). The enzymatic activity of N-acetylglucosaminidase was also found to be the highest at the 65% ammonium sulphate fraction (Figure 3.3).
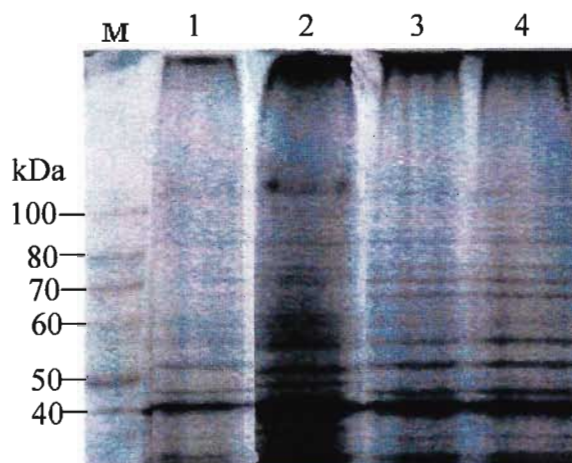
Figure 3.1: SDS-PAGE of *A. bisporus* total protein precipitated sequentially with different ammonium sulphate concentration. M: protein ladder (Norgen bioteck), 1: 75%, 2: 65%, 3: 55% of $(NH_4)_2SO_4$ and 4: unfractinated.
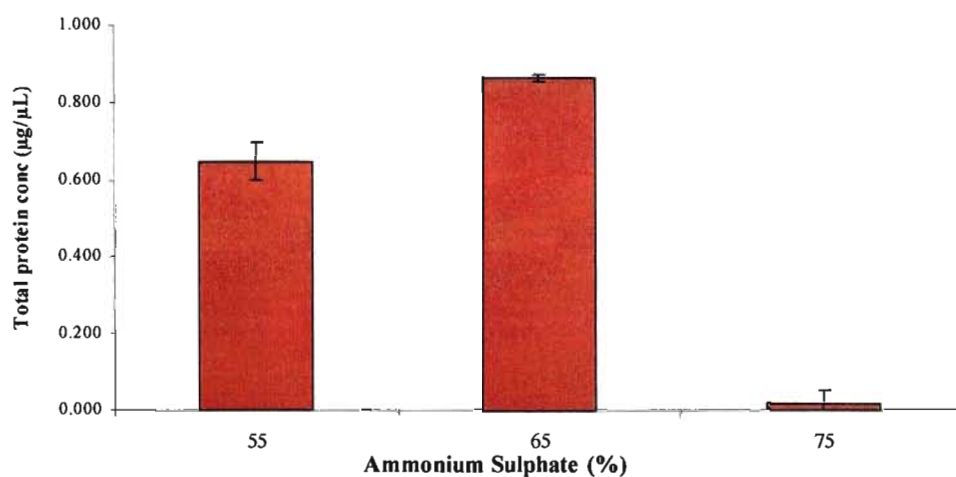
Figure 3.2: Yield of *A. bisporus* total protein precipitated sequentially with different ammonium sulphate concentrations (%).
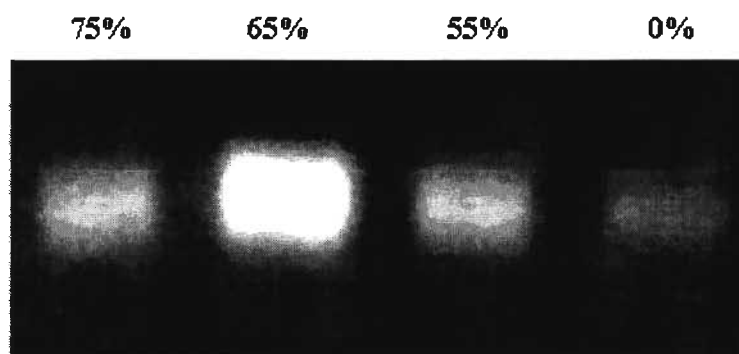
Figure 3.3: Activity of *A. bisporus* N-acetylglucosaminidase precipitated at different ammonium sulphate concentrations. SDS-PAGE gel was treated with [4-MU (GlcNAc)] and visualized under UV light.

3.1.2- Gel-filtration column chromatography

Total proteins isolated using ammonium sulphate at 65% was further purified using gel-filtration (Sephadex G-100). Twenty five different fractions were collected and tested for the N-acetylglucosaminidase activity. Out of the 25 fractions tested, fractions 8, 9, 10, 11 and 12 were found to have the highest chitinase activity (Figure 3.4). Furthermore, these fractions were separated on a 12% SDS-PAGE protein gel to test the quality of the purified proteins (Figure 3.5).
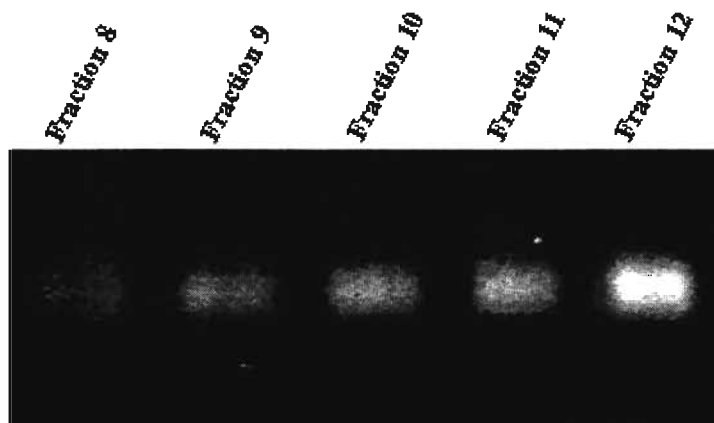
Figure 3.4: Activity of N-acetylglucosaminidase as detected in the different protein fractions collected from *A. bisporus* by gel filtration. SDS-PAGE gel was treated with [4-MU (GlcNAc)] and visualized under UV light.
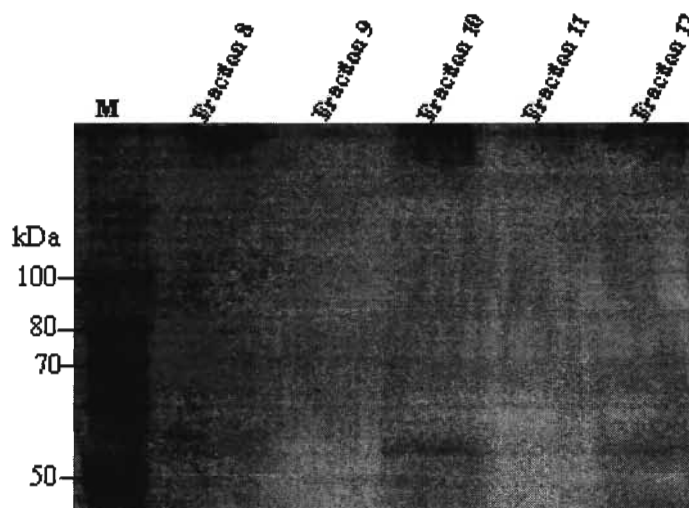
Figure 3.5: 12% SDS-PAGE gel. *A. bisporus* protein fractions collected by gel filtration.

3.1.3- Rotofor preparative isoelectrofocusing cell

The fractions with the highest chitinase activities collected from gel filtration were pooled and concentrated by lyophilisation. The resuspended pooled fractions were further fractionated based on isoelectric point (pI) using the Rotofor purification system. Twenty fractions were collected and tested for the N-acetylglucosaminidase activities. Out of the 20 fractions tested, fractions 3, 4, 5, 6 and 7 were found to have the highest N-acetylglucosaminidase activities (Figure 3.6). These fractions were then pooled and concentrated by freeze-dry lyophilisation. After that the resuspended pooled protein fractions were resolved on a 12% SDS-PAGE and stained with colloidal Coomassie blue (Invitrogen; http://www. Invitrogen.com).
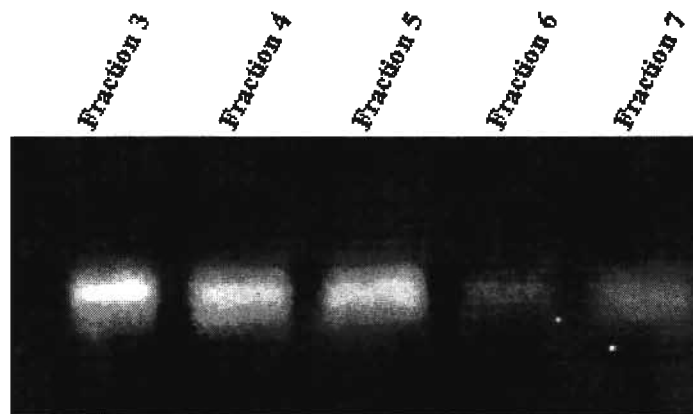
Figure 3.6: Enzymatic activity of N-acetylglucosaminidase as detected in the different protein fractions collected from *A. bisporus* by the Rotofor. SDS-PAGE gel was treated with [4-MU (GlcNAc)] and visualized under UV light.
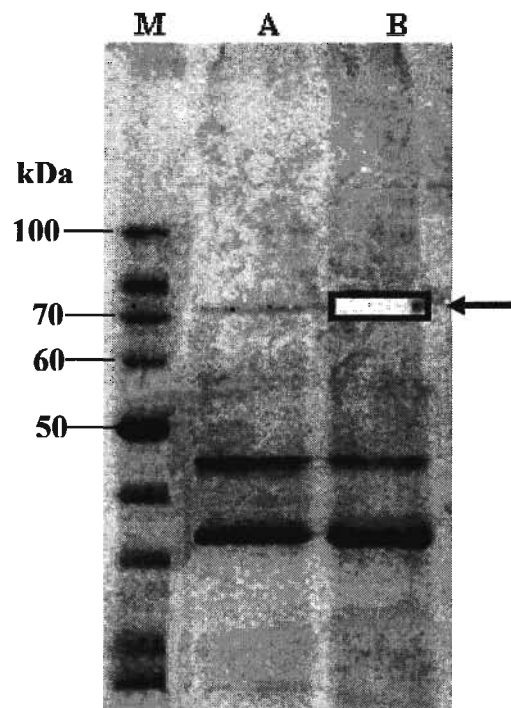
Figure 3.7: 12 % SDS-PAGE gel. (B) *A. bisporus* target protein N-acetylglucosaminidase was cut from the gel, after sample collected by Rotofor system.

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The protein band which positively correlated to N-acetylglucosaminidase activity (Figure 3.7) was excised from the gel and forwarded for sequence analysis performed at the Harvard Microchemistry facility (Harvard University) by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS; Thermo Scientific,Waltham, MA, USA) on a finnigan LCQ DECA XP Plus quadrupole ion trap mass spectrometer. The sequence data showed several different peptide sequences as shown in Appendix A. Five peptide sequences were chosen from the entire data (DVIIAYEPVWAIGTGK, LGAELLTPFGR, TVASKTEDQELQNR, NKTVVNNESSEIIR, and TITLEVESSDTXDNVK) based on the E –value. The obtained sequences showed low significant homology to fungal genes including known N-acetylglucosaminidase. Nevertheless, we proceeded to isolate DNAs based upon these peptide sequences.

3.1.4- N-acetylglucosaminidase gene isolation by PCR amplification

In an attempt to obtain the N-acetylglucosaminidase gene sequence, PCR primer sets were designed based on the obtained MS/MS peptide sequencing data as well as the consensus sequences of the N-acetylglucosaminidase gene from other fungi. These primer sets were used in a PCR reaction to amplify the N-acetylglucosaminidase gene from the *A. bisporus* genome. The PCR amplification is shown in Figure 3.8 and the amplified bands were excised from the gel, cleaned and sequenced. However, Blast search of the different obtained sequence data (Altschul *et al.*, 1997) did not show any significant homology with other fungal *N*-acetylglucosaminidases.
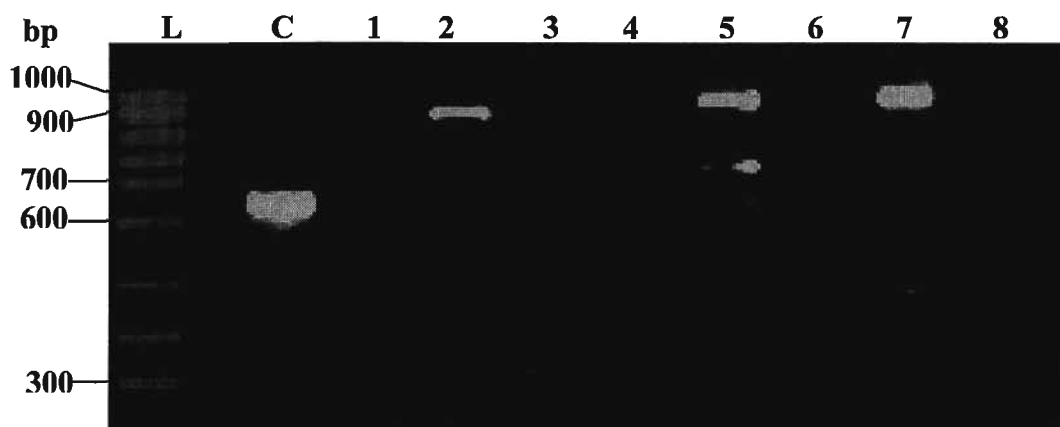
Figure 3.8: PCR amplification trials of N-acetylglucosaminidase gene using different primers sets were designed from obtained amino acid sequence and from other *Trichoderma* spp. (N-acetylglucosaminidase gene). L: PCR sizer Ladder (Norgen Biotek), C: *A. bisporus* DNA template (+control) and 1-8: amplification trials.

3.2- ISOLATION OF CELL WALL DEGRADING GENES FROM *TRICHODERMA*

AGGRESSIVUM

The hydrolytic enzymes cell wall degradation of *Trichoderma* species has been proposed as the major mechanism involving for the antagonistic activity against fungal pathogens. The mechanism of lytic enzymes induction in mycoparasitism is still a matter of speculation in many respects. *Trichoderma* spp. produce extracellular 1,3-glucanases, chitinases, and proteases when they are grown on cell walls of pathogenic fungi. In addition, several lines of evidence have shown that the production of some lytic enzymes is induced during the parasitic interaction between *Trichoderma* spp. and potential hosts.

Our starting hypothesis is that the *T. aggressivum* cell wall degrading enzymes play key roles during green mould disease development. The purpose of this part is to isolate and analyse the sequences of three cell wall degrading enzyme genes from *T. aggressivum* f. *aggressivum*. The genes that were targeted include the endochitinase *ech*42 gene, protease *prb*1 gene and a *β-1, 3 glucanase* gene. Although, these main hydrolytic enzymes genes were all isolated from *T. aggressivum* genomic DNA, relatively different procedures were used to obtain their full length sequences.

3.2.1- Methodology for chitinase *ech*42 gene recovery

3.2.1.1- Primer design

The initial step to design the primers was based on the alignment of different endo-chitinase *ech*42 gene sequences from other *Trichoderma* species (Table 3.1). The conservative regions between these different genes were used to obtain primer sequences.

Table 3.1: *Trichoderma* spp. that were used in the *ech*42 gene sequence alignment to identify the consensus regions for primers design.

Trichoderma spp.	NCBI Gene bank accession no
Hypocrea lixii	EF191273
Hypocrea virens	EU035808
Trichoderma viride	AF188924
Trichoderma hamatum	THU88560

3.2.1.2- Amplification of the initial gene fragment

In order to isolate the gene, the derived primer set ChF1 and ChR1 (Table 2.1) were used in a PCR reaction to amplify the initial *ech*42 gene fragment using the *T. aggressivum* genomic DNA as a template. The cycling conditions were melting at 95°C for 4 min, heating to 94°C for 45 second, annealing at 49°C for 45 second, extension at 72°C for 1min and finally, the reaction was held at 72°C for 5 min and incubated at 4°C afterwards. The amplification product was then visualized by agarose gel electrophoresis (Figure 3.9). The amplified DNA fragment (~ 700 bp) was excised from the agarose gel, cleaned and sequenced.
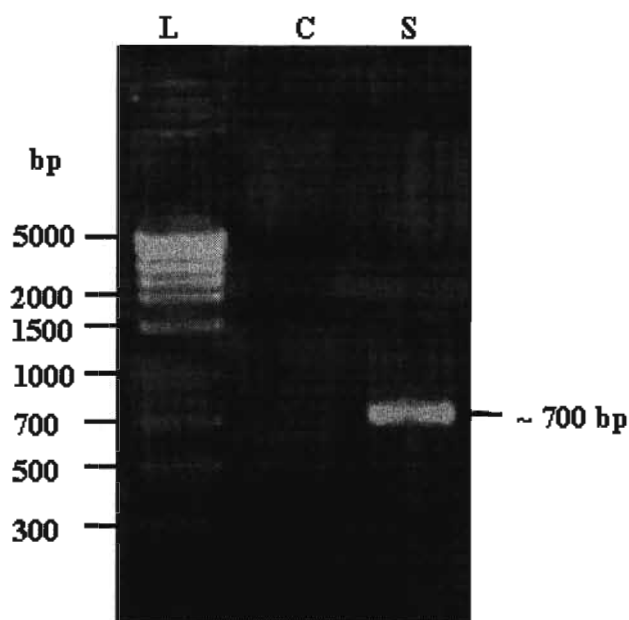
Figure 3.9: PCR amplification of the initial gene fragment of the *T. aggressivum ech*42 gene. (C) Negative control (no DNA template), (S) amplification product and (L) Norgen's MidRanger DNA ladder. The amplicon size as well as the size of the ladder bands is shown on the Figure.

3.2.1.3- Amplification of the full length gene

The sequencing result obtained from the initial gene fragment was used to design the next primer set *Ech*42-F1S and *Ech*42-R1S (Table 2.1). This primer set was used to amplify the flanking sequences of the initial fragment, by using an inverse PCR protocol (Ochman *et al.,* 2005) (Figure 3.10). In this protocol, the genomic DNA was digested by different restriction endonucleases include *Asc*I, *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III and *Nde*I that do not digest the initial amplification of *ech*42 gene fragment. The reaction mix was inactivated by heat and then T4 DNA ligase. The self-ligated DNA was then heat inactivated and used as a template for PCR amplification using the primer set *Ech*42-F1S and *Ech*42-R1S. The cycling conditions were (melting at 95°C for 4 min, heating to 94°C for 45 second, annealing at 60°C for 45 second, extension at 72°C for 1min and finally,

the reaction was held at 72°C for 5 min) and incubated at 4°C afterwards. The amplification product (~1800bp) was visualized by agarose gel electrophoresis (Figure 3.11). The band was then excised, cleaned and sequenced. Sequence data shown in Appendix B.

Digest DNA

Circularize

PCR

CYCLE 1 PRODUCTS

30 cycles of PCR

MAJOR PRODUCT
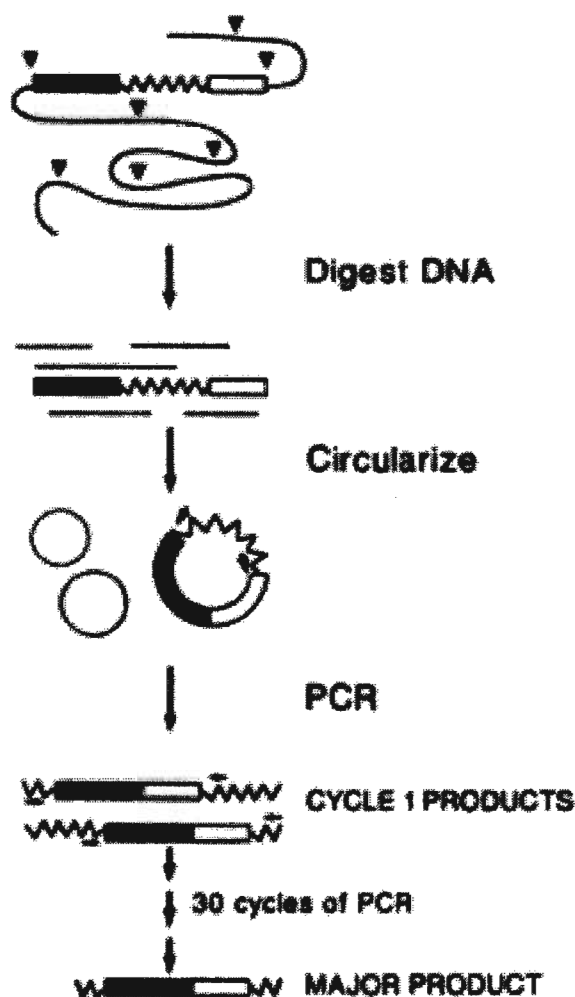
Figure 3.10: Inverse PCR strategy. The primers oriented in the reverse direction of the usual orientation. The template for the reverse primers is DNA has been digested and ligated upon itself to form a circle DNA (Ochman *et al.*, 2005).
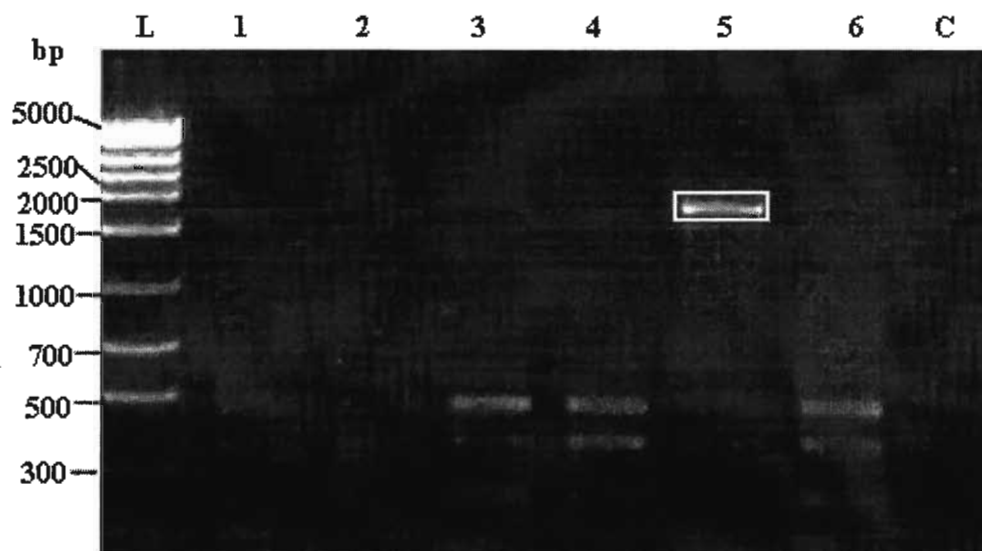
Figure 3.11: *T. aggressivum ech*42 First inverse PCR amplification with genomic DNA digested with different restriction enzymes and then religated. The restriction enzymes were: (1) *Asc*I, (2) *Bam*HI, (3) *Bgl*II, (4) *Eco*RI, (5) *Hind*III and *Nde*I. (C) Negative Control. Lane (L) is the MidRanger DNA ladder (Norgen Biotek).

Another set of primers Ech-F3Sc and Ech-R3S (Table 2.1) was designed based on the obtained sequence data and used in a second inverse PCR reaction to obtain the remaining gene sequence since the maximum sequencing capacity is 800-1000 bp (Applied Biosystems 337 DNA Sequencer limitation). The cycling conditions were melting at 95°C for 4 min, heating to 94°C for 45 second, annealing at 52°C for 45 second, extension at 72°C for 1min and finally, the reaction was held at 72°C for 5 min and incubated at 4°C. The agarose gel picture of the amplification product is shown in Figure 3.12. The DNA fragment (650 bp) was cleaned from the gel and sequenced afterwards.
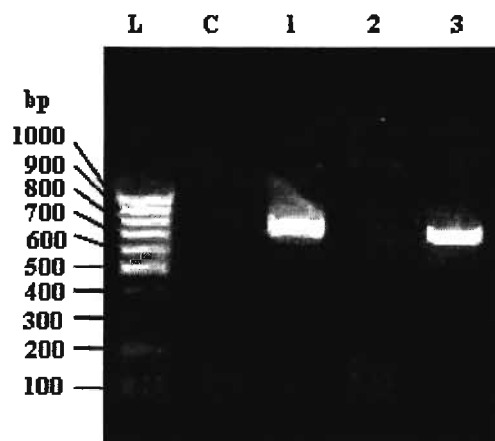
Figure 3.12: *T. aggressivum ech*42 Second inverse PCR amplification with genomic DNA digested with different restriction enzymes and then religated. The restriction enzymes were: (C) Control, (1) *Sac*I, (2) *Xba*I and (3) *Xho*I. Lane (L) is the PCR sizer DNA ladder. (Norgen Biotek).

3.2.1.4- Isolation of cDNA

Since the isolated genomic sequence gene has both introns and exons, and since our future experiments will be conducted on the gene expression level, therefore it was of a crucial importance to obtain the cDNA sequence of the gene. This sequence will be aligned against the sequence of the full length gene to determine the number and sequences of both the introns and exons. For this purpose, total *T. aggressivum* mRNA was isolated and the DNA contamination was digested by DNase I. The RNA sample was then cleaned to get rid of the DNase I. The success of the digestion step was tested by using PCR amplification using specific primers of the gene. Subsequently, the RNA sample was used in a reverse transcription (RT) reaction to synthesise the first strand cDNA. The product of the RT reaction was used as a template in a PCR reaction using the specific primer End (F-Fu) (Table 2.1) from the 5' end of the full length gene and oligo (dT) as a reverse primer. The cycling conditions were melting at 95°C for 4 min, heating to 94°C for 45 second, annealing at 61°C for 45 second, extension at 72°C for

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1min and finally, the reaction was held at 72°C for 5 min) and incubated at 4°C. The amplification product was visualized by agarose gel electrophoresis (Figure 3.13) and was cleaned, cloned and sequenced.
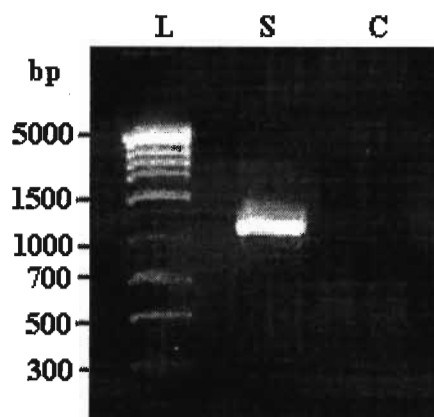
Figure 3.13: *T. aggressivum ech*42 RT-PCR amplification. (S) The amplification product of ~1200bp, (C) Negative Control (no DNA template) and Lane (L) is the MidRanger DNA ladder (Norgen Biotek).

3.2.1.5- Sequence analysis

The complete nucleotide sequence with the deduced amino acid sequences of genomic *ech*42 is shown in Figure 3.14. *Ech42* genomic sequence was verified as an endochitinase gene by average homology analysis (95% identity and e-value 0.0) with other fungal endochitinase genes. The full-length *ech*42 gene is 1468-bp, however, the corresponding cDNA sequence was 1290-bp due to the presence of three short introns (56, 67, and 55 bp), located near the N-terminus. The splice junctions were determined by comparing the sequence of *ech*42 with that of its corresponding translated sequence. Each intron began with GT and ended with AG, a common feature of fungal introns (Gurr *et al.*, 1987). The lariat sequences (CTGAC, CTAAT and CTAAG), characteristic of fungal introns, were also identified (Balance, 1986). The position of the introns is

conserved when compared with homologous genes from the hyperparasitic fungus *Aphanocladium album* (Blaiseau *et al.*, 1992) and *Trichoderma harzianum* (Carsolio *et al.*, 1994).

Promoter sequence of *ech*42 gene (-474 bp) contained different elements commonly found within fungal promoters. These included a TATATAA box located at 98-bp upstream of the translation start codon ATG. In addition, sequence, named *MYC1* (GCTTCA) that is postulated to be involved in mycoparasitism of *T. harzianum* (Cortes *et al.*, 1998) was detected. In the 3'untranslated region (871bp), three putative polyadenylation motifs (ATATAT at positions 2061 and 2154, and ATTAA at position 2092) were found.

Full-length *ech42* cDNA was 1492-bp with a predicted 1290 bp open reading frame (ORF) encoding a protein of 430 amino acids. The 5'non-coding, 3'non-coding, and poly (A^+) sequences were 54, 134 and 14 bp, respectively (Figure 3.15). The predicted peptide showed 95% identity to the amino acid sequences of other fungal endochitinases. The calculated molecular mass and theoretical p*I* of ech42 protein were 46.5 kDa and 5.6, respectively. Multiple alignments of ech42 predicted protein with other reported endochitinase sequences (Figure 3.16) highlighted a number of conserved motifs and structural similarities that are common to the fungal chitinases (Gan *et al.*, 2007). A signal peptide showed that a cleavage prediction would occur after Ala-22. The mature protein is 408 amino acids long, which has a calculated molecular mass of 44.3 kDa and a theoretical p*I* 5.4. Two potential N-glycosylation sites (NYS and NPS) were also identified.

A

T. aggressivum ech42 gene

DNA ————————————[atg ▨ ▨▨ ——————— taa]————————————
(-474)   (1)   (1467)   (2812)

B

```
   1  TGGAATGCCG CATATCGCCC AAAAGCATAT CAGAGCAACC GACTTCTTGC CGCCATTAGC AACGTTGCTA GACTTGGCGA GGGTATGAGA
  91  TGCCCGTAAT TCATATGGCG CTGAAGCTAA TCATGACCTC CACCTCGATT TGGTTCGCGC CGGGACATGC CTATCCATGC CAGTCGAGAG
 181  CCGCTGGACG TTGAGCACGG GACAGGGGTG GCACGCCTGG AGAACCATGA GCTCCGGGGC ATAACGTTGC CATTGATTGC GAGCTATAGC
 271  ACTATTGAAG AAGCATGGGT TACATGTTGG TGCCAAGGGC CTGAAAGGGA AGCTTCATAA GTGAGCATCA TGCTTTGTAG AGCATCTGGA
 361  AGGCTCCACA GCCACATATA TAAACATCGT TGGTTCGCAT CCAGCGCCTTC ACAAGAAAGT TTAATTCAGC ATCGGCAAGC TTGAACGATC
                                                         MYCI
                        M   L   S   F   L   G   K   S   V   A   L   L   A   A   L   Q   A   T   L   S   S   A
 451  TACCAACATC ACAAGCATTC TACCATGTTG AGCTTCCTCG GAAAATCTGT GGCCCTGTTG GCTGCGCTGC AGGCTACTCT CAGCTCTGCA
       S   P   L   A   T   E   D   R   S   V   E   K   R   A   N   G   Y   A   N   S   V   Y   F   T   N   W
 541  AGCCCTCTGG CTACCGAAGA TCGCTCTGTT GAGAAGAGAG CCAACGGATA CGCAAACTCC GTCTATTTCA CCAACTGgtg agtgaaacoa
                                          G   I   Y   D   R   N   F   Q   P   A   D   L   V   A   S
 631  ataagcttoa oatagattoa otgctgacta taagtgatta aagGGGCATC TACGACCGCA ACTTCCAGCC TGCCGATTTG GTGGCATCAG
       D   V   T   H   V   I   Y   S   F   M   N   L   Q   A   D   G   T   V
 721  ATGTCACTCA TGTCATCTAC TCCTTCATGA ACCTCCAGGC AGACGGCACA GTgtaagooa gatatoocgg agggaatata totooaaaag
                                          V   S   G   D   T   Y   A   D   F   E   K   H   Y   A   D   D   S   W
 811  oogotttota gttaotaatt gtgtttoagT GTCTCTGGCG ATACCTACGC TGATTTCGAG AAGCACTATG CCGATGATTC ATGGtgtaag
                                          N   D   V   G   T   N   A   Y   G   C   A   K   Q   L
 901  tttoootoot agataotaag taoaoataot taoaotoata atagotagAA TGATGTCGGC ACCAATGCCT ACGGCTGTGC CAAGCAGCTG
       F   K   V   K   K   A   N   R   G   L   K   V   L   L   S   I   G   G   W   T   W   S   T   N   F   P   S   A   A   S
 991  TTCAAGGTCA AGAAGGCCAA CCGAGGCCTC AAGGTTCTAC TCTCCATCGG TGGCTGGACC TGGTCACCA ACTTCCCTTC CGCAGCAAGC
       T   D   A   N   R   R   N   F   A   R   T   A   I   T   F   M   K   D   W   G   F   D   G   I   D   V   D   W   E   Y
1081  ACGGATGCCA ACCGAAGGAA CTTTGCGCGA ACTGCCATTA CCTTCATGAA GGATTGGGGT TTCGATGGTA TTGACGTCGA CTGGGAGTAC
       P   A   D   A   T   Q   A   S   N   M   V   L   L   L   K   E   V   R   S   Q   L   D   A   Y   A   A   Q   Y   A   P
1171  CCTGCCGATG CCACCCAGGC CTCCAACATG GTTCTTCTGC TCAAGGAAGT CCGATCTCAG CTGGATGCTT ATGCTGCCCA GTACGCCCCT
       G   Y   H   F   L   L   T   I   A   A   P   A   G   K   D   N   Y   S   K   L   R   L   A   D   L   G   Q   V   L   D
1261  GGCTACCACT TCCTCCTCAC CATTGCTGCC CCAGCTGGCA AGGACAACTA CTCCAAGCTG CGCCTGGCCG ATCTTGGCCA AGTCCTCGAC
       Y   I   N   L   M   A   Y   D   Y   A   G   S   F   S   P   L   T   G   H   D   A   N   L   F   A   N   P   S   N   P
1351  TACATCAACC TCATGGCCTA CGACTACGCT GGTTCCTTCA GCCCCCTCAC CGGCCACGAC GCCAACCTGT TTGCCAACCC GTCCAACCCC
       Y   G   R   S   F   Q   N   T   G   G   I   G   Q   S   Y   N   G   V   G   G   G   G   G   S   T   G   S   W   E
1441  AATGCCACCC CCTTCAACAC CGATTCTGCT GTCAGGGATT ATATCAACGG AGGTGTTCCC GCCAACAAGA TTGTTCTCGG CATGCCCATC
       A   G   I   W   D   Y   K   A   L   P   R   S   G   A   T   I   K   Y   D   D   V   A   K   G   Y   Y   S   Y   N   A
1531  TACGGACGAT CATTCCAGAA CACCGGTGGT ATTGGCCAGT CCTACAACGG AGTTGGAGGT GGAGGTGGTG GCTCTACTGG AAGCTGGGAG
       A   G   I   W   D   Y   K   A   L   P   R   S   G   A   T   I   K   Y   D   D   V   A   K   G   Y   Y   S   Y   N   A
1621  GCTGGTATCT GGGATTACAA GGCTCTTCCC AGGTCCGGCG CCACCATCAA GTACGATGAT GTCGCAAAGG GTTACTACAG CTACAACGCC
       N   T   K   E   L   I   S   F   D   T   P   D   M   I   N   T   K   V   A   Y   L   K   S   L   G   L   G   G   S   M
1711  AACACCAAGG AGCTCATCTC TTTCGATACC CCTGACATGA TCAACACACA GGTTGCCTAC CTCAAGTCTC TTGGCCTGGG AGGTAGCATG
       F   W   E   A   S   A   D   K   K   G   S   D   S   L   I   G   T   S   H   R   A   L   G   G   L   D   T   T   Q   N
1801  TTCTGGGAGG CCTCAGCCGA CAAGAAGGGA TCTGACTCTC TGATTGGAAC CAGCCACAGA GCTCTTGGAG GTCTGGCACAC GACTCAGAAC
       L   L   S   Y   P   N   S   K   Y   D   N   I   R   N   G   L   N   *
1891  CTGCTGAGCT ACCCCAACTC CAAGTATGAC AACATCAGGA ACGGTCTGAA CTAAGCTGCC TATTTTCAGG CGCTTCTTGT ACATTGAAGA
1981  TGTTACGGGG GGGAAGCTCT TGGAGGAGCA AAGCCATCAA GTAAATAGTT GTTTATATAC CTGTACATAG CCACATTCAT ATATATAGAT
2071  ACATGAATAG TATCTGAACT TATTTAAGAAA CCTTACAACA AGGCTACCTA CCATCTATAG GTGTCATCCT ATTTTGTACT GTGTATATAT
2161  CCTAGTTGAA CCTCGATGGG AATTACGCTA ATCTCTGTTC GTTTCGTCGC CTTACTTTAT ACAGAATAAC CAGGTGGAAA GGTCAATTTA
2251  TCCAAGAGGA TAGAGTTGCT TCGAAATATA AATCCCCTCG CTAGAACAGA GCAAAATTCC TCATCGCCCC GGAGCTAGCT TGGCTCGTCT
2341  GAGCTACCTT GCCCATCTCG GTGATGGTAG TAGAAGTCCC TGGAATGCTG GTACTGGAGC TGGAGTTGGG CCCCCGTACG ATATCGGATT
2431  GTGATGCCTC GAAAACACCA AGGCAATCCC TGCTGCCGCA GATCTGGGTC TCATCGCCGG TGCAGGCCAT GTTGCAGTCG CTCGCATTCG
2521  GCGTCCACTC ACCGACAACG TGACTACTGC ACCAACACTC GTTGCCCGCC TTGACGCCTG CAAATATGAA GTTTAAGCGG TAGCAGGCGT
2611  TTTGGCACTT GGGCATTGTG AGGGCGGCGT CTCCGCCCTT TTTACTGACG AGCTTCTCGA GTATTGGCCG ATTCGGTCTG TCAGCATAGC
2701  AGCCAAGGTC ATCCCACGCG GTTGGAGAAG GCCCCAGAGT GGCATGTCGTA GTGGTGCTGA TGGGTGCTGA ATTGCTGGTA ATAGTCGTTT
2791  TAAATGTCAA TCTATCAAGC TT
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Figure 3.14: Sequence of *T. aggressivum ech*42 gene. (A) Structural image of exon and intron positions in the putative *ech*42 gene. The unshaded portions represent exons. Introns are hash shaded. The letters (atg) and (taa) indicate the positions of the start and stop codon, respectively. (B) Nucleotide sequence of the *T. aggressivum* endochitinase gene (*ech*42). The deduced amino acid sequence is shown in one-letter code above the DNA sequence. Nucleotide coordinates are shown to the left of the sequence. The transcription start and termination are indicated by the first ATG and asterisk, respectively. Introns are shown in italic lowercase letters and t he lariat sequences characteristics of fungal introns are in bold and italic. The putative polyadenylylation signal sequences in the 3'untranslated region are shaded in black.

A *T. aggressivum* ech42 cDNA

FDGIDVDWE

SIGG   N   N

atg   taa

mRNA ——■□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■—— AAA

(-54) (1)   (1290) (1492)

B

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                                                  M  L  S  F  L     G  K  S     A  L  L
   1 TTAATTCAGC ATCGGCAAGC TTGAACGATC TACCAACATC ACAAGCATTC TACCATGTTG AGCTTCCTCG GAAAATCTGT GGCCCTGTTG
     A  A  L     Q  A  T  L     S  S  A     S  P  L     A  T  E  D     R  S  V     E  K  R     A  N  G  Y     A  N  S
  91 GCTGCGCTGC AGGCTACTCT CAGCTCTGCA AGCCCTCTGG CTACCGAAGA TCGCTCTGTT GAGAAGAGAG CCAACGGATA CGCAAACTCC
     V  Y  F     T  N  W  G     I  Y  D     R  N  F     Q  P  A  D     L  V  A     S  D  V     T  H  V  I     Y  S  F
 181 GTCTATTTCA CCAACTGGGG CATCTACGAC CGCAACTTCC AGCCTGCCGA TTTGGTGGCA TCAGATGTCA CTCATGTCAT CTACTCCTTC
     M  N  L     Q  A  D  G     T  V  V     S  G  D     T  Y  A  D     F  E  K     H  Y  A     D  D  S  W     N  D  V
 271 ATGAACCTCC AGGCAGACGG CACAGTTGTC TCTGGCGATA CCTACGCTGA TTTCGAGAAG CACTATGCCG ATGATTCATG GAATGATGTC
     G  T  N     A  Y  G  C     A  K  Q     L  F  K     V  K  R  A     N  R  G     L  K  V     L  L  S  I     G  G  W
 361 GGCACCAATG CCTACGGCTG TGCCAAGCAG CTGTTCAAGG TCAAGAAGGC CAACCGAGGC CTCAAGGTTC TACTCTCCAT CGGTGGCTGG
     T  W  S     T  N  F  P     S  A  A     S  T  D     A  N  R  R     N  F  A     R  T  A     I  T  F  M     K  D  W
 451 ACCTGGTCCA CCAACTTCCC TTCCGCAGCA AGCACGGATG CCAACCGAAG GAACTTTGCG CGAACTGCCA TTACCTTCAT GAAGGATTGG
     G  F  D     G  I  D  V     D  W  E     Y  P  A     D  A  T  Q     A  S  N     M  V  L     L  L  K  E     V  R  S
 541 GGTTTCGATG GTATTGACGT CGACTGGGAG TACCCTGCCG ATGCCACCCA GGCCTCCAAC ATGGTTCTTC TGCTCAAGGA AGTCCGATCT
     Q  L  D     A  Y  A  A     Q  Y  A     P  G  Y     H  F  L  L     T  I  A     A  P  A     G  K  D  N     Y  S  K
 631 CAGCTGGATG CTTATGCTGC CCAGTACGCC CCTGGCTACC ACTTCCTCCT CACCATTGCT GCCCCAGCTG GCAAGGACAA CTACTCCAAG
     L  R  L     A  D  L  G     Q  V  L     D  Y  I     N  L  M  A     Y  D  Y     A  G  S     F  S  P  L     T  G  H
 721 CTGCGCCTGG CCGATCTTGG CCAAGTCCTC GACTACATCA ACCTCATGGC CTACGACTAC GCTGGTTCCT TCAGCCCCCT CACCGGCCAC
     D  A  N     L  F  A  N     P  S  N     P  N  A     T  P  F  N     T  D  S     A  V  R     D  Y  I  N     G  G  V
 811 GACGCCAACC TGTTTGCCAA CCCGTCCAAC CCCAATGCCA CCCCCTTCAA CACCGATTCT GCTGTCAGGG ATTATATCAA CGGAGGTGTT
     P  A  N     K  I  V  L     G  M  P     I  Y  G     R  S  F  Q     N  T  G     G  I  G     Q  S  Y  N     G  V  G
 901 CCCGCCAACA AGATTGTTCT CGGCATGCCC ATCTACGGAC GATCATTCCA GAACACCGGT GGTATTGGCC AGTCCTACAA CGGAGTTGGA
     G  G  G     G  G  S  T     G  S  W     E  A  G     I  W  D  Y     K  A  L     P  R  S     G  A  T  I     K  Y  D
 991 GGTGGAGGTG GTGGCTCTAC TGGAAGCTGG GAGGCTGGTA TCTGGGATTA CAAGGCTCTT CCCAGGTCCG GCGCCACCAT CAAGTACGAT
     D  V  A     K  G  Y  Y     S  Y  N     A  N  T     K  E  L  I     S  F  D     T  P  D     M  I  N  T     K  V  A
1081 GATGTCGCAA AGGGTTACTA CAGCTACAAC GCCAACACCA AGGAGCTCAT CTCTTTCGAT ACCCCTGACA TGATCAACAC CAAGGTTGCC
     Y  L  K     S  L  G  L     G  G  S     M  F  W     E  A  S  A     D  K  K     G  S  D     S  L  I  G     T  S  H
1171 TACCTCAAGT CTCTTGGCCT GGGAGGTAGC ATGTTCTGGG AGGCCTCAGC CGACAAGAAG GGATCTGACT CTCTGATTGG AACCAGCCAC
     R  A  L     G  G  L  D     T  T  Q     N  L  L     S  Y  P  N     S  K  Y     D  N  I     R  N  G  L     N  *
1261 AGAGCTCTTG GAGGTCTGGA CACGACTCAG AACCTGCTGA GCTACCCCAA CTCCAAGTAT GACAACATCA GGAACGGTCT GAACTAAGCT

1351 GCCTATTTTC AGGCGCTTCT TGTACATTGA AGATGTTACG GGGGGGAAGC TCTTGGAGGA GCAAAGCCAT CAAGTAAATA GTTGTTTATA

1441 TACCTGTACA TAGCCACATT CATATATATA GATACATGAA AAAAAAAAAA AA
```

Figure 3.15: Sequence of *T. aggressivum* endochitinase *ech42* cDNA. (A) mRNA Model shows the conserved motif from family 18 of the glycosyl hydrolases (5'SIGG-3' and 5'FDGIDVDWE-3'). The possible signal sequence cleavage point is represented by the dark fragment at the 5' end of the mRNA. Potential N-glycosylation sites are indicated by the letter N and (B) cDNA nucleotide sequence and deduced amino acid sequence.
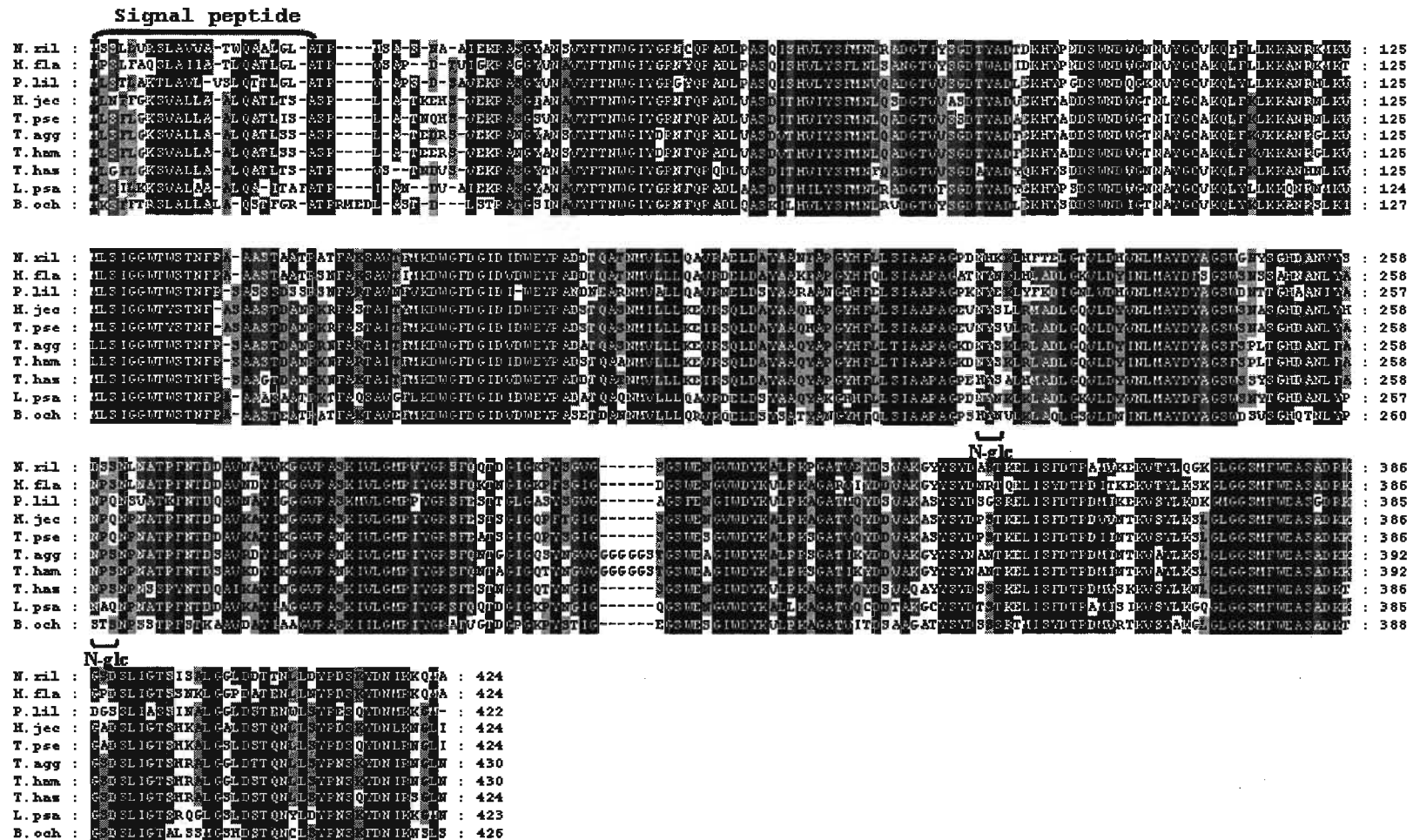
Figure 3.16: Predicted amino acid sequence alignment of *T. aggressivum* ech42 with closely related sequences *Nomuraea rileyi* (AAP04616), *Metarhizium flavoviride* (CAB44709), *Paecilomyces lilacinus* (ABP37997), *Hypocrea jecorina* (BAD44715), *Trichoderma pseudokoningii* (BAB40594), *Trichoderma hamatum* (AAC60385), *Trichoderma harzianum* (BAB40589) *Lecanicillium psalliotae* (ABQ57240), *Bionectria ochroleuca* (ABV57861), using ClustalX program. Conserved residues are shaded in black. Dark grey shading indicates similar residues in eight out of ten of the sequences and clear grey shading indicates similar residues in six out of ten of the sequences. Putative N-terminal signal sequences are indicated by "Signal peptide". The predicted N-glycosylation sites of endochitinase sequences are indicated by "N-glc".

3.2.2- Methodology for protease *prb*1 gene recovery

3.2.2.1- Primer design

Similar to the corresponding step in the design of the *ech*42 initial primers, the consensus regions obtained from the alignment of the protease gene sequences of different *Trichoderma* species (Table 3.2) were used to get the initial primers.

Table 3.2: *Trichoderma* spp. have used in the *prb*1 gene sequence alignment to identify the consensus regions for primer design.

Trichoderma spp.	NCBI Gene bank accession no
Trichoderma harzianum	M87516
Trichoderma hamatum	AY258899
Hypocrea virens	AY242844

3.2.2.2- Amplification of the initial gene fragment

The PCR amplification of the initial gene fragment from genomic DNA was performed with the designed primer *prb*1-Nter and *prb*1-Cter (Table 2.1). The cycling conditions were melting at 95°C for 4 min, heating to 94°C for 45 second, annealing at 56°C for 45 second, extension at 72°C for 1min and finally, the reaction was held at 72°C for 5 min and incubated at 4°C. The agarose gel picture of the amplification product (600 bp) is shown in Figure 3.17. The band was excised from the gel, cleaned and sequenced.
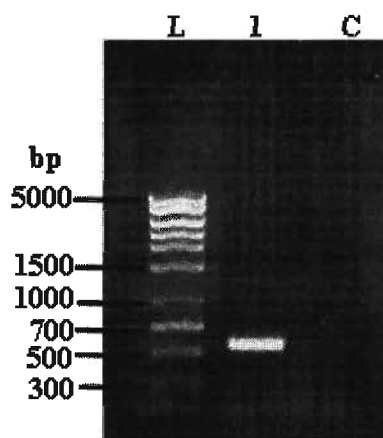
Figure 3.17: PCR amplification of the initial gene fragment of the *T. aggressivum prb*1 gene. (C) Negative control (no DNA template), (1) amplification product (600 bp) and (L) MidRanger DNA ladder (Norgen Biotek).

3.2.2.3- Amplification of the full length gene

Based on the sequence of the initial gene fragment, another primer set Prb1-F1S and Prb1-R1S (Table 2.1) were designed to be used in an inverse PCR reaction to obtain the sequences flanking the initial fragment. Cycling conditions were melting at 95°C for 4 min, heating to 94°C for 45 second, annealing at 50°C for 45 second, extension at 72°C for 1min and finally, the reaction was held at 72°C for 5 min and then at 4°C. The amplification product is shown on agarose gel in Figure 3.18 and was sequenced subsequently. This step provided the sequence of the 5' end of the gene. However, we were not able to get the sequence of the 3' end with the inverse PCR protocol. Therefore, we used the genome walker library technology (Figure 3.19) to get the sequence of the 3' end. In this protocol, genomic DNA was digested, in separate reactions, by four different restriction enzymes (*Dra*I, *Eco*RV, *Pvu*II and *Ssp*I). The DNA was cleaned afterwards using phenol-chloroform. Then, T4 DNA ligase was used to ligate the genome walker

adapter to each terminus of the cleaned digested product of each restriction digestion reaction. This ligation product was used as a template in a PCR reaction using specific primers (two primers were used) at the terminal end of the obtained gene sequence and the Ap1 primer (Table 2.1) (inside the genomic walker sequence). Cycling conditions were melting at 95°C for 3 min, heating to 94°C for 30 second, annealing at 58°C for 1 min, extension at 72°C for 2 min and finally, the reaction was held at 72°C for 10 min and incubated at 4°C. The amplification product was cleaned afterwards and used in another PCR reaction where the specific gene primer Prb-WKF and Prb-WKR was used with the Ap2 primer (Table 2.1). The PCR condition was the same as previously. The agarose gel picture of the second PCR amplification is shown in Figure 3.20. The amplified fragment (2500 bp) obtained from the second amplification was sequenced. Sequence information is provided in Appendix C.
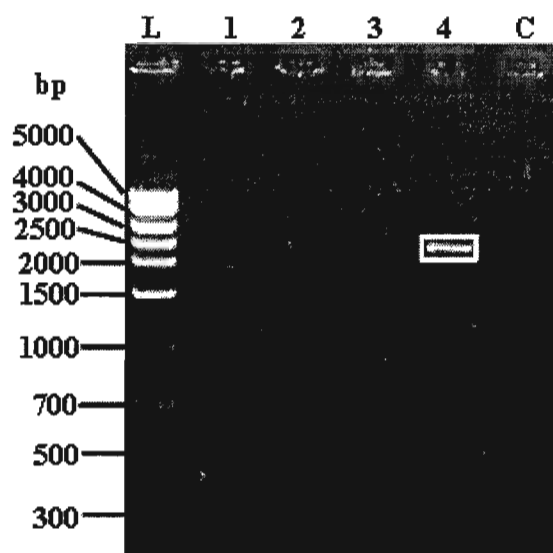
Figure 3.18: Inverse PCR amplification of *T. aggressivum prb*1 gene with different restriction enzymes. The used restriction enzymes are: (1) *NHe*I, (2) *Sac*I, (3) *Xba*I and (4) *Xho*I. (C) Negative control. (L) MidRanger DNA ladder (Norgen Biotek). The amplicon size indicated bands are shown on the side of the picture.

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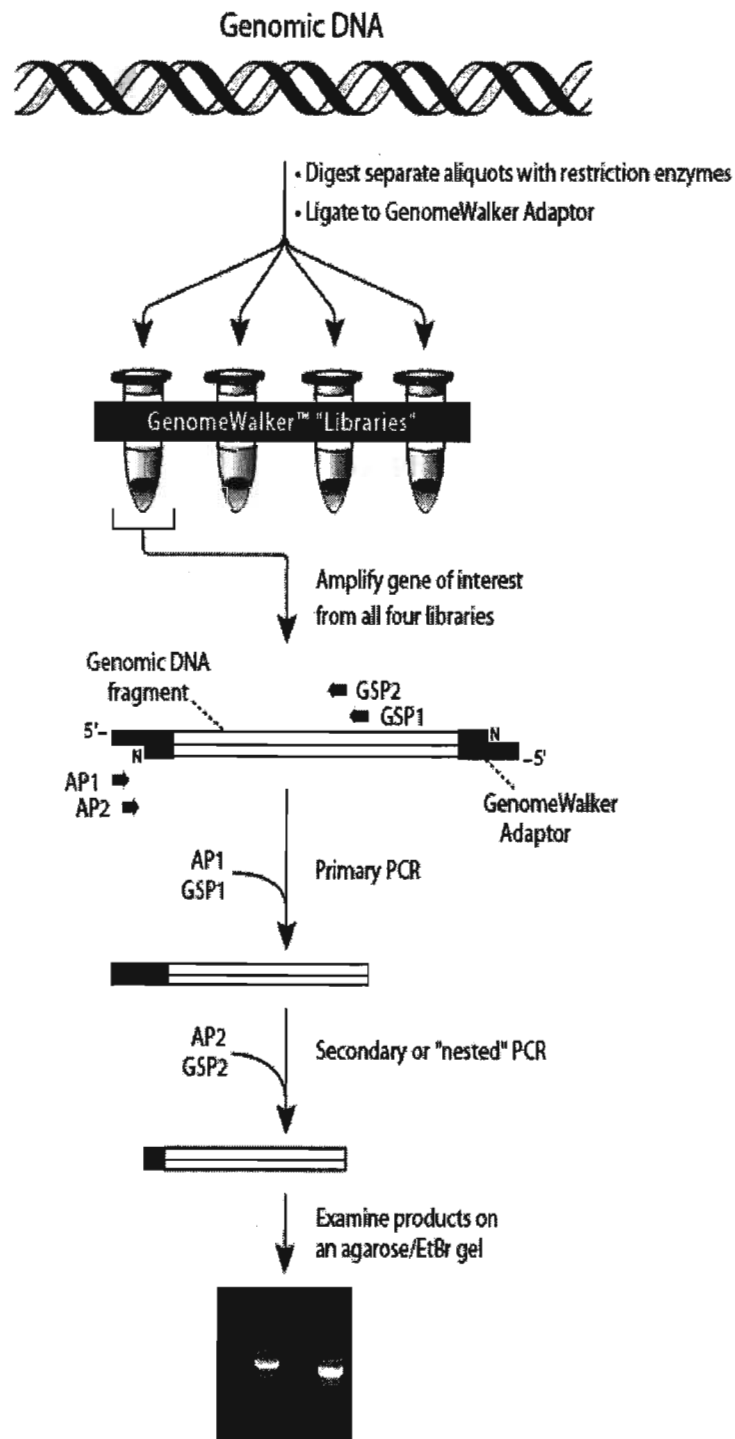
Figure 3.19: Illustration of the genomic walker library strategy.

http://www.clontech.com/images/pt/PT3042-1.pdf
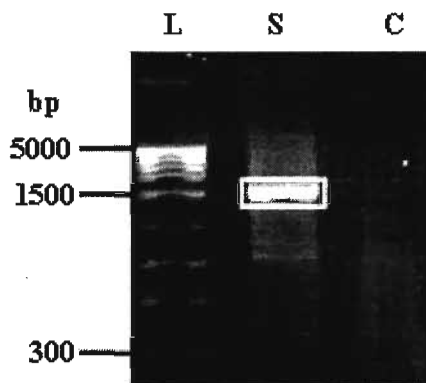
Figure 3.20: Genomic walker PCR amplification of *T. aggressivum prb*1 gene, (S) Amplification product (1500 bp), (C) Negative control (no DNA template) and (L) MidRanger DNA ladder (Norgen Biotek).

3.2.2.4- Isolation of cDNA

The cDNA sequence of the gene was obtained by the same steps described previously for *ech42* gene and from the same reverse transcription (RT) reaction product but with using specific primer *prb*1 (F-Fu) (Table 2.1) from the 5' end of the gene in addition to the oligo (dT), in the PCR reaction. Cycling conditions were melting at 95°C for 4 min, heating to 94°C for 45 second, annealing at 61°C for 45 second, extension at 72°C for 1min and finally, the reaction was held at 72°C for 5 min and incubated at 4°C. Agarose gel electrophoresis was used to visualize the amplification product (1500 bp) Figure 3.21), which was sequenced.
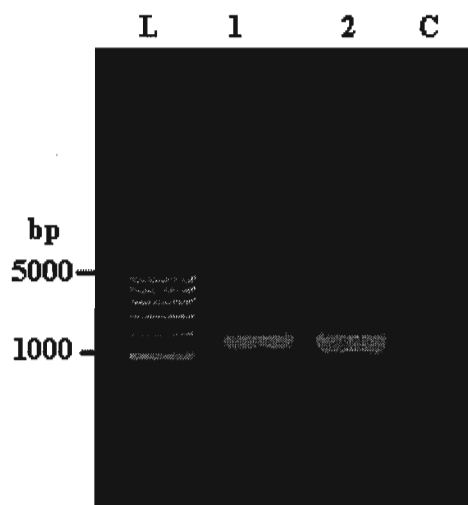
Figure 3.21: *T. aggressivum prb*1 RT-PCR amplification. (1) and (2) the amplification product (~1200bp) using different concentrations of RT reaction product of, (C) Negative Control and Lane (L) is the MidRanger DNA ladder (Norgen Biotek).

3.2.2.5- Sequence analysis

The complete nucleotide sequence with the deduced amino acid sequences of genomic *prb*1 is shown in Figure 3.22. *Prb1* genomic sequence was verified as a protease gene by homology analysis (96% identity and e-value 0.0) with other fungal protease genes. The full-length *prb*1 gene is 1365-bp, however, the corresponding cDNA sequence was 1227 bp. Comparison of the cDNA and genomic sequences revealed that *prb*1 gene contains two introns, one of 64-bp and the other of 74-bp in length, located near the middle of the gene sequence. The splice junctions were determined by comparing the sequence of *prb*1 with that of its corresponding translated sequence from cDNA. Each intron began with GT and ended with AG, a common feature of fungal introns (Gurr *et al.*, 1987). The lariat sequences (CTAAC and CTAAT) characteristic of fungal introns, were also identified (Balance, 1986).

Analysis of *prb1* promoter sequence (-1389 bp) identified numerous putative regulatory motifs previously reported in other fungal genes. A detailed summary of the motifs identified in the *prb*1 promoter region are shown on the Figure 3.22. These included a TATATAA box located at -558 bp upstream of the translation start codon ATG. In addition, two sequences, *MYC1* (GCTTCA) and *MYC2* (TTGGCAA), that are postulated to be involved in mycoparasitism (Cortes *et al.*, 1998) were detected. In the 3'UTR (876 bp), two putative polyadenylation motifs (ATTAA at positions 2810 and 3013) were found.

T. aggressivum prb1

A

DNA

B

(-1389) (1) (1365) (2141)

[DNA sequence and deduced amino acid sequence of the T. aggressivum prb1 gene, shown in numbered blocks from position 1 to 3511, with one-letter amino acid code above the coding regions and intron sequences in italic lowercase.]
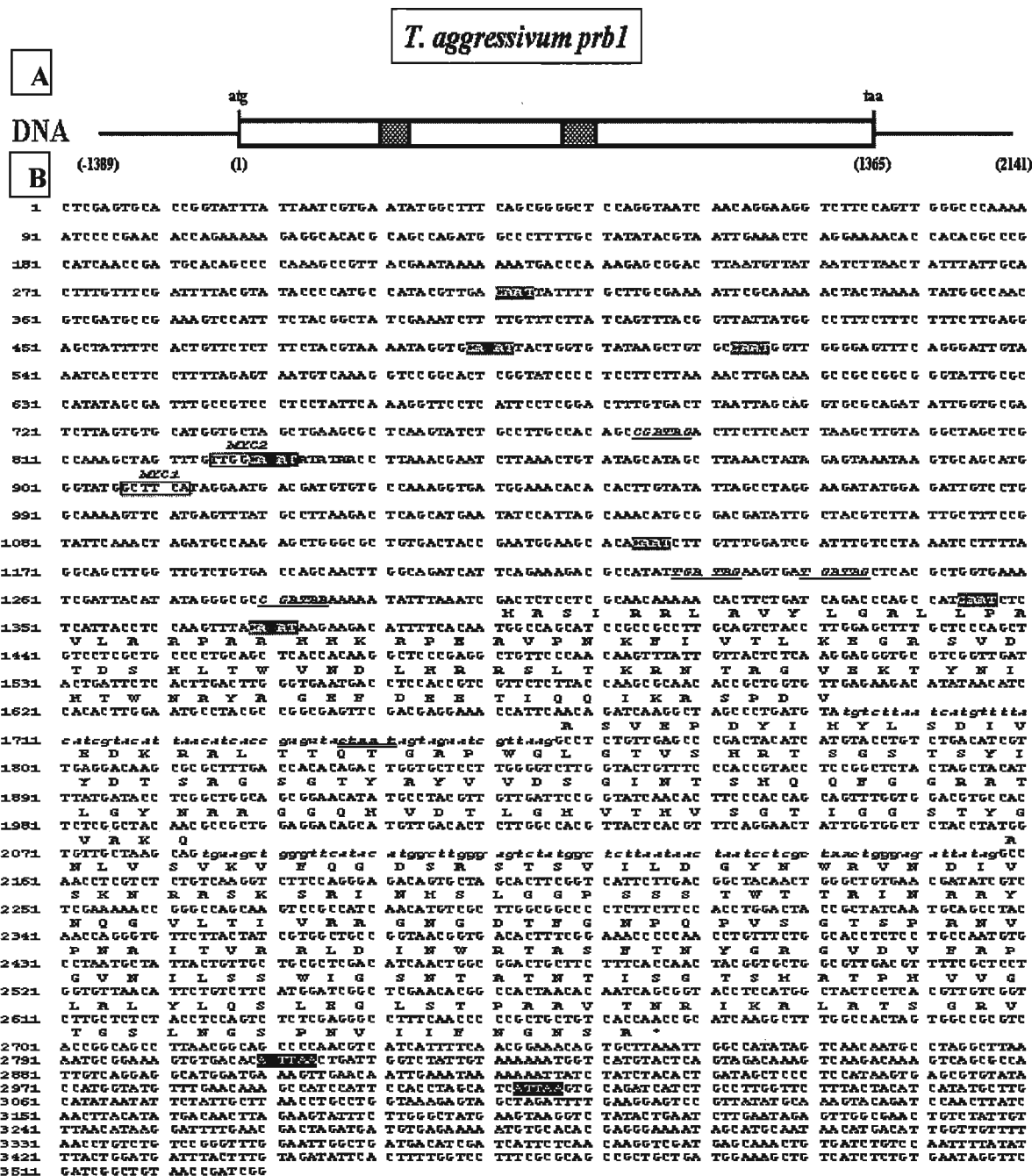
Figure 3.22: Sequence of *T. aggressivum prb*1 gene. (A) Structural image of exon and intron positions in the putative *prb*1 gene. The unshaded portions represent exons. Introns are hash shaded. The letters (atg) and (taa) indicate the positions of the start and stop codon, respectively. (B) Nucleotide sequence of the *T. aggressivum* protease gene (*prb*1). The deduced amino acid sequence is shown in one-letter code above the DNA sequence. Nucleotide coordinates are shown to the left of the sequence. The transcription start and termination are indicated by the first ATG and asterisk, respectively. Introns are shown in italic lowercase letters and the lariat sequences characteristics of fungal introns are in bold. The putative polyadenylylation signal sequences in the 3'untranslated region are shaded in black.

Full-length *prb*1 cDNA was 1401-bp with a predicted open reading frame (ORF) that encoded a protein of 409 amino acids. The 5'non-coding, 3'non-coding, and poly (A+) sequences were 85, 76 and 13 bp, respectively Figure 3.23. The predicted ORF showed 98% identity in amino acid sequence to other fungal proteases. The calculated molecular mass and theoretical *pI* of prb1 native protein were 42.5 kDa and 6.59, respectively. Multiple alignments of prb1 with other reported protease sequences Figure 3.24 revealed the identification of many conserved motifs that are common to the fungal proteases (Geremia *et al.*, 1993; Orozco *et al.*, 2002). A signal peptide showed that a cleavage prediction would occur after Ala-20. Mature protein was 389 amino acids long, which has a calculated molecular mass of 40.4 kDa and a theoretical *pI* of 6.26. Comparison of different protease sequences from various organisms revealed that the mature protein of all aligned sequences contains the five highly conserved regions (I to V) characteristic of the alkaline protease family (Geremia *et al.*, 1993). Consistent with fungal proteases (Orozco *et al.*, 2002), two potential N-glycosylation sites (NYS and NPS) were also identified.

Figure 3.23: Sequence of *T. aggressivum prb*1 cDNA. (A) mRNA Model shows the possible signal sequence cleavage point is represented by the dark fragment at the 5' end of the mRNA. Potential N-glycosylation sites are indicated by the letter N and (B) cDNA nucleotide sequence and deduced amino acid sequence.

```
                                                                                          M  A
   1  TCCTCGCAAC AAAAACACTT CTGATCAGAC CCAGCCATCA ATCTCTCATT ACCTCCAAGT TTACAATAAG AAGACATTTT CACAATGGCC
       S  I  R   R  L  A  V   Y  L  G   A  L  L   P  A  V  L   A  A  P   A  A  H   H  K  A  P   E  A  V
  91  AGCATCCGCC GCCTTGCAGT CTACCTTGGA GCTTTGCTCC CAGCTGTCCT CGCTGCCCCT GCAGCTCACC ACAAGGCTCC CGAGGCTGTT
       P  N  K   F  I  V  T   L  K  E   G  A  S   V  D  T  D   S  H  L   T  W  V   N  D  L  H   R  R  S
 181  CCCAACAAGT TTATTGTTAC TCTCAAGGAG GGTGCGTCGG TTGATACTGA TTCTCACTTG ACTTGGGTGA ATGACCTCCA CCGTCGTTCT
       L  T  K   R  N  T  A   G  V  E   K  T  Y   N  I  H  T   W  N  A   Y  A  G   E  F  D  E   E  T  I
 271  CTTACCAAGC GCAACACCGC TGGTGTTGAG AAGACATATA ACATCCACAC TTGGAATGCC TACGCCGGCG AGTTCGACGA GGAAACCATT
       Q  Q  I   K  A  S  P   D  V  A   S  V  E   P  D  Y  I   M  Y  L   S  D  I   V  E  D  K   R  A  L
 361  CAACAGATCA AGGCTAGCCC TGATGTTGCC TCTGTTGAGC CCGACTACAT CATGTACCTG TCTGACATCG TTGAGGACAA GCGCGCTTTG
       T  T  Q   T  G  A  P   W  G  L   G  T  V   S  H  R  T   S  G  S   T  S  Y   I  Y  D  T   S  A  G
 451  ACCACACAGA CTGGTGCTCC TTGGGGTCTT GGTACTGTTT CCCACCGTAC CTCCGGCTCT ACTAGCTACA TTTATGATAC CTCGGCTGGC
       S  G  T   Y  A  Y  V   V  D  S   G  I  N   T  S  H  Q   Q  F  G   G  R  A   T  L  G  Y   N  A  A
 541  AGCGGAACAT ATGCCTACGT TGTTGATTCC GGTATCAACA CTTCCCACCA GCAGTTTGGT GGACGTGCCA CTCTCGGCTA CAACGCCGCT
       G  G  Q   H  V  D  T   L  G  H   V  T  H   V  S  G  T   I  G  G   S  T  Y   G  V  A  K   Q  A  N
 631  GGAGGACAGC ATGTTGACAC TCTTGGCCAC GTTACTCACG TTTCAGGAAC TATTGGTGGC TCTACCTATG GTGTTGCTAA GCAGGCCAAC
       L  V  S   V  K  V  F   Q  G  D   S  A  S   T  S  V  I   L  D  G   Y  N  W   A  V  N  D   I  V  S
 721  CTCGTCTCTG TCAAGGTCTT CCAGGGAGAC AGTGCTAGCA CTTCGGTCAT TCTTGACGGC TACAACTGGG CTGTGAACGA TATCGTCTCG
       K  N  R   A  S  K  S   A  I  N   M  S  L   G  G  P  S   S  S  T   W  T  T   A  I  N  A   A  Y  N
 811  AAAAACCGGG CCAGCAAGTC CGCCATCAAC ATGTCGCTTG GCGGCCCCTC TTCTTCCACC TGGACTACCG CTATCAATGC AGCCTACAAC
       Q  G  V   L  T  I  V   A  A  G   N  G  D   T  F  G  N   P  Q  P   V  S  G   T  S  P  A   N  V  P
 901  CAGGGTGTTC TTACTATCGT GGCTGCCGGT AACGGTGACA CTTTCGGAAA CCCCCAACCT GTTTCTGGCA CCTCTCCTGC CAATGTGCCT
       N  A  I   T  V  A  A   L  D  I   N  W  R   T  A  S  F   T  N  Y   G  A  G   V  D  V  F   A  P  G
 991  AATGCTATTA CTGTTGCTGC GCTCGACATC AACTGGCGGA CTGCTTCTTT CACCAACTAC GGTGCTGGCG TTGACGTTTT CGCTCCTGGT
       V  N  I   L  S  S  W   I  G  S   N  T  A   T  N  T  I   S  G  T   S  M  A   T  P  H  V   V  G  L
1081  GTTAACATTC TGTCTTCATG GATCGGCTCG AACACGGCCA CTAACACAAT CAGCGGTACC TCCATGGCTA CTCCTCACGT TGTCGGTCTT
       A  L  Y   L  Q  S  L   E  G  L   S  T  P   A  A  V   N  R  I   K  A  L   A  T  S  G   R  V  T
1141  GCTCTCTACC TCCAGTCTCT CGAGGGCCTT TCAACCCCCG CTGCTGTCAC CAACCGCATC AAGGCTTTGG CCACTAGTGG CCGCGTCACC
       G  S  L   N  G  S  P   N  V  I   I  F  N   G  N  S  A  *
1261  GGCAGCCTTA ACGGCAGCCC CAACGTCATC ATTTTCAACG GAAACAGTGC TTAAATTGGC CATATAGTCA ACAATGCCTA GGCTTAAAAT

1321  GCGGAAAGTG TGACACATTA ACTGATTGGT CTATTGTAAA AAAAAAAAAA
```

Figure 3.24: Amino acid sequence alignment of *T. aggressivum* prb1 with closely related sequences *Trichoderma hamatum* (AAP15044), *Trichoderma atroviride* (Q03420), *Hypocrea virens* (AAO63588), *Aspergillus fumigatus* (EAL89613), *Aspergillus oryzae* (CAA35594), *Aspergillus flavus* (AAB29384), *Metarhizium anisopliae* (CAD11898), is using ClustalX program. Conserved residues are shaded in black. Dark grey shading indicates similar residues in seven out of nine of the sequences and clear grey shading indicates similar residues in five out of nine of the sequences. Putative N-terminal signal sequences are indicated by "Signal peptide". The predicted N-glycosylation sites of prb1sequences are indicated by "N-glc". The active site residues are indicated by the gray triangle.

3.2.3- Methodology for *β-1, 3 glucanase* gene recovery

3.2.3.1- Primer design

The same procedures used earlier to design primers for the other genes were used for the *β-1, 3 glucanase* gene. The gene sequences from different *Trichoderma* species (Table 3.3) were aligned and the primers were obtained from their consensus sequence and designated Glu-Nter and Glu-2 (Table 2.1).

Table 3.3: *Trichoderma* spp. used in the *β-1,3glucanase* gene sequence alignment to identify the consensus regions for primer design.

Trichoderma spp.	NCBI Gene bank accession no
Trichoderma asperellum	ABY19519
Hypocrea lixii	CAA05375
Trichoderma hamatum	AAP33112

3.2.3.2- Amplification of the initial gene fragment

The primers were used in a PCR reaction to amplify the initial gene fragment from genomic DNA. Cycling conditions were melting at 95°C for 4 min, heating to 94°C for 45 second, annealing at 60°C for 45 second, extension at 72°C for 1min and finally, the reaction was held at 72°C for 5 min and incubated at 4°C afterwards. The amplification product (850 bp) was visualized by agarose gel electrophoresis as shown in Figure 3.25. The amplified band was excised from the gel and sequenced.
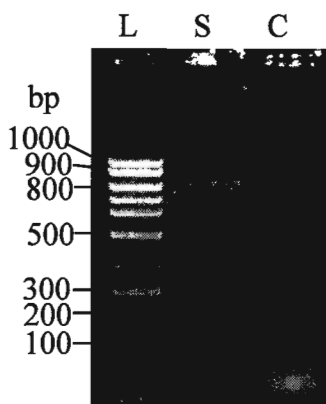
Figure 3.25: PCR amplification of the initial gene fragment of the *T. aggressivum β-1,3 glucanase* gene. (C) Negative control, (S) amplification product and (L) Norgen's PCR sizer DNA ladder. The amplicon size as well as the size of the ladder bands is shown on the figure.

3.2.3.3- Amplification of the full length gene

The sequence of the initial gene fragment was used to design a specific primers set (Glu walker F1, Glu walker R1, Glu walker F2 and Glu walker R2) (Table 2.1) that was used to get the full length gene by the genomic walker library protocol. These primers were used to get the 3' and 5' ends of the gene in two different genomic walker library steps, each by using one primer adjacent to the end to be amplified. The gel pictures of the two PCR amplifications to obtain the 3' end gene fragment (1500 bp) is shown in Figures 3.26 while the gel picture of the two PCR amplifications to obtain the 5' end gene fragment (800 bp) is shown in Figure 3.27. The two amplified gene fragments that flank the initial gene fragment were cleaned and sequenced. Sequence information is given in Appendix D.
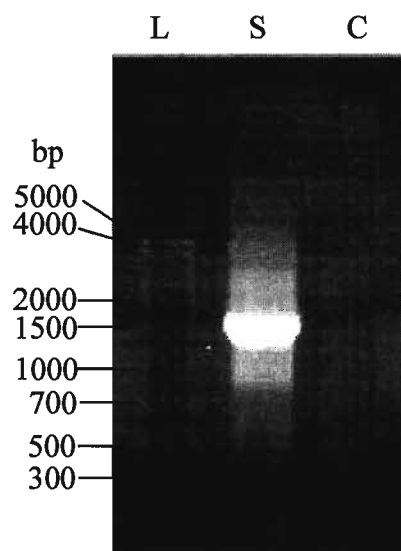
Figure 3.26: Genomic walker PCR amplification of *T. aggressivum β-1,3 glucanase* gene, to complete 3'. (S) Amplification product (C) Negative control (L) Norgen's MidRanger DNA ladder. The amplicon size as well as the size of the ladder bands is shown on the Figure.
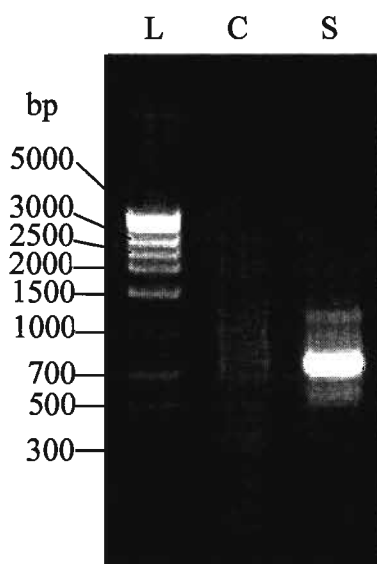
Figure 3.27: Complete 5' *T. aggressivum β-1,3 glucanase* gene, genomic walker PCR amplification. (S) Amplification product (C) Negative control (L) Norgen's MidRanger DNA ladder. The amplicon size as well as the size of the ladder bands is shown on the Figure

3.2.3.4- Isolation of cDNA

The same reverse transcription (RT) reaction product prepared earlier was used to amplify the cDNA of the *β-1,3 glucanase* gene in a PCR reaction using a specific primer Glu (F-Fu) from the 5' end of the gene and oligo(dT). The amplification product was visualised on an agarose gel (Figure 3.28). The amplified band was then excised, cleaned and sequenced.
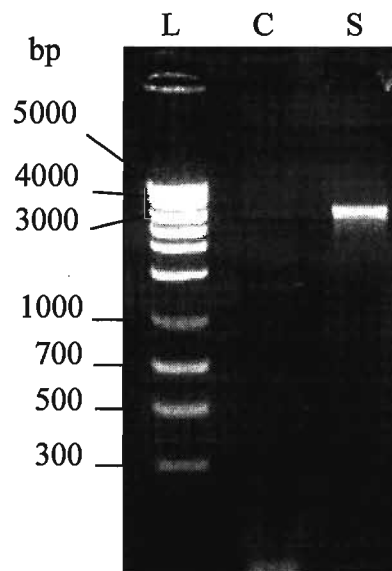
Figure 3.28: *T. aggressivum β-1, 3 glucanase* RT-PCR amplification. (S) Amplification product of ~3000bp, (C) Negative Control and Lane (L) is the MidRanger DNA ladder (Norgen Biotek).

3.2.3.5- Sequence analysis

The complete genomic nucleotide with the deduced amino acid sequences of *β-1,3-glucanase* is shown in Figure 3.29. *β-1, 3-glucanase* genomic sequence was verified as an exoglucanase gene by homology analysis (79% identity and e-value 0.0) with other

fungal *β-1, 3-exoglucanase* genes. The full-length *β-1, 3-glucanase* gene is 3588-bp, and the corresponding cDNA sequence was 3078-bp. The sequence of the genomic DNA coding region agreed perfectly with that of the cDNA. Comparison of the cDNA and genomic sequences showed that the gene is interspersed with 9 introns, which is consistent with several characterized glucanase genes of fungi. Each intron began with GT and ended with AG, a common feature of fungal introns (Gurr *et al.*, 1987). The lariat sequences (CTAAC and CTAAT) characteristic of fungal introns, were also identified (Balance, 1986).

Full-length *β-1, 3-glucanase* cDNA was 3248-bp with a predicted open reading frame (ORF) encoded a protein of 1026 amino acids. The 5'non-coding, 3'non-coding, and poly (A$^+$) sequences were 80, 70 and 20 bp, respectively (Figure 3.30). The predicted ORF showed 86% identity in amino acid sequence to other fungal β-1,3-glucanases. The calculated molecular mass and theoretical p*I*s of Glu native protein were 107.1 kDa and 4.85, respectively. Multiple alignments of Glu with other reported exo-glucanase sequences (Figure 3.31) revealed the identification of many conserved motifs that are common to the fungal glucanase. A signal peptide showed that a cleavage prediction would occur after Gly-20. Mature protein was 1006 amino acids long, which has a calculated molecular mass of 105 kDa and theoretical p*I* 4.77. Comparison of different β-1,3-glucanase sequences from various organisms revealed the presence of several putative asparagine glycosylation sites and phosphorylation sites.

A

β-1,3-glucanase

```
                   at                                                                        tg
DNA  ────────────[▯▦▬▬▬▬▦▬▬▬▦▬▬▬▦▯▬▦▬▬▬▬▬▦▬▬▯▦▯]────────
      (-364)     (1)                                                          (3588)(3788)
```
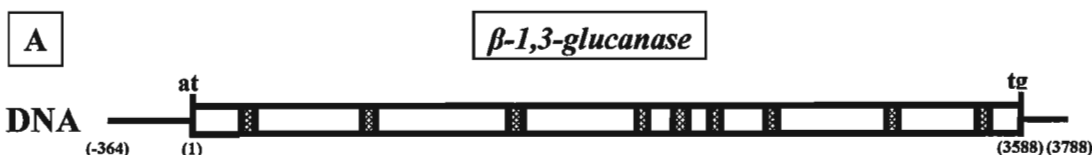
B

```
   1   TATTCTTTAT TGGGACCCTA TTTGTCTAGC TACGAGCAAG GTTACCGGGT AAGGGGTGCC GAGCTATTGC CAGCATAGCT CGTCTTTCGC
  91   AACGCAATCG TGATGACTTT CTCGGTCGTT CTCCATCTCC GGCTCATGGA AAGCTTCCTC GTGGTTAACT AAAAGGCCCT TTTCTGTTGT
 181   CTAACTGAAG CGCGGAGTCC TCAACTCCCT TTAAGCGTAA TTTATCCATG TGCACTTACC GTGTTTCAGT ATTCTTCTTC AAAATGATAT
 271   AAGAGCATGA ACCCCCCCAG GAAGCCTCTT CTGTCACCCT GCCACTTGGC TCATCCATTG TAATTGCGAA GCTGTGCAGA GAGCTCCTGT
               M  G  F  M  R  S  V  V  L  S  A  L  A  L  A  A  T  C  R  G  V  I  V  P  G  A  E  A  E
 361   CATCATGGGC TTCATGCGCT CCGTCGTGCT TTCGGCCCTG GCGCTTGCTG CGACCTGCCG GGGCGTGATT GTCCCTGGGG CCGAGGCTGA
          P  A  L  E  K  R  A  S  S  Y  W  Y  E  N  I  A  K  Q  G  V  A  P  F  A  P  S  G  Y  T  V
 451   ACCCGCTTTG GAAAAGCGTG CTTCGTCTTA TTGGTATGAG AATATTGCCA AACAAGGAGT TGCGCCGTTC GCACCTAGCG GTTACACCGT
          Y  R  N  V  K  D  F  G  A  K  G
 541   ATACCGCAAT GTCAAGGACT TTGGCGCAAA GGGTATATTA TTCTCCCTCA TTTCGTAAGG TTCCTTACAG TTACTGACGT AGAATGCTCA
          D  G  V  T  D  D  T  A  A  I  N  N  A  I  L  S  G  G  R  C  G  R  L  C  K  S  S  T  L
 631   AGGTGATGGT GTTACCGACG ACACGGCAGC AATTAACAAT GCCATCCTGT CAGGGGGTCG ATGTGGCCGA CTCTGCAAGT CCAGCACTTT
          T  P  A  I  V  Y  F  P  A  G  T  Y  L  I  S  T  P  I  I  D  Q  Y  Y  T  N  I  I  G  D  A
 721   GACTCCGGCC ATCGTGTACT TCCCAGCGGG AACATACCTG ATTTCGACTC CCATCATTGA TCAGTACTAT ACCAACATTA TCGGAGATGC
          T  N  L  P  T  I  K  A  T  A  N  F  N  G  I  A  L  I  D  G  D  T  Y  G  D  N  N  P  N
 811   GACCAATCTT CCCACCATAA AAGCGACGGC CAACTTCAAC GGTATTGCTC TCATTGATGG TGATACCTAT TATGGCGACA ACAATCCCAA
          D  P  N  W  I  S  T  N  V  F  Y  R  Q  V  R  N  F  K  L  D  M  T  S  M  P  A  S  A  P  R
 901   TGACCCCAAC TGGATTTCTA CCAACGTCTT CTATCGCCAA GTGCGCAACT TTAAGCTTGA TATGACATCT ATGCCGGCTT CGGCGCCCAG
          T  Y  G  I  H  W  P  T  A  Q  A  T  S  L  Q  N  I  Q  V  T  M  T  T  A  S  G  N  Q  Q  V
 991   GACGTATGGT ATCCATTGGC CGACAGCCCA GGCGACAAGC TTGCAGAACA TCCAGGTCAC AATGACTACT GCGTCTGGAA ATCAACAAGT
          G  L  F  V  E  N                                          G  S  A  G  F
1081   GGGCCTCTTC GTCGAAAACG GTATGTATTA CAGAGACACG GGACTCTTTC ATCTGTTCCT AGCTAATAGC TCCATAGGTT CTGCTGGATT
          L  T  D  L  T  F  N  G  G  L  I  G  A  A  V  G  N  Q  Q  F  T  M  R  N  L  V  F  N  N  C
1171   TCTCACAGAC TTGACTTTCA ACGGTGGCCT GATCGGTGCC GCTGTGGGTA ATCAACAGTT CACGATGCGC AACTTGGTAT TCAACAACTG
          G  T  A  I  V  S  G  F  T  W  E  W  V  Y  Q  G  I  S  I  N  N  C  G  L  G  I  D  I  S  S
1261   TGGGACAGCC ATTGTCAGTG GGTTTACCTG GGAGTGGGTT TACCAGGGCA TCAGCATCAA CAACTGTGGA TTGGGAATTG ACATATCGTC
          A  E  S  I  T  L  I  D  S  S  T  T  G  T  P  V  G  I  R  T  S  Y  S  A  N  Q  S  P  P  T
1351   CGCCGAGTCT ATTACACTGA TTGACAGTTC CACCACCGGG ACGCCAGTCG GAATCAGGAC CAGCTACAGT GCGAACCAGT CGCCCCCCAC
          S  N  S  L  I  I  E  N  L  S  L  N  N  V  P  V  A  V  Q  S  T  G  G  A  T  L  L  A  G  G
1441   ATCCAACAGC CTCATCATTG AGAACCTTTC TCTCAACAAT GTTCCAGTGG CCGTTCAATC GACGGGCGGC GCTACTCTTC TGGCTGGCGG
          T  T  T  I  A  A  W  G  Q  G  H  Q  Y  T  P  N  G  P  T  S  F  Q  G  T  I  A  A  N  A  R
1531   AACTACGACA ATCGCCGCAT GGGGCCAGGG CCACCAGTAT ACTCCAAATG GGCCGACGTC GTTCCAAGGC ACTATCGCAG CAAATGCCCG
          P  S  S  L  L  S  G  S  K  Y  Y  T  R  S  K  P  Q  Y  E  T  L  P  V  S  S  F  K  S  V  R
1621   TCCCTCGTCA CTCTTGAGCG GGTCCAAATA TTACACACGC TCAAAGCCTC AATATGAAAC CCTGCCGGTT TCTTCGTTCA AGAGCGTCCG
          S  A  G  A  T                                          G  N  A  V  T
1711   GTCTGCCGGT GCCACTGGTG AGTACGAGCC CCTGTGATTTG TAGAGATTTG AGTGCTAATC TAGTGTGCTA TTCTAGGTAA CGCGGTGAC
          D  D  T  V  A  L  Q  N  V  I  N  S  A  T  A  G  Q  I  V  Y  F  D  A  G  I  Y  R  I  T
1801   GGACGACACC GTAGCTCTAC AAAACGTCAT CAACAGTGCAA CCGCTGCAGG CCAAATTGT GTACTTCGAT GCTGGCATTT ACCGCATTAC
          S  T  L  K  I  P  P  G  A  K  L  V  G  E  E  Y  P  I  I  L  S  S  G  S  F  F  N  D  Q  N
1891   TAGCACACTCA AGATCCCTCC CGGTGCGAA GCTGGTCGGAG AGGAGTATCC CATCATCTT GTCTTCTGGA AGTTTCTTCA ATGACCAGAA
          N  P  K  P  V  V  Q  V  G  T  P  G  Q  T  G  Q  V  E  W  S  D  M  I  V  A  T  Q  G  T  Q
1981   CAATCCCAAGC CTGTTGTTCA AGTGGGTAC ACCTGGCCAGA CTGGGCAGGT GGAGTGGTC AGACATGATT GTTGCCACTC AAGGCACACA
```

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          A  G  A   V  L  I   E  W  N  L   A  T  S   G  T  P   S  G  M  W   D  V  H   T  R  I   G  G  F  K
2071  AGCTGGAGCT GTTCTTATCG AGTGGAATTT GGCTACTTCT GGAACTCCAA GTGGTATGTG GGATGTACAT ACCAGAATTG GTGGCTTCAA
          G  S  N   L  Q  L   A  Q  C  P   V  T  A   G  S  S   A  V  N  T   A  C  I   G  A  Y   M  S  M  H
2161  GGGGTCAAAC CTCCAGCTTG CACAATGCCC GGTCACTGCG GGCAGCTCGG CTGTCAACAC AGCTTGTATT GGCGCCTACA TGTCCATGCA
          I  T  S   G  A  S   N  L  Y  L   E  N                                                  N  W
2251  CATCACGTCT GGCGCAAGCA ACCTGTACTT GGAAAATGTG AGTCAATCTA GCATGGAAAC TTGGCCTACT AGCTAACTTG GATAGAACTG
          F  W  T   A  D  H   D  I  E  D   S  S  N   S  Q  I   T  V  F  S   G  R  G   L  Y  V   E  S  T  A
2341  GTTCTGGACG GCAGACCACG ACATTGAGGA CTCAAGCAAC TCACAAATCA CCGTCTTCAG CGGCAGAGGC TTGTATGTGG AGAGCACTGC
          G  T  F   W  L
2431  AGGAACGTTC TGGTTGTATG TGACCTCTTC TATCCGAGTT CCGAATTTTT TTCTTCTATT TTGCTTTGAG CTTTATGCTG ACACACTCTT
              V  G   T  A  V   E  H  H   V  L  Y  Q   Y  Q  F   A  N  T   Q  N  I  Y   A  G  V   I  Q  T
2521  CTAGTGTCGG AACCGCTGTT GAGCACCATG TTCTCTATCA GTATCAGTTT GCCAATACCC AAAACATCTA TGCAGGAGTT ATTCAGACAG
          E  T  P                                      Y   Y  Q  P   N  P  L  A   P  T  P
2611  AGACACCGTA GGTATATCAA TGCCCCTCAT TCGCTCCCGG CTAATAAATC CGACCAGGTA CTACCAACCA AATCCCCTCG CACCAACCCC
          F  T  V   N  T  A   I  N  D  P   D  F  A   A  S  C   A  G  K  S   G  R  C   A  E  A   W  G  L  R
2701  TTTCACCGTC AATACTGCTA TAAATGACCC CGACTTTGCA GCTTCCTGCG CCGGCAAATC TGGTCGATGC GCTGAGGCTT GGGGCCTGAG
          V  L  S   S  K  N   V  L  I  Y   A  A  G   L  Y  S   F  F  E  N   N  D  G   K  G
2791  AGTCCTTAGC TCCAAGAATG TTCTCATCTA TGCTGCTGGC TTATATTCTT TCTTCGAAAA CAACGACGGA AAAGGTAAAC CTGCTTTTGT
                 G   C  N  N  G   V  E  P   Q  N  C   Q  N  N  I   V  D  L
2881  TTTGTCCCTT GTATAAGAAT AAGCTAACGT GTAAAGGCT GTAACAACGG TGTAGAGCCG CAAAATTGCC AAAATAACAT CGTCGACCTC
          E  G  T   L  T  N  V   N  I  Y   N  L  G   T  V  G  V   V  N  S   I  V  E   N  G  N  V   L  A  A
2971  GAAGGCACGC TGACCAACGT TAACATCTAC AACTTGGGGA CAGTTGGTGT CGTCAATTCG ATTGTGGAGA ATGGAAATGT GCTGGCAGCT
          S  S  D   N  V  N  A   F  A  D   V  I  A   L  F  R  L   A  S  G   S  G  G   T  T  P  P   P  P  S
3061  TCTTCTGACA ATGTCAACGC CTTCGCTGAT GTTATAGCTC TGTTCCGACT TGCTTCTGGC AGTGGAGGCA CCACCCCCCC TCCTCCTAGC
          S  T  T   K  T  G  P   T  T  M   S  T  I   T  T  S  S   P  P  K   Q  T  G   W  N  F   L  G  C
3151  TCCACAACAA AGACAGGCCC GACAACAATG TCAACCATAA CCACTAGCTC GTCACCGCCG AAGCAAACTG GATGGAATTT CCTGGGATGC
          Y  S  D   N  V  N  G   R  T  L   A  N  Q   V  Q  V  P   G  G  A   S  A  M   S  I  E  A   C  E  T
3241  TACTCTGATA ATGTTAACGG TCGAACTCTG GCCAACCAGG TCCAAGTCCC AGGAGGTGCA TCAGCCATGT CGATCGAGGC GTGTGAGACG
          A  C  K   S  A  G  Y   T  I  A   G  L  E   Y  S  G  E   C
3331  GCATGCAAAT CCGCCGGATA TACTATTGCA GGTCTCGAAT ATTCGGGAGA ATGCTGTAAG TATTATAACG TGGACAACAA CACTCCGAAT
                 G   C  D  N   K  F  G   N  G  G  G   P  A  S   D  G  S  A   Q  C  T   M  T  C
3421  AGCACTAATG GTACCTAGGG TGCGATAACA AGTTCGGAAA CGGAGGCGGC CCTGCTTCTG ACGGAAGCGC TCAATGCACC ATGACCTGTA
          S  G  A  P   Q  E  T   C  G  G   P  N  R  L   D  V  Y   A  V  A   T  A  T  G   A  P  P   P  A  S
3511  GCGGTGCGCC ACAGGAAACC TGTGTGAGGCC CAAATCGCCT TGACGTGTAC GCAGTGGCGA CCGCAACTGG AGCTCCACCT CCCGCGTCCA
          T  G  W  T   L  K  G   C  Y  T   D  S  V  N   A  R  A   L  I  A   E  G  V  P   N  G  P   S  S  M
3601  CCGGATGGAC CTTGAAAGGA TGCTATACGG ATAGCGTCAA CGCTCGAGCA TTGATTGCTG AGGGTGTTCC CAACGGGCCA TCGTCCATGA
          T  V  E  A   C  Q  S   V  C  K   S  L  G  Y   I  L  A   G  V  E   F  G  D  E   C  C  K
3691  CTGTTGAGGC ATGTCAAAGT GTCTGCAAAA GCTTAGGCTA TATTCTTGCA GGCGTCGAAT TTGGCGACGA GTGCTGTAAG TCTTCTCCCA
                 G  C  N   S  L  A  N   G  A  T   I  A  P   D  G  N  A
3781  GTTCGTGATT CATTGGTTCT GGCTCTAACA AATTTCAGAT GTGGTAACA GCCTCGCCAA TGGTGCTACC ATCGCTCCAG ACGGAAACGC
          G  C  N   I  N  C   A  G  N  A   A  E  T   C  G  G   S  N  R  L   N  L  Y   S  Y  G   S
3871  TGGGTGCAAC ATAAAATTGTG CTGGCAACGC TGCTGAAACT TGTGGTGGCT CCAACAGACT GAACCTGTAT AGCTATGGCT CTTGATGTAT
```

Figure 3.29: Sequence of *T. aggressivum β-1,3-glucanase* gene. (A) Structural image of exon and intron positions in the putative *prb*1 gene. The unshaded portions represent exons. Introns are hash shaded. The letters (atg) and (taa) indicate the positions of the start and stop codon, respectively. (B) Nucleotide sequence of the *T. aggressivum* β-1,3-glucanase gene. The deduced amino acid sequence is shown in one-letter code above the DNA sequence. Nucleotide coordinates are shown to the left of the sequence and the introns are shown in italic lowercase letters.
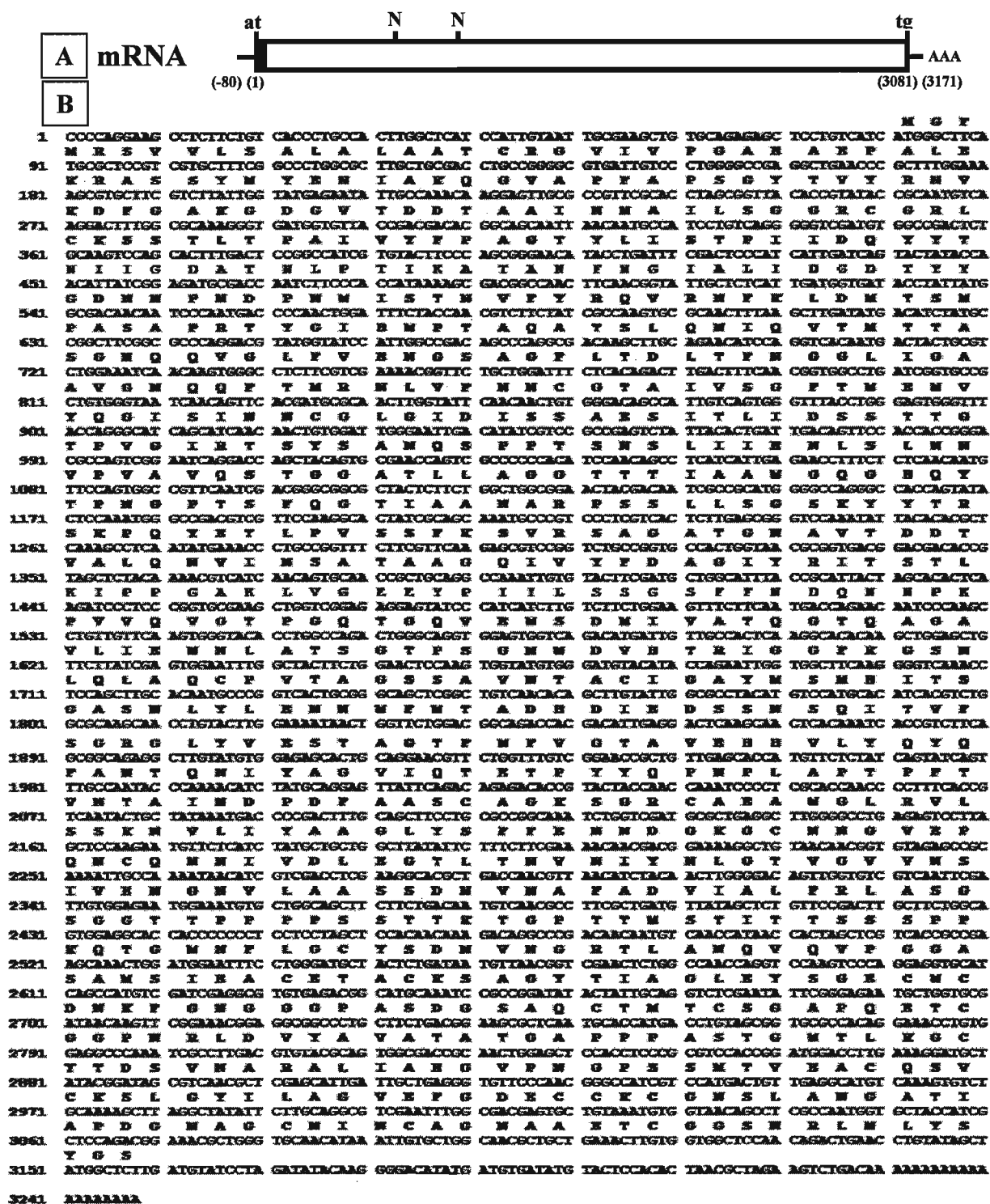
Figure 3.30: Sequence of *T. aggressivum β-1,3-glucanase* cDNA. (A) mRNA Model shows the possible signal sequence cleavage point is represented by the dark fragment at the 5' end of the mRNA. Potential N-glycosylation sites are indicated by the letter N and (B) cDNA nucleotide sequence and deduced amino acid sequence.

Signal peptide

T.asp : ... : 103
T.har : ... : 103
T.ham : ... : 103
T.agg : ... : 103
N.fis : ... : 136
A.fum : ... : 136
B.gra : ... : 103

T.asp : ... : 244
T.har : ... : 244
T.ham : ... : 244
T.agg : ... : 244
N.fis : ... : 273
A.fum : ... : 272
B.gra : ... : 233

T.asp : ... : 373
T.har : ... : 373
T.ham : ... : 373
T.agg : ... : 373
N.fis : ... : 408
A.fum : ... : 409
B.gra : ... : 370

T.asp : ... : 520
T.har : ... : 520
T.ham : ... : 520
T.agg : ... : 520
N.fis : ... : 555
A.fum : ... : 557
B.gra : ... : 517

T.asp	:		:	659
T.har	:		:	659
T.ham	:		:	659
T.agg	:		:	659
N.fis	:		:	668
A.fun	:		:	701
B.gra	:		:	656

T.asp	:		:	805
T.har	:		:	805
T.ham	:		:	805
T.agg	:		:	806
N.fis	:		:	804
A.fun	:		:	822
B.gra	:		:	767

T.asp	:	IITII-PPKQTGGNFLGCYIIHVNGRTLAHQVQVAGGAIAHIIEACETACESAGYIIAGVEISGEIGRDTKFQNGGGPASDGIAQCTHICIGAPQETCGGPNRLDVYILATATGIAIPPAATGGHFBGCYTDSVHARALIAESVPHGPSS	:	954
T.har	:	IITII-PPKQTGGNFLGCYIIHVNGRTLAHQVQVAGGAIAHIIEACETAIESAGYIIAGVEISGEIGRDTKFQNGGGPASDGIAQCTHICIGAPQETCGGPNRLDVYILATATGIAIPPAATGGHFBGCYTDSVHARALIAESVPHGPSS	:	954
T.ham	:	ITTIP-PPNQ	:	814
T.agg	:	ITTIIIPPKQTGGNFLGCYIDHVNGRTLAHQVQVPGGAIAHIIEACETAKHSAGYIIAALEYIGEIGRDHKFGNGGGPASDGIAQCTHICIGAPQETCGGPNRLDVYAVATATG-APPPAITGGILKGCYTDSVHARALIAESVPHGPSS	:	955
N.fis	:		:	-
A.fun	:		:	-
B.gra	:		:	-

T.asp	:	NTIEACQSVCKGLGYTLAGLEYAECCY-CGHILAHGATIAPDGHAGCHHHCAGHAAEYCGGPHHLDIYSYGQANGTQP-	:	1031
T.har	:	NTIEACQSVCKGLGYTLAGLEYAECCY-CGHILAHGATIAPDGHAGCHHHCAGHAAEYCGGPHHLDIYSYGQANGTQPL	:	1032
T.ham	:		:	-
T.agg	:	NTVEACQSVCKIGLGYILAGVHYGIECCKCGHILAHGATIAPDGHAGCHIHCAGHAAEYCGGBHLHLYSYGI-------	:	1027
N.fis	:		:	-
A.fun	:		:	-
B.gra	:		:	-

Figure 3.31: The predicted amino acid sequence alignment of *T. aggressivum* β-1,3 glucanase with closely related sequences *Trichoderma asperellum* (ABY19519), *Trichoderma harzianum* (CAA05375), *Trichoderma hamatum* (AAP33112), *Neosartorya fischeri* (XP_001258465), *Blumeria graminis* (AAL26904),„ is using ClustalX program. Conserved residues are shaded in black. Dark grey shading indicates similar residues in six out of seven of the sequences and clear grey shading indicates similar residues in four out of seven of the sequences. Putative N-terminal signal sequences are indicated by "Signal peptide". The predicted N-glycosylation sites of β-1,3 glucanase sequences are indicated by dotted line.

3.2.4- Phylogenetic analysis

The phylogenetic tree of *T. aggressivum* ech42 is shown in Figure 3.32. The phylogenetic of chitinases from various organisms was constructed using Clustal X multiple sequence alignment programs version 2.0 (Page, 2002). Clade I was further divided into three subclades: chitinases from mycoparasitic fungi (*Hypocrea* spp., *Trichoderma* spp.), including *ech*42, were clustered and formed the subclade Ia, and chitinases from different entomopathogenic fungi were grouped and formed the subclade Ib and Ic.

Phylogenetic analysis of *T. aggressivum* prb1 sequence with different proteins homologues as shown in Figure 3.33 from various organisms revealed that *T. aggressivum* prb1 belongs to S8 family-serine peptidase gene *prb*1 involved in mycoparasitism. Members of this family were initially cloned and characterized from *T. atroviride* (formerly *T. harzianum*) (Geremia *et al.* 1993), and *T. virens* (*tvsp*1) (Pozo *et al.* 2004).

The internal transcribed spacer (ITS) sequence from *T. aggressivum* was used. The sequence was aligned against the corresponding sequences from different *Trichoderma* species. The phylogenetic analysis of ITS (Figure 3.34) shows that *T. aggressivum* belongs to the same clade of the other aggressive *Trichoderma* species. According to the phylogenetic analysis of both *ech*42 and *prb*1 genes, *T. aggressivum* was found to be related to the same clade of the other aggressive *Trichoderma* species.
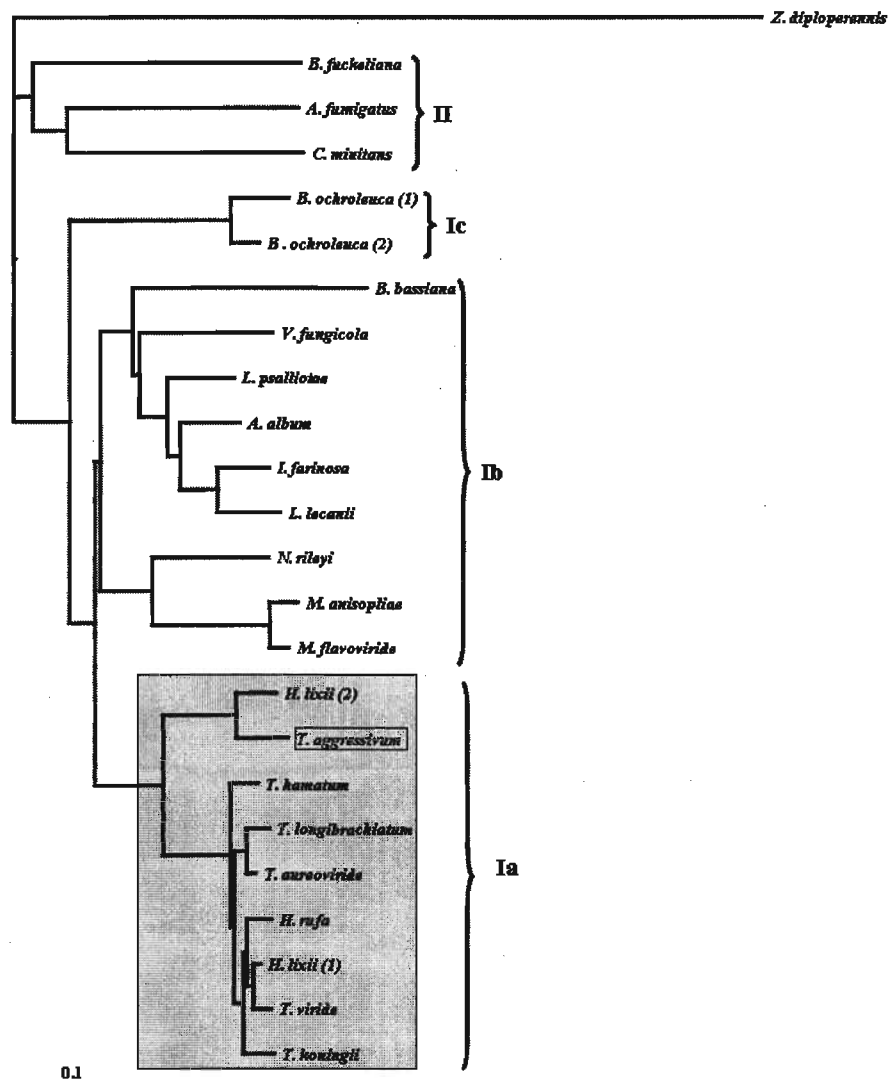
Figure 3.32: Phylogenetic tree showing the relationship between *ech*42 gene from *T. aggressivum* and other fungal spp., *Botryotinia fuckeliana* (gi|22255889), *Aspergillus fumigatus* (gi|30385694), *Coniothyrium minitans* (gi|9837268), *Bionectria ochroleuca* (gi|108744334), *Bionectria ochroleuca* (gi|157488917), *Lecanicillium psalliotae* (gi|148283178), *Aphanocladium album* (gi|1345774), *Verticillium fungicola* (gi|31414751), *Beauveria bassiana* (gi|24209902), *Isaria farinose* (gi|89242496), *Lecanicillium lecanii* (gi|56567522), *Nomuraea rileyi* (gi|37956598), *Metarhizium anisopliae* (gi|63098857), *Metarhizium flavoviride* (gi|5042254), *Hypocrea lixii* 2 (gi|1345775), *Trichoderma hamatum* (gi|2738109), *Trichoderma longibrachiatum* (gi|88191681), *Trichoderma asperellum* (gi|6630958), *Trichoderma aureoviride* (gi|56967602), *Hypocrea lixii* (gi|499085), Trichoderma *viride* (gi|6630948), *Hypocrea rufa* (gi|6630944), and *Trichoderma koningii* (gi|6630936). *Zea diploperennis* (gi|48093350) plant species is used outgroup. *T. aggressivum* is present in the same clade (Ia) as other parasitic *Trichoderma* spp. The tree was constructed using the Clustal X multiple sequence alignment programs version 2.0.
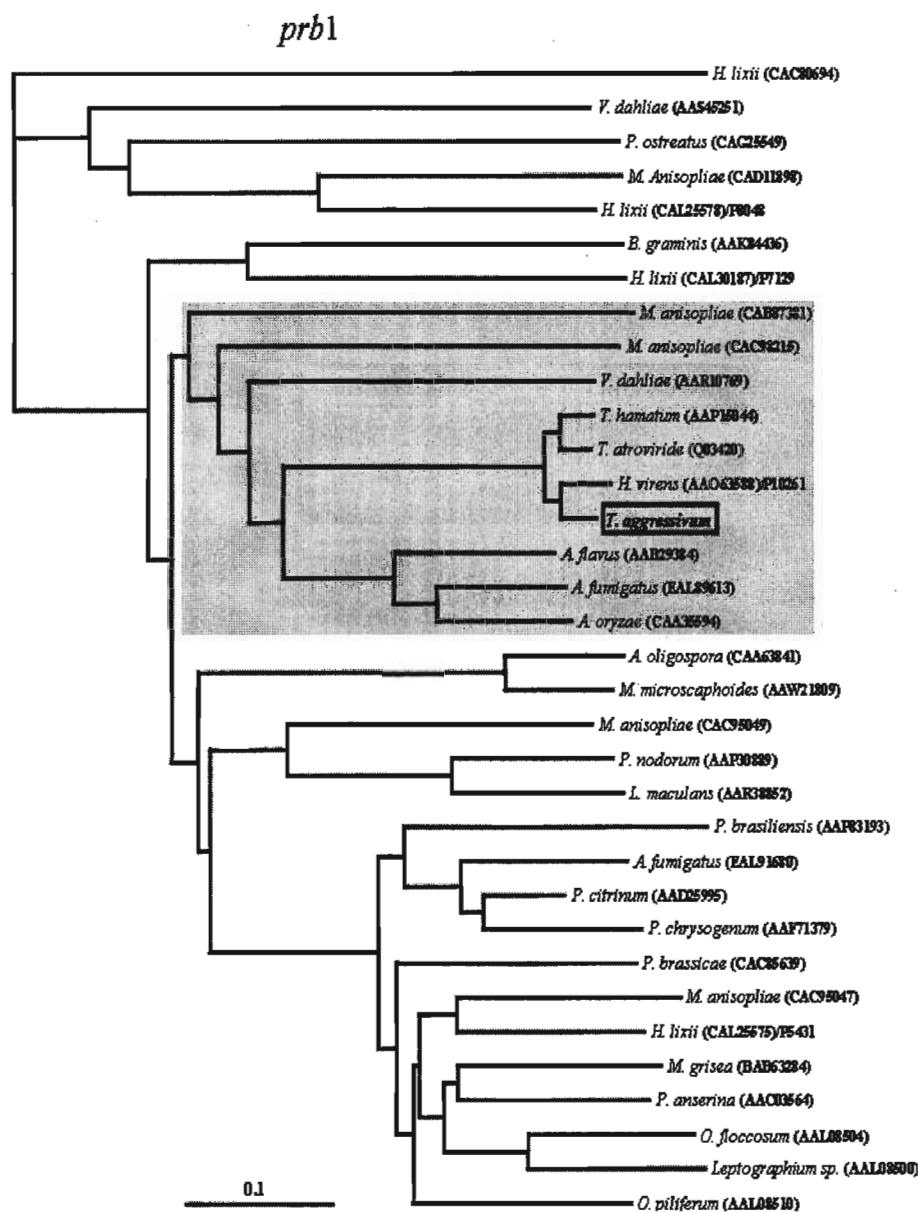
107

*prb*1

Figure: 3.33: Phylogenetic tree showing the relationship between *Prb1* gene from *T. aggressivum* and other fungal spp. *Metarhizium anisopliae* (CAD11898), *Hypocrea lixii* (CAL25578), *Pleurotus ostreatus* (CAG25549), *Verticillium dahliae* (AAS45251),*Trichoderma hamatum* (AAP15044), *Trichoderma atroviride* (Q03420), *Hypocrea virens* (AAO63588), *Verticillium dahliae* (AAR10769), *Aspergillus fumigatus* (EAL89613), *Aspergillus oryzae* (CAA35594), *Aspergillus flavus* (AAB29384), *Metarhizium anisopliae* (CAC98215), *Metarhizium anisopliae* (CAB87381), *Penicillium citrinum* (AAD25995), *Penicillium chrysogenum* (AAF71379), *Aspergillus fumigatus* (EAL91680), *Paracoccidioides brasiliensis* (AAP83193), *Hypocrea lixii* (CAL25575), *Magnaporthe grisea* (BAB63284), *Podospora anserine* (AAC03564), *Ophiostoma floccosum* (AAL08504), *Ophiostoma piliferum* (AAL08510), *Pyrenopeziza brassicae* (CAC85639), *Phaeosphaeria nodorum* (AAP30889), *Leptosphaeria maculans* (AAR38852), *Blumeria graminis* (AAK84436), *Arthrobotrys oligospora* (CAA63841), *Monacrosporium microscaphoides* (AAW21809), *Hypocrea lixii* (CAL30187), and *Hypocrea lixii* (CAC80694) The tree was constructed using the Clustal X multiple sequence alignment programs version 2.
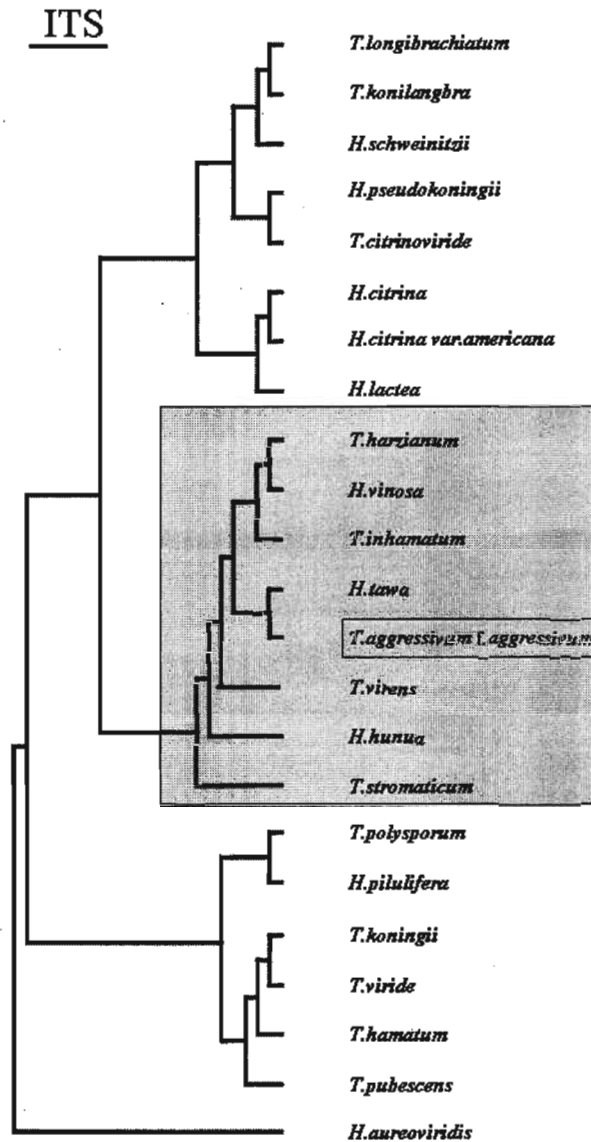
108

ITS

Figure 3.34: Phylogenetic relationships of internal transcribed spacer (ITS) sequences of ribosomal DNA among species of *Trichoderma*. *T. harzianum* (gi|22086343), *H. schweinitzii* (gi|1770505), *T. citrinoviride* (gi|1771797), *T. longibrachiatum* (gi|1518414), *T. pseudokoningii* (gi|1518421), *T. konilangbra* (gi|22086333), *H. aureoviridis* (gi|1770308), *H. citrine* (gi|22086319), *H. lacteal* (gi|22086317), *T. stromaticum* (gi|5230779), *T. longipile* (gi|22086251), *H. vinosa* (gi|6179953), *T. inhamatum* (gi|1771819), *H. tawa* (gi|22086327), *T. virens* (gi|7682674), *H. hunua* (gi|22086325), *T. oblongisporum* (gi|4102512), *T. polysporum* (gi|1771843), *H. pilulifera* (gi|1770344), *T. pubescens* (gi|4102515), *T. hamatum* (gi|1771822), *T. viride* (gi|5327280), and *T. koningii* (gi|2765665). The tree was constructed using the Clustal X multiple sequence alignment programs version 2.0.

3.3- GENE EXPRESSION PROFILING OF CELL DEGRADING ENZYMES

The gene expression profile of the three enzyme genes (*ech*42, *prb*1 and *β-1,3 glucanase*) of *T. aggressivum* was investigated during the co-cultivation of this fungus and *A. bisporus*. A primary step was the characterization of the basic expression levels of the three genes of *T. aggressivum* in both malt extract broth (MEB) (*T. aggressivum* medium) and the *Schizophyllum* complete yeast medium (SCM) medium (*A. bisporus* medium).

3.3.1- Effect of media on gene expression

Different media are used commonly in the laboratory to grow the two organisms and to study the interaction. Therefore, the levels of expression of these three genes were investigated in *T. aggressivum* grown solitary on both media to assess the contribution of the medium environment on gene regulation. Both the MEB and the SCM media were inoculated with *T. aggressivum* spores and grown for 7 days with collecting samples from both media every 24 h.

Total RNA was isolated from each sample and the DNA background was digested in all the samples by using DNase I. After cleaning the RNA samples from the digestion reaction components, the samples were quantified by the spectrophotometer and then equal amounts from each sample were used in a reverse transcription reaction. After that, the cDNA was used in a qPCR reaction in triplicate, to identify the expression levels of the different hydrolytic enzymes genes by using ED-WKF and ED-WKR specific primers for *ech*42, *prb*1 by using Prb-WKF and Prb-WKR primers, *β-1, 3 glucanase* by using BGlu-

WKF and BGlu-WKR primers as shown in (Table 2.1). A house keeping transcript for β-tublin was amplified with primers BTub-WKF and BTub-WKR (Table 2.1) and was used to normalize the Ct values from the hydrolytic enzyme genes. The value of the transcription was calculated using the $2^{\Delta\Delta Ct}$ formula. The transcription levels of the three genes dropped significantly when the fungus was grown on the SCM medium when compared to the normal *T. aggressivum* growth medium (MEB) Figures 3.35, 3.36 and 3.37).
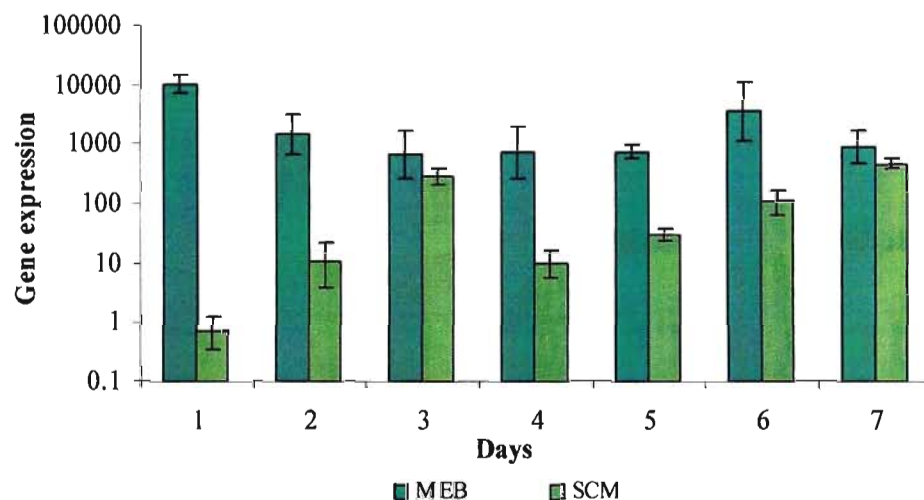

Figure 3.35: The transcription level of *T. aggressivum ech*42 gene over 7days growth on MEB and SCM medium.
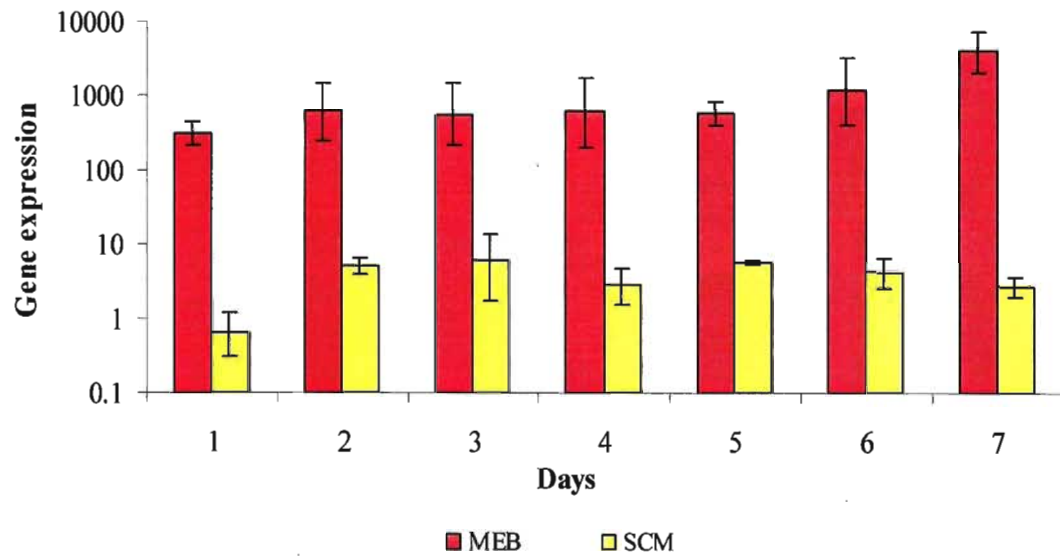
Figure 3.36: The transcription level of *T. aggressivum β-1,3-gucanase* gene over 7days growth on MEB and SCM medium.
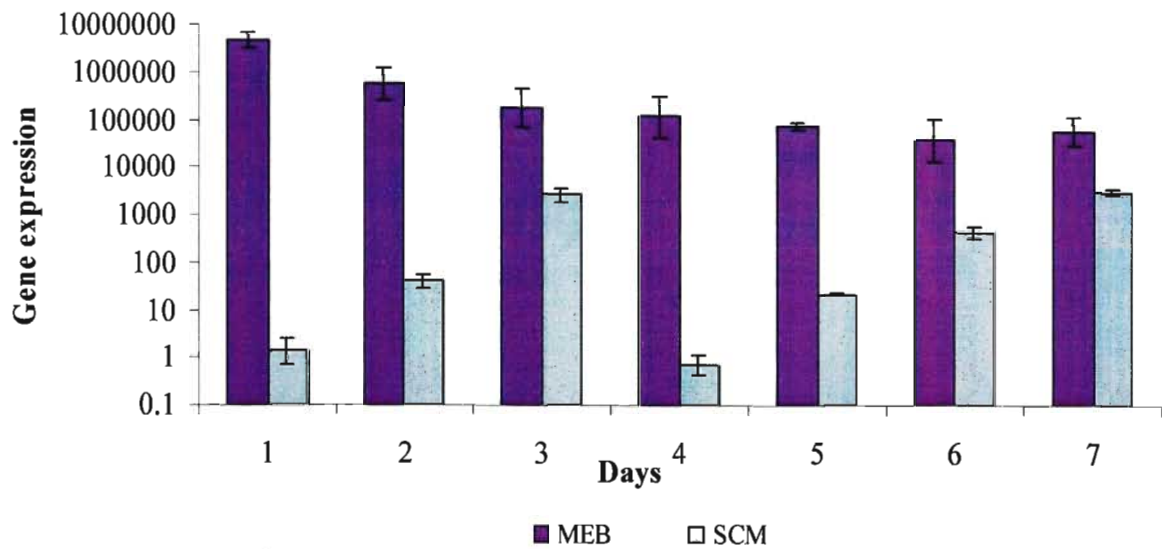

Figure 3.37: The transcription level of *T. aggressivum prb1* gene over 7days growth on MEB and SCM medium.

3.3.2- Gene expression during co-cultivation of *T. aggressivum* and *A. bisporus*

The study of the gene expression levels of the three hydrolytic enzymes of *T. aggressivum* will contribute to an understanding of their involvement and roles in the green mould disease development. Comparing the expression levels of the three hydrolytic enzymes in the solitary culture and the dual culture with *A. bisporus* is a useful approach to investigate the behaviour of these enzymes during green mould disease development. Two strains of *A. bisporus* were used, the white U1 strain and the brown SB65 strain. Both strains were grown on SCM medium and after that were transferred to MEB medium. Tests included *T. aggressivum* alone, *T. aggressivum* with U1 and *T. aggressivum* with SB65. Mycelia from the liquid cultures were harvested daily over a seven day period. This time frame was chosen because Guthrie and Castle (2006) showed that chitinase activities had stabilized by day seven in co-cultivation tests.

The isolated mycelia samples were used to isolate total RNA which was then treated with DNase I to get rid of residual DNA. After that, the RNA samples were cleaned by RNA clean-up and concentration Kit (Norgen Biotek) and quantified by the spectrophotometer. Equal amounts from each sample were used in a reverse transcription reaction and the cDNA was used in qPCR reactions run in triplicate to identify the expression levels of the three different genes. β-tubulin was used to normalize the obtained Ct values from the hydrolytic enzyme genes. The value of the expression was calculated using the $2^{\Delta\Delta Ct}$ formula.

Transcription of the *ech*42 and *prb*1 genes were down-regulated from days one to four and significantly up-regulated on days five to seven during the co-cultivation with either of the two *A. bisporus* strains. However, the increase was much more pronounced

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with the U1 strain than the SB65 strain (Figures 3.38 and 3.39). On the other hand, transcription of the *β-1, 3 glucanase* gene was upregulated but variable over the entire co-cultivation period. Similar to the previous observation with the two other genes, the increase was more pronounced in the U1 strain than the SB65 strain (Figure 3.40).
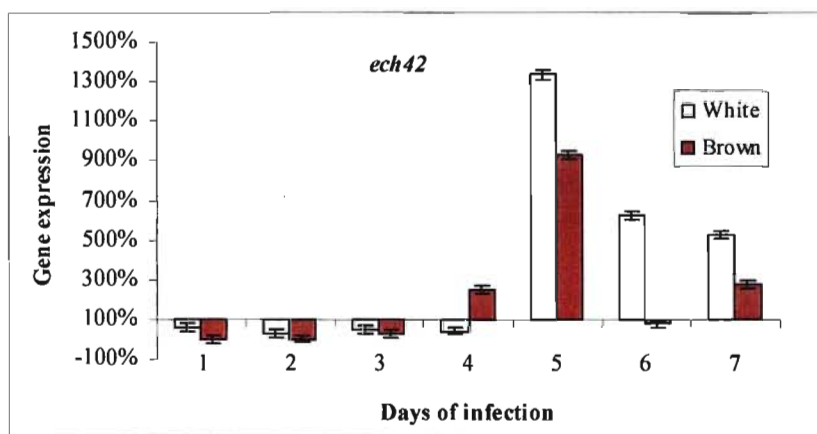
Figure 3.38: The expression level of *T. aggressivum ech42* gene over 7 days during co-cultivation with *A. bisporus* U1 and SB65 on MEB medium. The percentage change in gene expression was calculated by using the $2^{\Delta\Delta Ct}$. The gene expression in pure cultures of *T. aggressivum* or each of the test days was considered the baseline (100%).
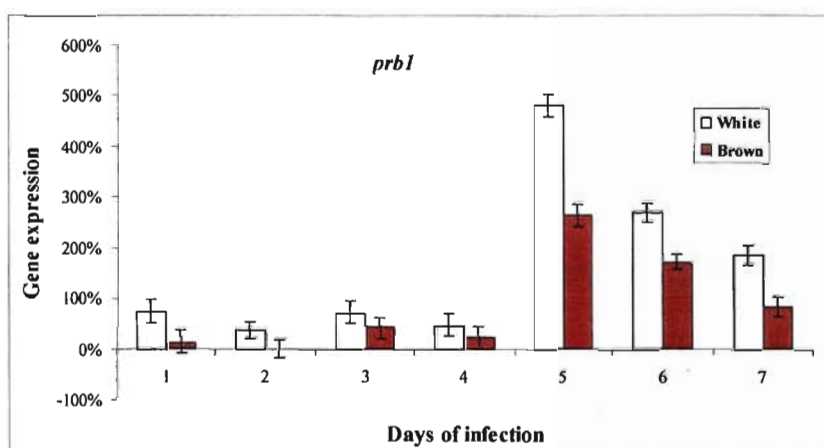
Figure 3.39: The expression level of *T. aggressivum prb1* gene over 7 days during co-cultivation with *A. bisporus* U1 and SB65 on MEB medium. The percentage change in gene expression was calculated by using the $2^{\Delta\Delta Ct}$. Gene expression in pure cultures of *T. aggressivum* or each of the test days was considered the baseline (100%).
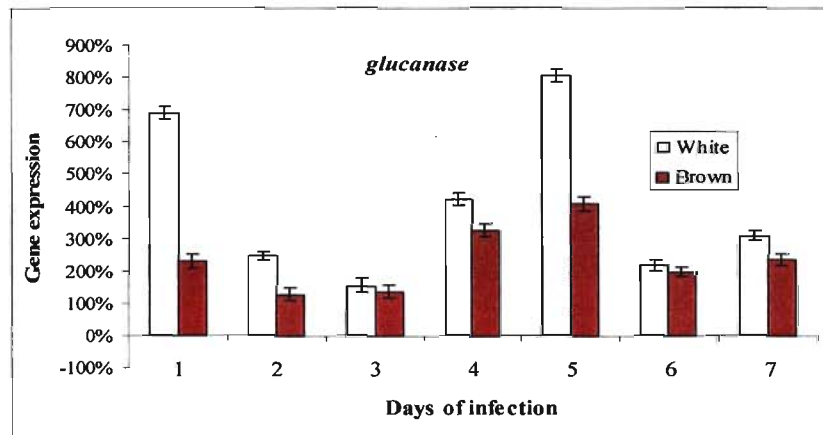
Figure 3.40: The expression level of *T. aggressivum β-1,3glucanase* gene over 7 days during co-cultivation with *A. bisporus* U1 and SB65 on MEB medium. The percentage change in gene expression was calculated by using the $2^{\Delta\Delta Ct}$. The gene expression in pure cultures of *T. aggressivum* or each of the test days was considered the baseline (100%).

3.4- Cell Wall Degrading Enzyme Genes Knock-Down

RNA interference (RNAi) is a powerful tool to search for gene function in various organisms. Researchers report the potency of this method and how it can suppress genes easily and quickly (Stipp *et al.*, 2003). RNA silencing using short interfering RNA (siRNA) or microRNA (miRNA) technology is a promising method for the study of filamentous fungi (Nakayashiki *et al.*, 2005). The process of RNA silencing involves many players such as Dicer and RNA-induced silencing complex (RISC), which are necessary to generate RNAi to silence a target gene (Figure 3.41). RNAi regulates gene expression either by inhibiting translation or by cleaving the messenger RNA (mRNA) (Tijsterman and Plasterk, 2004).

A common method of using RNAi is to generate short hairpin RNA (shRNA) and is performed using shRNA expression vectors (Wesley *et al.*, 2001 and Kim *et al.*,

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2006). Target gene silencing by shRNA has been reported in the ascomycete, *Magnaporthe oryzae* (Nakayashik *et al.*, 2005). Similarly, expression of select shRNAs reduced mRNA levels of target genes by at least 90% in the basidiomycete, *Coprinopsis cinerea* (Walti *et al.*, 2006). The target sequence which is synthesised as sense and anti-sense strands, which are complementary to each other and are separated by a spacer sequencer that creates a hairpin loop (Kim *et al.*, 2006).

In the present study, the expression vector, pSilent was used to generate a series of shRNA constructs to facilitate silencing of the three cell wall degrading genes of *T. aggressivum*. An *Agrobacterium* transformation system was used to introduce three constructs into *T. aggressivum*.
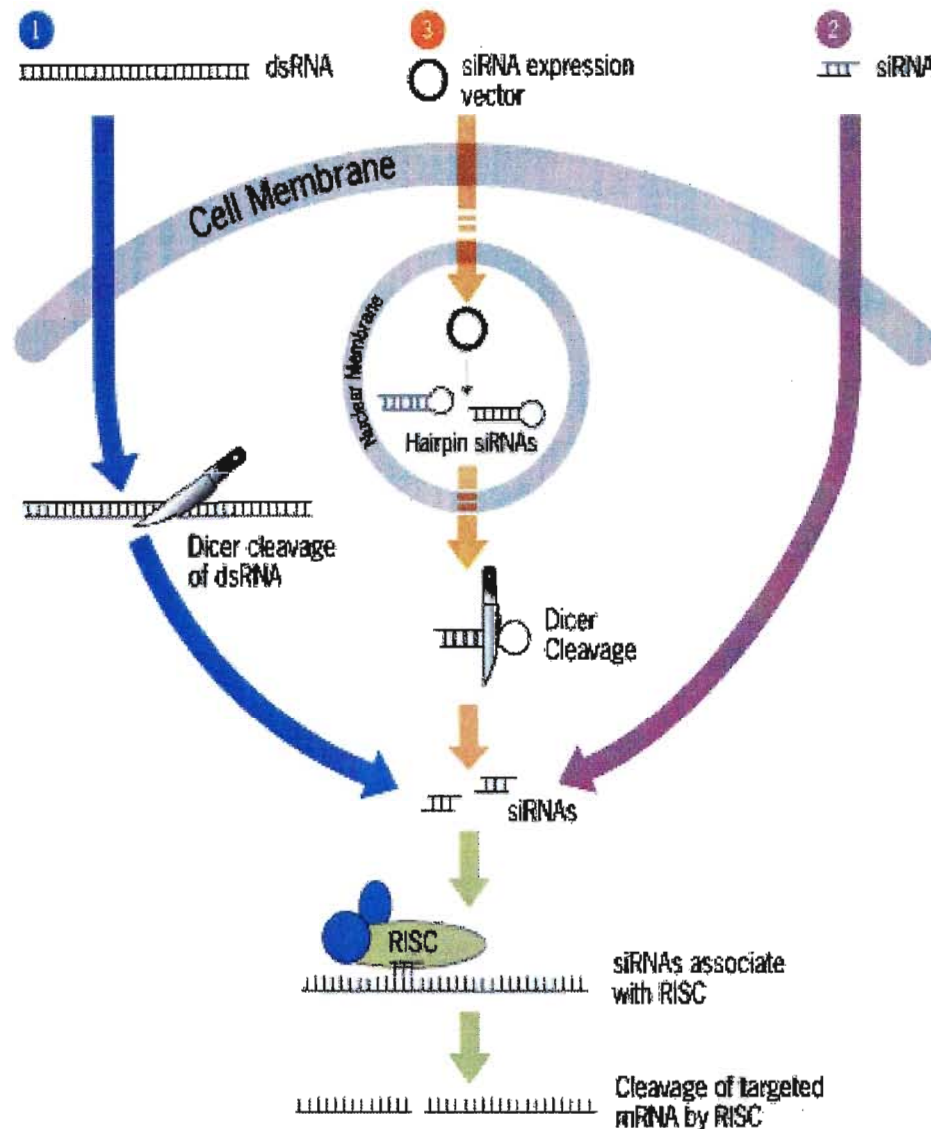
Figure 3.41: The RNA interference pathway. Long double-stranded RNA (dsRNA) or small hairpin RNA (shRNA) is processed by Dicer to form a small interfering RNA (siRNA), which associates with RNA-induced silencing protein complex (RISC) and mediates target sequence specificity for subsequent mRNA cleavage. (http://www.ambion.com /techlib/resources/RNAi/ overview/gfx/fig1.3.gif).

3.4.1- Development of an *Agrobacterium* transformation system for *T. aggressivum*

T. aggressivum had not been transformed prior to this work, so the first step was to develop a transformation system for this fungus. Transformation of the *T. aggressivum* T586 strain was performed using the *Agrobacterium tumefaciens*-mediated transformation system that has been described for filamentous fungi and *T. harzianum* (De Groot *et al.*, 1998; Yang *et al.*, 2007). *A. tumefaciens* is a plant-pathogenic soil bacterium which causes crown gall disease in plant tissues. This technique represents a relatively efficient method of transformation, with approximately 600-fold greater transformation frequency when compared to other techniques (De Groot *et al.*, 1998). Thus, the *Agrobacterium* binary vector allows for a stable and efficient transformation of filamentous fungi with the desired DNA sequence.

The binary vector pFBENGFP was used in the transformation experiment. The T-DNA includes a gene for resistance to (2-methoxycarbonylamino) benzimidazole, commonly known as benomyl (Seip *et al.*, 1990, Zeilinger *et al.*, 2004) that was tested for use as a selectable marker for *T. aggressivum*. The *benA3* allele of *Aspergillus nidulans,* confers resistance to benomyl (Goettel *et al.*, 1990), and the *hph* gene of *E. coli* confers resistance to hygromycin (Punt *et al.*, 1987). The *hph* gene was used a selectable marker in later transformation trials. Figure 3.42 shows the binary vector map.
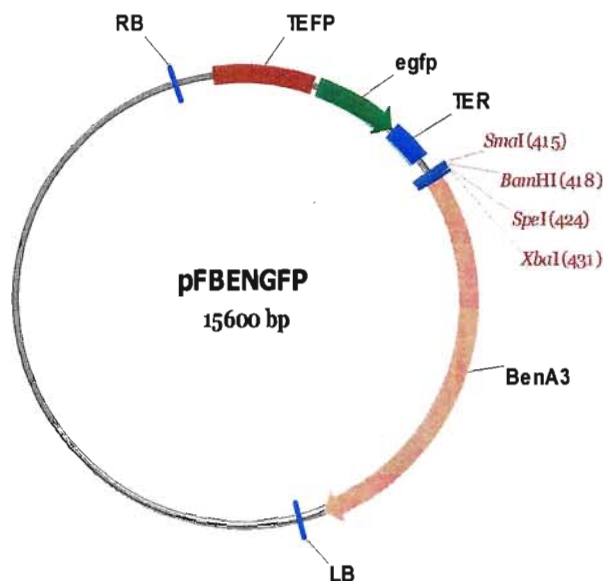
Figure 3.42: The *Agrobacterium* Binary vector (pFBENGFP) map. TEFP, promoter of translation elongation factor gene from *Aureobasidium pullulans*; eGFP, enhanced green fluorescent protein gene; TER, termination region of glucoamylase gene from *Aspergillus nidulans*; BENA3, benomyl resistance gene; RB and LB are right and left border of T-DNA, respectively (Fang *et al.,* 2006)

First, the benomyl concentration to be used for the selection of *T. aggressivum* transformation was optimized by testing *T. aggressivum* growth on various concentrations (1.0, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1 and 0.0 µg/mL) in ME growth medium. The radius of mycelial growth was measured after 48 hours. Three biological replicates were measured and graphed against benomyl concentrations as shown in Figure 3.43. No growth was observed at the concentration of 0.6 µg/mL or higher even at seven days.
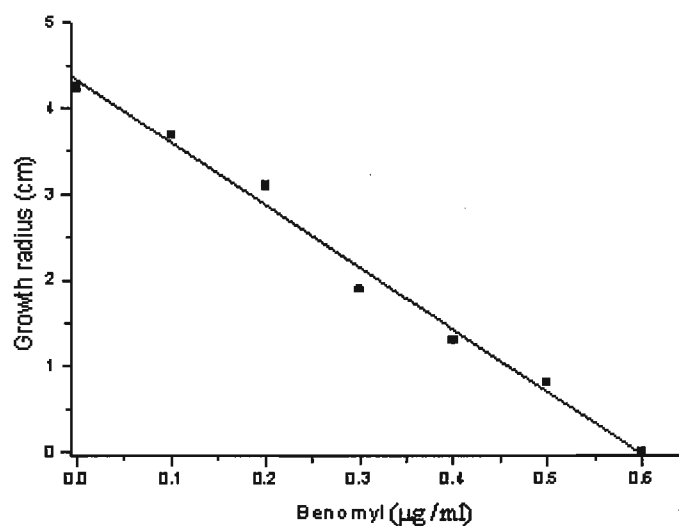
Figure 3.43: Growth radius of *T. aggressivum* at different benomyl concentrations. Colony diameters were taken after 48 hours growth. Each point represents the mean of three replicates.

The effect of *T. aggressivum* spore amount on the transformation efficiency was investigated by using different spore concentrations (10^6, 10^7 and 10^8 conidia/mL). The transformation efficiency was measured by counting the number of transformants per plate in three replicates. The mean transformant number was calculated and graphed against spore concentration (Figure 3.44). The concentration of (10^7 conidia/mL) showed the highest transformation efficiency.
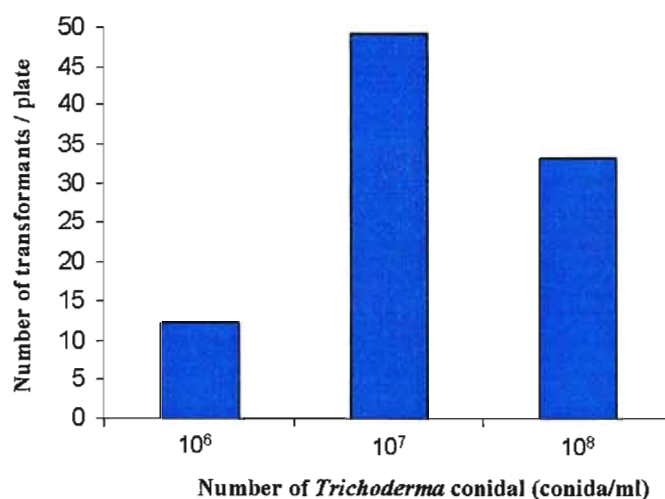
Figure 3.44: Transformation efficiencies of *T. aggressivum* using different conidial concentrations (conidia/ml).

Gene expression stability was monitored by visualizing green fluorescent protein (GFP) expression in the transformed mycelia using a confocal microscope (Nikon), over six days after replating on MEA lacking benomyl (Figure 3.45). GFP expression in the transformed *T. aggressivum* was positive for all days. The expression was proportional to the mycelial growth over the six days. No GFP expression appeared in the non-transformed *T. aggressivum*.
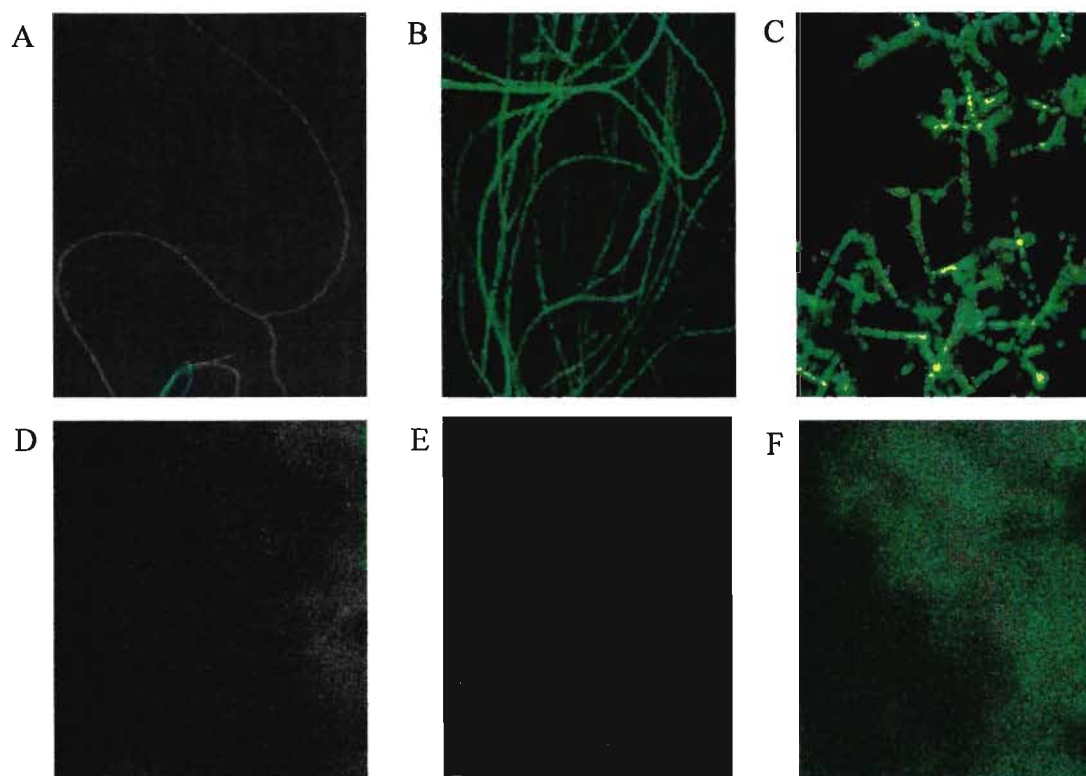
Figure 3.45: GFP expression in transformed *T. aggressivum*. A (2 day) B (4 days) and C (6 days) respectively. *T. aggressivum* untransformed D (2 day), (4 days) and F (6 days) (non-transformed). These images were taken by confocal microscope at a magnification of x400.

The stability of the transformants was attested throughout three successive generations by performing PCR amplification on the isolated DNA from transformed *T. aggressivum* grown on MEA, in the absence of the benomyl selection. Specific GFP primer sets F-eGFP and R-eGFP (Table 2.1) was used in the reaction and the agarose gel picture of the amplification product is shown in Figure 3.46. Positive amplification was obtained from the three generations.
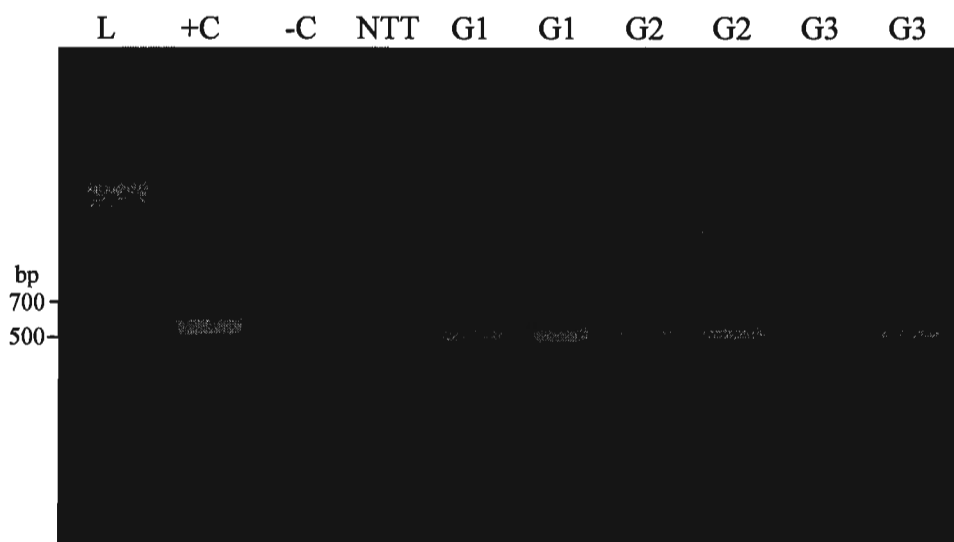
Figure 3.46: Transformant stability within different generations of *T. aggressivum* as detected by PCR amplification with GFP specific primers. L is the DNA ladder (Norgen's MidRanger), +C (pFBENGFP vector) and –C (no DNA template) are the positive and negative controls, respectively. NTT is the non-transformed *T. aggressivum*. G1 through G3 are 3 transformed *T. aggressivum* generations (duplicates) with an amplicon size of 530bp.

3.4.2- Short-hairpin RNA expression vectors construction

The knock-down of the three hydrolytic enzymes was based on the construction of three vectors that will produce the short hairpin RNA (shRNA) upon their transformation into the wild type *T. aggressivum*. This strategy will be used to obtain three new *T. aggressivum* phenotypes, where each phenotype contains a silenced gene. Therefore, three plasmids were constructed; each contains the sense and antisense sequences of one gene under the same promoter. The construction procedures contained two main steps, where the backbone plasmid was modified at first and then the second step contained the cloning of the sense and antisense sequences. The overall construction strategy is shown in Figure 3.47.
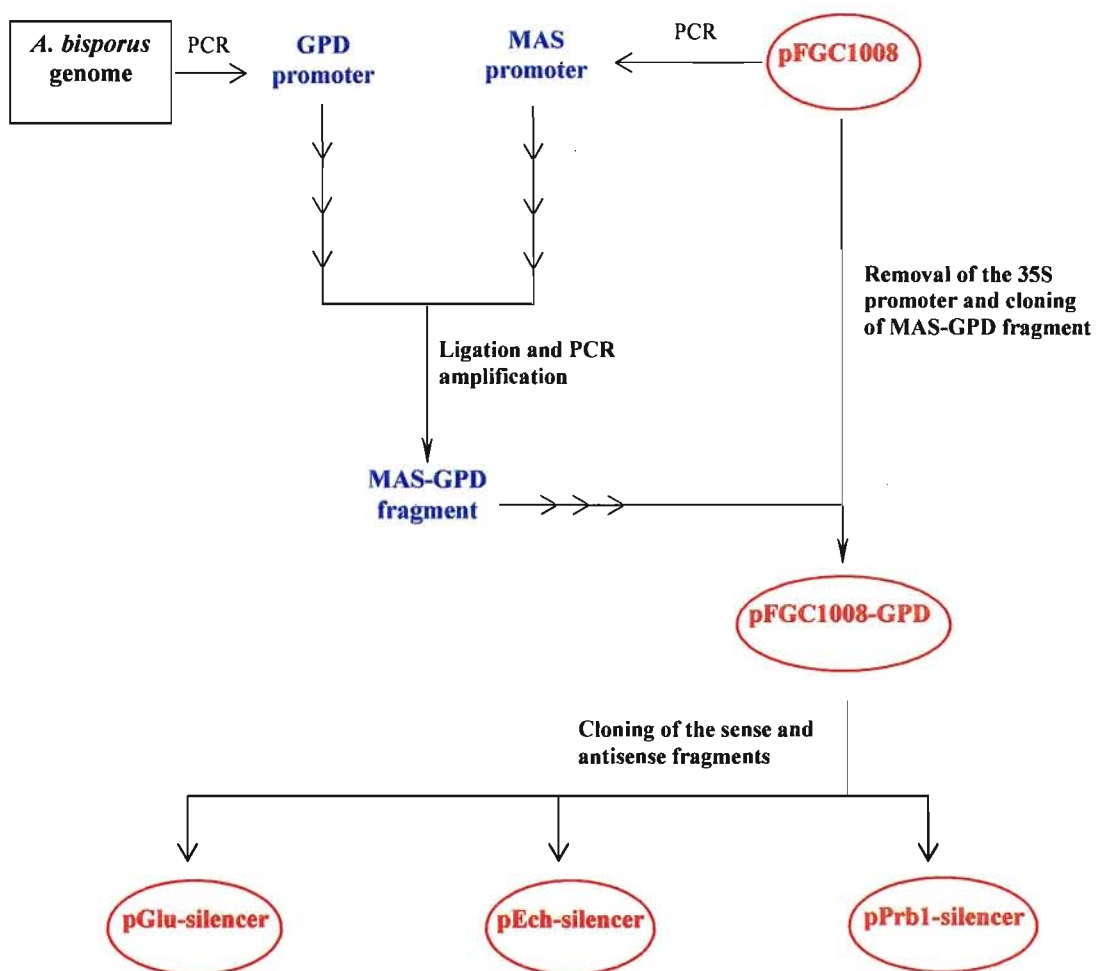
Figure 3.47: Overall construction strategy of the shRNA expression vectors.
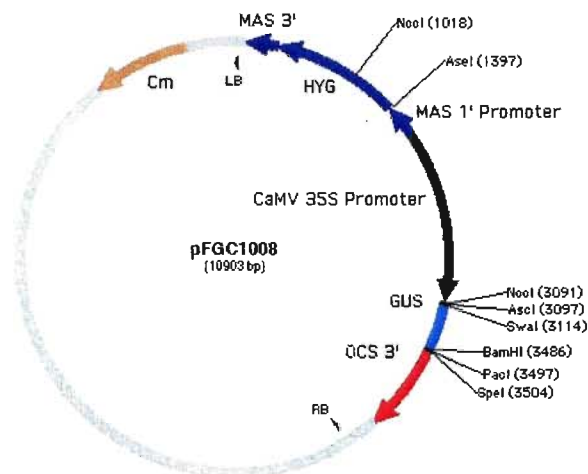
Figure3.48: pFGC1008 vector map

3.4.2.1- Construction of pFGC1008-GPD

The backbone plasmid pFGC1008 as shown in figure 3.48 is an RNAi vector obtained from the *Arabidopsis* Information Resource (TAIR). The vector was modified to suit the expression of shRNA in fungal cells by the replacement of the 35S promoter using the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter from *A. bisporus*. The fragment containing the 35S promoter was deleted from the plasmid through double restriction enzyme digestion by using *Xho*I and *Aat*II. However, the MAS promoter used to drive expression of the hygromycin resistance gene was deleted as well. In order to replace the MAS promoter as well as cloning the GPD promoter that will replace the 35S promoter and will be used to drive the expression of the shRNA, one DNA fragment with both MAS and GPD promoters was prepared. This fragment was obtained by the ligation of two PCR amplification products.

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The first PCR was carried out to amplify the MAS fragment from the backbone vector pFGC1008 by using the specific primer set MAS-F and MAS-R (Table 2.1). The second PCR was performed to obtain the GPD promoter from *A. bisporus* genome by using the specific primer set GPD-F and GPD-R (Table 2.1). Both PCR sets were phosphorylated to allow the downstream ligation of both fragments after their excision from agarose gel and subsequent cleaning. Upon ligation, the terminal PCR primers (GPD-R and MAS-R) of both fragments were used to amplify the correctly ligated MAS-GPD fragment. The three PCR amplifications are shown in Figure 3.49.

The MAS-GPD amplified fragment was cleaned and digested with *Sal*I and *Aat*II. The digested fragment was cleaned and ligated to the previously *Aat*II and *Xho*I digested pFGC1008 using T4 DNA ligase, and then transformed into *E.coli* DH5α. The plasmid was designated pFGC1008-GPD. The construction strategy is shown in Figure 3.50 and the restriction enzyme digestion pattern and the digestion picture used to confirm correct construction are shown in table 3.4 and Figure 3.51 respectively.
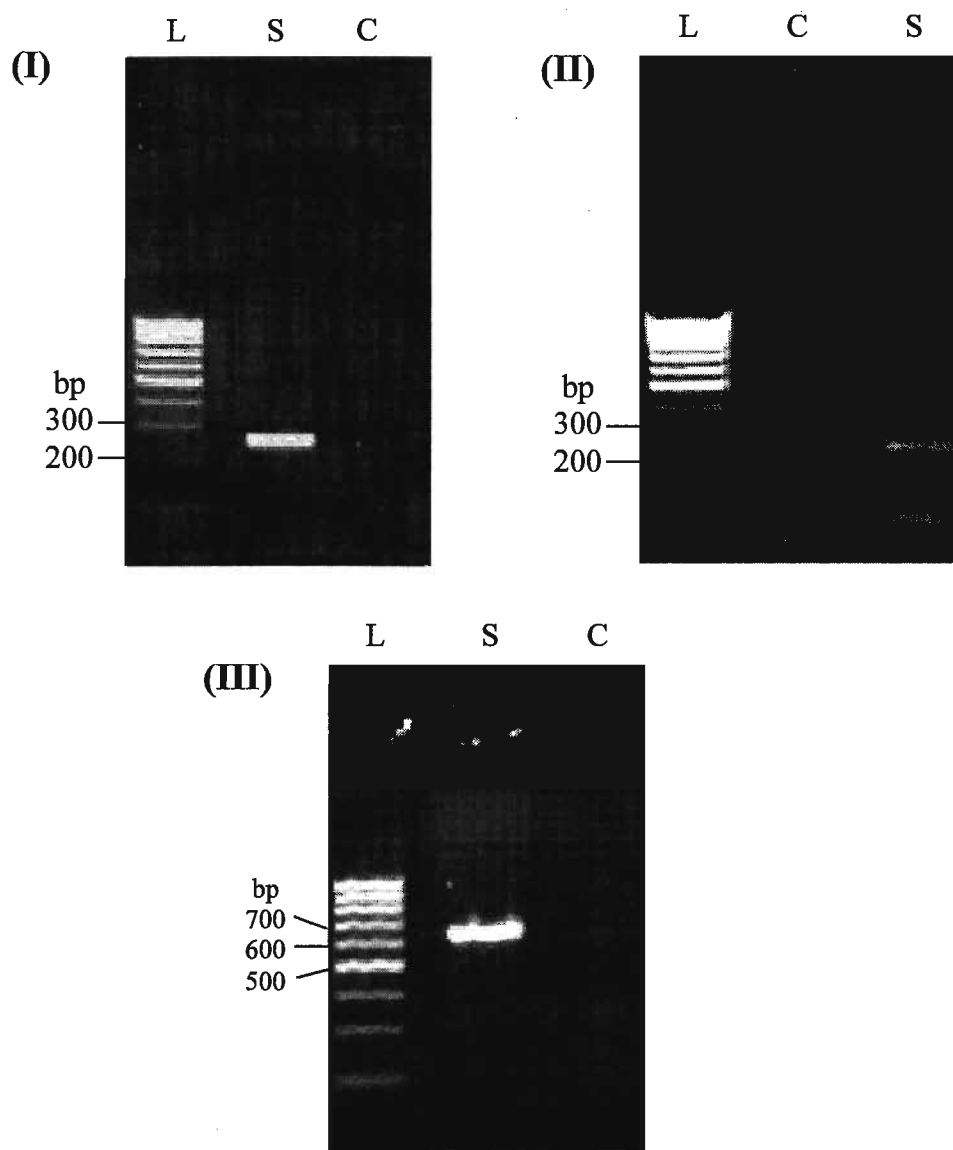
Figure 3.49: PCR amplifications for constructing the MAS-GPD fragment. (I) GPD promoter amplicon of *A. bisporus* (255bp), (II) MAS amplicon of pFGC1008 vector (279bp) and (III) MAS and GPD fragment (~552bp) C: no DNA template.

MAS Promoter

GPD II promoter

MAS-GPD
593 bp

Gel extracted PCR product
digested with *Stu*I *and Xho*I

LB

MAS

HYG

MAS Promoter

Stu I (2225)

35S Promoter

Xho I (3011)

TMV translation leader

Synthetic linker

GUS

Synthetic linker

Poly A

RB

pFGC1008
10907 bp

*Stu*I & *Xho*I digestion
& gel extraction of
10,121 bp fragment

Ligation

LB

MAS

HYG

MAS Promoter

GPD II promoter

TMV translation leader

Synthetic linker

GUS

Synthetic linker

Poly A

RB

pFGC1008-GPD
9826 bp

Figure 3.50: pFGC1008-GPD vector construction strategy.

Table 3.4: Restriction enzyme analysis of the pFGC1008-GPD.

Restriction enzyme	*Hind*III	*Bam*HI	*Eco*RI
Fragment size (bp)	9826	8293 1533	4820 3806 1200
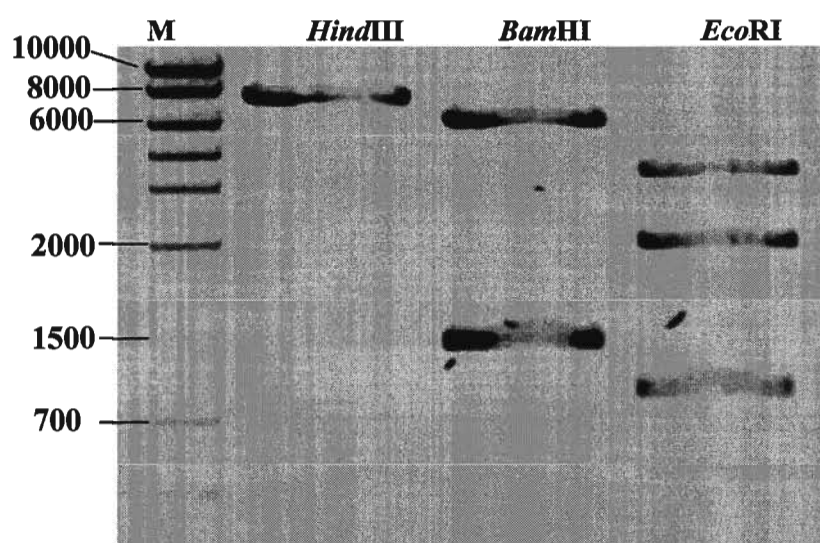
3.51: Restriction enzyme digestion profile of pFGC1008-GPD vector.

3.4.2.2- Cloning of the shRNA sense and antisense sequences

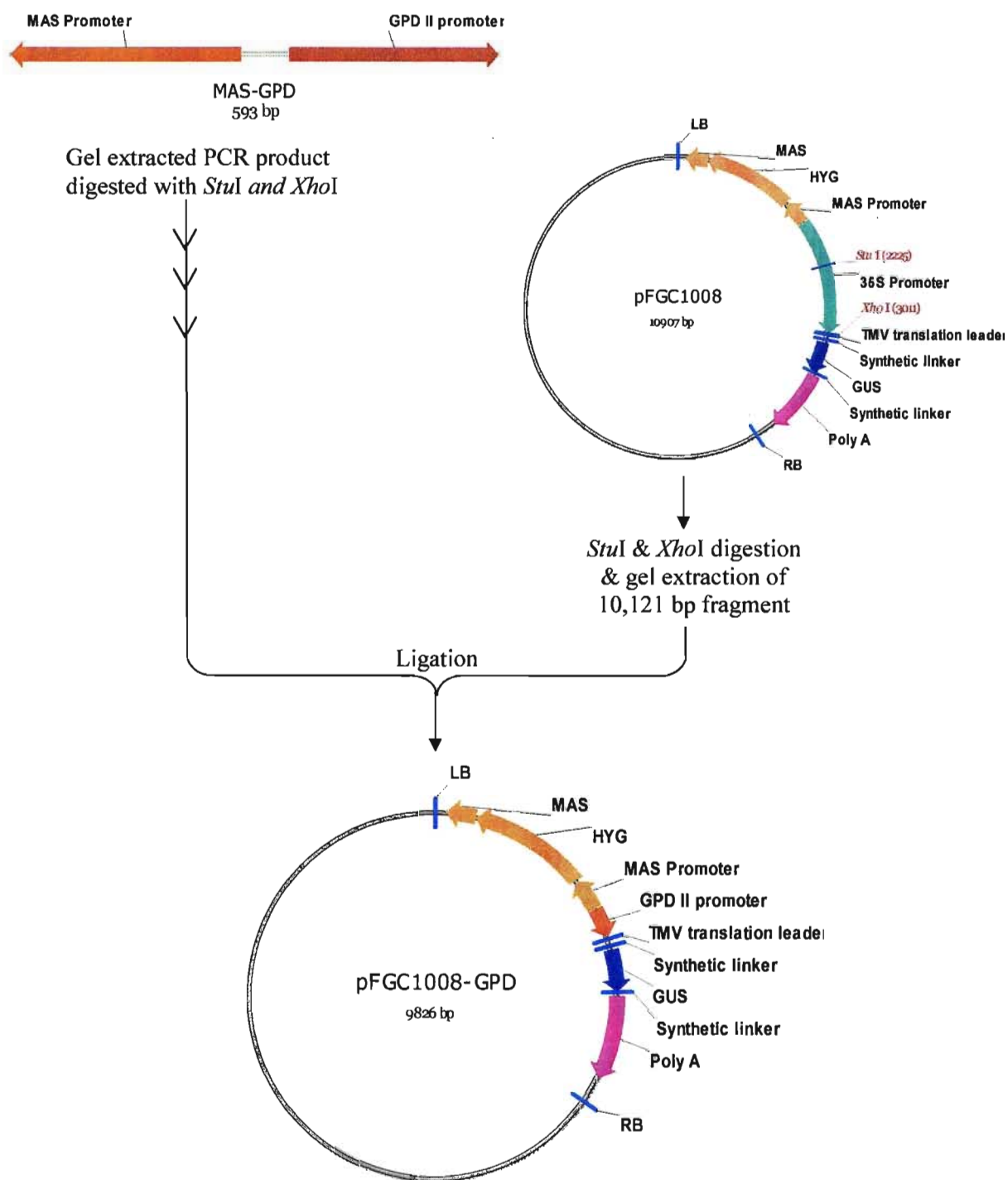The sequence of the sense and antisense strands of the three cell wall degrading enzyme genes as shown in Appendix E were obtained by analysing the cDNA sequences using the Ambion online siRNA target finder (http://www.ambion.com/techlib/misc/siRNA_finder.html). The fragments were obtained by PCR amplification using specific primer sets with two added restriction enzyme sites to the 5' end of each primer. After the amplification and cleaning of the PCR product, the internal restriction enzyme sites

(*Asc*I and *Swa*I) in each amplified fragment were digested when cloning the sense fragment. The terminal restriction enzyme sites *Spe*I and *BamH*I were used with cloning the antisense fragment. The sense and antisense cloning strategy is shown in Figures 3.52 and 3.53. The primer sets used to amplify the sense and antisense fragment from each gene were the same with the exception of using different restriction enzyme linkers as mentioned earlier. For *ech*42, the primers were siRNA-End (F) & siRNA-End (R) and the primers for *prb*1 were siRNA-Prb1 (F) & siRNA-Prb1 (R), while the primers for *β-1,3-glucanase* were siRNA-GLU (F) & siRNA-GLU (R) as showed in (Table 2.1).

The shRNA expression vector pFGC1008-GPD was digested with *Asc*I and *Swa*I when cloning the sense fragment. The vector was cleaned after the digestion and was ligated with the digested and cleaned sense fragment and transformed afterwards. The three plasmids that contained the sense strands of the three genes were used to clone the antisense fragments to create the shRNA silencing vectors of the three genes. The sense plasmids were digested with *Spe*I and *BamH*I, cleaned and ligated afterwards to the digested and cleaned antisense fragments. After transformation, the plasmids were designated pGlu-silencer, pEch-silencer and pPrb1-silencer. Table 3.5 contains the restriction enzyme digestion pattern and Figure 3.54 shows the agarose gel picture of the restriction enzyme digestion profile of the three plasmids. In addition, the correct construction of the three plasmids as confirmed by DNA sequencing.
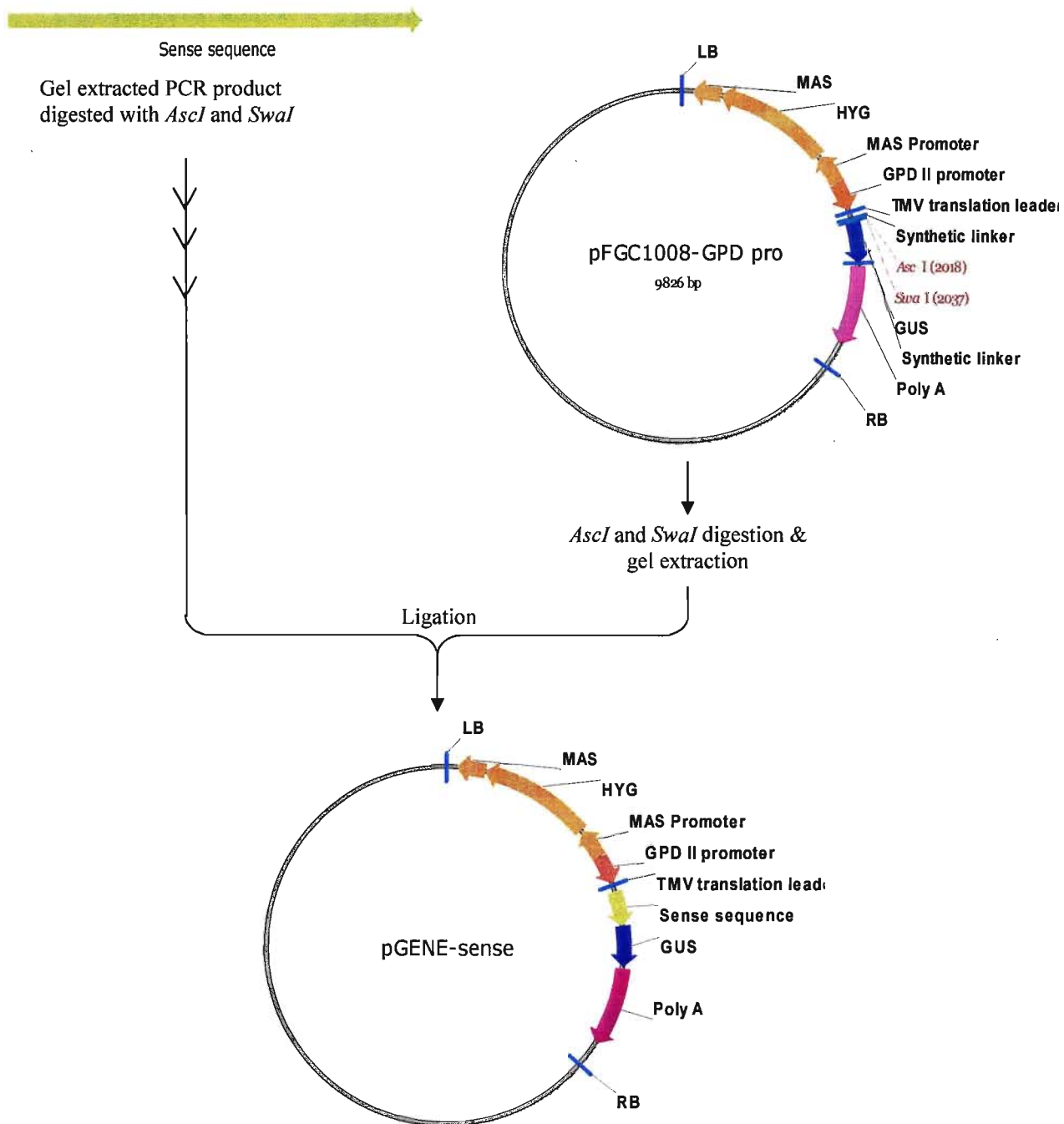
Figure 3.52: Sense cloning strategy. GENE indicates the used gene sequence.
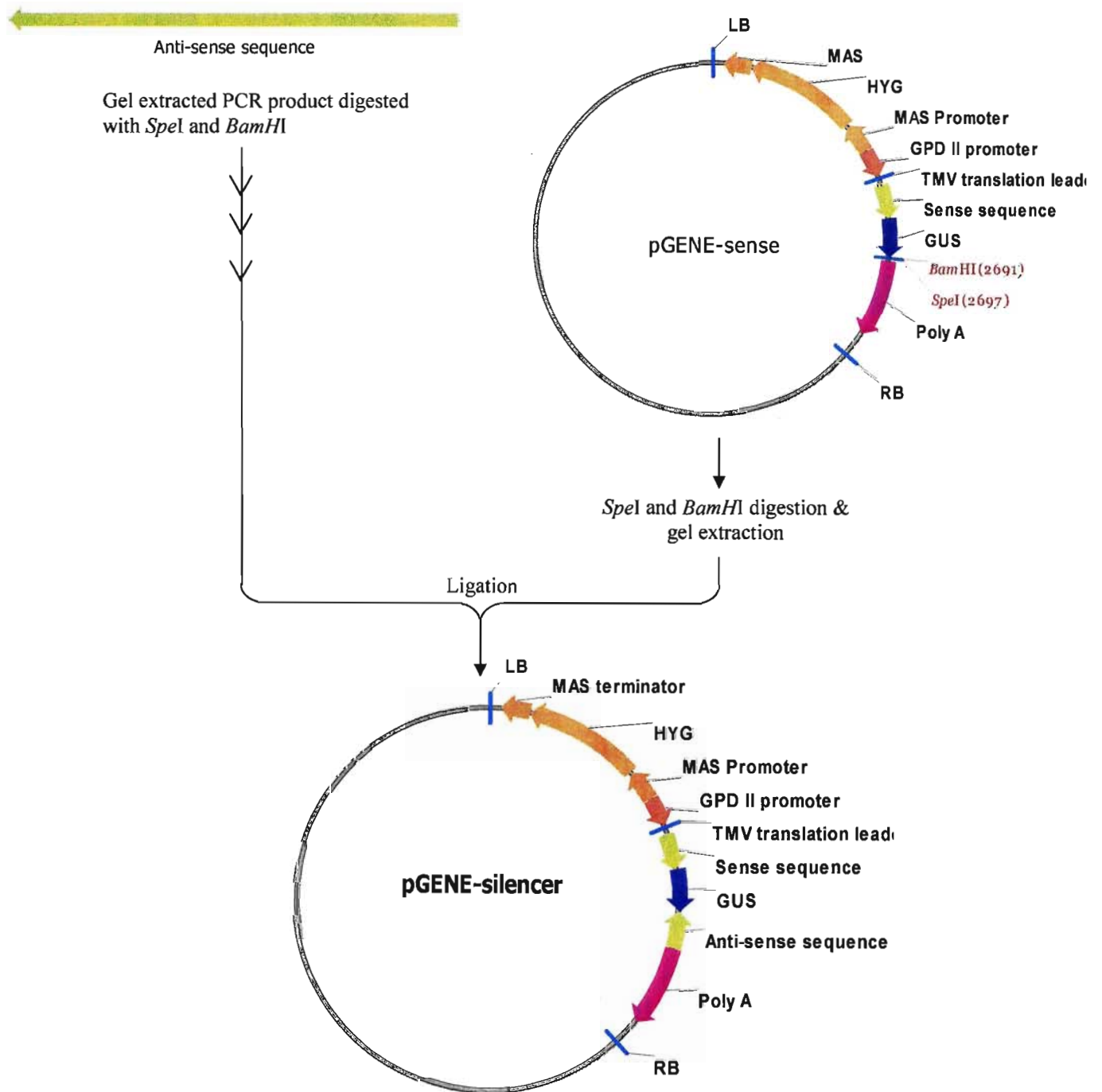
Figure 3.53: Anti-sense cloning strategy. GENE indicates the used gene sequence.

Table 3.5: Restriction enzyme analysis of the three silencer plasmids.

Restriction enzyme	*Xba*I	*Eco*RI	*Bam*HI
Fragment size (bp)	10037 389	9249 1171 15	5014 2258 1532 995 627
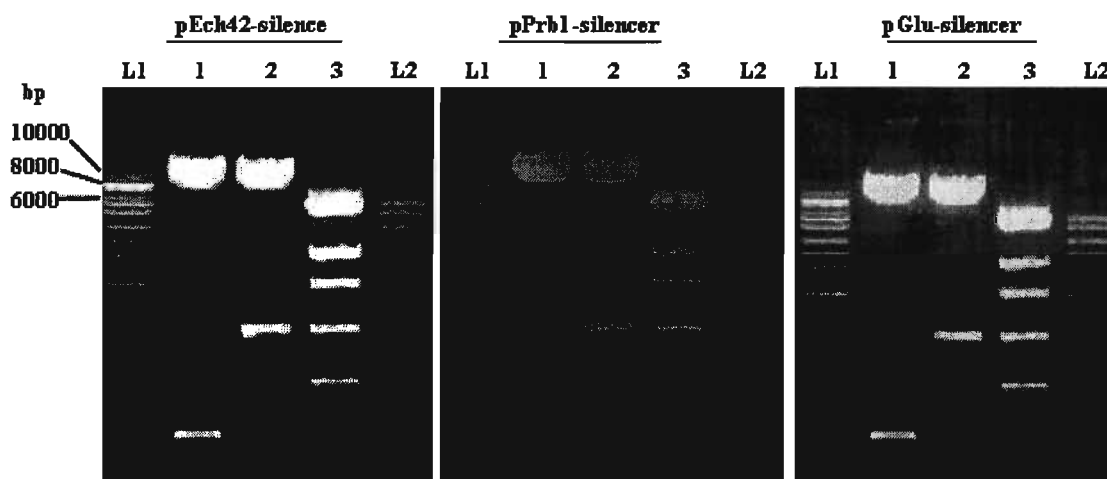
Figure 3.54: Restriction enzyme digestion profile of the three silencer plasmids. 1, 2 and 3 are pGENE-silencer was digested with *Xba*I, *Eco*RI and *Bam*HI respectively. L1 and L2 are Norgen's HighRanger and MidRanger DNA ladders, respectively.

3.4.3- Cultivation of cell wall degrading enzyme gene-silenced *Trichoderma* phenotypes

The three silencing plasmids (pGlu-Silencer, pEch-Silencer and pPrb1-Silencer) as shown in Figure 3.54 were transformed individually into the wild type *T. aggressivum* by using the *Agrobacterium* transformation system. Transformants with the empty plasmid pFGC1008-GPD were used as a negative control for siRNA effects. After the selection of the transformed *T. aggressivum* using hygromycin B, we evaluated the silencing efficiency of the new phenotypes as well as characterizing their growth.

3.4.3.1- Evaluation of the silencing efficiency

To evaluate the silencing efficiency, we isolated triplicate samples of total RNA from the new *T. aggressivum* phenotypes as well as the negative control and wild type over seven days. The isolated RNA samples were treated with DNase I to digest the DNA contamination and were cleaned afterwards. The concentrations of the RNA samples were then measured by using the spectrophotometer. Equal amounts of each sample were used in an RT reaction to synthesize the first strand cDNA. Then, the reverse transcription products were used in a qPCR reaction to evaluate the expression levels of the three silenced genes. The data were normalized by using the expression levels of the β-tubulin gene. The value of the expression of each cell wall degrading enzyme gene was calculated using the $2^{\Delta\Delta Ct}$ formula. The silencing efficiency of the *prb*1 gene was the highest when compared to that of the *ech*42 and *β-1,3 glucanase* genes. The latter showed the least silencing efficiency, although all three were silenced significantly (Figures 3.55; 3.56 and 3.57).
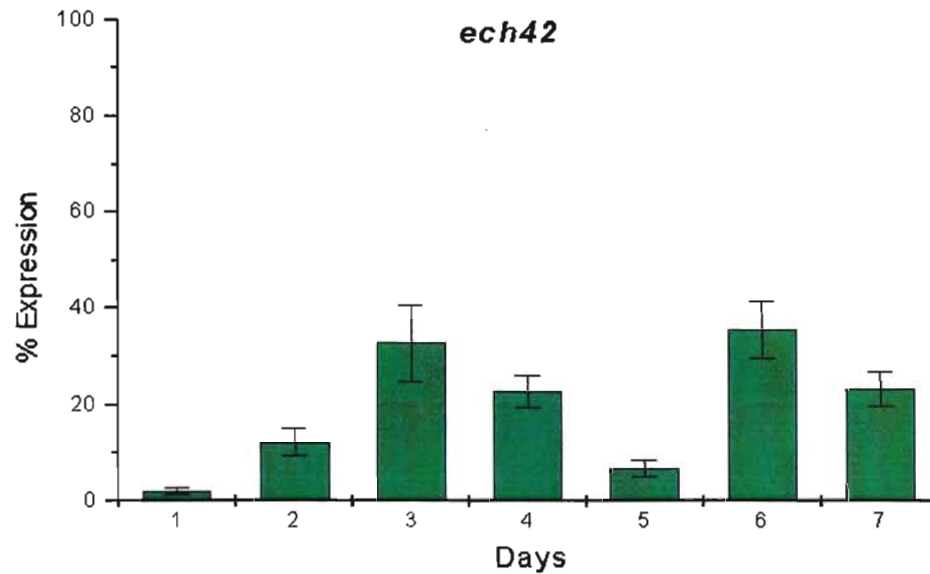
Figure 3.55: *T. aggressivum ech*42 gene expression in silenced phenotype over seven days. The percentage change in gene expression was calculated by using the $2^{\Delta\Delta Ct}$ and the expression of non-transformed *T. aggressivum* was normalized to 100%.
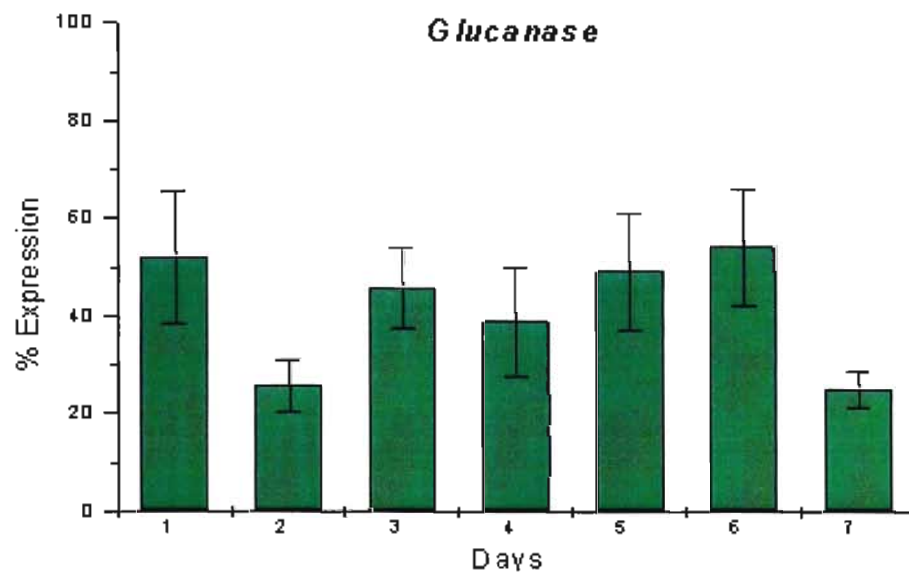
Figure 3.56: *T. aggressivum* glucanase gene expression in silenced phenotype over seven days. The percentage change in gene expression was calculated by using the $2^{\Delta\Delta Ct}$ and the expression of non-transformed *T. aggressivum* was normalized to 100%.
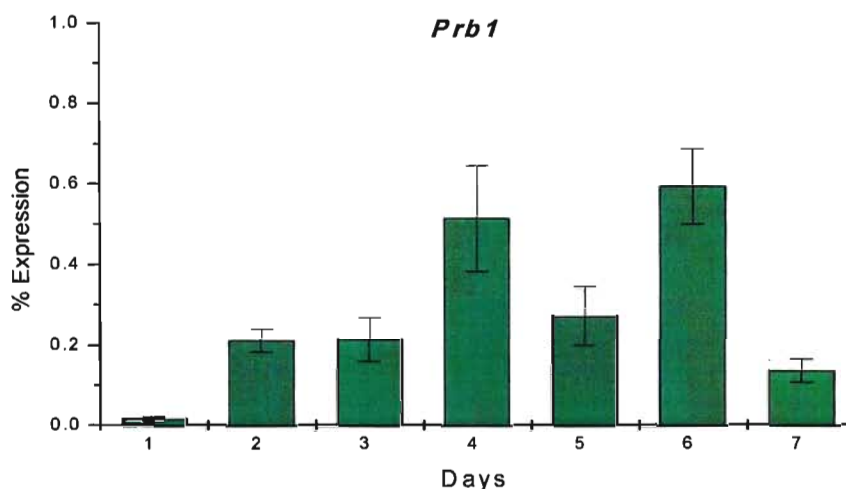
Figure 3.57: *T. aggressivum prb*1 gene expression in silenced phenotype over seven days. The percentage change in gene expression was calculated by using the $2^{\Delta\Delta Ct}$ and the expression of non-transformed *T. aggressivum* was normalized to 100%. Note: The Y axis was changed to 1% instead of 100% to better illustrate the magnitude of the expression changes.

3.4.3.2- Characterization of the radial growth of the new T. aggressivum phenotypes

The growth of the new phenotypes was investigated by measuring the growth radii of the new phenotypes and the wild type. The empty vector showed similar values to the wild type. In general, the silenced phenotypes showed lower growth radii than the wild type. When the wild type reached its highest growth of 90mm after day 6, both *ech*42 and *prb*1 were almost half the growth value while glucanase showed almost three quarters of the wild type growth at the same time point (Figure 3.58).
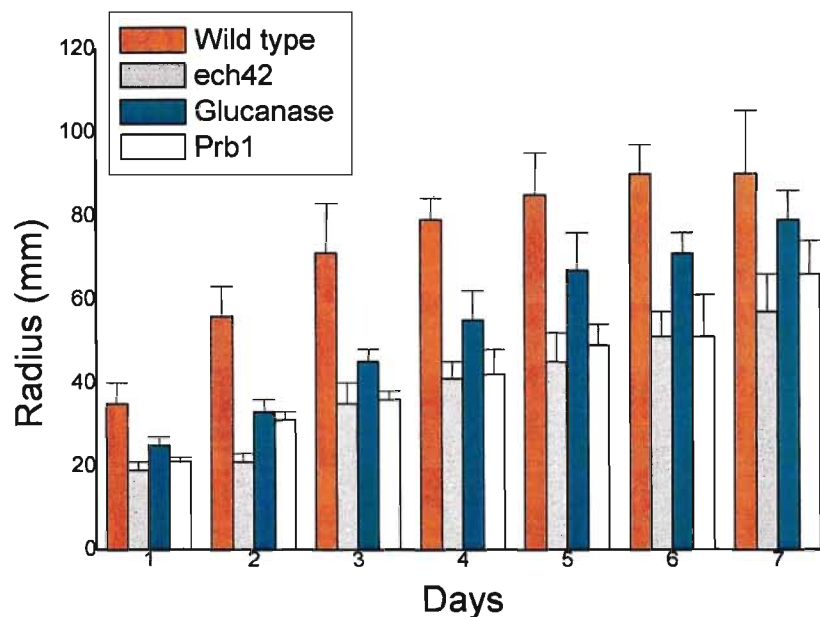
Figure 3.58: Growth of the wild type and silenced phenotypes of *T. aggressivum* over seven days.

3.4.4- Co-cultivation of gene knock-down *Trichoderma* phenotypes with *A. bisporus* U1 and SB65.

3.4.4.1- Evaluation of gene expression

The expression levels of the silenced genes in the three silenced phenotypes were estimated during their co-cultivation with both *A. bisporus* U1 and *A. bisporus* SB65. Total RNA samples were collected over 7 days and treated with DNase 1 to digest the DNA contamination of the samples. The RNA samples were cleaned and quantified by using the spectrophotometer. Equal amounts from each sample were used in an RT reaction to synthesize the first strand cDNA which was then used as a template for qPCR evaluate the expression levels of the three silenced genes during the co-cultivation event. As mentioned previously, the data were normalized by using the expression levels of the

β-tubulin gene and the value of the expression of each gene was calculated using the $2^{\Delta\Delta Ct}$ formula. The basic expression levels of the solitary culture wild type *T. aggressivum* were also collected over the seven days and were considered as 100%. The percentage expression levels of the silenced genes in the solitary silenced *T. aggressivum* as well as its dual culture with U1 and SB65 are shown in Figures 3.59 to 3.64.

In general, the expression levels of the three silenced genes were lower when compared to the non-silenced wild type in both the solitary and the dual cultures (U1 and SB65). *Ech42* gene showed fluctuating levels in the three culture conditions whereas that of glucanase gene showed more obvious differences. Glucanase expression levels can be arranged as follows: Solitary culture > U1 dual culture > SB65 dual culture. The silencing efficiency of *prb1* was the most efficient and was consistent in the three c- ultivation conditions.
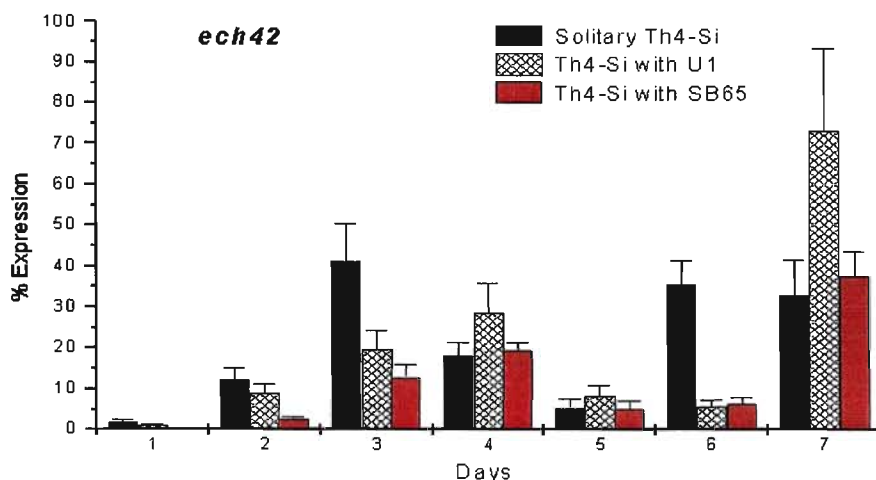
Figure 3.59: Expression levels of *ech42* gene of the silenced *T. aggressivum* phenotype in the solitary and dual culture (U1 and SB65) conditions.
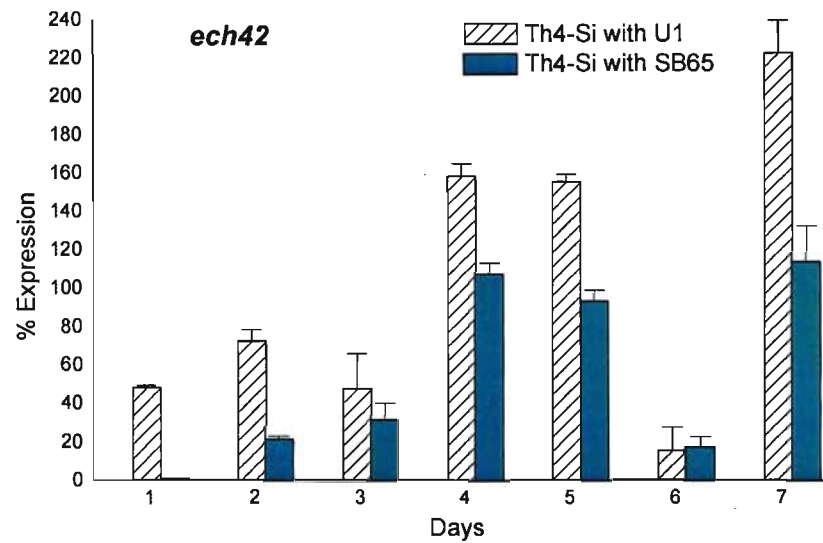
Figure 3.60: Expression levels of *ech*42 gene of the silenced *T. aggressivum* phenotype in the dual culture (U1 and SB65) conditions after normalization with the corresponding silenced phenotype solitary culture expression levels.
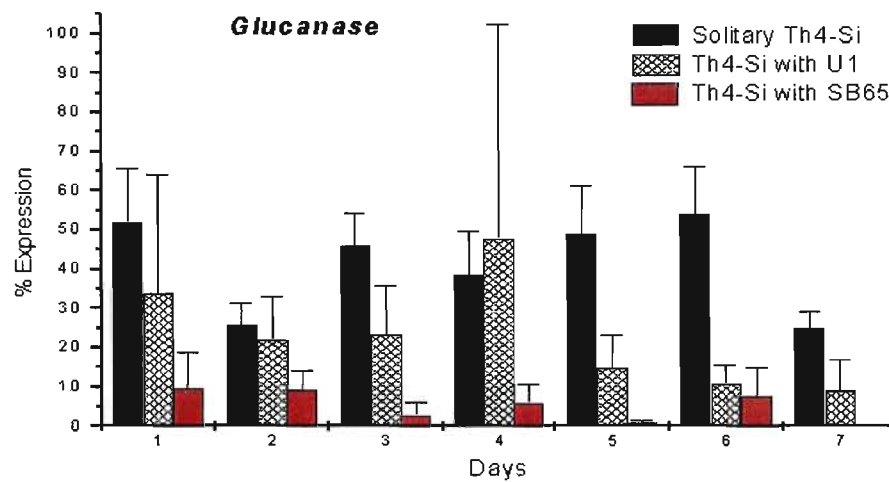
Figure 3.60: Expression levels of *glucanase* gene of the silenced *T. aggressivum* phenotype in the solitary and dual culture (U1 and SB65) conditions.
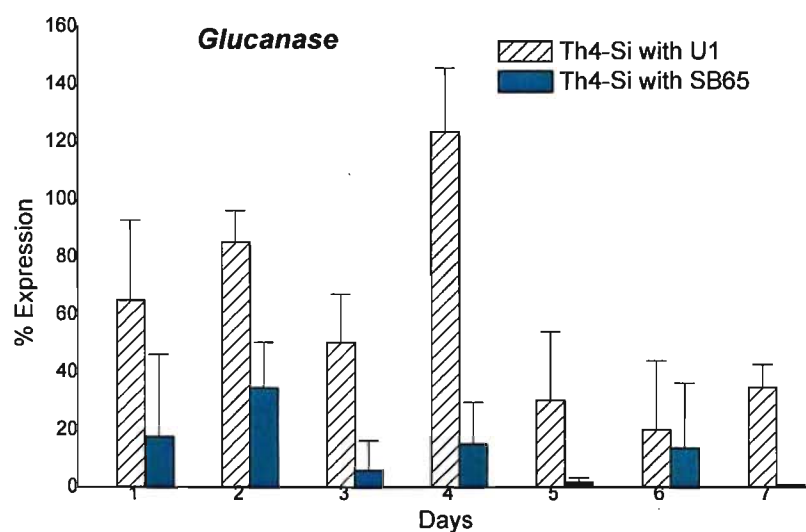
Figure 3.62: Expression levels of *glucanase* gene of the silenced *T. aggressivum* phenotype in the dual culture (U1 and SB65) conditions after normalization with the corresponding silenced phenotype solitary culture expression levels.
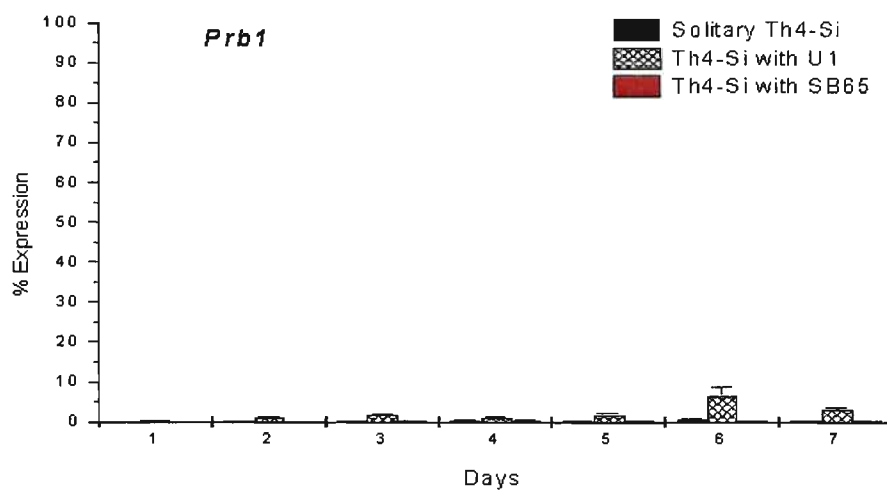
Figure 3.61: Expression levels of *prb*1 gene of the silenced *T. aggressivum* phenotype in the solitary and dual culture (U1 and SB65) conditions.
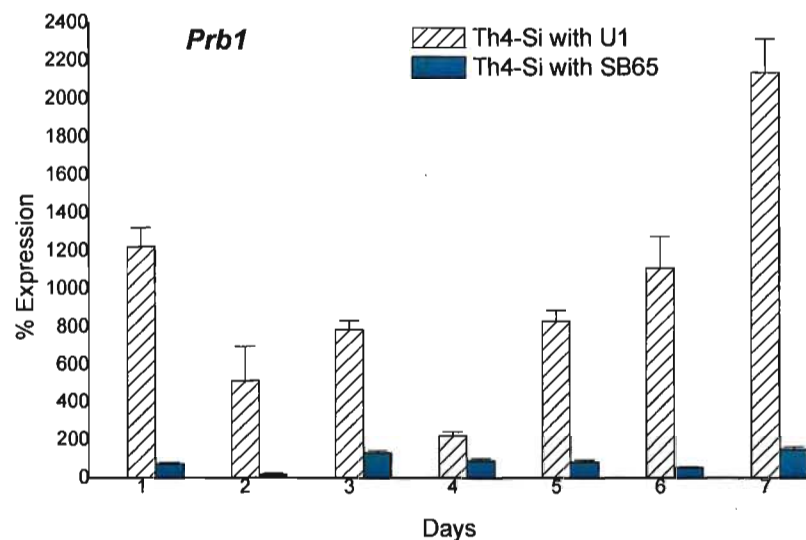
Figure 3.64: Expression levels of *prb1* gene of the silenced *T. aggressivum* phenotype in the dual culture (U1 and SB65) conditions after normalization with the corresponding silenced phenotype solitary culture expression levels.

3.4.4.2- Effect on green mould disease development

In this part, we investigated the effect of silencing the cell wall degrading enzymes on green mould disease development during commercial mushroom cultivation. Therefore, the three *T. aggressivum* silenced phenotypes were co-cultivated with a white strain A15 and both mushroom colonizationand green mould disease development were monitored overtime. Strain A15 was chosen because A15 is sensitive to green mould disease and we decided to use a commercial spawn product rather than making own with either U1 or SB65 strains.

Thirty-six compost trays containing the mushroom spawn were used in six inoculation tests of six trays each. The tests included wild type *T. aggressivum*, pFGC1008-GPD transformed *T. aggressivum* (empty vector), in addition to the three

silenced *T. aggressivum* phenotypes (Prb1, Ech42 and B-glucanase) as shown in Figure 3.65. Water was used as a negative control. Tables 3.6 show the competition on compost between the two organisms while table 3.7 shows the number of samples with *T. aggressivum* growth. *T. aggressivum* growth in compost on day 18 after co-inoculation is shown in Figure 3.61.

The positive control wild type *Trichoderma* T586 showed strong growth in most of the 6 trays on day 18 with an average growth radius of 18 cm (average growth radius was calculated for the 6 trays in all the different conditions). Same growth rate was observed on day 21, however on day 22 the average growth radius was 18.5 cm. Good colonization of *A. bisporus* was observed on day 18 and started to decline on days 21 and 22 to show weak colonization.

In the negative control (H_2O), no *T. aggressivum* growth was observed on day 18 in all of the 6 trays. Strong growth was observed in only one tray on day 21 with an average growth radius of 3.3 cm. Only two trays showed complete and weak growth on day 22 with an average growth radius of 5.7 cm. Strong colonization of *A. bisporus* was observed on day 18 with almost complete colonization on day 22.

The growth of *T. aggressivum* phenotype carrying the empty vector on day 18 was strong with an average growth radius of 16 cm. The average growth radius increased on day 21 to reach 18.3 cm and continues to increase on day 22 to show complete growth of an average radius of 21 cm. Good colonization of *A. bisporus* was observed on day 18 and started to decline on days 21 and 22 to show weak colonization.

The *prb*1-silenced *T. aggressivum* phenotype showed growth on day 18 only in 2 trays with an average growth radius of 4.8 cm. Another 2 trays started to show growth on

day 21 with an average growth radius of 10.5 cm. On day 22, growth was still observed in the same 4 trays with an average growth radius of 13.3 cm. Strong colonization of *A. bisporus* was observed on the three days in almost all the trays.

The *ech*42-silenced *T. aggressivum* phenotype showed growth on day 18 only in 2 trays with an average growth radius of 4.8cm. Growth was observed on days 21 and 22 from these 2 trays with an average growth radius of 7 cm. Strong colonization of *A. bisporus* was observed on the three days in almost all the trays.

The *β-1,3-glucanase* -silenced *T. aggressivum* phenotype showed growth on day 18 only in 2 trays with an average growth radius of 2.8 cm. Another 3 trays started to show growth on day 21 with an average growth radius of 9 cm. On day 22, growth was still observed in the same trays with an average growth radius of 15 cm. Weak colonization of *A. bisporus* was observed on the three days in all the trays.

Table 3.6: Co-cultivation of *A. bisporus* and *T. aggressivum* in compost on days 18, 21 and 22.

Treatment/Sample	*Agaricus* Growth	*Trichoderma* Growth	*Agaricus* Growth	*Trichoderma* Growth	*Agaricus* Growth	*Trichoderma* Growth
		18		21		22
Water Control/1	complete, weak	none	complete, strong	none	complete, strong	none
Water Control/2	complete, strong	none	complete, strong	none	complete, strong	weak, 13 cm
Water Control/3	complete, strong	none	complete, strong	none	complete, strong	none
Water Control/4	80%, weak	none	complete, strong	none	complete, strong	none
Water Control/5	80%, weak	none	80%, weak	strong, almost complete	80%, weak	complete, 21 cm
Water Control/6	60%, weak	none	80%, weak	none	80%, weak	none
Trichoderma T586/1	Good	complete, 21 cm	Good	complete, 21 cm	weak	complete, 21 cm
Trichoderma T586/2	Good	strong, 16 cm	Weak	strong, 16 cm	weak	weak, 16 cm
Trichoderma T586/3	Weak	weak, 14 cm	Weak	weak, 14 cm	weak	weak, 14 cm
Trichoderma T586/4	Good	strong, 15 cm	Good	strong, 15 cm	good	strong, 18 cm
Trichoderma T586/5	Good	complete, 21 cm	Weak	complete, 21 cm	weak	complete, 21 cm
Trichoderma T586/6	Good	complete, 21 cm	good around Ta spot	complete, 21 cm	weak	complete, 21 cm
Ta + Empty vector/1	Weak	weak, 17 cm	Weak	weak, 17 cm	weak	complete, 21 cm
Ta + Empty vector/2	Good	strong, 14 cm	Good	strong, 14 cm	good	complete, 21 cm
Ta + Empty vector/3	Weak	strong, 17 cm	Weak	strong, 19 cm	weak	complete, 21 cm
Ta + Empty vector/4	Good	complete, 21 cm	very weak	complete, 21 cm	very weak	complete, 21 cm
Ta + Empty vector/5	Weak	weak, 6 cm	Weak	strong, 18 cm	weak	complete, 21 cm
Ta + Empty vector/6	Weak	complete, 21 cm	Weak	complete, 21 cm	weak	complete, 21 cm

Treatment/Sample	Agaricus Growth	Trichoderma Growth	Agaricus Growth	Trichoderma Growth	Agaricus Growth	Trichoderma Growth
	18		21		22	
Ta + prb1 siCassette/1	complete, strong	complete, 21 cm	complete, strong	complete, 21 cm	complete, strong	complete, 21 cm
Ta + prb1 siCassette/2	complete, strong	none	complete, strong	none	complete, strong	none
Ta + prb1 siCassette/3	complete, strong	weak, 8 cm	complete, strong	strong, 18 cm	complete, strong	complete, 21 cm
Ta + prb1 siCassette/4	complete, strong	none	complete, strong	very weak, 3 cm	complete, strong	strong, 17 cm
Ta + prb1 siCassette/5	80%, strong	none	90%, strong	none	90%, strong	none
Ta + prb1 siCassette/6	60%, weak	none	90%, strong	complete, 21 cm	weak	complete, 21 cm
Ta + ech42 siCassette/1	60%, weak	complete, 21 cm	70%, strong	complete, 21 cm	weak	complete, 21 cm
Ta + ech42 siCassette/2	80%, strong	none	80%, strong	none	80%, strong	none
Ta + ech42 siCassette/3	complete, strong	none	complete, strong	none	complete, strong	none
Ta + ech42 siCassette/4	60%, weak	none	60%, weak	none	60%, strong	none
Ta + ech42 siCassette/5	complete, strong	weak, 8 cm	complete, strong	complete, 21 cm	complete, strong	complete, 21 cm
Ta + ech42 siCassette/6	complete, strong	none	complete, strong	none	complete, strong	none
Ta + b-glu siCassette/1	50%, weak	none	70%, good	weak, 8 cm	70%, good	strong, 16 cm
Ta + b-glu siCassette/2	50%, weak	none	50%, weak	weak, 8 cm	50%, weak	strong, 18 cm
Ta + b-glu siCassette/3	50%, weak	none	50%, weak	strong, 12 cm	50%, weak	strong, 16 cm
Ta + b-glu siCassette/4	40%, weak	none	50%, weak	none	50%, weak	weak, 19 cm
Ta + b-glu siCassette/5	40%, weak	weak, 14 cm	60%, weak	strong, 18 cm/poor sporul.	60%, weak	strong, 18 cm/poor sporul.
Ta + b-glu siCassette/6	40%, weak	weak, 3 cm	50%, weak	weak, 8 cm/poor sporul.	50%, weak	weak, two spots

Table 3.7: Number of samples with *T. aggressivum* growth in compost.

Treatment	Day 18	Day 21	Day 22
Water Control	0/6	1/6	2/6
Trichoderma T586	6/6	6/6	6/6
Ta + Empty vector	6/6	6/6	6/6
Ta + prb1 siCassette	2/6	4/6	4/6
Ta + ech42 siCassette	2/6	2/6	2/6
Ta + b-glu siCassette	2/6	5/6	6/6
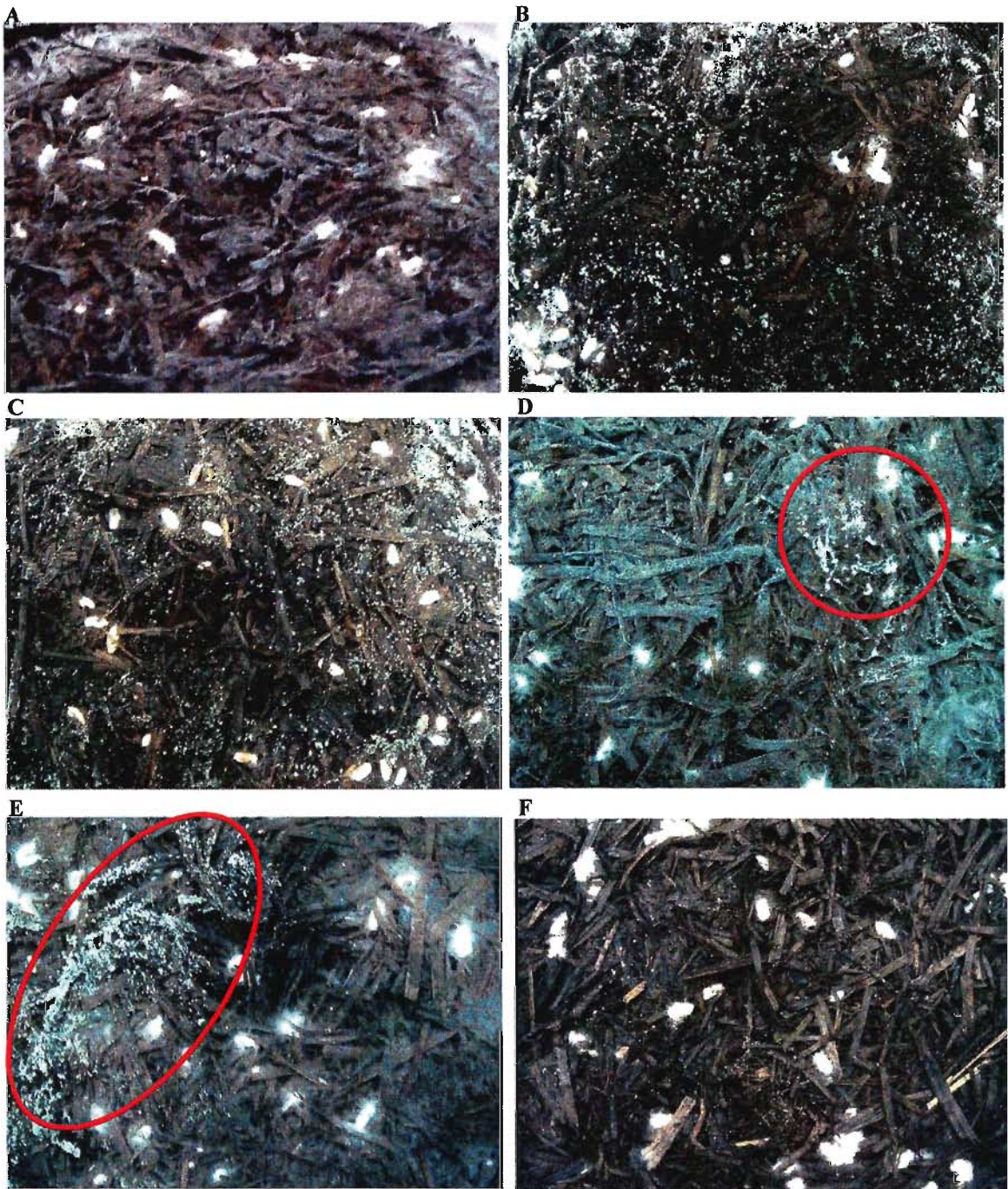
Figure 3.65: Co-cultivation *A. bisporus* and *T. aggressivum* on compost for 18 day. (A) H₂O control, (B) *T. aggressivum* wild type positive control, (C) *T. aggressivum* with empty vector, (D) silenced *Prb1*, (E) silenced *Ech42*, (F) silenced *β-1,3-glucanase* and the red circulated areas indicate the area of green mould disease infection by silenced *T. aggressivum*.

The confirmation of the *Trichoderma* type formed in the various tests was based on PCR performed on DNA samples isolated from the different trays on day 22. Different primers set were used, Alan 1 and Alan2 (Table 2.1) were used to confirm the identity of *T. aggressivum*, and other primers set were specific for the *T. aggressivum* shRNA cassette. The summary of the amplification results is shown in Table 3.8. All of the *Trichoderma* in the 36 trays were proven to be *T. aggressivum* and not a contaminant as shown from the positive PCR amplification. Amplification was observed from the second PCR test (Table 3.8) from two of the empty vector trays but not from any of the wild type or the water-treated trays. However, with the silenced phenotype trays, positive amplification was observed in all the tested samples except with one sample from each silenced phenotype.

Table 3.8: Identity confirmation of *T. aggressivum* and the insert shRNA cassette in *T. aggressivum* phenotypes.

Samples	Tray #	*T. aggressivum* identity	*T. aggressivum* contamination	Empty vector primers	Prb1 primers	Ech42 primers	β-glu primers
Water	5	+	−	−	−	−	−
Water	2	+	−	−	−	−	−
empty vector	4	+	−	+	−	−	−
empty vector	3	+	−	+	−	−	−
Wild type	1	+	+	−	−	−	−
Wild type	6	+	+	−	−	−	−
ech42	5	+	−	−	−	+	−
ech42	1	+	+	−	−	±	−
prb1	3	+	−	−	+	−	−
prb1	6	+	−	−	+	−	−
prb1	1	+	−	−	+	−	−
prb1	4	+	+	−	−	±	−
β-glu	3	+	−	−	−	−	+
β-glu	5	+	+	−	−	−	±
β-glu	1	+	−	−	−	−	+

CHAPTER IV

DISCUSSION

The commercial mushroom *Agaricus bisporus* is grown all over the world. This mushroom species has a high economic value and is the most valuable Canadian vegetable crop, generating an average of over 200 million dollars in revenue annually (adapted from source: Statistics Canada). Crop output however can be severely affected by green mould disease caused by *Trichoderma aggressivum* f. *aggressivum*. The development of several disease management procedures has allowed for a high degree of control; however to an understanding of the interaction mechanisms between the fungi may provide alternative methods for disease prevention.

Recent research in the field of molecular biology including the introduction of new molecular tools and the development of more effective techniques allow for better investigation and understanding of many host-disease interactions. In the present study, such molecular biology tools have been used to investigate the mechanism of interaction between *T. aggressivum* and *A. bisporus*. The interaction between the two organisms is based on the balance between two capabilities; the first is the ability of the *T. aggressivum* (the disease causing agent) to attack the host, and the second is the efficiency of the host organism (*A. bisporus*) in resisting the aggressiveness of the disease effect.

Different molecular techniques have been used in order to understand such disease-host interactions. In the present work, these include the isolation, characterization of the

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A. bisporus N-acetylglucosaminidase, isolation of the hydrolytic enzyme genes from *T. aggressivum*, and characterization of their expression profiles. RNA interference (RNAi) technology was used to further understand the possible roles of cell wall degrading enzymes in disease progression.

4.1- PURIFICATION AND ANALYSIS OF N-ACETYLGLUCOSAMINIDASE FROM *A. BISPORUS*

The interaction between *A. bisporus* strains and *T. aggressivum* showed various tolerance and disease resistance levels. The most commonly produced *A. bisporus* strains in North America are the brown (SB65) and white (U1) strains. Interestingly, the brown strain has an enhanced resistance against green mould disease compared to the white strain (Rinker and Alm 1996; Anderson *et al.,* 2001). The enhanced resistance of the brown strain may be due to its high expression levels of the hydrolytic enzyme N-acetylglucosaminidase during the disease interaction (Guthrie and Castle, 2006). Fungal chitinases have been previously shown to have protective roles in other interactions. For example, resistance of the fungus *Phascolomyces articulosus* to the mycoparasite *Piptocephalis virginiana* is due, in part, to chitinase activity produced by *P. articulosus* (Manocha and Zhonghua, 1997). In addition, transgenic plants expressing a *T. harzianum* endochitinase gene show increased resistance to several plant pathogenic fungi (Lorito, 1998). Understanding the role of this enzyme as a defensive agent against the green mould disease may enable the cloning and development of resistant phenotypes of the commercially valuable *A. bisporus* white strain.

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In order to sequence and characterize the defensive mechanism of the N-acetylglucosaminidase enzyme from the brown strain of *A. bisporus*, we carried out sequential purification methods starting with ammonium sulphate precipitation, gel filtration, and the Rotofor cell system. The molecular weight of the purified enzyme is ~75 kDa which is different from the molecular weight of the same enzyme reported previously by Guthrie and Castle (2006) who used native PAGE and the size was 96 kDa. This molecular weight difference is due to the the nature of the used PAGE since our experiments relied on the use of SDS PAGE. The protein was sequenced and the data were submitted to Gene Bank and the sequence homology showed low significance. Therefore, it is believed that we isolated a unique N-acetylglucosaminidase sequence. This was also supported by the results obtained on the gene sequence level, by PCR amplification using different primer sets designed from the homologous regions of N-acetylglucosaminidase genes in various fungi. The PCR amplification product was also unique when aligned against the other N-acetylglucosaminidase sequences.

The lack of genomic and proteomic information of *A. bisporus* limited our efforts in the investigation of the defensive mechanisms of the mushroom host against the green mould disease. Therefore, it is highly recommended that full genome sequencing of these strains should be performed. Such sequencing data will provide useful tools for subsequent molecular studies. An 8x coverage sequence of one of the nuclear types of the U1 strain is complete and will be released shortly.

The original aim of our project was to investigate the gene that provides resistance against *T. aggressivum* and N-acetylglucosaminidase has been previously suggested by

Guthrie and Castle (2006) to be involved in such resistance. However, the obtained information was not promising and was not pursued further without the availability of more data, such as the full genome sequence of *A. bisporus*, which make it possible to proceed in this direction. Since we were not able to follow up with our characterization of the *A. bisporus* defense mechanism, we examined the *T. aggressivum* cell wall degrading enzymes that are thought to attack *Agaricus* and mediate the disease development.

4.2- ISOLATION OF CELL WALL DEGRADING ENZYMES GENES FROM *TRICHODERMA AGGRESSIVUM*

The cell wall degrading enzymes of the disease causative agent, *T. aggressivum*, and their potential roles in diseases were the focus of the rest of the study. These hydrolytic enzymes genes include endochitinase *ech*42, protease *prb*1 and *β*-1, 3-glucanase. These genes may play a role in the disease-host interaction by degrading the cell wall of the host fungi. This supposition is based on previous reports on the activities of these enzymes in *T. harzianum* which is antagonism of several fungi (de la Cruz and Llobell 1999). In an attempt to investigate this possibility, we successfully isolated the three genes and their regulatory sequences. Isolation of the three genes from *T. aggressivum* was achieved by polymerase chain reaction (PCR) utilizing primers based on the conservative sequence of the similar enzymes genes from other *Trichoderma* spp. In turn, this similarity strengthens the initial assumption of the role of these enzymes in the interaction.

After the full sequences of the three genes were obtained, the sequence data from the DNA and cDNA were used in the characterization process. The characterization was based on the sequence comparison with *Trichoderma* species and other fungi. The comparison criteria included the length of the genes, promoter elements, intron positions and numbers and the putative protein elements.

The study of molecular characteristics of the interaction between *T. aggressivum* and *A. bisporus* will be very useful in improving our understanding of mycoparasitism. The obtained sequences were aligned against the sequences of other fungal species of known parasitic activity to characterize the different regulatory elements and parasitic domains. In addition, sequence data are essential to knockdown these genes in an attempt to further understand their involvement in the parasitic interaction. Such information will provide a more efficient control of phytopathogenic fungi, either through manipulation of the mycoparasite or as a source of genes which could be used to enhance mushroom resistance to green mould disease.

4.2.1- Endochitinase *ech*42

The putative protein sequence of *T. aggressivum* ech42 endochitinase has been identified according to the sequence data obtained from DNA as well as mRNA clones. Based on amino acid sequence similarity, all chitinase genes cloned and characterized to date have been classified into two different families, Family 18 and Family 19 (Henrissat, 1991 and Henrissat and Bairoch, 1993). Chitinases from bacteria, fungi, virus, animals, and class III and V chitinases from plants are grouped in Family 18, however, plant chitinases classes I, II, and IV belong to Family 19 (Sahai and Manocha,

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1993). Alignment of the predicted ech42 amino acid sequence with many other related homologues highlighted several sequence domains that are important for protein function. The catalytic domain that is responsible for the hydrolysis of chitin glycoside bonds was identified in the *ech*42 sequence. (Svitil and Kirchman, 1998).

Analysis of *T. aggressivum* ech42 amino acid sequence revealed, not surprisingly, a consensus sequences with the glycoside hydrolase Family 18 chitinases (Garcia *et al.,* 1994; Pishko *et al.,* 1995 and Lorito, 1998). Such consensus sequences include the lariat sequences (CTGAC, CTAAT and CTAAG), characteristic of fungal introns (Balance, 1986) and the position of the introns which is conserved when compared with homologous genes from *T. harzianum* (Carsolio *et al.,* 1994).

Chitinases are classified into two categories, either basic or acidic. Acidic chitinases are secreted extracellularly and accumulate in intercellular spaces, whereas basic chitinases are systemically induced by stress-related environments and accumulate intracellulary within the central vacuole (Samac *et al.,* 1990 and Beerhues and Kombrink 1994). Based on its isoelectric point (*pI*), ech42 is predicted to belong to the acidic chitinase category along with the large majority of fungal chitinases (Lu *et al.,* 2005). The existence of an N-terminal signal peptide in ech42 supports this categorization and is consistent with a possible role of this endochitinase in green mould disease. In addition, Guthrie and Castle (2006) showed that an endochitinase with an estimated mass of 40 kDa was secreted during the interaction with *A. bisporus*. This secreted endochitinase is most likely ech42. The *ech*42 promoter region contains the *MYC1* element which is one of the mycoparasitism response elements (MYREs) that have a major role in the

mycoparasitic interaction and have been previously identified in the promoter sequence of *T. atroviride ech*42 and *prb1* (Cortes *et al.* 1998).

4.2.2- protease *prb*1

T. aggressivum protease *prb*1, which belongs to the subtilisin family, has highly conserved signature elements associated with functional protease activity. These appear in the five highly conserved stretches assigned to subtilisin-type serine protease motifs (Geremia *et al.*, 1993). The presence of the glycosylation signals in the putative protein sequence indicates that prb1 may be glycosylated which is common among many secreted proteases in eukaryotes. This secretion of *prb1* would be required for function in green mould disease but has not yet been demonstrated. Four putative binding sites were identified, in which two of them (MYC1 and MYC2) have strong conservation with *T. atroviride* for global inducer of mycoparasitism-related genes (Cortes *et al.* 1998).

4.2.3- β-1, 3-glucanase

The full sequence of *T. aggressivum β-1, 3-glucanase* gene was obtained during the present study. In addition, the mRNA sequence of this gene was also identified. Alignment of the DNA and mRNA sequences revealed the presence of nine short intron sequences within the gene. The lack of sequence and regulatory domain characterization of *β-1,3-glucanase* genes from other fungal species did not allow for further characterization of the regulatory sequences within the 5' upstream sequence of the *T. aggressivum β-1, 3-glucanase* gene. However, the present sequence data can be helpful

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in future studies concerned with the characterization of the regulatory elements involved in *T. aggressivum β-1, 3-glucanase* gene expression.

4.3- GENE EXPRESSION PROFILING OF CELL WALL DEGRADING ENZYMES

The cell wall degrading enzymes of *T. aggressivum* have not been intensively investigated at the molecular level. The genetic regulation of these enzymes is not well studied. Understanding the expression of these genes during the pathogenic interaction is important to reveal their involvement in the parasitic mechanism.

In this part, the transcription levels of the three cell wall degrading enzymes genes (*ech*42, *β-1, 3-glucanase* and *prb*1) were investigated during the co-cultivation of *T. aggressivum* and *A. bisporus*. This profiling will help to understand the role of each of these enzymes in green mould disease and the interaction between the two organisms.

Media composition can alter gene expression (Morissette *et al.*, 2006); therefore we looked at the effect of the growth media on the expression of these *T. aggressivum* cell wall degrading enzymes genes, prior to their expression in the dual culture (co-cultivation).

Malt extract broth (MEB) medium is used to grow *T. aggressivum* and the *Schizophyllum* complete yeast medium (SCM) medium is used to grow *A. bisporus* therefore, it is of a great importance to look at the effect of media on the expression levels of *T. aggressivum* cell wall degrading enzymes. To achieve that, *T. aggressivum*

was grown on both media, total RNA was isolated over seven days, and the expression levels of the three enzymes were profiled by using RT-qPCR. Expression levels of the three hydrolytic enzymes were significantly reduced when the organism was grown on the SCM medium compared to the MEB medium. This experiment also showed that the growth rate of *Trichoderma* was decreased on SCM media compared to MEB.

The presence of dextrose (carbon source) in the SCM medium is the likely reason for the decreased expression levels of *T. aggressivum* cell wall hydrolytic enzymes. Such effect of the carbon source on the production of *Trichoderma* enzymes have been reported before on xylanase activity (Seyis and Aksoz, 2005). Since the SCM medium has higher carbon and nitrogen content than the MEB medium and different sources to supply the two elements, this might be responsible, at least in part, for their effect on the growth rate. It has been reported that the presence of glucose as a carbon source repressed the expression of *prb1* (Flores *et al.*, 1997) as well as the glucanase gene expression (Cohen-Kupiec *et al.*, 1999) in the *Trichoderma* spp. More specifically, *T. harzianum* showed repressed *prb1* and *β-1, 3 glucanase* expression in the presence of glucose (Geremia *et al.*, 1993; Ulhoa and Pederby, 1991 and Geremia *et al.*, 1993). In addition, the expression of these enzymes is activated under the starvation conditions of the carbon or nitrogen content (Viterbo *et al.*, 2002), which agrees with our results that show lower gene expression in the carbon-rich and nitrogen-rich in SCM medium.

Another complication that has been encountered with this approach is the suitability of the MEB medium for mushroom growth. The growth of *A. bisporus* is significantly reduced on MEB medium. For this reason, we performed the co-cultivation experiment

by growing the mushroom on its SCM medium and when the growth is almost complete, we transferred the mushroom to the MEB medium following the immediate inoculation of *T. aggressivum*. This allowed us to avoid the negative effect of the MEB medium on the early growth stages of *A. bisporus* and also to guarantee the growth of *T. aggressivum* on its optimum medium.

Two common mushroom strains are the white strain (U1) and the brown (SB65) strains. Both the white and the brown mushroom strains are susceptible to the green mould disease infection however the brown strain shows more resistance to the disease than the white strain (Rinker and Alm, 1996). In the co-cultivation experiment, we studied the effect of both strains on the expression levels of the hydrolytic enzymes of *T. aggressivum*. This allowed for understanding the involvement of these enzymes in the interaction and resistance mechanisms.

The expression levels of the three cell wall degrading enzymes over seven days, during the co-cultivation, were compared to their expression in *T. aggressivum* grown in solitary culture. The expression levels of both the *ech*42 and the *prb*1 genes decreased during the first four days of the interaction but started to increase on day five to day seven. This was seen for both white and brown strains. Similar results were previously seen for these two hydrolytic enzymes in *T. harzianum* during its co-cultivation with *Crinipellis perniciosa* (De Marco *et al.*, 2003). The expression profiles of prb1 and ech42 are very similar. Interestingly, the characterization of the promoter elements in the two genes revealed that both have the MYC1 and MYC2 sequences which is a good confirmatory sign of the role of these genes in green mould disease.

Several *T. harzianum* chitinases were induced by the host fungus *Botrytinia cinerea, S. rolfsii* or *R. solani* (De La Cruz *et al.*, 1993; Carsolio *et at.*, 1994; Haran *et al.*, 1996). Also, it has been reported that the *ech*42 enzyme is over expressed during the interaction between *T. atroviride* and *R. solani* (Kulling *et al.*, 2000). This is similar to our findings in *T. aggressivum ech*42 in presence of *A. bisporus*. Also, the production of protease is common among parasitic fungi in order to attack their host. Therefore, the regulation of gene expression of these enzymes during the parasitic interaction is very important. For instance, the fungus *Metarhizium anisopliae* as well as *Arthrobotrys oligospora* produce protease during their interaction with their host to facilitate their parasitic activity (St Leger *et al.*, 1987 and Goettel *et al.*, 1989).

The *T. aggressivum prb*1 gene was found to be upregulated during co-cultivation with *A. bisporus*. This implies the involvement of the enzyme in the mechanism of mycoparasitism. Overexpression of *prb*1 gene in transgenic *T. harzianum* carrying multiple copies of the gene has been used to improve the biological control of soil-borne plant pathogens and *prb1* has been shown to be directly related to mycoparasitism (Flores *et al.*, 1997). It has been reported that the induction of the *prb1* gene expression is mediated by the presence of the host fungi or its cell wall (Geremia *et al.*, 1993). Overexpression of the two cell wall degrading enzyme genes, *prb1* and *ech*42, in *T. harzianum* has been reported by Cortes and co-workers (1998). Both enzymes were expressed in dual cultures of the fungus and its host, even under the limited fungal contact. Furthermore, the investigation of such induction revealed that it is contact-independent and is mediated by a diffusible compound produced by the host fungus.

In contrast, the expression level of the *β-1,3-glucanase* gene increased over the entire co-cultivation period (seven days), when either mushroom strains were used. This indicates that the β-glucanase gene is under the control of different regulatory mechanisms compared to *ech*42 and *prb1* genes.

The induced levels of *β-1,3-glucanase* in our co-cultivation study also suggest its involvement in the mycoparasitic interaction. Previous studies showed that various *β-glucanase* activity levels were induced against cell-wall preparations from several pathogens (Markovich *et al.*, 2003). Moreover, these enzymes play a role in the *Trichoderma* antagonism of *Pythium* (Thrane *et al.*, 1997). *Pythium*, an oomycete contains a cell wall that is a mixture from ß-1,3-glucans and 1,6-glucans, in addition to cellulose, but not chitin.

When comparing *T. aggressivum* hydrolytic enzymes gene expression over seven days, increases and decreases in expression of the three hydrolytic enzymes are similar with both white and brown strains of mushroom. However, when comparing the two mushroom strains, *T. aggressivum* enzymes expression is always much lower in the brown strain than the white strain. The resistance of the brown strain to green mould disease might be due, in past, to the reduced induction of the *T. aggressivum* enzymes.

4.4- HYDROLYTIC ENZYMES KNOCKDOWN

In order to further understand the function of the three *T. aggressivum* cell wall hydrolytic enzymes genes in the green mould disease, we utilized RNAi to construct three *T. aggressivum* phenotypes, each with a silenced hydrolytic enzyme gene. Gene

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expression of the new phenotypes was evaluated in solitary cultures as well as co-cultivations with the white and the brown commercial mushroom strains. To ensure a successful outcome, the development of an efficient delivery and integration system and the precise design of the sense and antisense sequences under a suitable promoter were mandatory.

Several transformation methods such as polyethyleneglycol, electroporation, and biolistic techniques have previously been performed with a variety of organisms (Goldman *et al.*, 1990; Fungaro *et al.*, 1995 and Herzog *et al.*, 1996). However, they were either not successful or were not reproducible for different reasons with different fungal species. Difficulties arose from the generation of false positives, low level of integration of DNA, poor expression of foreign sequences and DNA modification after integration (Mikosch *et al.*, 2001). *Agrobacterium* transformation system is more suitable for the transformation of filamentous fungi, with up to 100 fold greater efficiency than other transformation systems (De Groot *et al.*, 1998). Therefore, the use of plant-pathogenic soil bacterium *A. tumefaciens* was deemed promising as a transformation method.

A. tumefaciens induces disease by transferring part of its DNA, called transferred DNA (T-DNA) which is located between the right (RB) and the left (LB) borders in the Tumor-inducing (Ti) plasmid. A single strand of T-DNA can integrate randomly through non-homologous recombination into the host genome. Genes that are naturally located within this T-DNA can induce tumorgenesis by expression of the oncogenes on the T-DNA (Sheng and Citovsky, 1996).

The Ti-plasmid contains a number of virulence genes (*vir*) normally found outside the T-DNA region, that are thought to be essential for the recognition of host cells, transfer of T-DNA into the host cell, as well as its integration into the host-cell genome (Hajdukiewicz *et al.*, 1994). *A. tumefaciens* transformation systems utilize a group of small, stable *Agrobacterium* binary vectors that contain a selectable marker gene and multiple cloning sites, so that the desired sequence can be inserted between the left and right borders of the Ti-plasmid (De Groot *et al.*, 1998). Furthermore, the two components of the binary system, the T-DNA and the virulence regions, are placed on two separate plasmids to allow the genetic manipulation of the binary vector.

A. tumefaciens-mediated transformation system was chosen in our study because of its high transformation efficiency and the transformed DNA is mitotically and meiotically stable. However, the *A. tumefaciens* transformation has not been used before for *T. aggressivum*. Therefore, we relied on green fluorescence protein (GFP) and antibiotic selection to evaluate the transformation efficiency of *T. aggressivum* by the *A. tumefaciens* system.

The pFBENGFP vector (Fang *et al.*, 2006) was used in the initial optimization for the ease of monitoring the transformation efficiency using GFP. Since the selectable marker encoded on this vector is benomyl resistance due to an altered β-tubulin gene, it was important to optimize the benomyl concentration required for positive selection due to the high sensitivity of *T. aggressivum* to this antibiotic (Royse *et al.*, 2003). This step is essential since the selection concentration of benomyl varies between the different fungal species (Bollen and Fuchs, 1970; Bollen and Scholten, 1971). A benomyl

concentration of 0.6μg/mL resulted in the absence of *T. aggressivum* growth and was therefore used during the initial development of the transformation system that was monitored by GFP expression. The silencing vectors contain a different selectable marker hygromycin resistance; therefore a similar optimization step was performed to optimize the required hygromycin concentration.

The transformation efficiency and stability were confirmed by using two different methods (Vanden Wymelenberg *et al.*, 1997), the first relied on the detection of GFP expression by confocal microscopy and the second relied on PCR amplification of the GFP gene to detect the physical presence of the gene inside *T. aggressivum*. Since the transcription of GFP will appear only if the T-DNA is integrated inside the *T. aggressivum* genome, the GFP expression over the six days post-transformation proved that the delivery and the integration events were successful.

After the development of the *Agrobacterium*-mediated transformation system of *T. aggressivum*, three plasmids were constructed. Each plasmid contains the sense and antisense sequences of each of the hydrolytic enzymes genes. The promoter used to drive the transcription of these sequences was the *glyceraldehyde-3-phosphate dehydrogenase (GPD)* promoter which is a strong fungal promoter for numerous fungi (Paoletti and Clave, 2007) and should therefore be suitable to drive transcription in *T. aggressivum*.

The silencing plasmids (pEch42-Silence, pPrp1-silence and pGlu-silence) were transformed into *Agrobacterium* which was used subsequently to transform *T. aggressivum*. This would generate three new phenotypes, presumably, with knocked down hydrolytic enzymes genes. However, to validate the efficiency of the silencing, we

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looked at the transcription levels of these hydrolytic enzyme genes in the new phenotypes compared to the wild type *T. aggressivum*. All the new phenotypes had reduced transcription levels indicating that RNAi knockdown was successful. This was the first ever reported gene knockdown in *T. aggressivum* which indicates that, the components of the RNAi machinery, such as Dicer and argonaute proteins, are present and functional.

Silencing efficiency is directly proportional to the loss in mRNA levels as detected by RT-qPCR. The transcription of all these genes was knockdown effectively with ~98% silencing for *prb1*, ~70% for *ech*42 and ~50% silencing for β-1,3-glucanase. In addition, *prb*1 showed more consistent silencing over the seven days of the experiment compared to fluctuating silencing efficiencies with the other two genes.

In addition to the estimated silencing efficiency, the new phenotypes showed reduced mycelia growth compared to the wild type *T. aggressivum*. This important observation suggests the involvement of these enzymes in the fungal growth. Chitinases have been reported to be involved in the growth of *Trichoderma* hyphae (Lorito, 1998).

The expression levels of the three silenced hydrolytic enzymes in the new phenotypes over seven days, in solitary cultures and in co-cultivation with the white and brown commercial mushroom strains, were compared to their expression in the wild type *T. aggressivum* grown on the solitary culture and graphed as percent expression levels. In general, the expression levels of the three hydrolytic enzymes genes of the three phenotypes decreased during the entire seven days either in the solitary culture or under co-cultivation.

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Co-cultivation of the silenced *Trichoderma* phenotypes revealed that the three genes have a direct role in the disease development. The severity of the disease was reduced when mushroom was exposed to any of the knockdown phenotypes compared with the wild-type control. The disease progression required a longer incubation period compared to the wild type. The detected contamination with the wild type *T. aggressivum* in some trays is due to conidia carry over during tray monitoring and data collection. However, such contamination did not affect our overall results since we used several replicates. These results are in agreement with the previous gene expression analysis and both indicate that the three enzymes included in our study are crucial for the growth of *T. aggressivum*. Silencing of these enzymes reduced the growth rate of the new phenotypes compared to the wild type. Also, these enzymes have a role in the interaction between *T. aggressivum* and *A. bisporus*. Silencing of *prb*1 or *ech*42 allowed the growth of *A. bisporus* mycelium, which did not take place with the wild type *Trichoderma*. This indicates that these two genes encodes for proteins that attack or inhibit the growth of *A. bisporus*.

Our results showed that the knockdown of *T. aggressivum* cell wall degrading enzymes *ech*42 or *prb*1 are sufficient to cause significant decrease in green mould disease progression and does not affect *A. bisporus* colonization on compost. This indicates that both enzymes have a very important role in the disease development and their synergistic effect might be essential for green mould disease pathway.

The β-1,3-glucanase showed weak growth of both organisms. The only explanation for such a result is that, β-1,3-glucanase is much more important for the growth of *T. aggressivum*. However, it is not clear if it is involved in the interaction with *A. bisporus*.

The weak *A. bisporus* growth might be due to the production of the other two enzymes *prb*1 and *ech*42 that are highly involved in the interaction. The weak β-glucanase growth suggests greater efficiency of these two enzymes even at low *Trichoderma* growth levels.

In conclusion, the obtained data suggest that the three *T. aggressivum* cell wall degrading enzymes investigated in this study are involved in the parasitic interaction against *A. bisporus*. The role of these enzymes is most likely the main mechanism of the parasitic interaction between the *Trichoderma* and its host. The interaction is mediated by the ability of these enzymes to degrade the cell wall of the host fungus. These enzymes play a role as virulence factors in the disease incidence.

The involvement of these enzymes in the *Trichoderma* life cycle is mainly as growth factors that facilitate the growth and spread of the fungal mycelia (Sahai and Manocha, 1993). However, in the presence of the host fungus, *A. bisporus*, the expression levels of these enzymes increased significantly. This indicates that at this stage the cell wall degrading enzymes plays a dual role: growth factors and virulence factors. Tha latter role is the most crucial during the pathogenic interaction as indicated from our knock-down results. These enzymes are secreted from the parasitic fungus to damage the cell wall of the host's hyphae without even physical contact between the two organisms to facilitate the host organism's hyphal lysis.

Expression of these enzymes in mushroom compost is responsible for the aggressive mycoparasitic action of *T. aggressivum* against *A. bisporus*. This mycoparasitic action is more pronounced in the white mushroom strain than the brown mushroom strain. In compost study showed that *prb*1 gene is more effective in the

interaction between the two fungi than the other two genes (*ech*42 and β-1,3-glucanase). Therefore, the control of the expression levels of these enzymes is a promising tool in the prevention and treatment of green mould disease. Further understanding and controlling of these cell wall degrading enzymes genes might provide promising tool for the treatment and management of the green mould disease infection.

SUMMARY

It is now possible to formulate a preliminary view of the mechanisms of *T. aggressivum* competition with *A. bisporus*. Based upon previous literature, *Trichoderma* species can inhibit the growth of other fungal species by attachment and direct penetration of host hyphae, by the secretion of cell wall degrading enzymes and by the production of toxic secondary metabolites that inhibit fungal growth. The first mechanism has not been observed in the *T. aggressivum* - *A. bisporus* interaction despite extensive searching by several groups including our own. The present work showed that three cell wall degrading enzymes produced by *T. aggressivum* are likely to be key components in the antagonism of *A. bisporus*. Lastly, our group has shown that *T. aggressivum* produces methyl-isocoumarin derivatives that are inhibitory to *A. bisporus*. Thus, the last two mechanisms probably work together to impair *A. bisporus* growth in compost and lead to green mould disease.

Commercial brown strains of *Agaricus* show greater resistance to green mould disease than white or off-white strains. This resistance can be attributed to increased production of an N-acetylglucosaminidase that may inhibit the growth of *T. aggressivum*, a reduced induction of *T. aggressivum* cell wall degrading enzymes as compared to white strains and a lower inhibition of growth by the methyl-isocoumarin secondary metabolite. The last effect may be due to increased production of enzymes such as laccases that may degrade the secondary metabolite. Increased extracellular laccase activity is produced the Shiitake mushroom, *Lentinula edodes*, in response to secondary metabolites produced by *T. harzianum* (Savoie *et al.*, 1998).

Green mould disease onset and severity appears to be a complex series of interactions between the two fungi. These interactions include attempts by either fungus to inhibit the growth of the competitor, and the exchange of signals that induce responses from each fungus.

POSSIBLE FUTURE STUDIES

1- Knockdown of double and triple *T. aggressivum* cell wall degrading enzyme genes at the same time by using different constructs or a single construct with multiple shRNA cassettes. This will help to understand whether or not the effect of these genes is additive and can provide more information about the synergistic effect of the different genes.

2- The use of specific inhibitors against the three *T. aggressivum* cell wall degrading enzymes, especially *prb*1, as a protection and treatment of green mould disease. The successful inhibition of the key enzymes that are responsible for the Pathogenicity of *T. aggressivum* can provide an applicable tool to control and treat green mould disease.

3- Further investigate the involvement of other *T. aggressivum* cell wall degrading enzymes, especially proteases, in the mycoparasitic interaction pathway. Such study will provide more data about the role of other cell wall degrading enzymes and their participation or not in the pathogenic interaction.

4- Study of the regulatory signals and factors responsible for the transactivation of the expression of *T. aggressivum* cell wall degrading enzymes. This can reveal other possible levels to control green mould disease by repressing the expression of these genes.

LITERATURE CITED

Adams, D. J. (2004). Fungal cell wall chitinases and glucanases. *Microbiology*. 150: 2029-2035.

Altschul, S. F.; Madden, T. L.; Schaffer, A. A.; Zhang, J.; Zhang, Z.; Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*. 25: 3389-3402.

Anderson, M. G.; Beyer, D. M. and Wuest, P. J. (2001). Yield comparison of hybrid *Agaricus* mushroom strains as a measure of resistance to *Trichoderma* green mould. *Plant Disease*. 85: 731-734.

Baek, J. M.; Howell, C. R. and Kenerley, C. M. (1999). The role of an extracellular chitinase from *T. virens* Gv29-8 in the biocontrol of *Rhizoctonia solani*. *Current Genetics*. 35: 41-50.

Ballance D. J. (1986). Sequences important for gene expression in filamentous fungi. *Yeast*. 2: 229–236.

Beerhues, L. and Kombrink, E. (1994). Primary structure and expression of mRNAs encoding basic chitinase and 1,3-beta-glucanase in potato. *Plant Molecular Biology*. 24: 353- 367.

Benhamou, N. and Chet, I. (1993). Hyphal interactions between *Trichoderma harzianum* and *Rhizoctonia solani*: ultrastructure and gold cytochemistry of the mycoparasitic process. *Phytopathology*. 83:1062-1071.

Blaiseau, P. L.; Kunz, C.; Grison, R.; Bertheau, Y. and Brygoo, Y. (1992). Cloning and expression of a chitinase gene from the hyperparasitic fungus *Aphanocladium album*. *Current Genetics*. 21: 61-66.

Bollen, G. J. and Fuchs, A. (1970). On the specificity of the in vitro and in vivo antifungal activity of benomyl. *Netherlands Journal of Plant Pathology*. 76: 299-312.

Bollen, G. J. and Scholten, G. (1971). Acquired resistance to benomyl and some other systemic fungicides in a strain *of Botrytis cinerea* in cyclamen. *Netherlands Journal of Plant Pathology*. 77: 83- 90.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. 7: 248-254.

Carsolio, C. A.; Gutierrez, A.; Jimenez, B.; Van Montagu, M. and Herrera-Estrela, A. (1994). Characterization of *ech*-42, a *Trichoderma harzianum* endo-chitinase gene expressed during mycoparasitism. *Proceedings of the National Academy of Sciences. USA*. 91: 10903-10907.

Castle, A.; Speranzini, D.; Rghei, N.; Alm, G.; Rinker, D. and Bissett, J. (1998). Morphological and molecular identification of *Trichoderma* isolates on North American mushroom farms. *Applied and Environmental Microbiology*. 64: 133-137.

Chang, S. T. (1999). World production of cultivated and medicinal mushrooms in 1997 with emphasis on *Lentinus edodes* (Berk.) Sing. In China. *International Journal of Medicinal Mushrooms*. 387-409.

Chaverri, P.; Castlebury, L. A.; Samuels, G. J. and Geiser, D. M. (2003). Multilocus phylogenetic structure of *Trichoderma harzianum/Hypocrea lixii* complex. *Molecular Phylogenetics and Evolution*. 27: 302-313.

Chen, X.; Romaine, C. P.; Ospina-Giraldo, M. D. and Royse, D. J. (1999). A polymerase chain reaction-based test for the identification of *Trichoderma harzianum* biotypes 2 and 4, responsible for the worldwide green mould epidemic in cultivated *Agaricus bisporus*. *Applied Microbiology and Biotechnology*. 52: 246-250.

Chet, I., Harman, G. E. and Baker, R. (1981). *Trichoderma hamatum*: its hyphal interactions with *Rhizoctonia solani* and *Pythium* spp. *Microbial Ecology*. 7: 29-38.

Chet, I. (1987). *Trichoderma*-application, mode of action and potential as a biocontrol agent of soilborne plant pathogenic fungi. *John Wiley. P*: 137-160.

Claydon, N.; Allan, M.; Hanson, J. R. and Avent, A. G. (1987). Antifungal alkyl pyrones of *Trichoderma harzianum*. *Transactions of the British Mycological Society*. 88: 503-513.

Cohen-Kupiec, R.; Broglie, K. E.; Frisem, D.; Broglie, R. M. and Chet, I. (1999). Molecular characterization of a novel ß-1,3-exoglucanase related to mycoparasitism of *Trichoderma harzianum*. *Gene*. 226: 147-154.

Cortes, C.; Gutierrez, A.; Olmedo, V.; Inbar, J.; Chet, I. and Herrera-Estrella, A. (1998). The expression of genes involved in parasitism by *Trichoderma harzianum* is triggered by a diffusable factor. *Molecular and General Genetics*. 260: 218-225.

Crisan, E. W. and Sands, A. (1978). Nutritional value. In: Chang ST, Hayes WA (eds) The biology and cultivation of edible mushrooms. *Academic Press. P:* 172-189.

De Groot, M.; Bundock, P.; Hooykaas, P. and Beijersbergen, A. (1998). *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. *Nature Biotechnology*. 16: 839-842.

De la Cruz, J. A.; Hidalgo-Gallego, J. M.; Lora, T.; Benı́tez, J. A.; Pintor-Toro and Llobell, A. (1992). Isolation and characterization of three chitinases from *Trichoderma harzianum. European Journal of Biochemistry.* 206: 859-867.

De la Cruz, J.; Rey, M.; Lora, J. M.; Hidalgo-Gallego, A.; Domínguez, F.; Pintor-Toro, J. A.; Llobell, A. and Benítez, T. (1993). Carbon source control on β-glucanase, chitobiase and chitinase from *Trichoderma harzianum. Archives of Microbiology.* 159: 316-322.

De la Cruz, J.; Pintor-Toro, J. A.; Benítez, T. and Llobell, A. (1995). Purification and characterization of an endo-beta-1,6-glucanase from *Trichoderma harzianum* that is related to its mycoparasitism. *Journal of Bacteriology.* 177: 1864-1871.

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

De Marco, L.; Valadares-Inglis, C. and Felix, R. (2003). Production of hydrolytic enzymes by *Trichoderma* isolates with antagonistic activity against *Crinipellis perniciosa*, the causal agent of witches' broom of cocoa. *Brazilian Journal of Microbiology.* 34: 33-38.

Deane, E. E.; Whipps, J. M.; Lynch, J. M. and Peberdy, J. F. (1998). The purification and characterization of a *Trichoderma harzianum* exochitinase BBA Protein Struct. *Molecular Enzymology.* 1383: 101-110.

Draborg, H.; Kauppinen, S.; Dalboge, H. and Christgau, S. (1995). Molecular cloning and expression in *S. cerevisiae* of two exochitinases from *Trichoderma harzianum. Biochemistry and Molecular Biology International.* 36: 781-791.

Dubos, B. (1987). Fungal antagonism in aerial agrobiocenoses. In: Innovative approaches to plant disease control. Wiley, New York. P. 107-135.

Elad, Y.; Chet, I. and Henis, Y. (1982). Degradation of plant pathogenic fungi by Trichoderma harzianum. *Canadian Journal of Microbiology.* 28: 719-725.

Elad, Y.; Barak, R.; Chet, I. and Henis, Y. (1983). Ultrastructural studies of the intraction between *Trichoderma* spp. *Phytopathogenic.* 107: 168-175.

Elad, Y.; Barak, R. and Chet, I. (1984). Parasitism of *Sclerotium rolfsii* by *Trichoderma harzianum. Soil Biology and Biochemistry.* 16: 381-386.

Elad, Y. and Kapat, A. (1999). The role of *Trichoderma harzianum* protease in the biocontrol of *Botrytis cinerea. European Journal of Plant Pathology.* 105: 177-189.

El-Katatny, M. H.; Gudelj, M.; Robra, K. H.; Elnaghy, M. A. and Gübitz, G. M. (2001). Characterization of a chitinase and an endo-ß-1,3- glucanase from *T. harzianum*

Rifai T-24 involved in control of the phytopathogen *Sclerotium rolfsii*. *Applied Microbiology and Biotechnology*. 56: 137-143.

Fang, W.; Pei, Y. and Bidochka, M. J. (2006). Transformation of *Metarhizium anisopliae* mediated by *Agrobacterium tumefaciens*. *Canadian Journal of Microbiology*. 52: 623-626.

Faull, J. L.; Graeme-Cook, K. A. and Pilkington, B. L. (1994). Production of an isonitrile antibiotic by an UV-induced mutant of *Trichoderma harzianum*. *Phytochemistry*. 36: 1273-1276.

Fergus, C. L.; Sinden, J. W.; Schisler, L. C. and Sigel, E. M. (1963). Possible detrimental effect of *Pythium artotrogos* on the cultivated mushroom. *Phytopathology*. 53: 1360-1362.

Fletcher, J. Y. and Yarham, D. J. (1976). The incidence of benomyl tolerance in *Verticillium fungicola*, *Mycogone perniciosa* and *Hypomyces rosellus* in mushroom crops. *Annals of Applied Biology*. 84: 343-353.

Fletcher, J. T.; Connolly, G.; Mountfield, E. X. and Jacobs, L. (1980). The disappearance of benomyl from mushroom casing. *Annals of Applied Biology*. 95: 73-82.

Fletcher, J. T.; Smewin, B. J. and O'Brien, A. (1990). *Pythium oligandrum* associated with a cropping disorder of *Agaricus bisporus*. *Plant Pathology*. 39: 603-605.

Flores, A.; Chet, I. and Herrera-Estrella, A. (1997). Improved biocontrol activity of *Trichoderma harzianum* by over-expression of the protease-encoding gene *prb*1. *Current Genetics*. 31: 30-37.

Fungaro, M. H. P.; Rech, E.; Muhlen, G. S.; Vainstein, M. H.; Pascon, R. C.; deQueiroz, M. V.; Pizzirani-Kleiner, A. A. and de Azevedo J. L. (1995). Transformation of *Aspergillus nidulans* by microprojectile bombardment on intact conidia, *FEMS Microbiology Letters*. 125: 293-298.

Gan, Z.; Yang, J.; Tao, N.; Yu, Z. and Zhang, K. Q. (2007). Cloning and expression analysis of a chitinase gene Crchi1 from the mycoparasitic fungus *Clonostachys rosea* (syn. *Gliocladium roseum*). *Journal of Microbiology*. 45: 422-430.

Garcia, I.; Lora, J. M.; De la Cruz, J. T.; Benı́tez, T.; Llobel, A. and Pintor-Toro, J. A. (1994). Cloning and characterization of a chitinase (CHIT 42) cDNA from the mycoparasitic fungus *Trichoderma harzianum*. *Current Genetics*. 27: 83-89.

Gaze, R. H.; Calvo-Bado, L.; Challen, M. P.; Adie, B. A. T. and Romaine, C. P. (2000). A new virus disease of *Agaricus bisporus*. *Mushroom science*. 15: 701-705.

Geremia, R. A. Goldman, G. H.; Jacobs, D.; Ardiles, W.; Vila, S. B.; Van Montagu, M. And Herrera-Estrella, A. (1993). Molecular characterization of the protease-

encoding gene, *prb*1, related to mycoparasitism by *Trichoderma harzianum*. *Molecular Microbiology*. 8: 603-613.

Ghisalberti, E. L. and Rowland, C. Y. (1993). Antifungal metabolites from *Trichoderma harzianum*. *Journal of Natural Products*. 56: 1799-1804.

Goettel, M.; St.Leger, R.; Rizzo, N.; Staples, R. and Roberts, D. (1989). Ultrastructural localization of a cuticle-degrading protease produced by the entomopathogenic fungus *Metarhizium anisopliae* during penetration of host (*Manduca sexta*) cuticle. *J.Gen.Microbiol*. 135: 2233-2239.

Goettel, M.; St. Leger, R.; Bhairi, S.; Jung, M.; Oakley, B.; Roberts, D. and Staples, R. (1990). Pathogenicity and growth of *Metarhizium anisopliae* stably transformed to benomyl resistance. *Current Genetics*. 17: 129-132.

Goldman, G. H.; Geremia, R.; vanMontagu, M. and Herrera-Estrella, A. (1990). Transformation of filamentous fungi by high voltage electroporation, Bulletin 1352, *Bio-Rad Laboratories, Hercules CA*.

Grogan, H. M. and Gaze, R. H. (2000). Fungicide resistance among *Cladobotryum* spp. causal agents of cobweb disease of the edible mushroom *Agaricus bisporus*. *Mycological Research*. 104: 357-364.

Grogan, H. (2009). Challenges facing mushroom disease control in the 21st century. (pest control). *Mushroom News*.

Gurr, S. J.; Unkles, S. E and Kinghorn, J. R. (1987). The structure and organisation of nuclear genes of filamentous fungi. Gene Structure in Lower Eukaryotes. *Oxford, IRL Press*. 93-139.

Guthrie, J. L.; Khalif, S. and Castle A. J. (2005). An improved method for detection and quantification of chitinase activities. *Canadian Journal of Microbiology*. 51: 491-495.

Gutherie, J. L. and Castle, A. J. (2006). Chitinase production during interaction of *Trichoderma aggressivum* and *Agaricus bisporus*. *Canadian Journal of Microbiology*. 52: 961-967.

Hajdukiewicz, P.; Svab, Z. and Maliga, P. (1994). The small, versatile pPXP family of *Agrobacterium* binary vectors for plant transformation. *Plant Molecular Biology*. 25: 989-994.

Haran, S.; Schickler, H.; Pe'er, S.; Logemann, S.; Oppenheim, A. and Chet, I. (1993). Increase constitutive chitinase activity in transformed *Trichoderma harzianum*. *Biological Control*. 3: 101-108.

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Haran, S.; Schickler, H.; Oppenheim, A. B. and Chet, I. (1995). New components of the chitinolytic system of *Trichoderma harzianum*. *Mycological Research.* 99: 441-446.

Haran, S.; Schickler, H. and Chet, I. (1996). Molecular mechanisms of lytic enzymes involved in the biocontrol activity of *Trichoderma harzianum*. *Microbiology.* 142: 2321-2331.

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

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

Herzog, R. W.; Daniell, H.; Singh, N. K. and Lemke, P. A. (1996). A comparative study on transformation of *Aspergillus nidulans* by microprojectile bombardment of conidia and a more conventional procedure using protoplasts treated with polyethylene glycol (PEG). *Applied Microbiology and Biotechnology.* 45: 333-337.

Ingold, C. T. and Hudson, H. J. (1993). The biology of fungi. 6th Ed. Chapman and Hall, New York, N. Y.

Inoue, H.; Nojima, H. and Okayama, H. (1990). High efficiency transformation of *Escherichia coli* with plasmids. *Gene.* 96: 23-28.

Jeffries, P. and Young, TWK. (1994). Interfungal parasitic relationships. *Wallingford,* Oxon UK: CAB.

Kendrick, B. (2000). The Fifth Kingdom, 3rd Ed. *Focus Publishing, Massachusetts.*

Kim, J.; Kim, H.; Lee, Y.; Yang, K.; Byun, S. and Han, K. (2006). A simple and economical short-oligonucleotide-based approach to shRNA generation. *Journal of Biochemistry and Molecular Biology.* 39: 329-334.

Koenraadt, H.; Somerville, S. C. and Jones, A. L. (1992). Characterization of mutations in the beta-tubulin gene of benomyl-resistant field strains of *Venturia inaequalis* and other plant pathogenic fungi. *Phytopathology.* 82: 1348-1354.

Koga, K.; Iwamoto, Y.; Sakamoto, H.; Hatano, K.; Sano, M. and Kato, I. (1991). Purification and characterization of beta-*N*-acetylhexosaminidase from *Trichoderma harzianum*. *Agricultural Biology and Chemistry.* 55: 2817-2823.

Kollar, R.; Petrakova, E.; Ashwell, G.; Robbins, P. W. and Cabib, E. (1995). Architecture of the yeast cell wall; The linkage between chitin and β (1-3)-glucan. *Journal of Biological Chemistry.* 270: 1170-1180.

Krämer A. (1996). The structure and function of proteins involved in mammalian pre-mRNA splicing. *Annual Review of Biochemistry.* 65: 367-409.

Krupke, O. A.; Castle, A. J. and Rinker, D. L. (2003). The North American mushroom competitor, *Trichoderma aggressivum f. aggressivum*, produces antifungal compounds in mushroom compost that inhibits mycelial growth of the commercial mushroom *Agaricus bisporus. Mycological Research.* 107: 1467-1475.

Kulling, C.; Mach, R. L.; Lorito, M. and Kubicek, C. P. (2000). Enzyme diffusion from *T. atroviride* (=*T. harzianum* P1) to *R. solani* is a prerequisite for triggering of *Trichoderma ech*42 gene expression before mycoparasitic contact. *Applied and Environmental Microbiology.* 66: 2232-2234.

Lieckfeldt, E.; Kullnig, M.; Kubicek, C. P.; Samuels, G. J. and Börner, T. (2001). *Trichoderma aureoviride*: Phylogenetic position and characterization. *Mycological Research.* 105: 313-322.

Lora, J. M.; de la Cruz, J.; Benitez, T.; Llobell, A. and Pintor-Toro, A. (1995). Molecular characterization and heterologous expression of an endo-ß-1,6-glucanase from the mycoparasitic fungus *T. harzianum. Molecular and General Genetics.* 28: 478-483.

Lorito, M. (1998). Chitinolytic enzymes and their genes. *Trichoderma* and *Gliocladium. Taylor and Francis Ltd., London, UK.* 2: 73-99.

Lorito, M.; Harman, G. E.; Hayes, C. K.; Broadway, R. M.; Tronsmo, A.; Woo, S. L. and Di Pietro, A. (1993). Chitinolytic enzymes produced by *Trichoderma harzianum*: antifungal activity of purified endochitinase and chitobiosidase. *Phytopathology.* 83: 302-307.

Lorito, M.; Hayes, C. K.; Di Pietro, A.; Woo, S. L. and Harman, G. E. (1994a). Purification, characterization, and synergistic activity of a glucan 1,3-b-glucosidase and an *N*-acetyl-b-glucosaminidase from *Trichoderma harzianum. Phytopathology.* 84: 398-405.

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

Lu, Z. X.; Laroche, A. and Huang, H. C. (2005). Isolation and characterization of chitinases from *Verticillium lecanii. Canadian Journal of Microbiology.* 51: 1045-1055.

Manocha, M. S. and Zhonghua, Z. (1997). Immunocytochemical and cytochemical localization of chitinase and chitin in the infected hosts of a biotrophic mycoparasite, *Piptocephalis virginiana. Mycologia.* 89: 185-194.

Markovich, N. A. and Kononova, G. L. (2003). Lytic enzymes of *Trichoderma* and their role in plant defense from fungal disease*s. Applied Biochemistry and Microbiology.* 39: 341-351.

McKay, G. J.; Egan, D.; Morris, E.; Scott, C. and Brown, A. E. (1999). Genetic and morphological characterization of *Cladobotryum* species causing Cobweb disease of mushrooms. *Applied and Environmental Microbiology.* 65: 606-610.

Michalenko, G. O.; Hohl, H. R. and Rast, D. (1976). Chemistry and architecture of the mycelial wall of *Agaricus bisporus. Journal of General Microbiology.* 92: 251-265.

Migheli, Q.; Gonzales-Candelas, L.; Dealessi, L.; Camponogara, A. and Ramon-Vidal, D. (1998). Transformants of *Trichoderma longibrachiatum* overexpressing the β-1,4 endoglucanase gene *egl*1 show enhanced biocontrol of *Pythium ultimum* on cucumber. *Phytopathology.* 88: 673-677.

Mikosch, T.; Lavrijssen, B.; Sonnenberg, A. and van Griensven, L. (2001). Transformation of the cultivated mushroom *Agaricus bisporus* (Lange) using T-DNA from Agrobacterium tumefaciens. *Current Genetics.* 39: 35-39.

Moore-Landecker, E. (1996). Fundamentals of the fungi. 4th ed. Prentice-Hall International Limited, London.

Morais, M. H.; Ramos, A. C.; Matos, N. and Santos-Oliveira, E. J. (2000). Note: production of shiitake mushroom *(Lentinus edodes)* on ligninocellulosic residues. *Food Science and Technology International.* 6: 123-128.

Morissette. D.; Seguin, P. and Jabaji, S. (2006). Expression regulation of the endochitinase-encoding gene sechi44 from the mycoparasite *Stachybotrys elegans. Canadian Journal of Microbiology.* 52: 1103-9.

Mullis, K.; Faloona, F.; Scharf, S.; Saiki, R.; Horn, G. and Erlich, H. (1986). Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. *Cold Spring Harbor Symposium on Quantitative Biology.* 1: 263-273.

Mumpuni, A., Sharma, H. S. S. and Brown, A. E. (1998). Effect of metabolites produced by *Trichoderma harzianum* biotypes and *Agaricus bisporus* on their respective growth radii in culture. *Applied and Environmental Microbiology.* 64: 5053-5056.

Muthumeenakshi, S.; Mills, P. R.; Brown, A. E. and Seaby, D. A. (1994). Intraspecific molecular variation among *Trichoderma harzianum* isolates colonizing mushroom compost in the British Isles. *Microbiology.* 140: 769-777.

Muthumeenakshi, S. and Mills, P. R. (1995). Detection and differentiation of fungal pathogens of *Agaricus bisporus. Mushroom Science.* 14: 603-610.

Muthumeenakshi, S.; Brown, A. E. and Mills, P. R. (1998). Genetic comparison of the aggressive weed mould strains of *Trichoderma harzianum* from mushroom

compost in North America and the British Isles. *Mycological Research.* 102: 385-390.

Nakayashiki, H. (2005). RNA silencing in fungi: Mechanisms and applications. *FEBS Letters.* 579: 5950-5957.

National Agricultural Statistics Service (2009). Mushrooms. United States Department of *Agriculture. Online, publication Pr 2.*

Noronha, E. F. and Ulhoa, C. J. (1996). Purification and characterization of an endo-β-1,3-glucanase from *Trichoderma harzianum. Canadian Journal of Microbiology.* 24: 1039-1044.

Noronha, E. F. and Ulhoa, C. J. (2000). Characterization of a 29-kDa β- 1,3-glucanase from *Trichoderma harzianum. FEMS Microbiology Letters.* 183: 119-123.

Ochman, H.; Gerber, A.S. and Hartl, D.L. (1988). Genetic applications of an inverse polymerase chain reaction. *Genetics.* 120: 621-623.

Orozco, I.; Ortiz, L.; Elorza, M. V.; Ruiz-Herrera, J. and Sentandreu, R. (2002). Cloning and characterization of *PRB1*, a *Candida albicans* gene encoding a putative novel endoprotease B and factors affecting its expression. *Research in Microbiology.* 153: 611-620.

Ospina, M.; Royse, D.; Chen, X. and Romaine, C. (1999). Molecular phylogenetic analyses of biological control strains of *Trichoderma harzianum* and other biotypes of *Trichoderma* spp. Associated with mushroom green mould. *Phytopathology.* 89: 308-313.

Page, R. D. (2002). Visualizing phylogenetic trees using TreeView. *Current Protocols in Bioinformatics.* 6: Unit 6.2.

Paoletti, M. and Clave, C. (2007). The Fungus-Specific HET Domain Mediates Programmed Cell Death in *Podospora anserine. Eukaryot Cell.* 11: 2001-2008.

Peberdy, J. F. (1990). *Biochemistry of Cell Walls and Membranes in Fungi,* Kuhn, P. J., Trinci, A. P. J., Jung, M. J., Goosey, M. W. and Copping, L. G., Eds., Heidelberg: *Springer.* 5-24.

Peterbauer, C. K.; Lorito, M.; Hayes, C.K.; Harman, G. E. and Kubicek, C. P. (1996). Molecular cloning and expression of the nag1 gene (*N*-acetyl-beta-D-glucosaminidase-encoding gene) from *Trichoderma harzianum* P1. *Current Genetics.* 30: 325-331.

Pishko, E. J.; Kirkland, T. N. and Cole, G. T (1995). Isolation and characterization of two chitinase-encoding genes (*cts1*, *cts2*) from the fungus *Coccidioides immitis. Gene.* 167: 173-177

Pitson, S. M.; Seviour, R. J. and McDougall, B. M. (1993). Non cellulolytic fungal *β-glucanases*: their physiology and regulation. *Enzyme and Microbial Technology.* 15: 178-192.

Pozo, M. J.; Baek, J. M.; García, J. M. and Kenerley, C. M. (2004). Functional analysis of *tvsp1*, a serine protease-encoding gene in the biocontrol agent *Trichoderma virens. Fungal Genetics and Biology.* 41: 336-348.

Punt, P.; Oliver, R.; Dingemanse, M.; Pouwels, P. and van den Hondel, C. (1987). Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli. Gene.* 56: 117-124.

Ramot, O.; Cohen-Kupiec, R. and Chet, I. (2000). Regulation of β-1,3- glucanase by carbon starvation in the mycoparasite *T. harzianum. Mycological Research.* 104: 415-420.

Ranzani, M. R. and Sturion, G. L. (1998). Amino acid composition evaluation of *Pleurotus* spp cultivated in banana leaves. *Archivos Latinoamericanos de Nutricion.* 48: 339-348.

Rifai, M. A. (1969). A revision of the genus *Trichoderma. Mycological Papers.* 116: 1-56.

Rinker, D. (1993). Disease management strategies for *Trichoderma* mould: a summary of seminar by Don Betterley. *Mushroom World.* 4: 3-5.

Rinker, D. L. and Alm, G. (1996). Are brown mushroom strains more resistant to green mould? *Mushroom Green Mould Round Table, Pennsylvania State University.*

Rinker, D. L.; Alm, G. and Castle, A. (1997). Distribution of green mould on infected mushroom farms. *Mushroom World.* 8: 71-75.

Rinker, D. L. and Alm, G. (1998). Efficacy and limitations of mushroom grain spawn treated with benomyl against green mould disease of the cultivated mushroom. *Mushroom News: Science and Technology.* 11: 6-11.

Rinker, D. and Chalmers, W. (1998). Specialty mushroom industry in Canada. *Mushroom World.* 9: 7-8.

Rinker, D. L. and Castle, A. (2005). Green moulds and bacterial blotch of the cultivated mushroom, *Agaricus bisporus.* From April 8-12, 2005, *the Fifth International Conference on Mushroom Biology and Mushroom Products* (ICMBMP), *Shanghai, China.*

Romaine, C. P.; Royse, D. J. and Schlagnhaufer, B. (2005). Superpathogenic *Trichoderma* resistant to TopsinM found in Pennsylvania and Delaware. *Mushroom News.* 53: 6-9.

Royse, D. J. and Romaine, C. P. (2003). Spawn application of fungicides for control of *Trichoderma* green mould of mushrooms. *Fungic. Nematicide Tests.*

Sahai, A. S. and Manocha, M. S. (1993). Chitinases of fungi and plants: their involvement in morphogenesis and host–parasite interaction. *FEMS Microbiology Reviews.* 11: 317-338.

Samac, D. A.; Hironaka, C. M.; Yallaly, P. E. and Shah, D. M. (1990). Isolation and Characterization of the Genes Encoding Basic and Acidic Chitinase in *Arabidopsis thaliana. Plant Physiology.* 93: 907-914.

Sambrook, J.; Fritsch, E. F. and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, *Cold Spring Harbor, NY.*

Samuels, G. J.; Pardo-Schultheiss, R.; Hebbar, P.; Lumsden, R. D.; Bastos, C. N.; Costa, J. C. and Bezerra, J. L. (2000). *Trichoderma stromaticum*, sp. nov., a parasite of the cacao witches broom pathogen. *Mycological Research.* 104: 760-764.

Samuels, G. J.; Dodd, S. L.; Gams, W.; Castlebury, L. A. and Petrini, O. (2002). *Trichoderma* species associated with the green mould epidemic of commercially grown *Agaricus bisporus. Mycologia.* 94: 146-170.

Savoie, J. M.; Mata, G. and and Billette, C. (1998). Extracellular laccase production during hyphal interactions between *Trichoderma* sp. and Shiitake, *Lentinula edodes. Applied Microbiology and Biotechnology.* 49: 589-593.

Schirmbock, M.; Lorito, M.; Wang, Y. L.; Hayes, C. K.; Arsian-Atac, I.; Scala, F.; Harman, G. E. and Kubicek, C. P. (1994). Parallel formation and synergism of hydrolytic enzymes and peptabiol antibiotics: molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against phytopathogenic fungi. *Applied and Environmental Microbiology.* 60: 4364-4370.

Seaby, D. A. (1987). Infection of mushroom compost by *Trichoderma* species. *Mushroom Journal.* 179: 355-361.

Seaby, D. A. (1989). Further observations on *Trichoderma. Mushroom Journal.* 197: 147-151.

Seaby, D. A. (1996a). Differentiation of *Trichoderma* taxa associated with mushroom production. *Plant Pathology.* 45: 905-912.

Seaby, D.A. (1996b). Investigation of the epidemiology of green mould of mushroom (*Agaricus bisporus*) compost caused by *Trichoderma harzianum. Plant Pathology.* 45: 913-923.

Seip, E.; Woloshuk, C.; Payne, G. and Curtis, S. (1990). Isolation and sequence of a beta-tubulin gene from *Aspergillus flavus* and its use as a selectable marker. *Applied and Environmental Microbiology.* 56: 3686-3692.

Seyis, I. and Aksoz, N. (2005). Effect of carbon and nitrogen sources on xylanase production by *Trichoderma harzianum* 1073 D3. *International Biodeterioration and Biodegradation*. 55: 115-119.

Sheng, J. and Citovsky, V. (1996). *Agrobacterium*-plant cell DNA transport: Have virulence proteins, will travel. *The Plant Cell*. 8: 1699-1710.

Sinden, J. and Hauser, E. (1950). Report on two new mushroom diseases. *Mushroom Science*. 1: 96-100.

Sinden, J. and Hauser, E. (1953). Nature and control of three mildew diseases of mushrooms in America. *Mushroom Science*. 2: 177-180.

Sivan, A. and Chet, I. (1989). Degradation of fungal cell walls by lytic enzymes of *Trichoderma harzianum*. *Journal of General Microbiology*. 135: 675-682.

Small, C. L.; Donaldson, N. and Bidochka, M. J. (2004). Nucleotide sequence variation does not relate to differences in kinetic properties of neutral trehalase from the insect pathogenic fungus *Metarhizium anisopliae*. *Current Microbiology*. 48: 428-234.

Stipp, D. (2003). Biotech's Billion Dollar Breakthrough: A technology called RNAi has opened the door to major new drugs. Already it's revolutionizing gene research. *Fortune*. 147: 196.

Svitil, A. L. and Kirchman, D. L. (1998). A chitin-binding domain in a marine bacterial chitinase and other microbial chitinases: implications for the ecology and evolution of 1,4-beta-glycanases. *Microbiology*. 144: 1299-1308.

Thornton, C. R.; Pitt, D.; Wakley, G. E. and Talbot, N. J. (2002). Production of a monoclonal antibody specific to the genus *Trichoderma* and closely related fungi, and its use to detect *Trichoderma* spp. in naturally infested composts. *Microbiology*. 148: 1263-1279.

Thrane, C.; Tronsmo A. and Jensen D. F. (1997). Endo-1,3-β-glucanase and cellulase from *Trichoderma harzianum* : purification and partial characterization, induction of and biological activity against plant pathogenic *Pythium* spp. *European journal of plant pathology*. 103: 331-344.

Tijsterman, M. and Plasterk, R. (2004). Dicers at Risc: The mechanism of RNAi. *Cell*. 117: 1-4.

Turner, D.; Kovacs, W.; Kuhls, K.; Lieckfeldt, E.; Peter, B.; Arisan-Atac, I.; Strauss, J.; Samuels, G. J.; Börner, T. and Kubicek, C. P. (1997). Biogeography and phenotypic variation in *Trichoderma* sect. *Longibrachiatum* and associated *Hypocrea* species. *Mycological Research*. 101: 449-459.

Ulhoa, C. J. and Peberdy, J. F. (1991). Regulation of chitinase synthesis in *Trichoderma harzianum*. *Journal of General Microbiology*. 137: 2163-2169.

Vanden Wymelenberg, A. J.; Cullen, D.; Spear, R.; Shoenike, B. and Andrews, J. (1997). Expression of green fluorescent protein in *Aureobasidium pullulans* and quantification of the fungus on leaf surfaces. *Biotechniques* 23: 686-690.

Vazquez-Garciduenas, S.; Leal-Morales, C.A. and Herrera- Estrella, A. (1998). *Applied and Environmental Microbiology*. 64: 1442-1446.

Viterbo, A.; Haran, S.; Friesem, D.; Ramot, O. and Chet, I. (2001). Antifungal activity of a novel endochitinase gene (chit36) from *T. harzianum* Rifai TM. *FEMS Microbiology Letters*. 200: 169-174.

Viterbo, A.; Ramot, O.; Chemin, L. and Chet, I. (2002). Significance of lytic enzymes from *Trichoderma* spp. in the biocontrol of fungal plant pathogens. *Antonie Van Leeuwenhoek*. 81: 549-556.

Viterbo, A.; Harel, M. and Chet, I. (2004). Isolation of two aspartyl proteases from *Trichoderma asperellum* expressed during colonization of cucumber roots. *FEMS Microbiology Letters*. 238: 151-158.

Walti, M.; Villalba, C.; Buser, R.; Grunler, A.; Aebi, M. and Kunzler, M. (2006). Targeted gene silecing in the model mushroom *Coprinopsis cinerea* (*Coprinus cinereus*) by expression of homologous hairpin RNAs. *Eukaryotic Cell*. 5: 732-744.

Wesley, S.; Helliwell, C.; Smith, N.; Wang, M.; Rouse, D.; Liu, Q.; Gooding, P.; Singh, S.; Abbott, D.; Stoutjesdijk, P.; Robinson, S.; Gleave, A.; Green, A. and Waterhouse, P. (2001). Construct design for effective and high-throughput gene silencing in plants. *Plant Journal*. 27: 581-590.

Williams, J.; Clarkson, J. M.; Mills, P. R. and Cooper, R. M. (2003). Saprotrophic and mycoparasitic components of aggressiveness of *Trichoderma harzianum* groups toward the commercial mushroom *Agaricus bisporus*. *Applied and Environmental Microbiology*. 69: 4192-4199.

Woo, S.; Donzelli, B.; Scala, F.; Mach, R.; Harman, G.; Kubicek, C.; Sorbo, G. and Lorito, M. (1999). Disruption of the *ech*42 (endochitnase-encoding) gene affects biocontrol activity in *Trichoderma harzianum* P1. *Molecular Plant-Microbe Interactions*. 12: 419-429.

Wuest, P. J.; Duffy, M. D. and Royse, D. J. (1985). Six Steps to Mushroom Growing. *The Pennsylvania State University Extension Bulletin, Special Circular*. 268.

Wuest P. J.; Anton, L. A. and Beyer, D. M. (1996). Mushroom crop losses associated with *Trichoderma* green mould when compost was infested prior to casing was CAC's or deepscratched. *Mushroom Green Mould Round Table*. PSU. 43.

Yang, Q.; Yang, L.; Liu, P.; Li, S. and Song, J. (2007). *Agrobacterium tumefaciens-*mediated transformation of *Trichoderma harzianum. KMITL Science and Technology Journal.* 7: S2.

Yarden, O.; Salomon, R.; Katan, J. and Aharonson, N. (1990). Involvement of fungi and bacteria in enhanced and nonenhanhanced biodegradation of carbendazim and other benzimidazole compounds in soil. *Canadian Journal of Microbiology.* 36: 15-23.

Zeilinger, S.; Galhaup, C.; Payer, K.; Woo, S. L.; Mach, R. L.; Fekete, C.; Lorito, M. and Kubicek, C. P. (1999). Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. *Fungal Genetics and Biology.* 26: 131-140.

Zeilinger, S. (2004). Gene disruption in *Trichoderma atroviride* via *Agrobacterium-*mediated transformation. *Current Genetics.* 45: 54-60.

APPENDIX

A- The *A. bisporus* N-acetylglucosaminidase sequence. The data showed several different peptide sequences.

Ion Current (Sum): 1.8e7
Ion Current (Avg): 3.1e6
MS/MS Spectra: 52

Sample: BH222-GT (a) **User:** Abubaker, K. **Db:** nr (01/03/06)
Sample ID: 25612 **Dir:** BH222-GT (a)
Sample: BH222-GT2 (b) **Db:** nr (01/03/06)
Sample ID: 25612 **Dir:** BH222-GT2 (b)
Sample: BH222-GT3 (c) **Db:** nr (01/03/06)
Sample ID: 25612 **Dir:** BH222-GT3 (c)
Sample: BH222-GT (d) **Db:** est_others (03/25/05)
Sample ID: 25612 **Dir:** BH222-GT_500 (d)
Ion Current (Sum): 1.8e7
Ion Current (Avg): 3.1e6
 # MS/MS Spectra: 52
A gi|10282058 MS/MS **Spectra: 3 Sum TIC: 1.5e5 Avg TIC: 5.0e4**
AB061 Primordium cDNA library Agaricus bisporus cDNA 5' similar to Tyrosinase, mRNA sequence
Sequence Reference TIC Sf D Scan
(-) EWAQAAEDLR gi|10282058 4.1e4 0.85 d 3274
(-) EWAQAAEDLR gi|10282058 7.1e4 0.82 d 3281
(-) LDIVDFVR gi|10282058 3.6e4 0.74 d 4491
B gi|19914831 MS/MS Spectra: 3 Sum TIC: 1.4e5 Avg TIC: 4.6e4
Fmn oxidoreductase protein [Methanosarcina acetivorans str. C2A] gi|20089879|ref|NP_615954.1| Fmn oxidoreductase protein [Methanosarcina acetivorans C2A]

Sequence Reference TIC Sf D Scan
(K) YGGSLENR gi|19914831 +30 3.1e4 0.76 c 1701
(R) TDKYGGSLENR gi|19914831 +18 9.3e4 0.57 a 2170
(R) TDKYGGSLENR gi|19914831 +18 1.4e4 0.08 c 1766
C gi|181402 MS/MS Spectra: 8 Sum TIC: 1.1e5 Avg TIC: 1.4e4
epidermal cytokeratin 2 [Homo sapiens] gi|2565257|gb|AAB81946.1| keratin 2e [Homo sapiens] gi|547754|sp|P35908|K22E_HUMAN Keratin,
type II cytoskeletal 2 epidermal (Cytokeratin-2e) (K2e) (CK 2e)
Sequence Reference TIC Sf D Scan
(K) NVQDAIADAEQR gi|181402 +1 1.5e4 0.90 a 3134
(K) VDLLNQEIEFLK gi|181402 +1 2.3e4 0.86 a 4831
(K) GGSISGGGYGSGGGK gi|181402 +2 2.6e3 0.80 a 1971
(K) GGSISGGGYGSGGGK gi|181402 +2 5.3e3 0.75 c 1641
(R) TAAENDFVTLK gi|181402 +2 1.6e4 0.70 a 3301
(R) HGGGGGGFGGGGFGSR gi|181402 +1 1.6e4 0.34 a 2622
(R) YLDGLTAER gi|181402 +1 2.8e4 0.30 a 3138
(R) SKEEAEALYHSK gi|181402 +1 6.2e3 0.15 a 2267
D gi|20260807 MS/MS Spectra: 4 Sum TIC: 1.0e5 Avg TIC: 2.6e4
Hsp70 protein 1 [Rhizopus stolonifer]
Sequence Reference TIC Sf D Scan
(K) NGLESYAYNLR gi|20260807 +5 2.5e4 0.85 b 3012
(K) NGLESYAYNLR gi|20260807 +5 4.6e4 0.83 c 3011
(K) NGLESYAYNLR gi|20260807 +5 3.1e4 0.65 a 3777
(K) YKAEDEAAASR gi|20260807 2.3e3 0.34 a 1841
E gi|7428712 MS/MS Spectra: 5 Sum TIC: 9.2e4 Avg TIC: 1.8e4
keratin 1, type II, cytoskeletal - human
Sequence Reference TIC Sf D Scan
(R) GGGGGGYGSGGGSSYGSGGGSYGSGGGGGGGR gi|7428712 +2 1.5e4 0.98 a 2315
(K) NM*QDM*VEDYR gi|7428712 +3 4.7e3 0.74 a 2404
(K) QISNLQQSISDAEQR gi|7428712 +4 4.3e4 0.68 a 3377
(K) NMQDM*VEDYR gi|7428712 +3 1.8e4 0.62 a 2877
(R) M*SGECAPNVSVTVSTSHTSISGGGSR gi|7428712 1.2e4 0.09 a 3007
F gi|1051292 MS/MS Spectra: 5 Sum TIC: 8.6e4 Avg TIC: 1.7e4

NADP-dependent glutamate dehydrogenase (NADP+) [Agaricus bisporus] gi|1706404|sp|P54387|DHE4_AGABI NADP-specific glutamate
dehydrogenase (NADP-GDH) (NADP-dependent glutamate dehydrogenase)
Sequence Reference TIC Sf D Scan
(R) VQYNSALGPYK gi|1051292 +40 1.9e4 0.79 a 2986
(R) VTWEDDQGKPQVNR gi|1051292 4.5e3 0.79 a 2688
(K) NALTGLSM*GGGK gi|1051292 +10 2.1e4 0.63 a 2743
(R) VTWEDDQGKPQVNR gi|1051292 2.7e4 0.50 a 2672
(K) GGALEAIVDDLGAGYTYHAGK gi|1051292 1.4e4 0.45 a 5124
G gi|62899650 **MS/MS Spectra: 3 Sum TIC: 7.4e4 Avg TIC: 2.5e4**
Agaricus bisporus lectin (ABL) (Gal-beta-1,3-GalNac binding lectin) (Agaricus bisporus agglutinin) (ABA) (TF antigen-binding lectin)
Sequence Reference TIC Sf D Scan
(R) ENQLTSYNVANAK gi|62899650 +2 2.4e4 0.96 a 2846
(R) FAIEYTVTEGDNLK gi|62899650 +1 2.9e4 0.94 a 3886
(K) YANGGTWDEVR gi|62899650 +2 2.1e4 0.52 a 3061
H gi|3355324 **MS/MS Spectra: 3 Sum TIC: 7.3e4 Avg TIC: 2.4e4**
putative aldehyde dehydrogenase (NAD+) [Agaricus bisporus] gi|12230028|sp|O74187|ALDH_AGABI Aldehyde dehydrogenase (ALDDH)
Sequence Reference TIC Sf D Scan
(K) NFGQVIETDEK gi|3355324 1.4e4 0.90 a 3119
(K) VAFTGSTLVGR gi|3355324 +6 3.3e4 0.85 a 3362
(R) IFVQEGIYDK gi|3355324 +1 2.6e4 0.83 a 3372
I gi|433180 **MS/MS Spectra: 2 Sum TIC: 7.0e4 Avg TIC: 3.5e4**
heat shock protein
Sequence Reference TIC Sf D Scan
(K) KTQTFSTYSDNQPGVLIQVYEGER gi|433180 +2 2.3e4 0.90 a 4448
(K) FELSGIPPAPR gi|433180 +99 4.7e4 0.90 a 3821
J gi|435476 **MS/MS Spectra: 3 Sum TIC: 6.3e4 Avg TIC: 2.1e4**
cytokeratin 9 [Homo sapiens] gi|81175178|sp|P35527|K1C9_HUMAN Keratin, type I cytoskeletal 9 (Cytokeratin-9) (CK-9) (Keratin-9) (K9)
Sequence Reference TIC Sf D Scan
(R) GSRGGSGGSYGGGGSGGGYGGGSGSR gi|435476 +2 3.4e3 0.86 a 1930

(R) GGSGGSYGGGGSGGGYGGGSGSR gi|435476 +2 3.9e3 0.68 a 2005			
(K) TLLDIDNTR gi|435476 +3 5.5e4 0.46 a 3457			
K gi|28317 MS/MS Spectra: 3 Sum TIC: 5.3e4 Avg TIC: 1.8e4			
unnamed protein product [Homo sapiens]			
Sequence Reference TIC Sf D Scan			
(R) SGGGGGGGGGCGGGGGGVSSLR gi|28317 +2 9.6e3 0.77 a 2252			
(R) NVSTGDVNVEM*NAAPGVDLTQLLNNMR gi|28317 +5 1.1e4 0.67 a 5072			
(K) YENEVALR gi|28317 +9 3.3e4 0.43 a 2636			
L gi|4104248 MS/MS Spectra: 2 Sum TIC: 3.6e4 Avg TIC: 1.8e4			
glucose regulated protein [Laccaria bicolor]			
Sequence Reference TIC Sf D Scan			
(K) LAEVQGVVNPITTK gi|4104248 1.6e4 0.88 a 3528			
(R) LSQEEIDR gi|4104248 +35 2.0e4 0.71 c 1821			
M gi|58219326 MS/MS Spectra: 2 Sum TIC: 2.2e4 Avg TIC: 1.1e4			
ribonuclease T2 [Agaricus bisporus] gi|58219324|dbj|BAD88711.1| ribonuclease T2 [Agaricus bisporus]			
Sequence Reference TIC Sf D Scan			
(K) GPGVDVFIK gi|58219326 1.6e4 0.84 a 3611			
(R) FFQNFPTFK gi|58219326 5.9e3 0.75 a 4247			
Single, ungrouped sequences MS/MS Spectra: 6 Sum TIC: 1.7e7 Avg TIC: 2.8e6			
Sequence Reference TIC Sf D Scan			
(K) DVIIAYEPVWAIGTGK gi|39983609 2.5e4 0.95 c 3766			
phosphoglycerate kinase/triosephosphate isomerase [Geobacter sulfurreducens PCA] gi|39996728|ref|NP_952679.1| phosphoglycerate			
kinase/triosephosphate isomerase [Geobacter sulfurreducens PCA]			
(K) LGAELLTPFGR gi|57225990 +1 1.7e7 0.89 c 3422			
acid phosphatase, putative [Cryptococcus neoformans var. neoformans JEC21] gi|58265204|ref|XP_569758.1| acid phosphatase, putative			
[Cryptococcus neoformans var. neoformans JEC21]			
(K) LGAELLTPFGR gi|57225990 +1 2.5e4 0.88 b 3357			
acid phosphatase, putative [Cryptococcus neoformans var. neoformans JEC21] gi|58265204|ref|XP_569758.1| acid phosphatase, putative			
[Cryptococcus neoformans var. neoformans JEC21]			

(K) TVASKTEDQELQNR gi|72006297 +1 4.3e4 0.85 b 1907
PREDICTED: similar to talin 1 [Strongylocentrotus purpuratus]
(-) NKTVVNNESSEIIR gi|23036340 3.8e4 0.81 d 3577
AU273738 sea urchin cDNA library, gastrula Hemicentrotus pulcherrimus cDNA clone 001a02r_D05 5', mRNA sequence
(K) TITLEVESSDTXDNVK gi|3789940 1.1e4 0.79 a 3754
tetra-ubiquitin [Saccharum hybrid cultivar H32-8560]
Dir: kabubakerbh222-gt (a) **Files:** 060209Ykabh222-gt (02/12/06-02/12/06) **Enz:** Trypsin
kabubakerbh222-gt2 (b) 060225Ykabh222-gt2 (02/25/06-02/25/06) Trypsin
kabubakerbh222-gt3 (c) 060226Ykabh222-gt3 (02/26/06-02/26/06) Trypsin
kabubakerbh222-gt_500 (d) 060209Ykabh222-gt (02/25/06-02/25/06) Trypsin
Oper: wsl **Date:** 2/26/06 **Report:** BH222-GTsnr (wsl) R52/D8/C18 **MR:** 2

188

B- *T. aggressivum ech*42 gene sequence data

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```

C- *T. aggressivum prb*1 gene sequence data

>**T.Prb1.D**
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D- *T. aggressivum β-1,3 glucanase gene* sequence data

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E- *T. aggressivum* shRNA sense and antisense sequences

>SiRNA-End Fragment

GTCATCTACTCCTTCATGAACCTCCAGGCAGACGGCACAGTTGTCTCTGGCGATACCTACGCTGATTTCGA
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AGGTCAAGAAGGCCAACCGAGGCCTCAAGGTTCTACTCTCCATCGGTGGCTGGACCTGGTCCACCAACTTC
CCTTCCGCAGCAAGCACGGATGCCAACCGAAGGAACTTTGCGCGAACTGCCATTACCTTCATGAAGGATTG
GGGTTTCGATGGTATTGACGTCGACTGGGAGTACCCTGCCGATGCCACCCAGGCCTCCAACATGGTTCTTC
TGCTCAAG

```
                 SpeI    AscI
- End (F): agactagtggcgcgccgtcatctactccttcatgaac


            BamHI   SwaI
- End (R): ctggatccatttaaatcttgagcagaagaaccatgtt
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>SiRNA-Prb1 Fragment

GTTTCAGGAACTATTGGTGGCTCTACCTATGGTGTTGCTAAGCAGGCCAACCTCGTCTCTGTCAAGGTCTT
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ATCAATGCAGCCTACAACCAGGGTGTTCTTACTATCGTGGCTGCCGGTAACGGTGACACTTTCGGAAACCC
CCAACCTGTTTCTGGCACCTCTCCTGCCAATGTGCCTAATGCTATTACTGTTGCTGCGCTC

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                 SpeI    AscI
- Prb1 (F): agactagtggcgcgccgtttcaggaactattggtgg


            BamHI   SwaI
- Prb1 (R): ctggatccatttaaatgagcgcagcaacagtaatagc
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>SiRNA-GLU.Fragment

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CACCATAAAAGCGACGGCCAACTTCAACGGTATTGCTCTCATTGATGGTGATACCTATTATGGCGACAACA
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                 SpeI    AscI
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            BamHI   SwaI
- GLU (R): ctggatccatttaaatcatatcaagcttaaagttgcg
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