Development of fungal expression plasmids to facilitate promoter profiling and gene silencing studies in *Coprinopsis cinerea*

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Declaration: “I hereby declare that this project is entirely my own work and that it has not been submitted for any other academic award, or part thereof, at this or any other education establishment”.

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Abbreviations

ABL – A. bisporus Lectin

ATPD - A-Subunit of ATP-Synthase

BCCM - Belgian Co-ordinated Collections of Micro-Organisms

BLAST - Basic Local Alignment Search Tool

*CGL*1 - *C. cinerea* Galectin Protein I

*CGL*2 - *C. cinerea* Galectin Protein II

CTAB - Cetyltrimethylammonium Bromide

CYPA - Cytochrome P450

ECM - Extracellular Matrix

EDTA - Ethylenediaminetetraacetic Acid

EXP1 - Expansionless 1

GFP – Green Florescent Protein

HMG-CoA - 3-Hydroxy-3-Methylglutaryl-Coenzyme A

HYPA - Hydrophobin A

HYPB - Hydrophobin B

HYPC - Hydrophobin C

LB - Luria Broth

LSD - Lysergic Acid Diethylamide

MA - Malt Agar

MCS – Multiple cloning Site

MS - Murashige and Skoog Basal Medium

NCBI - National Centre for Biotechnology Information

OAT - Ornithine Aminotransferase

PCR - Polymerase Chain Reaction

SEPA - Septin

SPR - Serine Proteases

YMG - Yeast Malt Glucose

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I also want to thank my family, friends and colleagues who have supported and helped me throughout this project.

Abstract

The ink cap mushroom *Coprinopsis cinerea* (formally *Coprinus cinereus*) has long been regarded as one of the best model systems for the study of basidiomycete fungi. Most attention in *C. cinerea* has been focused on processes such as mating type determination and on meiosis. Little detail is known about the other aspects of the biology of this fungus, such as the cellular mechanisms involved in the development of fruiting structures. This research describes the development of fungal expression plasmids to facilitate promoter profiling and gene silencing studies in *C. cinerea*.

The main focus of this research was the development of expression cassettes to study the *C. cinerea* serine protease enzymes (SPRs). Serine proteases have been shown to be significant in both post-harvest spoilage of mushrooms and nutrient acquisition from compost. These expression cassettes will enable the elucidation of the role these enzymes play in mushroom fruiting body development and nutrient acquisition.

Initial work focused on the development of a rapid DNA extraction method for fungal samples which was central to this work. A protocol using the Precellys Bead Mill Homogeniser was successfully developed and a technical report was produced and published online, which is available at: https://homogenizers.net/pages/p-dna-extraction-from-fungal-organisms.

Early stages in the construction of expression cassettes included the identification, isolation and analysis of the promoters of the serine protease genes and of selected fruiting body development genes (*Agaricus bisporus* lectin (*abl*), *C. cinerea* ornithine transferase (*oat)*, and *C. cinerea* galectin genes (*cgl*1 and *cgl*2). In particular, work focused on the development of a GFP-promoter fusion plasmid containing the *C. cinerea* serine protease 10592 promoter. The plasmid was designed and constructed to link the promoter to efficient Green Fluorescent Protein (eGFP) producing the peGFPi10592 construct. The construction of this vector is a significant addition to the *C. cinerea* molecular toolkit.

In addition to this, GFP fusion cassettes were also designed for the SPR promoters 04562 and 10615. The results presented here include work undertaken to identify and isolate the *C. cinerea* SPR promoter regions of 04562 and 10615 and the preparation of plasmid backbones for cloning these fragments. Antisense cassettes were also designed for the SPR genes 10592 and 04562. The results seen here illustrate the successful isolation of

these genes and the generation of the corresponding plasmid backbones ready for cloning experiments. This research will form a foundation for future experiments to study *C. cinerea* SPRs and fruiting body development genes in the fungal life cycle.

Dissemination List

Conferences

Poster presentation at Environ conference (2015), Sligo Institute of Technology. "Investigation of Endogenous Serine Proteases in the Basidiomycete *Coprinopsis Cinerea*". Granahan AM, Bailey A, Foster GD, and Heneghan MN.

Poster presentation at Environ conference (2016), Limerick University. "Investigation the role of serine proteases in basidiomycetes fruiting body development and nutrient acquisition from humic- rich environments". Granahan AM, Bailey A, Foster GD, and Heneghan MN.

Poster presentation at European fungal genetics conference (2016), Paris, France. "Investigation of the role of serine proteases in basidiomycetes fruiting body development and nutrient acquisition from humic- rich environments". Granahan AM, Bailey A, Foster GD, and Heneghan MN.

Chapter 1:

1.1 Introduction

The ink cap mushroom *Coprinopsis cinerea* (formally *Coprinus cinereus*) is the most widely used model system for the study of basidiomycete fungi. However, to date, research into the cellular mechanisms involved in the development of fruiting structures is limited. This research describes the development of fungal expression plasmids to facilitate promoter profiling and gene silencing studies in *C. cinerea*. These expression cassettes will enable future research studies to determine the role that particular enzymes play in mushroom fruiting body development and nutrient acquisition.

Serine proteases have been shown to be significant in both post-harvest spoilage of mushrooms and nutrient acquisition from compost. Previous research has primarily focused on the *A. bisporus* serine proteases, particularly SPR1. This enzyme has been shown to be essential in fruiting body development, post-harvest senescence, and in nutrient acquisition from humic associated material in compost. Through genome mining Heneghan *et al.*, (2009) have identified seven putative SPRs in *C. cinerea*. An expression profile confirmed the presence of extracellular serine proteases, while in fruiting body development serine protease activity was low in the primordium, karogamy, meiosis and immature stages but rapidly increased at the mature stage (Heneghan *et al*. 2009). A combinatorial approach utilising promoter profiling and gene silencing would help elucidate the functional role of these endogenous *C. cinerea* SPRs.

Expression profiles for these enzymes can be developed through promoter profiling. This involves the identification and isolation of the regulatory region upstream from the encoding gene and linking this putative promoter to eGFP, resulting in the construction of a fungal expression plasmid. This plasmid could subsequently be transformed into *C. cinerea,* fruiting initiated and eGFP fluorescence monitored throughout the life cycle. The fluorescence observed would indicate the expression of the SPR protein and would provide a profile of the enzyme during the complete life cycle of the basidiomycete. The response of these enzymes to available nutrients could also be examined through a series of plate based and biochemical assays. Transformants harbouring the promoter-eGFP constructs could be grown on a range of media containing different supplements, and

eGFP fluorescence monitored. This Master's thesis focuses on the identification and isolation of the *C. cinerea* SPR promoter regions, and the construction of *C. cinerea* SPR promoter:GFP fusion plasmids.

Gene silencing is a powerful technique that can be used to study gene functionality. Previous reports have demonstrated that silencing in *A. bisporus* and *C. cinerea* results in the production of a range of transformants that display different levels of silencing (Heneghan *et al*. 2007, Heneghan *et al*. 2016). This is a particularly useful technique for studying proteins involved in cellular functions, where total inactivation may result in lethality to the fungus. The study of the *C. cinerea* SPR genes functionality using antisense technology would involve the isolation of the SPR genes and the generation of antisense cassettes, followed by transformation into *C. cinerea*. Biochemical assays could be performed on the resulting transformants to monitor SPR activity. Transformants could be fruited and the number of mushrooms produced compared to the wild type *C. cinerea*. Biochemical assays could also be performed at each developmental stage in the life cycle to study the response of down regulation of the enzyme to available nutrients. The use of antisense constructs for rapid and high-throughput silencing would provide essential data regarding the functionality of these *C. cinerea* genes. This Master's thesis will focus on the generation of silencing cassettes to contain the *C. cinerea* SPR genes in the antisense orientation under the control of the *A. bisporus* glyceraldehyde-3-phosphate dehydrogenase (*gpd*II) promoter.

To elucidate the basidiomycete fruiting body process, candidate fruiting body gene promoters will be identified and isolated. These promoters could be linked to eGFP and transformed into *C. cinerea* to produce GFP expressing transformants. These transformants could be fruited and GFP fluorescence monitored throughout the life cycle of the basidiomycete. These experiments could provide essential information regarding what genes are expressed at each stage of the fruiting process. The data generated by these profiles would form the foundation for future silencing experiments, with the potential to silence SPR genes at specific developmental stages. This MSc thesis will focus on the study of fruiting body development promoters to include the identification and isolation of a selection of fruiting body promoters and the subsequent construction of promoter:GFP expression plasmids.

1.2 Biotechnological and ecological significance of fungi

The kingdom Fungi (super kingdom eukaryotes) is predicted to comprise millions of species (Badotti *et al.* 2018) which includes mushrooms, yeasts, moulds, smuts, rusts and puffballs. Fungi are distinctly different from the animal and plant kingdom due to the presence of chitin in their cell wall and their mode of nutrient acquisition, which is absorptive rather than digestive (Walker and White 2017). Fungi have a clear ecological role, where the depolymerisation of biopolymers is a key process in the cycling of carbon, with litter decomposition in temperate forests mainly driven by fungal activity. They constitute a major fraction of the living biomass responsible for efficient degradation of many recalcitrant organic compounds in soil litter and the humic layer (Bhatnagar *et al.* 2018). Fungi are economically significant, not only in terms of mushroom production cultivated for human consumption, but they are also employed in many industries such as brewing and baking. Fungi play an important role in many biotechnological processes, including the production of antibiotics, alcohols, enzymes, organic acids, and numerous pharmaceuticals (Zhang *et al.* 2018). The discovery of recombinant DNA technology and large scale genomic analysis has increased the use of yeasts and filamentous fungi in commercial applications (Chan *et al.* 2018).

Fungi have existed on earth for at least one thousand million years (Heckman *et al.* 2001). For much of that time, they have been exploited and evolved secondary metabolism for the production of bioactive compounds (Boruta 2018). While primary metabolites are essential for growth and development, secondary metabolites are not required for growth and are usually produced during the stationary phase. They are often associated with sporulation, are structurally low molecular mass molecules (He *et al.* 2018, Cary *et al.* 2018, Palazzotto and Weber 2018, Boruta 2018) and can have profound biological activities. The characteristics that differentiate them from primary metabolites include: retaining a variety of biosynthetic origins and structures, existence tends to be limited to a small number of organisms and can differ amongst isolated strains of the same species, and their production is characterized by groups of closely linked compounds, which could have very dissimilar biological properties (Atanasova-Penichon *et al.* 2018). These secondary metabolites are used in many applications. The antirejection/immunosuppressant drug Cyclosporine A is produced by the fungal species *Tolypocladium inflatum*. It acts by obstructing the production of interleukin-2 by T-lymphocytes, thus inhibiting the immune response produced naturally against

transplanted organs (Yang *et al.* 2018). Cholesterol-lowering drugs such as statins, act by inhibiting the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which catalyses the reduction of HMG-CoA to mevalonate during the synthesis of cholesterol. Examples of statins include lovastatin, which is produced by the fungal species *Monascuc ruber* (Manzoni *et al.* 1999, Subhan *et al.* 2017) and mevastatin which is produced by the fungus *Penicillium citrinum* (Zheng *et al.* 2014, Martín 2017). Alkaloids are organic compounds and synthetically produced lysergic acid diethylamide (LSD) is one of the most potent mood-changing chemicals. It is a semisynthetic product of lysergic acid, produced by the ergot fungus *Claviceps purpurea* (Liu and Jia 2017) Passie *et al*. 2008). Furthermore, isolates of *Claviceps paspali* also have the capacity to produce lysergic acid amides (Bushley *et al.* 2013, Liu and Jia 2017). Penicillin is a well-known β-lactam antibiotic derived from the mould *Penicillium chrysogenum* (Kumar *et al.* 2018).

Genes encoding the biosynthesis of these secondary metabolite compounds are arranged in clusters and encode for regulatory, transporter and signalling proteins. The expression of all of these secondary metabolites is dependent on environmental conditions such as temperature, nutrient availability, and pH, all of which control a complex regulatory network involving multiple proteins and complexes. The regulation of secondary metabolism gene clusters arises at several levels, some entirely specific for the respective pathway, while others display a more global regulation of secondary metabolism (Hautbergue *et al.* 2018, Kjærbølling *et al.* 2018, Rokas *et al.* 2018).

Fungal pathogens are causative agents responsible for numerous human, animal, and plant diseases (Daszak *et al.* 2000, Cunningham *et al.* 2017). Human diseases resulting from fungal infection include pneumonia, (*Aspergillus* spp. and *Cryptococcus neoformans*), (Ravi Kumar *et al.* 2018), septicaemia (*Candida fabianii)*, (Bano *et al.* 2019) and meningitis (*Cryptococcus neoformans)* (Lam *et al.* 2017). Fungal pathogens of plants, namely fruits and vegetables, can result in harvest losses of between 35–55% (Fortunati *et al.* 2017). *Botrytis cinerea* is a common plant pathogen infecting a wide range of species including strawberries and grapes (Singh and Sharma 2018).

1.3 Classification of fungi

Fungal classification is in a constant state of flux and numerous different classifications have been proposed (Hawksworth 1991, Hibbett *et al.* 2007). Dated fungal classifications relied heavily on morphological and ecological techniques. Strains of fungi that were used in most cases were not carefully referenced (Guarro *et al.* 1999). In 2007 Hibbett proposed a new classification system based on phylogenetic techniques (Hibbett *et al.* 2007). Hibbett's (2007) classification recognises a kingdom (Fungi), one subkingdom (Dikarya), seven phyla (Microsporidia, Blastocladiomycota, Neocallimastigomycota, Chytridiomycota, Glomeromycota, Basidiomycota and Ascomycota, two of which are in the subkingdom dikarya) and four subphyla (Kickxellomycotina, Zoopagomycotina, Entomophthoromycotina and Mucoromycotina) (Hibbett *et al.* 2007).

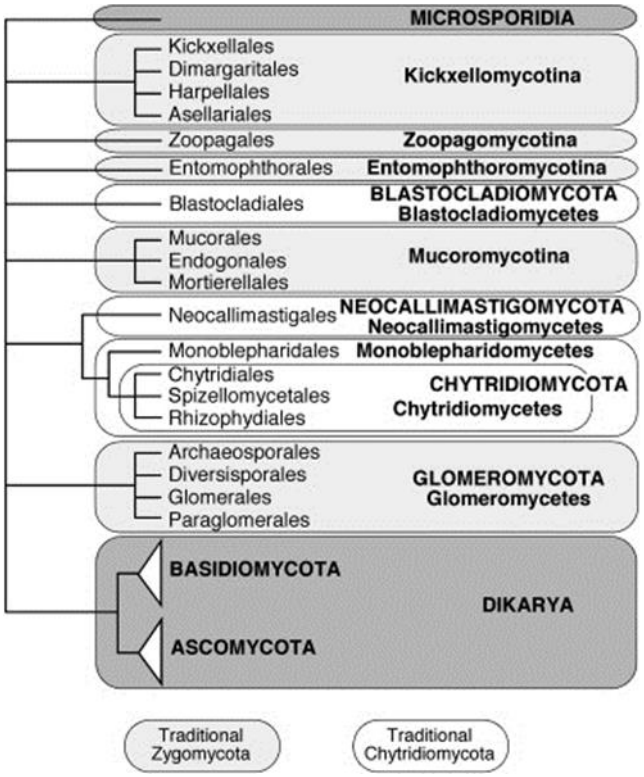


Figure 1. 1: Hibbett's phylogeny and classification of fungi (Hibbett *et al.* 2007).

Microsporidia were included in Hibbett's classification and ranked as a phylum, but no further subdivision was proposed in this report. Microsporidia (James *et al.* 2006a, James *et al.* 2006b) have been identified in diverse environments ranging from deep-sea vents (Sapir *et al.* 2014) to dust (Favet *et al.* 2013). They are endoparasitic organisms of

animals and humans. A significant characteristic of these organisms is the elaborate mechanism by which the spores are quickly injected into the host's cytoplasm via a thin tube (Corsaro *et al.* 2016).

Blastocladiomycota are related to the Chytridiomycota phylum and are parasites of fungi, algae, plants and invertebrates (James *et al*. 2006b). These fungi are identified by a zoospore with a single flagellum. The reproduction of these species is achieved by both asexual and sexual means (James *et al*. 2006b).

Neocallimastigomycota are related to chytrids (phylum Chytridiomycota) (James *et al.* 2006b, Hibbett *et al.* 2007), and are a diverging lineage of the zoosporic fungi. These fungi are found in the digestive tract of mammalian herbivores (Gruninger *et al*. 2014), and possibly in other terrestrial and aquatic anaerobic environments (Hibbett *et al*. 2007). They are distinguished by the presence of flagella and the mechanism they employ to degrade plant matter (Gruninger *et al.* 2014). These anaerobic fungi reproduce asexually, producing flagellated zoospores from sporangia (Heath *et al.* 1986), with no sexual reproductive life stage identified to date (Gruninger *et al*. 2014).

Chytridiomycota are found in water or soil. These fungi are recognised by having zoospores (motile cells) and are microscopic in size. Zoospores have a single, posterior whiplash flagellum. The majority of them are parasites to algae and animals (James *et al.* 2006b) and can reproduce by both asexual and sexual methods.

Glomeromycota from hyaline globose to subglobose spores (Błaszkowski *et al.* 2012). Morton (1988) states that spores may be formed singly, in clusters, or in morphologically distinct "fruitbodies" called sporocarps (Morton 1988, Redecker and Raab 2006). These fungi are biotrophic and unlike other fungal species, they lack saprotrophic abilities. Glomeroycota reproduces asexually and to date, there is no evidence to support that sexual reproduction can take place (VanKuren *et al.* 2013).

A subkingdom, dikarya, houses two of the seven phyla, Ascomycota and Basidiomycota. Ascomycota is a diverse fungal phylum of eukaryotes, occurring in many niches. Their primary function is the decay of organic matter (dung, leaf litter and wood) (Schoch *et al.* 2009). Ascomycetes reproduce asexually (Schoch *et al*. 2009), where meiosis occurs in a cell called the ascus and the resulting ascospores develop inside the cell (Casselton and Olesnicky 1998).

Basidiomycota is the second phylum of dikarya and they constitute a large, diverse fungal group including the rusts, smuts and mushrooms. The basidiomycete fungi are so-called because meiosis occurs in specialized cells called basidia and the resulting spores, named the basidiospores, are produced outside the cell (Casselton and Olesnicky 1998).

Hibbett (2007) was the first to introduce phylogenetic classification for fungi and since then many revised classifications have been proposed (Spatafora *et al.* 2016, Spatafora *et al.* 2017, Zhao *et al.* 2017, Wijayawardene *et al.* 2018). The most recent reclassification by Tedersoo *et al.*, (2018) utilised a comprehensive system of phylogenetic, monophyletic and divergence time approaches. Tedersoo classification recognises a kingdom (Fungi), nine subkingdoms; (Dikarya, Mucoromyceta, Zoopagomyceta, Chytridiomyceta, Aphelidiomyceta, Basidiobolomyceta, Blastocladiomyceta, Olpidiomyceta, Rozellomyceta), 18 phyla (Rozellomycota, Aphelidiomycota, Blastocladiomycota, Chytridiomycota, Neocallimastiomycota, Ascomycota, Basidiomycota, Zoopagomycota, Mucoromycota, Entorrhizomycota, Kickxellomycota, Basidiobolomycota, Calcarisporiellomycota, Glomeromycota, Entomophthoromycota, Monoblepharomycota, Mortierellomycota and Olpidiomycota) and 23 subphyla (Tedersoo *et al.* 2018). The last eight phyla were classified as lower-level fungi and Tedersoo raised them to phylum level following the criteria of monophyly and comparable divergence time. Tedersoo's higher-level classification is due to the introduction of monophyletic and divergence time methods applied to improve existing phylogenetic classification of the kingdom fungi.

1.4 Mushroom industry

Mushrooms are the single most important crop in Irish horticulture, with 80% of production being exported to Britain. The farm gate value of the mushroom crop in Ireland is considerable, valuing €136 million in 2017 (Teagasc 2017). Ireland is self-sufficient in mushroom production with only a few exotic species of mushroom being imported into the country. *Agaricus bisporus*, the button/closed cup mushroom holds the largest share of the market.

1.5 *Agaricus bisporus*

The basidiomycete *Agaricus bisporus* (Figure 1.2) is naturally a leaf litter degrading fungus. It is ecologically significant as it participates in the carbon and nitrogen cycle by degrading leaf and needle litter that occurs on the forest floor (Kerrigan *et al.* 2013). This mushroom is the most cultivated worldwide (Stoop and Mooibroek 1999). The biotechnological applications of the fungus have increased due to the establishment of a successful transformation system (Chen *et al*. 2000) and the development of a molecular toolkit (Burns *et al*. 2006). *A. bisporus* naturally grows on composted material or animal dung (Miles and Chang 2004) and has a life cycle of 24-36 days.

Figure 1.2: *Agaricus bisporus*, the button mushroom, the most cultivated mushroom worldwide (Morin *et al.* 2012).

A. bisporus is amphitallic and can be homothallic or heterothallic, which are homo or heterokaryotic, respectively (Largeteau *et al.* 2011). The life cycle initiates with the germination of its homokaryotic or heterokaryotic spores. The hyphae grow, forming a large network of mycelium; here cells differentiate to establish the tissues needed to induce the mycelium to produce fruit bodies (Umar and Van Griensven 1999, Kues 2000, Kues and Liu 2000). Fruiting bodies of *A. bisporus* develop and grow directly from the onset of primordial. Once they mature, they produce spores, before entering senescence (Figure 1.3) (Umar and Van Griensven 1999, Kues and Liu 2000, Cheng *et al.* 2013). Umar and Van Griensvan (1997) conducted morphological studies on the life span, developmental stages, senescence and death of *A. bisporus* fruit bodies. They found that

by using artificial conditions to mimic the environment and ensuring it was free from diseases and pests, that the maximum life span of *A. bisporus* increased to 36 days, as opposed to 24 days without any protective measures (Umar and Van Griensven 1997). Umar and Griensven (1997) describe in detail the five successive stages of the *A. bisporus* life cycle. Stage I, the primordial stage, is undifferentiated hyphal knots that ends when the knots reach a size of 6mm. This takes approximately 2 days. Stage II, the histo-organogenetic stage, lasts approximately 3 days and is identified by the appearance of morphogenetic cell death, followed by basidial differentiation and the formation of the lamellae/gills. The maturation stage (stage III) takes approximately 4 days and involves cell extension to mature fruiting body and basidiospore formation. The basidia produce and bear spores that are ready to fall. In stage IV, the basidia shed spores for approximately 10 days. Stage V, senescence, is the gradual deterioration of the fruiting body resulting in death. It is recorded as lasting up to 18 days (Umar and Van Griensven 1997).

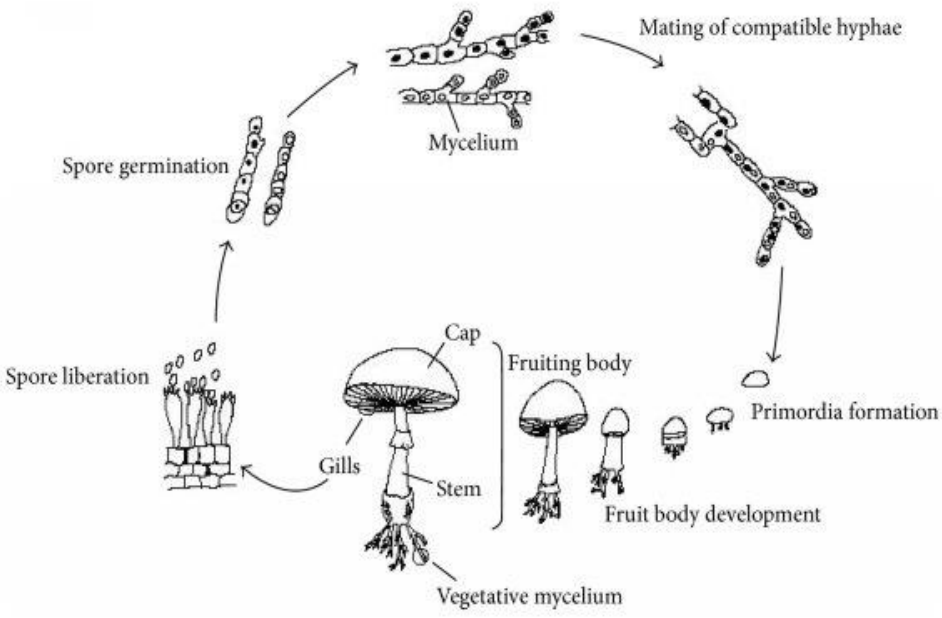


Figure 1.3: The life cycle of *A. bisporus* (Rahi and Malik 2016).

In *A. bisporus*, three sexual reproductive modes have been identified, namely intramixis, heteromixis and homomixis (Figure 1.4). The corresponding life cycles are pseudohomothallism, heterothallism and homothallism (Kamzolkina *et al.* 2006). Pseudohomothallic reproduction (Figure 1.4(1)) is described by Le Tocan *et al.,* (2016)

as requiring two strains to mate, but the nuclei that result from meiosis are organized so that each mating type is present in each ascospore (Le Tacon *et al.* 2016). Julca *et al.*, (2016) describes heterothallic reproduction (Figure 1.4(2)) as involving the interaction of two partners of opposite mating types (mating types *Mat1*-1 and *Mat1*-2) with compatible *Mat* idiomorphs (two non-allelic sequences on the same locus on the chromosome) (Julca *et al.* 2016). Le Tocan *et al.*, (2016) describe homothallic reproduction (Figure 1.4(3)) as involving fungi that have both *Mat* genes in each strain. Both haploid strains produce male and female structures that are able to mutually fertilize (Le Tacon *et al*. 2016).

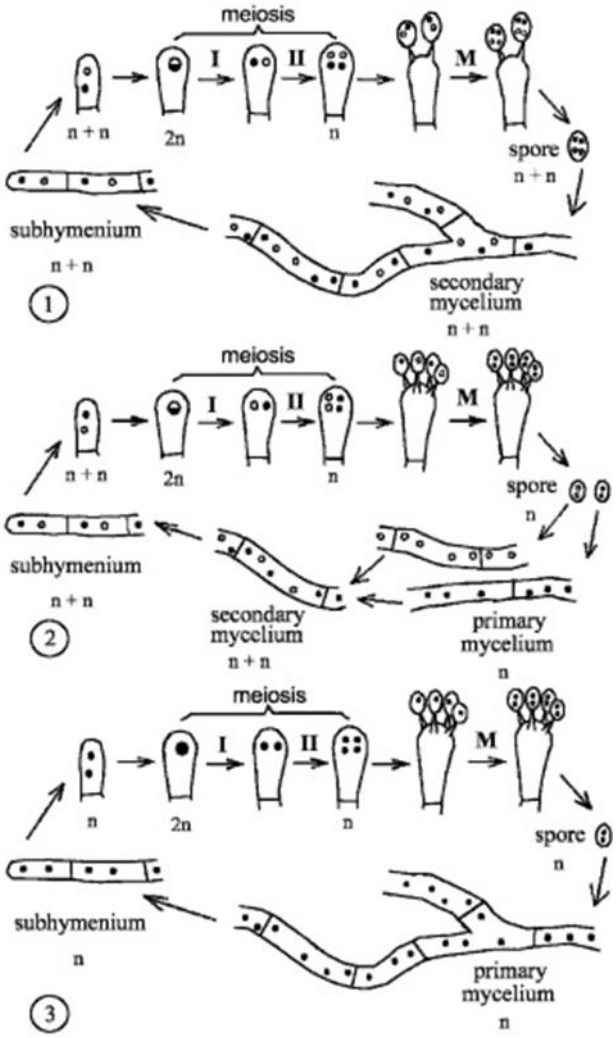


Figure 1.4: The three types of *A. bisporus* life cycle. 1: pseudohomothallism. 2: heterothallism, 3: homothallism (Kamzolkina *et al*. 2006).

1.6 *Coprinopsis cinerea*

The inkcap mushroom, *Coprinopsis cinerea* (Figure 1.5), (formally *Coprinus cinerea*) has emerged as the model basidiomycete. It is found naturally on heaps of mixed dung,

rotten straw or vegetable refuse, and it can complete its full life cycle in 14-21 days (Moore and Pukkila 1985). Furthermore, the genome has been fully sequenced and has been made available since 2003 (https://www.ncbi.nlm.nih.gov/bioproject/1447). This makes it the perfect model mushroom for studying gene expression in the life cycle stages of fruiting body development, somatic recombination and mating type determination. *C. cinerea* reproduces sexually and is regulated by the A and B mating type genes (Papazian 1954, Parag 1962, Raper 1966, Casselton and Olesnicky 1998, Kues and Liu 2000, Raudaskoski and Kothe 2010).

Figure 1.5: The ink cap mushroom *C. cinerea*, is the model basidiomycete due to its ease of lab manipulations and short life cycle (14-21 days) (Stajich *et al.* 2010).

Two forms of *C. cinerea* mycelia exist. Asexual mycelium (monokaryon) is fungal hyphae in which each cell contains a single nucleus (mononuclear spore or cell) (Kues 2000). Dikaryon is the sexual mating of two haploid homokaryons containing different mating type alleles (Banuett 2015). Fruiting bodies develop from dikaryon mycelia (Kues and Liu 2000, Kues 2000), and their formation is the result of a very complicated process changing radically from mycelia to a multihyphal structure resulting in many different cell types (Figure 1.6). Cap maturation is achieved 4 to 5 days after the fruiting process commences (Kues and Liu 2000).

Environmental factors have an important part to play in the successful formation of the fruiting body structure. Light and dark periods are the most vital parameters that encourage the development of this structure. In Figure 1.6, the light and dark stages that are required for the development of a mushroom are highlighted. The fruiting process starts in the dark, forming hyphal knots (Figure 1.6) (Moore 1981, Kues *et al.* 1998). This stage proceeds in the light and globose fruiting body initials are formed. Cellular differentiation starts here and continues forming primordial tissue. This is a crucial step due to the formation of the unique cellular tissues of the *C. cinerea* fruiting body (Morimoto and Oda 1973, Lu 1974). Following a second dark period, the light enables meiosis to conclude. This stage includes stipe elongation and cap maturation (Figure 1.6). This is followed by basidiospore formation and cap opening before autolyzation occurs (Muraguchi *et al.* 1999).

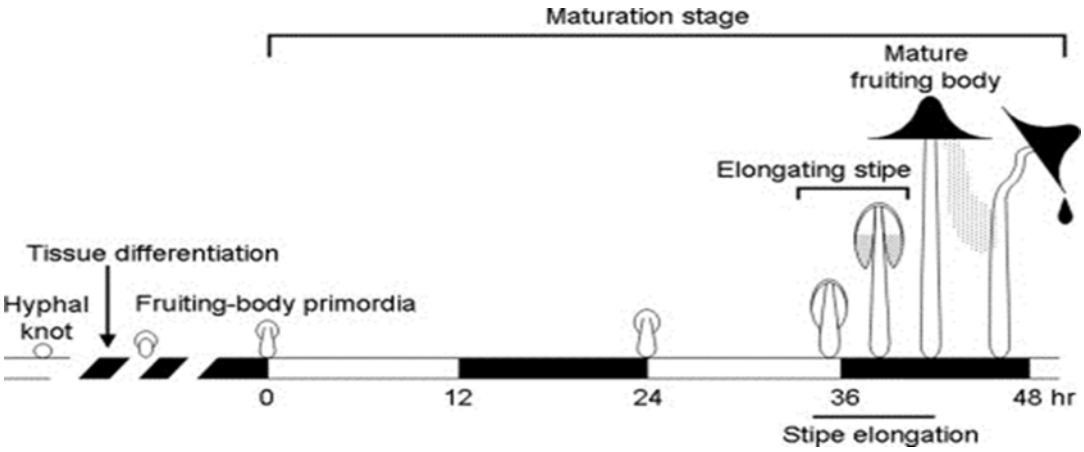


Figure 1.6: Fruiting body formation in the basidiomycete *Coprinopsis cinerea* is a developmental process that occurs as a response of the mycelium to external stimuli. The bar beneath the figure indicates the light and dark cycles that induce the development of *C. cinerea* fruiting bodies (Kues 2000).

Temperature conditions are a critical parameter in the formation of basidiomycete fruiting bodies. In *C. cinerea,* the optimal temperature range is 25-28°C while in *A. bisporus* the optimum range is 16-18°C. Other environmental factors affecting fruiting body development include humic conditions and carbon concentration (Kues and Liu 2000; Kues 2000) (see Section 1.7).

1.7 Control of fruiting body development

There are many proteins involved in the fruiting body process. These include hydrophobins (HYPA/ABH1, HYPB/ABH3, and HYPC/ABH2), a-subunit of ATP-synthase (ATPD), septine (SEPA), cytochrome P450 (CYPA), ornithine aminotransferase (OAT), lectin (ABL), expansionless 1(EXP1), galectin proteins (CGL1 and CGL2) and serine proteases (SPR).

Hydrophobins are small cysteine rich proteins present in large quantities in the outer surface of the fungal cell wall, and have been identified as key proteins involved in *A. bisporus* fruiting body formation (Linder *et al.* 2005, De Groot *et al.* 1996). HYPA (ABH1) line air channels within the fruiting body and are responsible for the hydrophobicity of the fruiting body surface (Lugones *et al.* 1996). ABH1 prevents water from entering the air channels, and the air channels remain capable of gas exchange within the fruiting body. ABH1 is highest in concentration in the outer tissue of the cap (Lugones *et al.* 1999). HYPA (ABH1) and HYPB (ABH3) are responsible for cap expansion in the basidiomycetes, especially in the peel tissue (De Groot *et al*. 1996, Lugones *et al*. 1999). The elucidation of the genomic structure of *hyp*A (ABH1) and *hyp*C (ABH2) has previously been determined (De Groot *et al*. 1996), with *hyp*A (ABH1) located 1.8kb upstream of the *hyp*C (ABH2) gene. The *hyp*A and *hyp*C genes are typically expressed in fruiting bodies of the heterokaryon and not in the substrate mycelium (Lugones *et al.* 1996, De Groot *et al.* 1996). De Groot *et al*., (1999) also elucidated the genomic structure of *hyp*B (ABH3). The translated amino acid sequence showed little identity to HYPA, apart from the arrangement of eight cysteine residues that are a typical characteristic of hydrophobins (De Groot *et al*. 1999). HYPB (ABH3) is found during early development in the formation of aerial hyphae (Wösten *et al.* 1999). This gene is specifically expressed in the substrate mycelium (Lugones *et al.* 1996, De Groot *et al.* 1996, Lugones *et al.* 1998) . It has also been identified in the mature mushroom, located between the cap and stipe tissue (De Groot *et al.* 1999). Sequences analysis of the three gene showed that HYPA (ABH1) shows an overall sequence identity of 78% with HYPC (ABH2), whereas HYPB (ABH3) showed only 37% identity to the other hydrophobins of *A. bisporus* (De Groot *et al.* 1996, Lugones *et al.* 1996, Lugones *et al.* 1998).

The genes *atpD, cypA* and *sepA* have had little research carried out to date; they are all expressed in the vegetative phase of the life cycle (De Groot *et al.* 1999). *AtpD* has been reported to have a probable function in the electron transport chain (De Groot *et al.* 1999).

ATP synthesis is up-regulated during fruit body formation, thus indicating the increasing need for ATPD activity to provide energy for the developing mushroom (De Groot *et al.* 1997). CYPA is very closely related to ATPD and belongs to the cytochrome p450 family of enzymes. CYPA are known in fungi to be involved in the oxidative metabolism of a large number of compounds (Eastwood *et al.* 2001). *SepA* encodes septin, which is a cell division control protein involved in the formation of septa (De Groot *et al.* 1998).

The OAT protein catalyses the reversible transfer of an amino group from ornithine to glutamate, which was demonstrated for all fruiting body tissues except the gills (Schaap *et al.* 1997). A transcript profile of *oat* was established during fruiting body development and post-harvest senescence. These studies have shown that *oat* is needed to drive fruiting body formation and is responsible for the redistribution of metabolites in the developing mushroom (Burton *et al*. 1994, Donker and Van As. 1999, Wagemaker *et al*. 2007b).

Lectins are carbohydrate-binding proteins found in a variety of organisms (Wang *et al.* 1996, Nagata 2000, Li *et al.* 2008,). Pemberton studied more than 400 mushroom species and discovered that 50% of them contained lectins (Pemberton 1994, Singh *et al.* 2015). Lectins play an important role in cell adhesion, dormancy, growth, morphogenesis, morphological changes consequent of parasitic infections and molecular recognition during the early stages of mycorrhization (Guillot and Konska 1997, Ng 2004, Zhang *et al.* 2009). The majority of research carried out on the *A. bisporus* lectin (*ABL*) has concentrated on immunomodulatory, antitumor, cytotoxic and antiproliferative activities (Wang *et al.* 1996, Wang *et al.* 1998, Wang *et al.* 2000, Wang *et al.* 2002, Han *et al.* 2004, Ngai and Ng 2004, Liu *et al.* 2006). Due to this, ABL has drawn significant interest from the scientific community due to its potential importance in cancer research and little work has focused on the role of ABL in fruiting body development. The few studies that have been performed revealed that lectin expression is closely correlated with fruiting body formation and could be a significant feature in regulating fruiting body development (Presant and Kornfeld 1972, Crenshaw *et al.* 1995, De Groot *et al.* 1997, Lakkireddy *et al.* 2011,). In mushrooms, lectins are present in the cap, stipe, spores, and mycelia (Ng 2004, Eastwood *et al.* 2008, Zhang *et al.* 2009).

Expansionless (EXP1) is a transcriptional regulator controlling the final phase of fruiting body morphogenesis and is involved in cap expansion and autolysis (Liu *et al.* 2006, Muraguchi *et al.* 2008, Sakamoto *et al.* 2009, Ohm *et al.* 2011). The research conducted

on EXP1 to date is limited and has primarily focused on *C. cinerea*, *Lentinula edodes* and *Schizophyllum commune*.

Fungal galectins are extracellular matrix (ECM) proteins that are differentially expressed in fruiting body development. Two galectins CGL1 and CGL2 have been isolated from *C. cinerea*. CGL1 is expressed in primordial and mature fruiting bodies while CGL2 is produced during hyphal-knot formation and expression is maintained through to maturation of the fruiting body (Boulianne *et al.* 2000). Galectin expression is dependent on light and dark cycles, with *cgl1* being induced by light while *cgl2* is repressed by light (Kues and Liu 2000, Kues 2000).

Another key group of enzymes that have been identified in the fruiting body formation process and post-harvest senescence of mushrooms is serine proteases (Burton *et al.* 1997) which are discussed in more detail in section 1.7 below.

1.8 Basidiomycete contribution to the carbon and nitrogen cycles

Basidiomycetes play a key role in the process of carbon cycling through the depolymerisation of biopolymers. A key feature of *A. bisporus* adaptation to humic rich ecosystems is the high protein degradation and nitrogen scavenging abilities of the basidiomycete. Due to global climate change, scientific interest has increased in an ecosystems sequestration potential (Strickland and Rousk 2010). The sequencing of the *A. bisporus* genome revealed genetic and enzymatic mechanisms governing adaptation to the humic-rich ecological niche formed during plant degradation, further defining the critical role such fungi contribute to soil structure and carbon sequestration in terrestrial ecosystems (Hättenschwiler 2005, Steffen *et al.* 2007).

Two enzymes, in particular, have been studied extensively in basidiomycetes in relation to their role in carbon and nitrogen cycling, namely ornithine aminotransferase (OAT) and serine protease (SPR).

The *A. bisporus* OAT (synonyms ornithine δ-aminotransferase and ornithine-oxo-acid aminotransferase) was the first fully reported homobasidiomycete ornithine aminotransferase protein sequence. This protein is regulated in response to carbon and nitrogen (Wagemaker *et al*. 2007a). OAT is a key enzyme in the urea cycle (Figure 1.7), and its function is the conversion of ornithine into glutamate. Glutamate is required for diverse functions such as synthesising of proteins, the cell wall and other nitrogenous

compounds, all of which are key components needed to drive fruiting body tissue development. Urea also facilitates the translocation of water and metabolites in fruit bodies, which is required for the production of spores (Burton *et al.* 1994, Donker and Van As 1999, Wagemaker *et al.* 2005, Wagemaker *et al.* 2007a). The greater expression of OAT in the latter stages of development suggests that OAT is responsible for the redistribution of metabolites in the developing mushroom (Donker and Van As 1999, Wagemaker *et al*. 2007b).

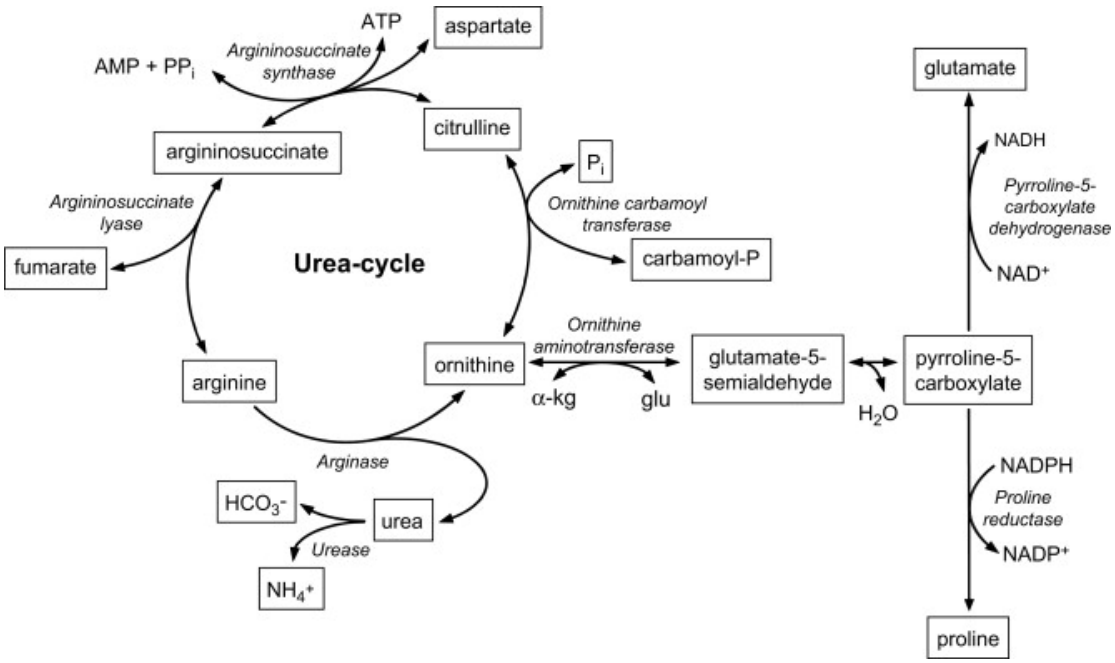


Figure 1.7: A schematic of the urea cycle. This depicts the presence of ornithine aminotransferase (OAT) (Wagemaker *et al*. 2007b).

Four serine proteases have been identified in *A. bisporus* (Burton *et al*. 1993a). SPR1 has been studied extensively and studies have demonstrated that the protein has an essential role in nutrient acquisition from humic associated material in compost, along with a clear role in fruiting body development and postharvest senescence (Burton *et al.* 1994, Burton *et al.* 1997, Burton 1997, Heneghan *et al.* 2009, Morin *et al.* 2012). The SPR1 promoter was linked to eGFP, transformed into both *A. bisporus* and *C. cinerea* and the expression of SPR was monitored by eGFP fluorescence throughout fruiting body development. eGFP expression was observed throughout the *C. cinerea* life cycle, demonstrating that serine proteinase can be active in all stages of fruiting body development. Expression was most concentrated during the development of young tissue, which may be indicative

of high protein turnover during cell differentiation (Heneghan *et al*. 2009). In *A. bisporus* fruiting bodies, GFP activity was localized to the stipe of postharvest senescing sporophores. While previous research has focused mainly on *A. bisporus* SPR1, it has been shown that *A. bisporus* SPR2, SPR3 and SPR4 are present in much lower concentrations compared to SPR1. The activity of these SPRs indicates possible roles in humic nutrient acquisition (Morin *et al*. 2012, Heneghan *et al*. 2016).

In both the *A. bisporus* and *C. cinerea* SPR regulatory sequences, CreA and several Nit2/AreA transcription factor-binding sites were identified. SPR1 expression in response to available nitrogen sources was studied, by growing the *A. bisporus* and *C. cinerea* SPR1 promoter-eGFP transformants on media containing nitrogen sources. eGFP expression was observed in *A. bisporus* and *C. cinerea* transformants grown in ammonia free media supplemented with humic fraction, milk, or glutamate (Burton 1997, Heneghan *et al*. 2009). These experiments demonstrated that in response to available nitrogen, both *C. cinerea* and *A. bisporus* produce serine proteases (Heneghan *et al.* 2009).

Heneghan *et al.,* (2016) silenced SPR1 in *A. bisporus* to study the effects of the protein on nutrient acquisition and adaption of the fungus to the humic-rich ecological niche during biomass degradation. The silencing experiment yielded numerous transformants displaying different levels of silencing. Some strains failed to colonise the compost, some failed to produce mushrooms, while other strains displayed a significant reduction in mushroom yield compared to wild type *A. bisporus*. From these results, it appears that SPR1 is a vitally important enzyme and has an essential role in nutrient acquisition from humic associated material in compost. This protease plays a role in *A. bisporus* accessing nitrogen and carbon sources during degradation of plant material in the soil (Heneghan *et al*. 2016).

1.9 Molecular toolkit

A "molecular toolkit" was developed to facilitate the transformation of basidiomycetes. This toolkit was designed to allow the relatively easy exchange of promoters and gene elements for subsequent analysis of gene expression (Burns *et al.* 2005, Burns *et al.* 2006, Heneghan *et al.* 2007, Heneghan *et al.* 2009, Collins *et al.* 2010, Heneghan *et al.* 2016).

Burns *et al.*, (2005) constructed a range of plasmids containing different promoters linked to either green fluorescence protein (GFP), β-glucuronidase (GUS) or luciferase (LUC) (Figure 1.8 A), to assess the efficacy of these promoters in *C. cinerea* and *A. bisporus* (Burns *et al*. 2005). GFP, GUS and LUC are reporter genes that are established markers used in other systems (Jefferson 1989, Millar *et al.* 1992, Sheen *et al.* 1995, De Ruijter *et al.* 2003). A codon-optimised GFP gene (efficient GFP, (eGFP)) was used in these plasmids as this version of the gene had previously been shown to express green fluorescent protein in fungi (Cubitt *et al.* 1995, Chiu *et al.* 1996, Yang *et al.* 1996).

Co-transformations of plasmids into *C. cinerea* with pCc1001 resulted in a high recovery of *trp*+ transformants, but no fluorescence was detected. The plasmid pCc1001 is the pUC9-based vector carrying the *C. cinerea* tryptophan synthetase gene (*trp1*) and is used as a selectable marker for co-transformation in *C. cinerea* (Binninger *et al.* 1987, Skrzynia *et al.* 1989). A binary GFP plasmid pGR4-GFP containing hygromycin (*hyg*) as the selectable marker was constructed for transformation studies in *A. bisporus* (Figure 1.8 C). This also resulted in *hyg*+ colonies, but no GFP fluorescence was observed. Burns *et al.*, (2005) concluded that an extra element was required to facilitate efficient gene expression. A 5′ intron was introduced to the plasmids as Ma *et al.*, (2001) had previously demonstrated that an intron was needed for gene expression in the white rot basidiomycete *P. chrysosporuim.* *C. cinerea* plasmids were constructed to contain promoters, linked to the intron-eGFP-UTR region from pUGiGM3 (Figure 1.8 B) (Ma *et al.* 2001). For transformation studies in *A. bisporus*, the binary vector pGF4-4iGM3 was constructed (Figure 1.8 C). Following transformation, GFP expressing colonies were recovered for both *C. cinerea* and *A. bisporus.* These results indicated that a 5′ intron is required for GFP expression in these basidiomycetes. The *C. cinerea trp*1 promoter resulted in 21% of *C. cinerea* transformants expressing GFP, the *A. bisporus trp*2 promoter resulted in 34% of *C. cinerea* transformants expressing GFP, while the *A. bisporus gpd*II promoter resulted in 49% of *C. cinerea* transformants expressing GFP. This was the first report of GFP expression in *A. bisporus* and *C. cinerea* (Burns *et al.* 2005), and indicated that the *A. bisporus gpd*II promoter was a strong promoter for driving GFP expression in these basidiomycetes.

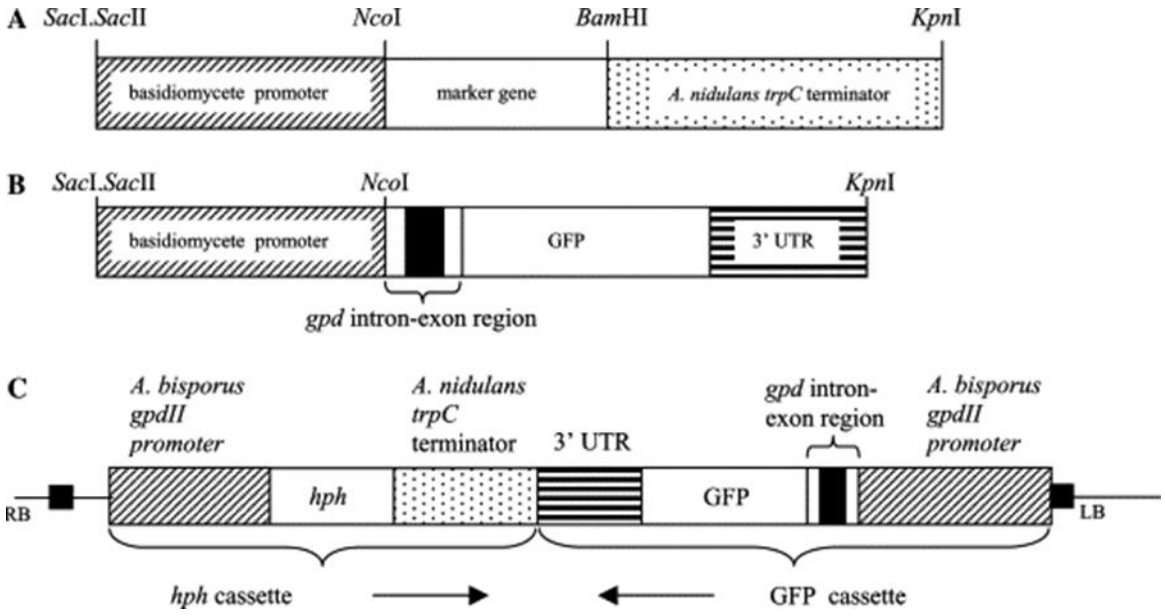


Figure 1.8: Construction of a molecular toolkit to facilitate transformations in *C. cinerea* and *A. bisporus*. All constructs developed by Burns *et al.*, (2005) used the *trpC* terminator from *A. nidulans* and the cassettes were cloned into pBluescript SK (Stratagene). This schematic shows the construction of the molecular toolkit utilising GFP as a marker gene. (A) A plasmid toolkit with a range of restriction sites for the rapid exchange of promoters and marker genes. (B) Toolkit plasmids containing basidiomycete promoters linked to the 5′ intron-GFP-terminator amplicon of pUGiGM3 (Ma *et al*. 2001). (C) Binary GFP plasmids for transformation in *A. bisporus* (Burns *et al*. 2005).

Burns *et al.*, (2005) plasmid toolkit facilitates molecular studies in *A. bisporus* and *C. cinerea* as it allows the relatively easy exchange of promoters and gene elements and contains three different basidiomycete promoters that are capable of driving gene expression. However, one limitation with the toolkit was the small number of restriction sites that were available for the exchange of DNA elements. Collins *et al*., (2010) extended the toolkit by constructing the plasmids pMCSi004 and pMCS004 (Figure 1.9). Both plasmids were engineered to have a multiple cloning site (MCS) downstream of the promoter providing a broader range of restriction enzyme sites for gene cloning. Both vectors contain the *A. bisporus gpd*II promoter and *A. nidulans trpC* terminator (Collins *et al.* 2010). The vector pMCSi004 has a 5′ intron upstream of the MCS to facilitate efficient gene expression (Figure 1.9 A).

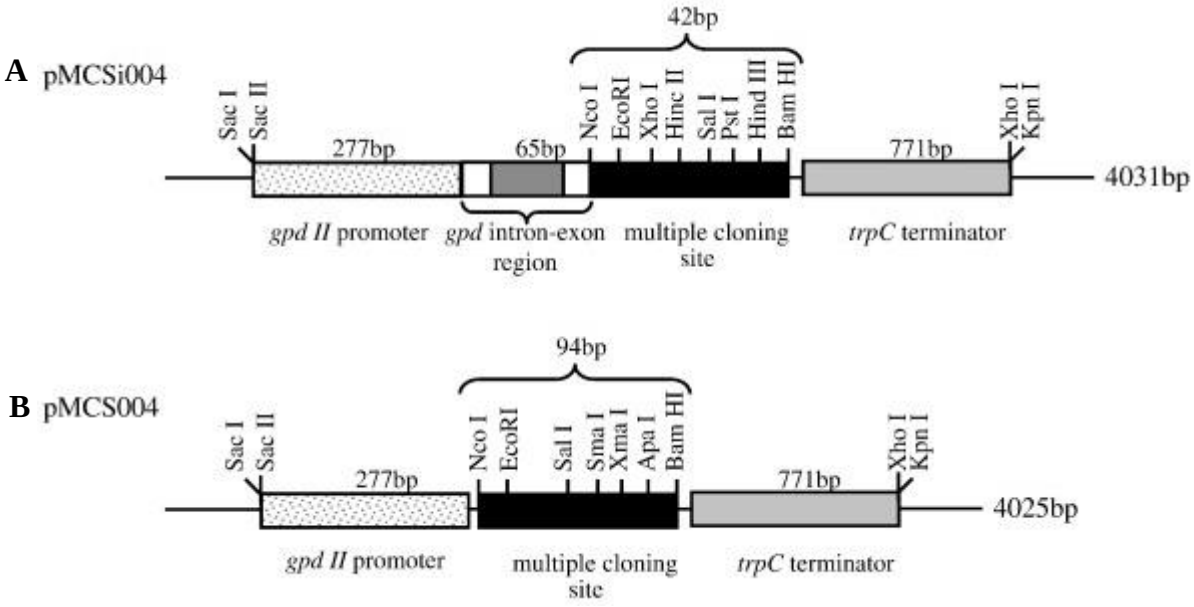


Figure 1.9: *C. cinerea* toolkit plasmids pMCSi004 and pMCS004. Regulatory sites are the *A. bisporus gpd*II promoter and the *A. nidulans trp*C terminator. A: pMCSi004 has an intron upstream of the multiple cloning site. B: pMCS004 does not contain an intron (Collins *et al.* 2010).

The plasmid peGFPi004 (Figure 1.10) was constructed as a positive control to confirm the efficiency of pMCSi004. GFP was excised from an existing toolkit plasmid and ligated into pMCSi004, resulting in peGFPi004. PCR analysis confirmed that 65% of *C. cinerea* transformants had the construct present and 33% visually displayed GFP expression (Collins *et al.* 2010).

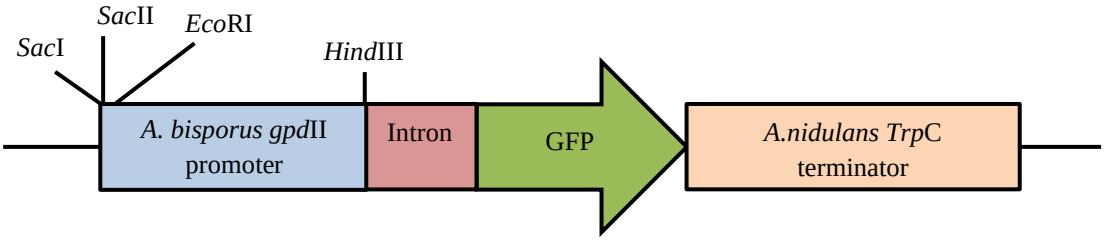


Figure 1.10: The vector peGFPi004. eGFP was ligated into the plasmid pMCSi004 to construct peGFPi004. This vector contains the *A. bisporus gpd*II promoter, a 5′ intron, GFP and the *A. nidulans TrpC* terminator.

Collins *et al.,* (2010) tested a range of marker genes using pMCSi004 and pMCS004 and this was the first report of mRFP and DsRed expression in *C. cinerea*. PCR confirmed the presence of intact cassettes (pYES-hph-RFP004, pYES-hph-RFPi004, pYES-hph-DsRed004 and pYES-hph-DsRedi004) in transformants. 40% of the transformants containing the pYES-hph-RFPi004 plasmid exhibited mRFP fluorescence while no fluorescence was detected for transformants containing the intronless plasmid (pYES-hph-RFP004). 44% of the transformants containing the pYES-hph-DsRedi004 plasmid exhibited DsRed fluorescence while no fluorescence was detected for transformants containing the intronless plasmid (pYES-hph-DsRed004). The results of this study further demonstrated the requirement for an intron for gene expression in basidiomycetes, as no gene expression was detected in transformants harbouring the intronless plasmid. These plasmids enhance the existing toolkit by providing vectors with and without introns that enable the easy exchange of genes through a range of restriction sites.

Gene silencing is a particularly useful technique for studying proteins involved in cellular functions, where total inactivation (gene knockout) may result in lethality to the fungus. To induce gene silencing, unstable mRNA or double-stranded mRNA that the cell recognises as non-sense is required. This aberrant mRNA then triggers silencing pathways (González *et al.* 2001, Wang *et al.* 2006, Heneghan *et al.* 2007). Gene expression in basidiomycetes requires a 5′ intron to be present in the expression plasmid. It has been suggested that this 5′ intron provides stability to the mRNA transcript following transcription (Burns *et al.* 2005, Heneghan *et al.* 2007). Therefore, construction of expression plasmids without a 5′ intron is more likely to trigger the silencing pathways.

Heneghan *et al*., (2007) evaluated three different silencing cassettes for their functionality and ease of construction. Antisense and untranslatable sense vectors were constructed based on the plasmid pMCS004. GFP was selected as the gene for this study and was excised from peGFPi004 (Figure 1.11 A). GFP was engineered to contain a stop codon after the translational start codon. This was cloned into pMCS004, producing pGFPStop004, the untranslatable sense construct (Figure 1.11 B). The antisense construct involved excising eGFP from peGFPi004 and ligating it into pMCS004 in the antisense orientation to produce pGFPAnti004 (Figure 1.11 C). A hairpin construct was also produced to contain sense and antisense fragments separated by a non-functional ‘loop’ sequence (Figure 1.11 D).

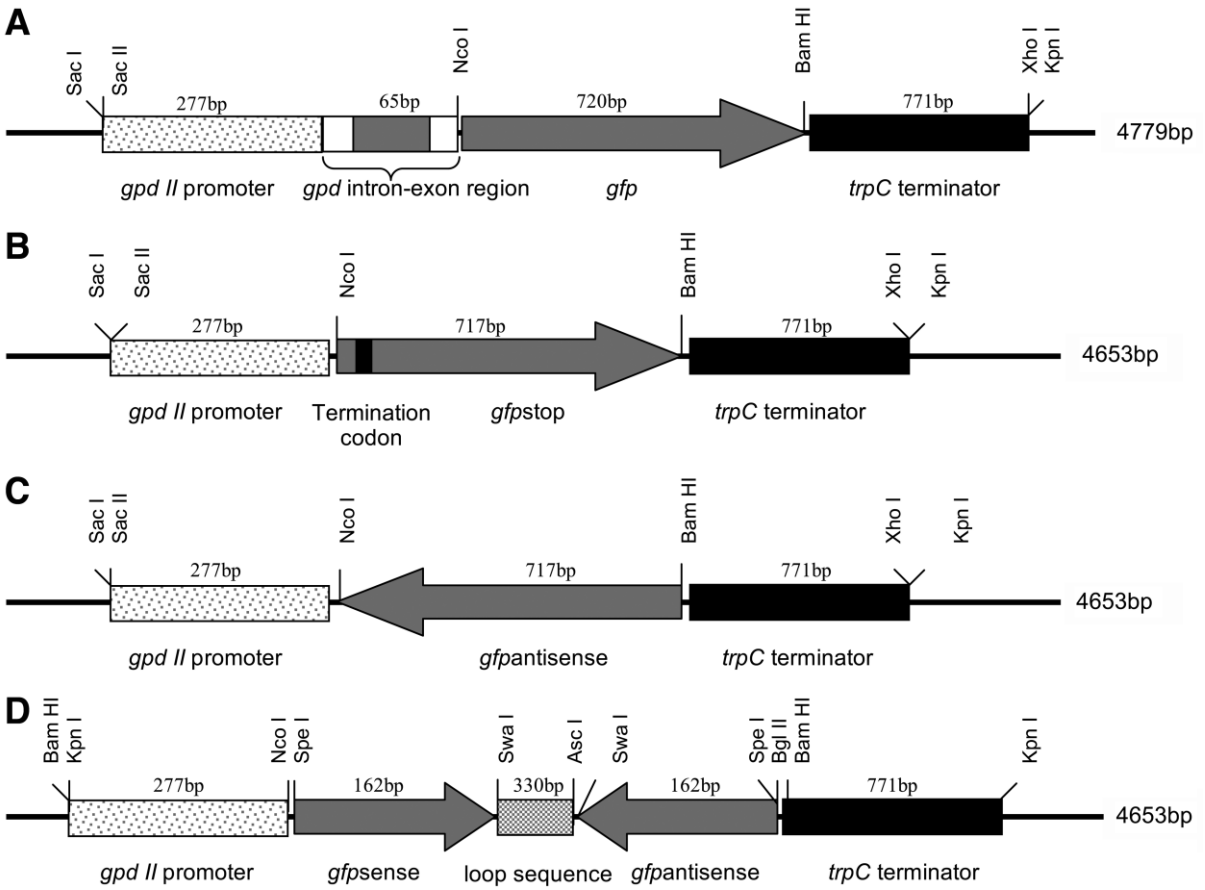


Figure 1.11: GFP silencing plasmids. (A) peGFPi004 contains an intron upstream from GFP. (B) pGFPStop004 contains an engineered stop codon immediately after the transcriptional start. (C) pGFPAnti004 contains GFP in the antisense orientation. (D) pRNAiAC005 contains 162bp of eGFP sense and antisense fragments separated by 330bp of non-functional *uidA* sequence (Heneghan *et al.* 2007).

Co-transformations with the three constructs into a strain of *C. cinerea* expressing eGFP yielded transformants that exhibited silencing of the eGFP gene. The study demonstrated that 18.75% of untranslatable sense transformants, 87.5% of antisense transformants and 55% of hairpin transformants exhibited gene silencing (Heneghan *et al.* 2007). As construction of an antisense cassette involved a rapid one-step cloning method and resulted in the highest number of downregulated transformants, Heneghan proposed this construct design as the most efficient for use in high throughput silencing (Heneghan *et al.* 2007).

The molecular toolkit was employed to study the expression of serine protease 1 (SPR1) in *A. bisporus*. A promoter:GFP fusion cassette was constructed to contain a 5′ intron,

transformed into *A. bisporus* and *C. cinerea*, and GFP expression was monitored through the life cycle of the basidiomycetes. GFP expression was high in the primordium stage, which may be due to the large cytoplasm content in the developing primordium. Fluorescence was seen throughout the karogamy and meiosis stages. GFP was most concentrated at the base of the gills in the karogamy stage and upward to the cap in the meiosis stage. This suggested that the SPR1 promoter activity was enhanced during the development of young tissue and indicated a high protein turnover during cell differentiation. In the immature stage, GFP was detected in the stipe close to the cap. In the mature body, GFP was observed most concentrated at the stipe-cap junction. This again indicated that SPR1 is critical in the development of young tissue and high protein turnover. During autolysis, the highest GFP activity was seen in senescing stipe tissues before the export of nutrients to the reproductive spores (Heneghan *et al.* 2009).

Gene silencing was also employed to analyse the *A. bisporus* SPR1 gene. Silencing cassettes were based on the molecular toolkit and did not contain an intron. *A. bisporus* transformants displaying a diverse range of silenced phenotypes from total inactivation of SPR1 to a minor reduction in gene expression. To specifically test for SPR activity, transformants were subjected to liquid culture supplemented with humic acid, as this had previously been shown to induce SPRs activity (Burton *et al.* 1997). 83% of transformants grown on humic acid had a reduction in SPR activity compared to *A. bisporus* wildtype. This suggested that SPR1 would normally account for the majority of protease activity during growth of the fungus on purified humic fraction. Fruiting induced transformants were assessed to study the effect of SPR1 silencing on *A. bisporus* growth in compost and fruiting. This resulted in one transformant (AS5) that was unable to colonize the compost, whereas the transformants that could colonize the compost demonstrated a 60% reduction in mushroom yield (Heneghan *et al.* 2016). The combinatorial approach of promoter-GFP profiling and gene silencing of the SPR1 provided a key insight into the functionality of this protein in *A. bisporus*.

1.10 Aims and objectives

The aim of this MSc thesis was the development of fungal expression plasmids to facilitate promoter profiling and gene silencing studies in *C. cinerea* . These plasmids could be employed in future research to fully elucidate the role of the *C. cinerea*

endogenous serine proteases in fruiting body development and nutrient acquisition. The experimental work presented includes the following:

- Development of a rapid genomic DNA extraction method. This was paramount to the research in order to obtain DNA template for PCR amplification of promoter and gene fragments.
- Construction of the *Coprinopsis cinerea* serine protease promoter plasmids peGFPi10592, peGFPi04562 and peGFPi10615. GFP fusion cassettes were designed for the SPR promoters 10592, 04562 and 10615. Work included identification and isolation of the *C. cinerea* SPR promoter regions and the preparation of plasmid backbones ready for future cloning experiments. The *C. cinerea* SPR 10592 promoter was ligated into the peGFPi004 backbone to produce the intact expression cassette peGFPi10592.
- Construction of the *C. cinerea* serine protease antisense gene plasmids pAS10592 and pAS04562. Antisense cassettes were designed for the SPR genes 10592 and 04562. This work involved PCR isolation of the genes and the generation of the corresponding plasmid backbones ready for future cloning experiments.
- Design and development *C. cinerea* promoter plasmids to facilitate the analysis of fruiting body genes enzymes. Work involved identification and isolation of promoters and the design of plasmid backbones for future cloning experiments.

Chapter 2: Materials and Methods

2.1 Routine Chemicals and Reagents

The following is a list of routine chemicals used in this research. Unless otherwise stated, chemicals were obtained from VWR: agar powder, agarose (Amresco), aminobenzoic acid (Thermo Fisher Scientific Ireland), ammonium acetate (Amresco), ampicillin (Sigma Aldrich), β-mercaptoethanol (Sigma Aldrich), bromophenol blue (Sigma Aldrich), cetyltrimethylammonium bromide (CTAB) (AppliChem, Panreac ITW Companies), cefotaxime sodium salt (Thermo Fisher Scientific Ireland), chloroform (Sigma Aldrich), chloroform: isoamyl 24:1 (SEVAG) (AppliChem, Panreac ITW Companies), E.N.Z.A SP Fungal DNA Mini Kit D5542-01, ethylenediaminetetraacetic acid (EDTA) free acid (AppliChem, Panreac ITW Companies), gel red, glucose (D) anhydrous (AppliChem, Panreac ITW Companies), Instaclone PCR cloning kit (Fisher Scientific), 1 kilobase (kb) molecular weight ladder, 100 base pair (bp) molecular weight ladder (New England Bio labs, Brennan & Company, Ireland), luria bertani agar (Sigma Aldrich), luria bertani broth (Sigma Aldrich), malt extract (Thermo Fisher Scientific Ireland), Monarch® Acid Purification Kits (New England Bio labs, Brennan & Company, Ireland), Murashige and Skoog Basal Medium, PCR *taq* 1.1X mastermix, 1-pentanol (Sigma Aldrich), phenol liquid non water-saturated (AppliChem, Panreac ITW Companies), potassium acetate (Sigma Aldrich), potassium hydroxide (KOH) (Sigma Aldrich), 2-propanol (Sigma Aldrich), Rely+On™ Virkon®, sodium acetate (Amresco), sodium chloride, sucrose, peptone mycological agar (Thermo Fisher Scientific Ireland), T4 DNA ligase (VWR), TAE (trisbase, acetic acid, EDTA), tris hydrochloride (Amresco), tryptophan (AppliChem, Panreac ITW Companies) and yeast extract (Sigma Aldrich).

2.2 Microbial Strains and Growth Media

2.2.1 *E.coli*

Escherichia coli One Shot® Top10 Chemically Competent cells (DH10B™) and *E.coli* JM109 (*end*A1 *gln*V44 *thi*-1 *rel*A1 *gyr*A96 *rec*A1 *mcr*B+ Δ(*lac-pro*AB) e14- [F' *tra*D36 *pro*AB+ *lac*Iq ZΔM15] hsdR17(rK-mK+) were used for bacterial transformation.

E.coli glycerol stocks were prepared from liquid LB cultures with a final concentration of 15% glycerol and stored at -40°C.

2.2.2 *Agaricus bisporus*

Agaricus bisporus A15 was sourced from the lab of Dr Helen Groghan, Ashtown Food Research Centre, Teagasc, Ashtown, Dublin 15, Ireland. *A. bisporus* A15 was routinely cultured on malt mycological peptone (MMP) agar, (5g malt extract, 2.5g mycological peptone and 7.5g agar) and incubated at 25°C for 25-30 days (Sonnenberg *et al.* 1988), Leach *et al.* 2004). Subculturing was performed by aseptically transferring 1cm^2 plugs of the fungus to fresh media. Plates were wrapped in parafilm to prevent drying out.

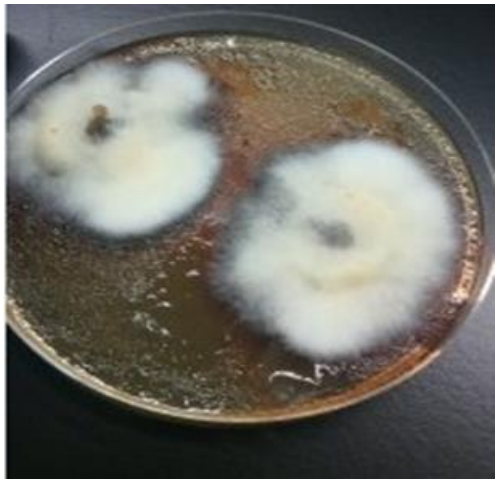


Figure 2.1: *A. bisporus* A15 grown on MMP media cultured at 25°C.

MMP broth was prepared as described above with the omission of agar. A 1cm^2 plug of *A. bisporus* mycelia from solid culture was aseptically transferred to 100ml MMP broth. Cultures were incubated at 25°C with shaking at 180 rpm for 25-30 days (Sonnenberg *et al.* 1988; Leach *et al.* 2004). Glycerol stocks were prepared by aseptically submerging 1cm^2 plugs of the fungus in 15% glycerol and storing at -40°C.

Figure 2.2: *A. bisporus* A15 grown in liquid culture (MMP broth) at 25°C.

2.2.3 *Coprinopsis cinerea*

Coprinopsis cinerea FA2222 mycelium was sourced from the lab of Dr Andy M. Bailey, School of Biological Sciences, Bristol University, UK. *C. cinerea* mycelia were routinely grown at 37ºC on yeast malt glucose (YMG) agar (4g/L glucose, 4g/L yeast extract, 10g/L malt extract and 12g/L agar). The pH was adjusted to 7.2 with KOH (Moore and Pukkila 1985). Following autoclaving, the media was supplemented with 100µg/mL tryptophan (Binninger *et al.* 1987), as *C. cinerea* FA2222 is a tryptophan auxotroph.

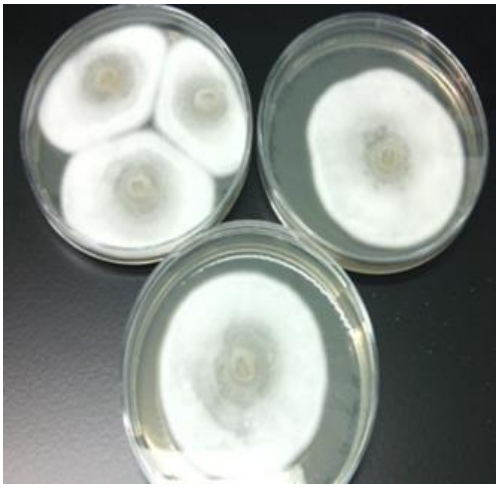


Figure 2.3: *C. cinerea* FA2222 grown on YMG agar supplemented with 100µg/mL tryptophan at 37°C.

YMG broth was prepared as above but with the omission of agar. Media was then supplemented with 100µg/mL tryptophan. A 1cm^2 plug of *C. cinerea* mycelia from solid culture was aseptically transferred to 100mL YMG broth. Cultures were incubated at 37°C with shaking at 180 rpm for 4 days (Binninger *et al.* 1987). Glycerol stocks were prepared by aseptically submerging 1cm^2 plugs of the fungus in 15% glycerol and storing at -40°C.

Figure 2.4: *C. cinerea* FA2222 grown in liquid YMG supplemented with tryptophan (YMGT) at 37°C.

2.2.4 *Hyphaloma faciculare*

The *Hyphaloma faciculare* fungal strain was sourced from the Belgian Co-ordinated Collections of Micro-Organisms (BCCM). *H. faciculare* mycelia were routinely grown at 20°C on Malt Agar (MA) (50g /L Malt). Plates were poured post autoclaving and plugs from slant agar stocks were inoculated onto fresh media.

Figure 2.5: *H. Faciculare* grown on malt agar at 20°C.

2.2.5 *Tyromyces stipticus*

The *Tyromyces stipticus* strain was sourced from the BCCM. *T. stipticus* mycelium was routinely grown at 20°C on Murashige and Skoog Basal Medium (MS) (21g MS and 14g/L agar). Plates were poured post autoclaving and plugs from slant agar stocks were inoculated onto fresh media.

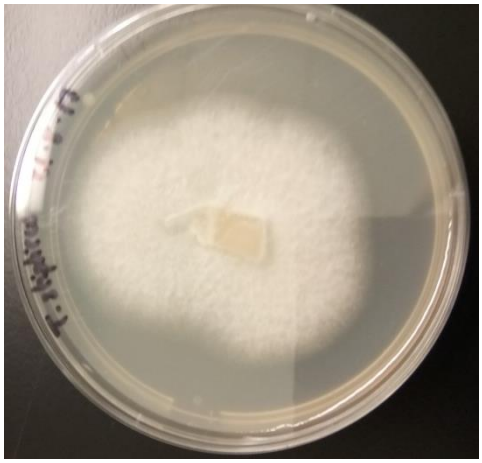


Figure 2.6: *T stipticus* grown on Murashige and Skoog Basal Medium at 20°C.

2.3 Vectors

Plasmid peGFPi004 (Figure 2.7) was sourced from the lab of Dr Andy M. Bailey, School of Biological Sciences, Bristol University, Bristol, UK. pCR®2.1-TOPO (Figure 2.8) and pTZ57R/T (Figure 2.9) were sourced from Thermo Fisher Scientific Ireland.

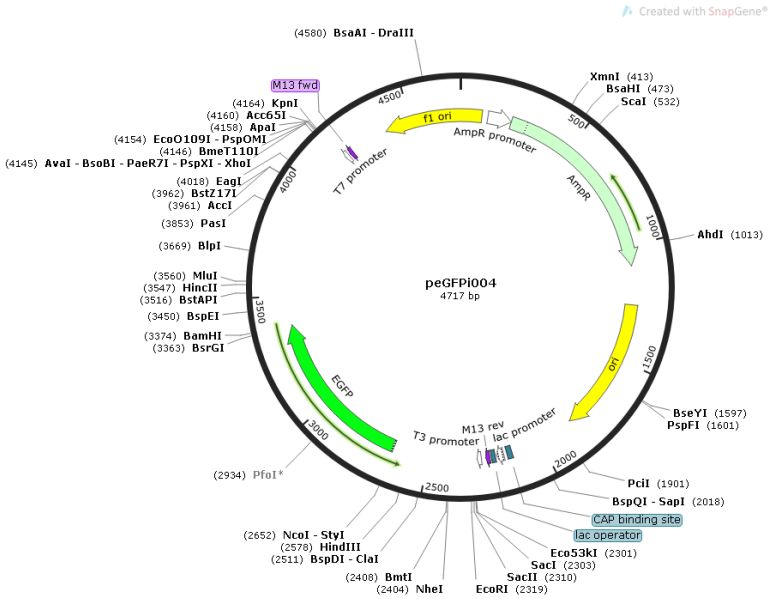


Figure 2.7: A plasmid map of peGFPi004 (Heneghan *et al.* 2007).

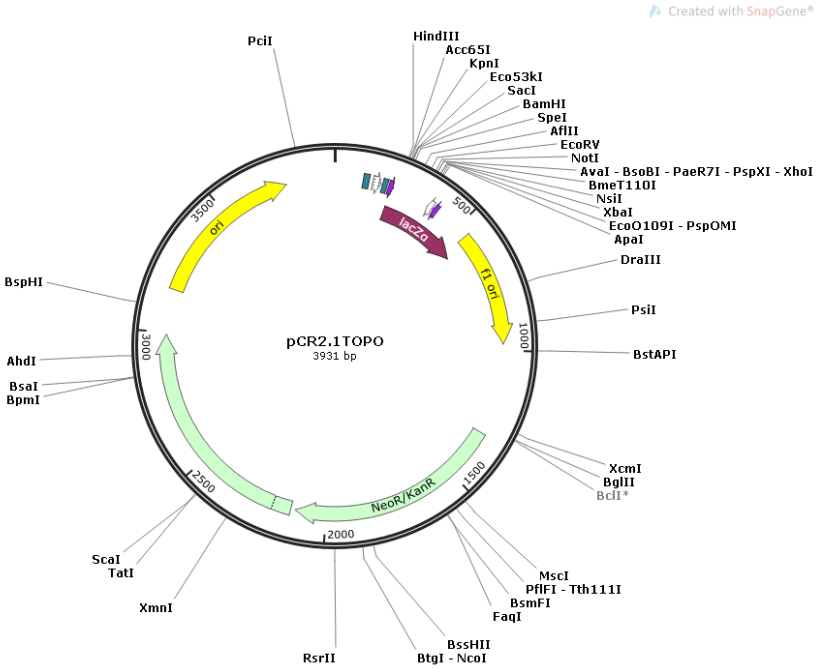


Figure 2.8: A plasmid map of pCR®2.1-TOPO (ThermoFisher).

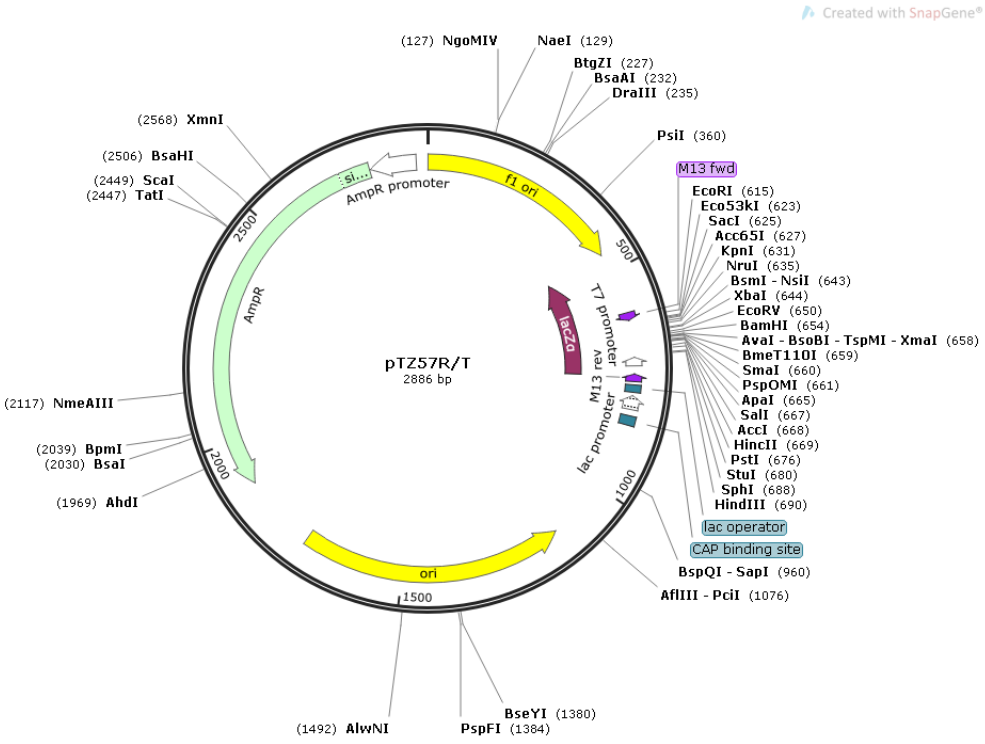


Figure 2.9: A plasmid map of the pTZ57R/T vector (ThermoFisher).

2.4 DNA Cloning

DNA fragments were subcloned into pCR®2.1-TOPO or pTZ57R/T following the manufacturer's instructions and subsequently transformed into *E.coli*.

The SPR 10592 promoter was cloned into the peGFPi004 expression construct (after the removal of *A. bisporus gpd*II promoter (*Sac*II-*Hind*III)) using the InsTAclone kit with the addition of T4 DNA ligase in ligase buffer following the manufacturer's instructions and subsequently transformed into *E.coli*. The molar ratio of the vector (peGFPi004) to insert (SPR 10592 promoter) used was 1:5.5.

2.5 *E.coli* Transformations

The pCR®2.1-TOPO cloning kits and *E.coli* chemically competent cells (Invitrogen TOP10cells) were used for transformation of promoters or gene DNA fragments. Plasmid DNA was chemically transformed using Top10 *E.coli* following the manufacturer's instructions, spread on Luria Broth (LB) agar supplemented with 100 µg/mL ampicillin and incubated overnight at 37ºC. Individual colonies were selected and used to inoculate 5mL LB broth (supplemented with 100µg/mL ampicillin) and incubated at 37ºC in a shaking incubator overnight. Alternatively, plasmids were transformed using *E.coli* JM109 cells following the manufacturer's instructions using the InsTAclone PCR Cloning Kit. The JM109 *E.coli* cells were made competent by inoculating a single bacterial colony in 2mL of pre-warmed C-medium (supplied in the kit) and incubating for 20 minutes in a shaking incubator at 37°C. Cells were pelleted by centrifugation for 1 minute and the supernatant was discarded. T-solution (supplied with the kit) was prepared by mixing equal volumes of T-solution A and B (following the manufacturer's protocol) which was then kept on ice. 300µL of T-solution was used to resuspend the cells and they were then incubated on ice for 5 minutes. 2.5µL of ligation mixture containing the vector was added into a fresh microcentrifuge tube and chilled on ice for 2 minutes. 50µL of the prepared *E.coli* cells were added to the tube containing the DNA vector; the contents were mixed and incubated on ice for 5 minutes. The cells were plated on LB agar supplemented with ampicillin and incubated overnight at 37°C. These are two similar cloning kits that were utilised throughout this MSc project. These kits made use of two separate strains of *E. coli* which served the same purpose. This was dictated primarily by availability of kits to our laboratory.

2.6 Nucleic Acid Extraction

2.6.1 Plasmid DNA extraction

Purification of plasmid DNA from *E.coli* cells was performed using the ENZA miniprep kit or the Monarch® Acid Purification Kit following the manufacturer's guidelines. For miniprep extraction, *E.coli* cells containing the appropriate plasmid were cultured in 5mL LB supplemented with the appropriate antibiotic and incubated at 37°C in a shaking incubator overnight.

Midiprep extractions were performed on 50mL cultures using the Qiagen midiprep kit or the Monarch® Acid Purification Kit, as per manufacturer's instructions. The kits used in these extractions were dictated primarily by availability of kits to the laboratory.

2.6.2 Gel extraction and purification of PCR products

Omega bio-tek gel extraction and purifications were performed following the manufacturer's guidelines. This kit was also employed for direct purification of PCR products.

2.6.3 Extraction of Genomic DNA from *Coprinopsis cinerea*

In order to establish the most efficient method for rapid isolation of DNA, two separate methods of DNA extraction were trialled.

2.6.3.1 Method I: DNA extraction using the Zolan and Pukkila method

DNA was extracted following the procedure of Zolan and Pukkila (1986) with the following adjustments: Mycelia from 100mL liquid cultures were filtered through muslin cloth and all the liquid and moisture was squeezed from the culture. Mycelium was ground to a fine powder under liquid nitrogen using a mortar and pestle. 600µL extraction buffer (0.7M NaCl, 50mM Tris pH8, 10mM EDTA pH8 and 1% w/v CTAB) along with 6µL β-mercaptoethanol was then added to 40mg of sample, which was vortexed briefly and incubated at 60°C for 30 minutes. 600µL of SEVAG (24:1 chloroform:isoamyl alcohol) was added to the sample. The sample was then vortexed and centrifuged at 13,000rpm for 5 minutes. The top layer was transferred to a fresh 1.5mL microcentrifuge tube (lower phase discarded) and re-extracted with SEVAG as before. The top layer was again transferred to a clean 1.5mL micro-centrifuge tube and an equal volume of isopropanol was added to the sample. The tube was mixed by inversion and centrifuged for 1 minute at 13,000 rpm. The isopropanol supernatant was discarded and the sample centrifuged for 1 minute, removing any remaining isopropanol. The pellet was resuspended in 300µL TE. 3µL RNase A was added and the tube was incubated at 37°C for 30 minutes. 300µL of 25:24:1 phenol:chloroform:pentanol (PCP) was added, the sample was vortexed, and then centrifuged at 13,000rpm for 5 minutes. The upper phase was transferred to a fresh tube. 50µL ammonium acetate (7.5M) and 2 volumes of ice-cold 100% ethanol were then added. The sample was mixed by inversion and incubated at -40°C for 15 minutes, before centrifugation at 13,000rpm for 1 minute. The supernatant was removed and the remaining pellet was resuspended in 300µL 0.2M ammonium

acetate. 2 volumes of ice-cold ethanol was added and the sample incubated at -40°C for 15 minutes. The sample was then centrifuged at 13,000rpm for 1 minute. The final pellet was washed with 100µL 70% ethanol and air-dried before resuspending in 50µL sterile water or TE.

2.6.3.2 Method II: The Minilys® and Precellys® beads DNA extraction method

This rapid method uses a bead mill, Minilys® personal homogenizer, in conjunction with a fungal DNA extraction kit. 0.4g of liquid culture (filtered through a muslin cloth) or 0.5g of solid culture mycelia (scraped from an agar plate), were placed into the precellys® vial containing ceramic/glass beads, using a sterile pipette tip to help push mycelia into contact with the contents of the tube. Samples were shaken for 60 seconds in the Minilys® bead mill homogenizer at the low/medium setting until the contents were seen to be completely broken down. 600µL SFG1 Buffer and 4µL of RNaseA (E.N.Z.A SP Fungal DNA Mini kit D5542-01) were added to the vial, and vortexed to ensure that samples were suspended and that no clumps remained. An extraction was subsequently carried out from step 3 of the E.N.Z.A SP Fungal DNA Mini kit (D5542-01), according to the manufacturer's instructions.

2.7 Restriction Digests

Restriction enzymes were obtained from Sigma, Promega, NEB (Brennan & Company) and Fermentas (VWR). Standard reaction conditions used for analytical and preparative digests are outlined in Table 2.1. Appropriate buffers for double digests were selected using online tools (NEB double digest finder, Promega double digest finder or Fermentas double digest finder).

Table 2.1: Standard reaction conditions for restriction digest analysis.

Reagent	**Single digest**	**Double digest**
Buffer (10X)	1µL	1µL
Restriction enzyme(100u/µL)	0.75µL	0.75µL
Restriction enzyme(100u/µL)	0µL	0.75µL
DNA(0.15ng/µL - 97.62ng/µL)	1µL	1µL
Sterile H_2O	17.25µL	16.6µL
Total volume	20µL	20µL

2.8 Polymerase Chain Reaction

2.8.1 Primer Design

Primers were designed to amplify the *C. cinerea* serine protease promoters (Table 2.2), the serine protease genes (Table 2.3) and the fruiting body promoters (Table 2.4). Restriction enzyme sites were incorporated to aid in cloning experiments. Universal primers used in this study are detailed in Table 2.5. All primers were received lyophilised (Sigma) and were resuspended in the appropriate volume of nuclease free H_2O to a final concentration of 100µM.

Table 2.2: Primers designed to amplify the serine protease promoters of *C. cinerea.* Restriction sites are underlined.

***C. cinerea* promoter**	**Restriction Enzyme**	**Sequence (5'-NNNN-3')**	**Primer name**
CC1G_ 04562	*Sac*I	GAGCTCGGAGGAGCGCTTTCGAGAGTCTCG	SPR04562 fwd
CC1G_ 04562	*Hind*III	AAGCTTCATGGCAGAGACAGGGAAGCGAC G	SPR04562 rev
CC1G_ 07792	*Sac*I	GAGCTCCGAGGCTATCTGGAAATGGGGT	SPR07792 fwd
CC1G_ 07792	*Hind*III	AAGCTTGCATGGTAGAGGATGGAAGCAG	SPR07792 rev
CC1G_ 10592	*Sac*II	CCGCGGGCGATATCTAATGTTCGAGATA	SPR10592 fwd
CC1G_ 10592	*Hind*III	AAGCTTGCATAGTGAAGCTGCTAGAGG	SPR10592 rev
CC1G_ 10606	*Eco*R1	GAATTCGTGATGAAGCTACAACGTGTATC	SPR10606 fwd
CC1G_ 10606	*Hind*III	AAGCTTGCATCCTGGGGTCTGGTTGGGC	SPR10606 rev
CC1G_ 10615	*Sac*II	CCGCGGACCCTATGTTATGAAAACATATGT C	SPR10615 fwd
CC1G_ 10615	*Hind*III	AAGCTTTCATCGTGAAGAGAAGGGGCGA	SPR10615 rev
CC1G_ 3122	*Eco*R1	GAATCCAACCACTTCCCGGAGAGCGATG	SPR3122 fwd
CC1G_ 3122	*Hind*III	AAGCTTTCCGCATGGCTGGCTGCAGCTGATT T	SPR3122 rev
CC1G_ 4470	*Eco*R1	GAATTCCCGCAGGGAAGAAAGTGAATCC	SPR4470 fwd
CC1G_ 4470	*Hind*III	AAGCTTGACATGGTAGTGGTTCAGGAG	SPR4470 rev

Table 2.3: Primers designed to amplify the serine protease genes (for the construction of antisense expression vectors). Restriction sites are underlined.

***C. cinerea* genes**	**Restriction enzyme**	**Sequence (5'-NNNN-3')**	**Primer name**
CC1G_10592	*BamHI*	GGATCCACGCGTAACGAGAATCTTAC	Antisense10592 forward
CC1G_10592	*HindIII*	AAGCTTTTCCAACGTCATCTCTGCCT	Antisense10592 reverse
CC1G_04562	*XmaI*	GGATCCTATCATGTACACCATGTCCC	Antisense04562 forward
CC1G_04562	*AatI*I	AAGCTTTGCCAACACTTCTCCCGCAA	Antisense04562 reverse

Table 2.4: Primers designed to amplify the fruiting body promoters. Restriction sites are underlined.

***C. cinerea* promoter**	**Restriction Enzyme**	**Sequence (5'-NNNN-3')**	**Primer name**
OAT	*Sac*II	CCGCGGAGTCGTGTCGGGACAACGGC	*OAT* forward
OAT	*Hind*III	AAGCTTCACCAATGCCTGTACGAGGT	*OAT* reverse
*CGL*1	*Sac*I	GAGCTCTACACGTTTCAGCTTTAGCT	*CGL*1 forward
*CGL*1	*Cla*I	ATCGATGGTAGAGCATGATGTTGAAC	*CGL*1 reverse
*CGL*2	*Sac*II	CCGCGGGCTGCTATAGCGAGATTTGT	*CGL*2 forward
*CGL*2	*ClaI*	ATCGATGTGGTAGAGCATGATGTTAG	*CGL*2 reverse
ABL	*SacI*	GAGCTCCTCAACGGATTAACATGGAT	*ABL* forward
ABL	*Cla*I	ATCGATCCATGGCTGCTCAACGTTATGAGTTA	*ABL* reverse

Table 2. 5: M13 universal primer sequences and primers designed to amplify the ITS region of plasmids.

Name	**Sequence (5'-NNNN-3')**
M13 forward	CTGGCCGTCGTTTTAC
M13 reverse	GTCCTTTGTCGATACTG
ITS1	TCCGTAGGTGAACCTGCGG
ITS4	TCCTCCGCTTATTGATATGC

2.8.2 PCR components

A standard 50µL PCR reaction mix was composed of: 25µL PCR *taq* mastermix at a final concentration of 0.55X (containing Extender™ DNA Polymerase Blend, dNTPs, electrophoresis tracking dye, and a non-mutagenic EZ-Vision® visualization dye; VWR), 0.5µL of 10µm forward primer, 0.5µL of 10µm reverse primer, 0.5µL template DNA (0.15ng/µL - 100ng/µL) and 24µL sterile water.

2.8.3 PCR conditions

A typical PCR programme is detailed in Table 2.6. The extension time of a PCR reaction depends on the product length and is generally calculated at 1 minute per kb. The annealing temperature (Ta) is determined by the sequence of the specific primer pair and is calculated using the following formula:

$Ta^{o}C = Tm°C-5^{o}C$

where Tm°C (Melting Temperature) is calculated using the following equation:

$Tm^{o}C = 4(G+C) +2(A+T)$

Table 2.6: Standard PCR conditions.

PCR step	Temperature	Time	Cycles
Lid heating	110°C	1 minute	1
Initial denaturation	95°C	5 minutes	1
Denaturation	95°C	30 seconds	30
Annealing	50°C	30 seconds	
Extension	72°C	45 seconds	
Final extension	72°C	10 minutes	1
Holding time	4ºC	∞	1

2.8.4 Rapid PCR screening method

Fungal colony PCR was performed as described by Walch *et al*., 2016. This method involved using a sterile pipette tip to scrap a sample of mycelia from solid cultures, which then served as the DNA template for the colony PCR. This mycelium was added to 17µL of PCR *taq* mastermix (1.1X) (containing Extender™ DNA Polymerase Blend, dNTPs, electrophoresis tracking dye, and a non-mutagenic EZ-Vision® visualization dye; VWR) and 1µL of forward and reverse ITS primers (10nm). The PCR was performed with an annealing step of 55°C for 1 minute and an extension step of 72°C for 1 minute, as per section 3.8.3.

2.9 DNA Analysis

Agarose gels were cast and run in TAE buffer at a concentration of 1X for DNA visualisation. To prepare a 1% gel, 0.4g of agarose was added to 40ml 1X TAE buffer. The solution was heated in a microwave in 30-second intervals with intermittent mixing until the agarose was completely dissolved. The agarose solution was then cooled to approximately 50°C and 4µL of gel red (final concentration 1X) was added. Bromophenol blue was used as a loading/tracking dye. Analytical gels were run at 145V for 45 minutes and preparative gels at 90V for 1 hour and 30 minutes. Gels were visualised by UV light and recorded using the ChemiDoc™ MP Bio-Rad imaging machine. DNA was quantified using the DeNovix Spectrophotometer DS-11 (spectrum analysis 190-840 nm) following the manufacturer's guidelines.

2.10 DNA Sequencing, Sequence analysis and Bioinformatics

2.10.1 Sanger Sequencing Services

DNA was quantified using the DeNovix DS-11 nanodrop and 10µg was transferred to a sterile 1.5mL eppendorf. The DNA was then sent to Source Bioscience for sequencing. Sequencing primers were supplied by Source BioScience, Ireland.

2.10.2 Sequence retrieval

Sequence information was obtained from the NCBI (National Centre for Biotechnology Information) database (https://www.ncbi.nlm.nih.gov/), a retrieval system for searching several linked databases. The Genbank database is a public source of DNA sequences assembled from data submissions, that is hosted through the NCBI website. This database was used to retrieve nucleotide sequences for the genes and promoters regions used in this study.

2.10.3 Sequence analysis

Sequences were aligned using BLAST (Basic Local Alignment Search Tool) software, which can search for and align sequences using various interfaces. These interfaces include BLASTN (searches nucleotide databases using a nucleotide query), BLASTP (searches protein databases using a protein query), BLASTX (searches protein databases using a nucleotide sequence), TBLASTN (aligns protein sequences to a nucleotide sequence) TBLASTX (searches translated nucleotide databases using a translated nucleotide query), and Primer-BLAST (generates primers to a specific nucleotide template sequence).

The EMBL-EBI MUSCLE (a multiple sequence comparison by log-expectation) programme allows multiple sequence alignments. Both amino acid and nucleotide sequences can be aligned using this programme (http://www.ebi.ac.uk/Tools/msa/muscle/). The reverse complement of a sequence was obtained using an online tool at http://reverse-complement.com.

Webcutter (http://rna.lundberg.gu.se/cutter2/), Serial cloner 2.1 and Snapgene software were used to predict where restriction enzymes sites are located in a sequence, generating results as a graphical image of a plasmid vector. These programmes were also used for plasmid mapping and to open sequencing information in a chromatogram format.

Chapter 3: Construction of the *Coprinopsis cinerea* serine protease 10592 and antisense plasmid

3.1 Introduction

Serine proteases (SPRs) have been established as playing a significant role in both post-harvest spoilage of mushrooms and nutrient acquisition from compost (Burton *et al.* 1994, Heneghan *et al.* 2016). In particular, previous research has indicated a critical role of the *A. bisporus* SPR1 enzyme in the carbon and nitrogen sequestration abilities of basidiomycetes (Heneghan *et al.* 2016). Through genome mining, Heneghan *et al.*, (2009) have identified seven putative SPRs in *C. cinerea.* Analysis of these putative *C. cinerea* SPRs genes revealed significant homology to the *A. bisporus* SPRs (Heneghan *et al.* 2009). It has been previously suggested that the *C. cinerea* genes are serine proteases, belonging to the subtilism family (Heneghan *et al.* 2009). In particular, *C. cinerea* SPR 10592 showed 57% homology when aligned with *A. bisporus* SPR1 and 54% homology when aligned with *A.bisporus* SPR2. This research proposes to investigate the role of the endogenous *C. cinerea* SPR 10592, using promoter profiling and gene silencing. Promoter profiling involves amplification of the SPR 10592 promoter, linking this putative promoter fragment to the eGFP reporter gene using the basidiomycete molecular toolkit kit (see Section 1.9) (Burns *et al.* 2005, Heneghan *et al.* 2009, Collins *et al.* 2010) to generate plasmid peGFPi10592 which can subsequently be used in transformation experiments in *C. cinerea*. Gene silencing involves construction of a plasmid designed to contain the gene in the antisense orientation under the control of a strong constitutive promoter.

The first step in this process was to design the promoter plasmid peGFPi10592. Figure 3.1 represents a schematic overview of this design. The plasmid peGFPi004 is based on the commercial pBluescript vector and contains the *A. bisporus* glyceraldehyde-3-phosphate dehydrogenase (*gpd*II) promoter, an intron that is required for gene expression in *C. cinerea*, eGFP and the *A. nidulans Trp*C terminator (Figure 3.1 A). The restriction enzymes *Sac*II and *Hind*III were identified to remove the 300bp *A. bisporus gpd*II promoter leaving the rest of the plasmid intact (Figure 3.1 B). The SPR promoter was then PCR amplified to include the restriction sites *Sac*II and *Hind*III for directional cloning (Figure 3.1 C). The *gpd*II promoter was replaced by the *C. cinerea* SPR 10592 promoter forming the SPR promoter plasmid peGFPi10592 (Figure 3.1 D).

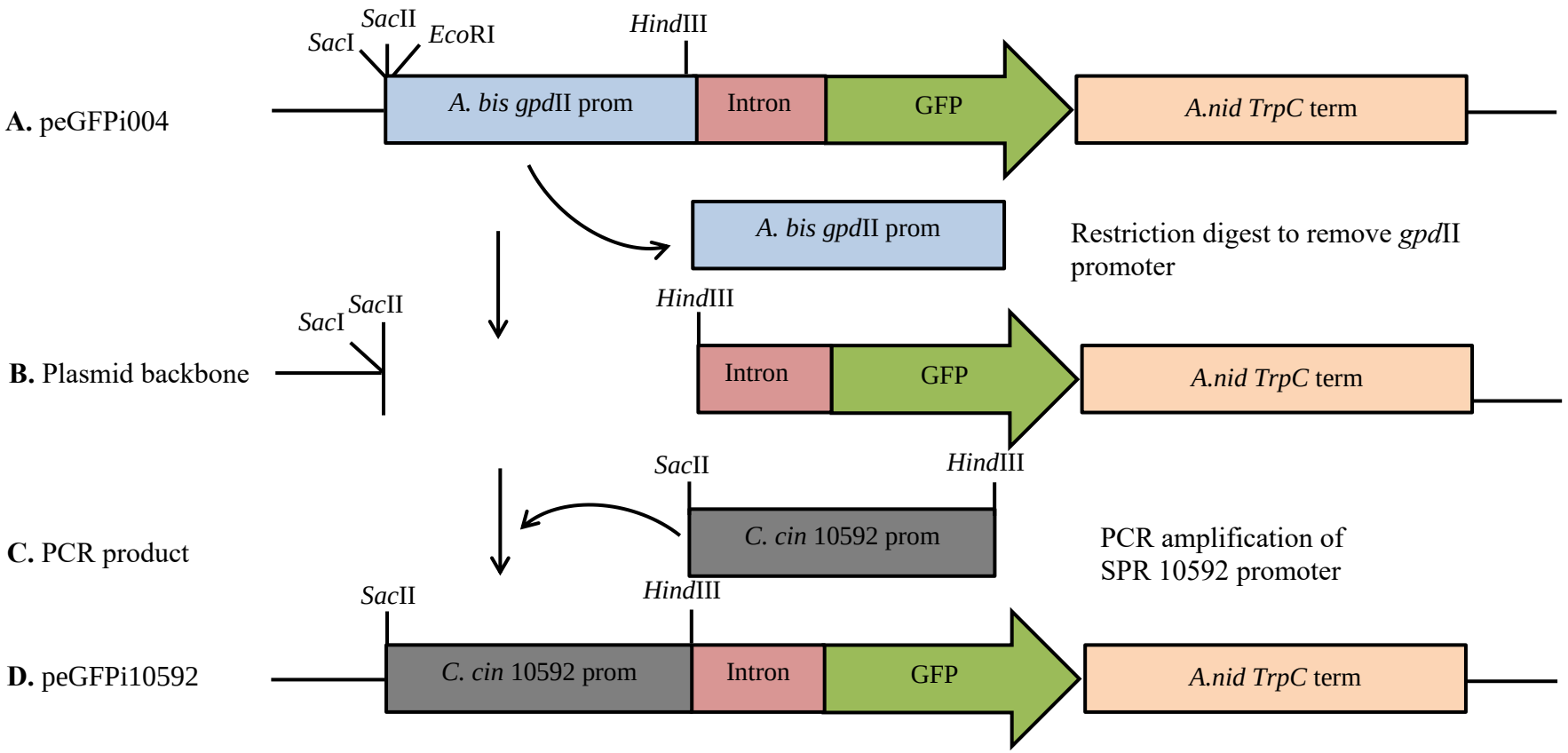


Figure 3.1: A schematic representation of the design and construction of recombinant vector peGFPi10592. A: The intact peGFPi004 plasmid containing the original *A. bisporus gpd*II promoter (*A.bis gpd*II prom), intron, eGFP and *A.nidulans trpC* terminator (*A.nid TrpC* term). B: The plasmid digested with restriction enzymes *Sac*II and *Hind*III to remove the *gpd*II promoter leaving the plasmid backbone. C: The amplified *C. cinerea* SPR 10592 promoter flanked with *Sac*II and *Hind*III restriction sites. D: The intact peGFPi10592 contains the *C. cinerea* SPR 10592 promoter ligated into the peGFPi004 backbone

3.2 DNA extraction-The Zolan and Pukkila method

An important requirement to facilitate this research was the sourcing of a DNA template for the PCR amplification of the *C. cinerea* SPR promoter. For high throughput screening and analysis, the development of a rapid DNA extraction method for fungal samples was central to this work as previously published methods are time consuming, laborious and often result in low yield (Zolan *et al*. 1986).

Initially, a large scale method, based on the published method of Zolan and Pukkila (1986) was employed for gDNA extraction (as per Section 2.6.3.1) from *C. cinerea* liquid cultures and 5µL of gDNA was analysed by agarose gel electrophoresis, as depicted in Figure 3.2. DNA was successfully extracted as indicated by the high molecular weight bands seen in lane 2 and 3 of the figure below.

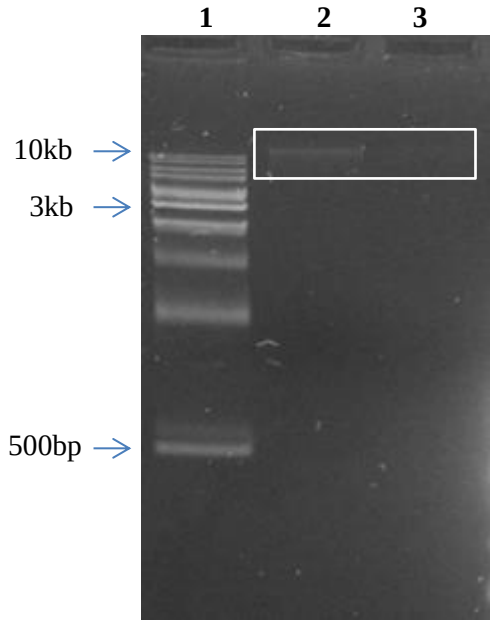


Figure 3.2: *C. cinerea* genomic DNA (extracted using the Zolan and Pukkila method) electrophoresed on a 1% agarose gel at 120V for 45 minutes. Lane 1: 1kb NEB ladder; lanes 2 and 3: 3µL genomic DNA from two separate mini preps. The white box indicates the location of the DNA.

3.3 DNA extraction-The Minilys® and the Precellys® beads method

Next, an alternative DNA extraction method was tested on *C. cinerea* mycelia harvested from liquid culture employing a combination of the Minilys® personal homogenizer,

Precellys® bead vials and the ENZA fungal DNA extraction kit (see Materials and Methods, Section 2.6.3.2). Following extraction, 3µL of DNA was analysed by agarose gel electrophoresis, as shown in Figure 3.3. As can be seen below, DNA was successfully extracted as shown by the presence of the high molecular weight bands in lane 3 and 4.

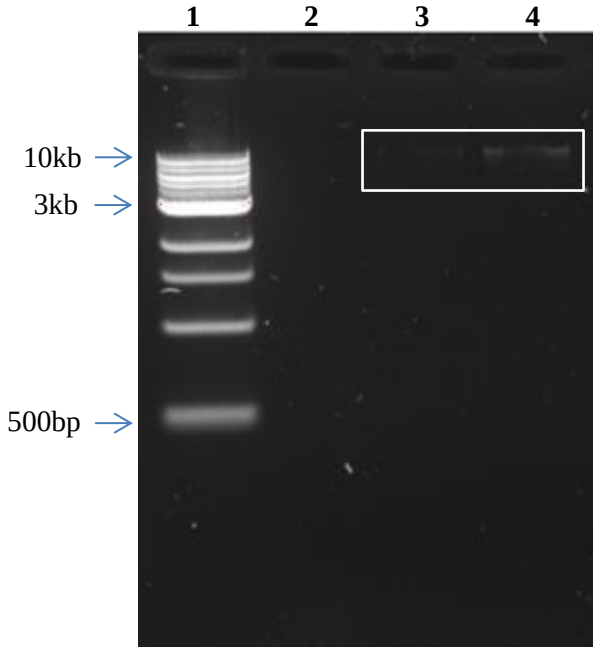


Figure 3.3: *C. cinerea* genomic DNA (extracted using the Minilys® personal homogenizer and the Precellys® beads) electrophoresed on a 1% agarose gel at 120V for 45 minutes. Lane 1: 1kb NEB ladder; lane 2: blank; lanes 3 and 4: 3µL genomic DNA from two separate mini preps. The white box indicates the location of the *C. cinerea* DNA.

This rapid method was also tested on *C. cinerea* and *A. bisporus* mycelia, scraped from solid culture and 3µL of gDNA was analysed as before. The results are depicted in Figure 3.4 and 3.5. As can be seen from the analysis, DNA was successfully extracted from solid culture, thus eliminating the need for liquid cultivation. This was a welcome research outcome, reducing the time and labour intensive method of Zolan and Pukkila (1986). This rapid DNA extraction method was published as a technical report and is available at: https://homogenizers.net/pages/p-dna-extraction-from-fungal-organisms. A copy of this report can also be seen in Appendix A.

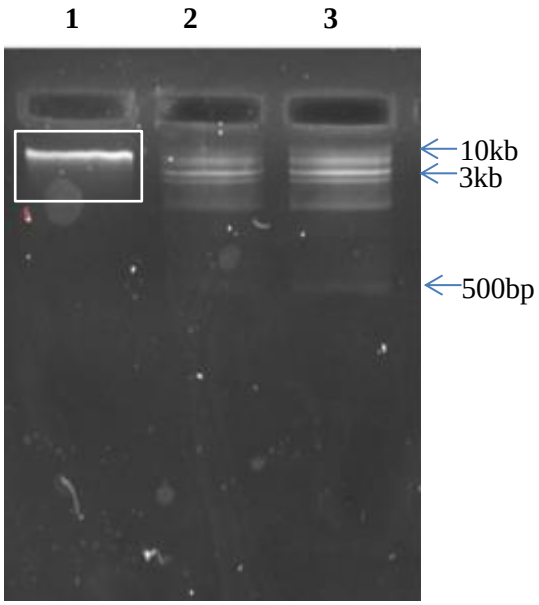


Figure 3.4: *C. cinerea* genomic DNA (extracted using the Minilys® personal homogenizer and the Precellys® beads) electrophoresed on a 1% agarose gel at 120V for 45 minutes. Lane 1: 3µL genomic DNA; lanes 2 and 3: 1kb NEB ladder. The white box indicates the location of the *C. cinerea* DNA.

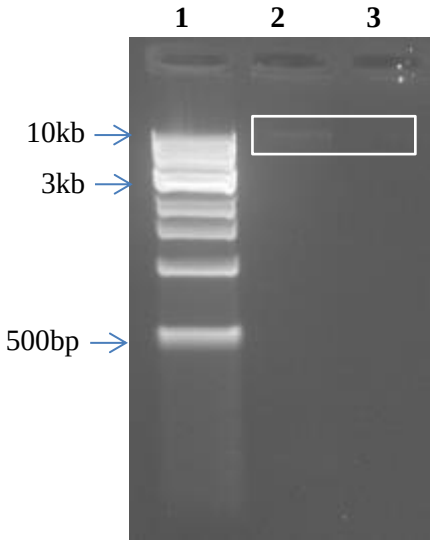


Figure 3.5: *A. bisporus* genomic DNA (extracted using the Minilys® personal homogenizer and the Precellys® beads) electrophoresed on a 1% agarose gel at 120V for 45 minutes. Lane 1: 1kb NEB ladder; lanes 2 and 3: 3µL genomic DNA from two separate mini preps. The white box indicates the location of the *A. bisporus* DNA.

3.4 Rapid PCR screening method

A method for the rapid PCR screening of fungi was also employed (Walch *et al.* 2016). This method allows the rapid amplification of DNA fragments from fungi as well as the screening of fungal transformants without the genomic DNA extraction step. This method was tested on three basidiomycetes; *C. cinerea* FA2222, *Hypholoma fascicular* (Kampichler *et al.* 2004, Jafari *et al.* 2018) and *Tyromyces Stipticus* (Liaud *et al.* 2014*)*.

The sourcing of this rapid reliable method was an important requirement to facilitate this research. In this PCR screening method , Internal Transcribed Spacer (ITS) primers were used as test primers and the method was used on different strains to establish the reliability of it.

Briefly, the method involved using a sterile pipette tip to scrap mycelia from a solid culture. 1µL of a forward ITS1 primer and 1µL of a reverse ITS4 primer was utilised and the PCR was performed with an annealing step at 55°C for 1 minute and an extension step at 72°C for 1 minute (as per Materials and Method, Section 2.8.3 Table 2.6). Following PCR, 5µL of the products were analysed by agarose gel electrophoresis to check for migration of the fragments and the results can be seen in Figure 3.6, lane 2-5. The rapid PCR screening method successfully amplified the 600bps ITS region of *C. cinerea* FA2222, *H. fascicular* and *T. Stipticus*. This method allows the use of fungal material directly from a solid culture in a PCR, thus eliminating the need for genomic DNA extraction when performing high throughput screening.

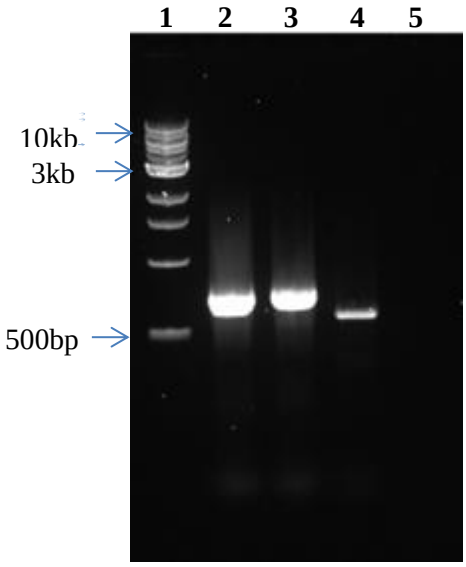
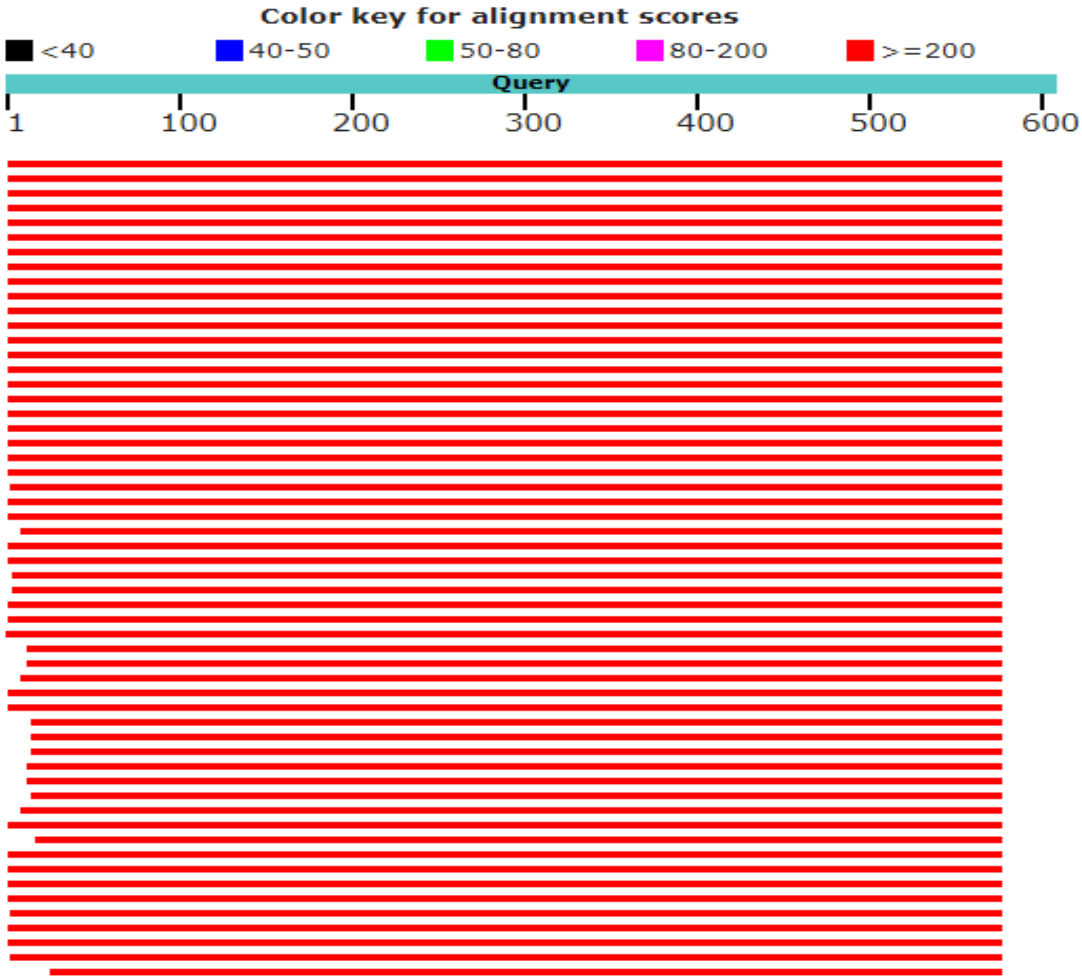


Figure 3.6: The ITS region of *C. cinerea* FA2222 *H. faciculare* and *T. Stipticus* electrophoresed on 1% agarose gel at 120V for 45 minutes. Lane 1: 1kb NEB ladder; lane 2: 5µL *C. cinerea* FA2222; lane 3: 5µL *H. faciculare*, lane 4: 5µL *T. Stipticus* and lane 5: negative control.

The fragments amplified from *C.cinerea* and *H. fasciculare* were next sequenced using ITS primers and the results were analysed using NCBI BLAST. The BLAST software results confirmed the identity of both *C. cinerea* and *H. faciculare* (Figures 3.7 A and 3.8 A, respectively). MUSCLE software assembles multiple sequence alignment (identical nucleotides are denoted by the symbol *) and generates a "Percent Identity Matrix", which determines the number of identical bases between two sequences in an alignment and gives a measure of similarity between sequences. This measurement is presented as a percentage value. A percent identity matrix is presented for each species in Figure 3.7 B and 3.8 B, indicating identity values of 99%.

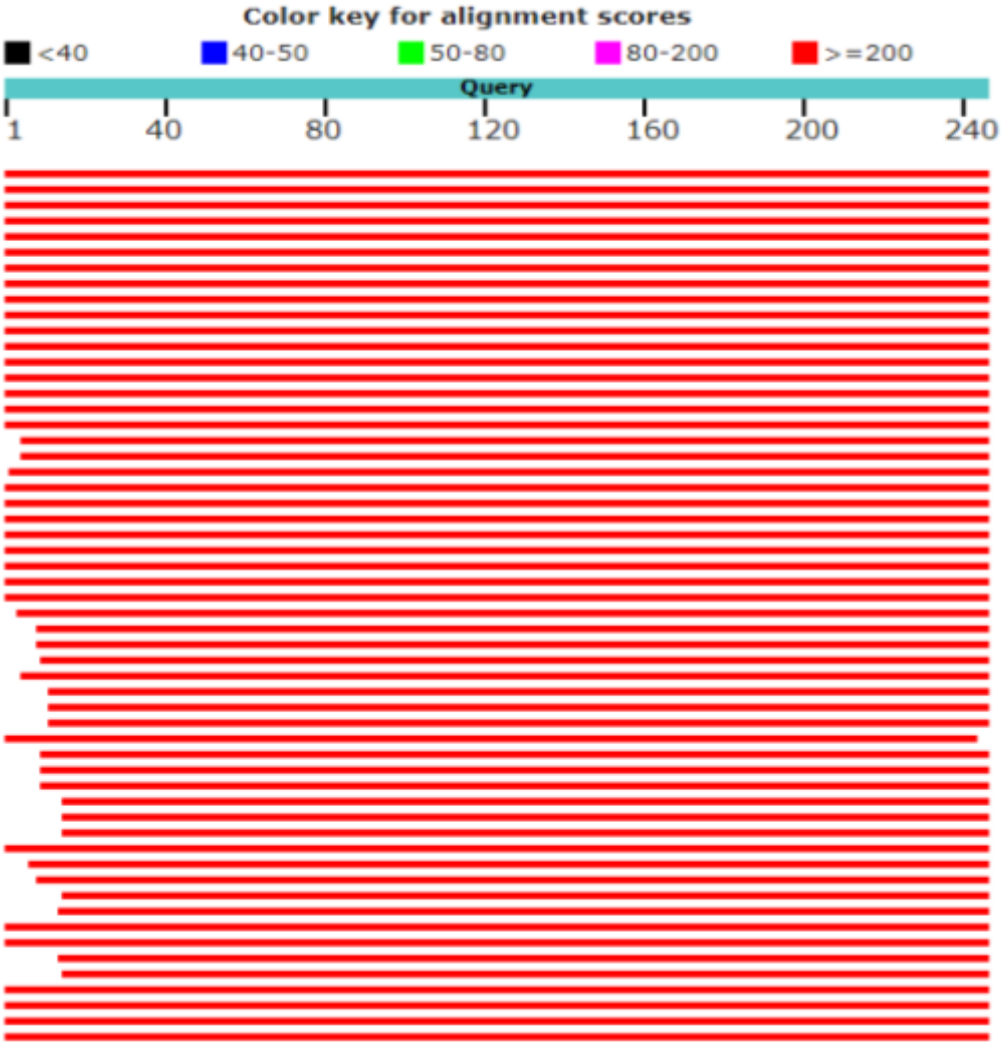
A.



B.

Figure 3.7: A: A graphical representation of the BLAST results to confirm the identity of *C. cinerea*. B: The percent identity matrix for *C.cinerea* indicating an identity value of 99% as highlighted by the yellow box.

A.



B.

Figure 3.8: A: A graphical representation of the BLAST results to confirm the identity of *H. faciculare*. B: The percent identity matrix for *H. fasciculare* indicating an identity value of 99%, as highlighted by the yellow box.

A combination of the rapid gDNA extract method and PCR screening method was used throughout this thesis to isolate DNA fragments.

3.5 Preparation of the SPR 10592 promoter fragment

3.5.1 Sequence retrieval and analysis

Having established a reliable protocol for gDNA extraction, the next step in the process was the preparation of the SPR 10592 promoter fragment in advance of cloning into peGFPi004. The SPR 10592 gene was first identified using the Genbank database, and a 1kb sequence upstream of the ATG start site was selected. Promoters are often recognised by specific motifs that are present in a sequence such as CAAT regions and/or TATA boxes. A 1000bps of the putative SPR 10592 promoter sequence was analysed for these sequences. Such motifs were identified and are highlighted in bold and underlined in Figure 3.9.

AATGGTTAGTTTCCTGTTCTGTGCGGTGATTTCTCAAGTGTGCCTTATGCAAGCGAATAGTCC
ATGCGTTTTGTTCAGAAAATGGACACTCAGATTGGGTTCCTCTTGGGTGCATACCCATGGAC
GCGACGGTCCGCATTGAACCATGACCATGAAGGGCGATGGCGGCTTCGTTCCCAGAAATGA
TTGCGCGATATCTAATGTTCGAGATAACAGGCGAGTGGTGACGCTTGCCATACCAACTTCTT
CTGTTCTAACGGGTGTTCTTCTCCACTATCAAGGCCAACGCACACGTTCACTTGA**CAAT**ATTC
ATGTGGTGGCGCTTGGATATAGCACCCAGGCCCAACGCACGATTGGTAGTATCCATGTCGAG
CTCGTCCTGTTCGACTACACCTGACTCCATCGCAGTGCTTCTCTGCGTCC**CAAT**TGGTAATAA
ACACCACAGGAACCCCACAAACACCCGTCTC**CAAT**CAGGATATGGCGTTGTGAGCAGCGAC
GGTACAGACCGGCCCATCGTTTCCCGCGACCCGCGTTAGTTATCCTATGCAACGCACGAGCC
TAGACACTTGACGCCCAGTTCTCTTGATGAAGGCCGCAACAGATAGGTTGTCAAACAGTGAA
ACGCGCGATCTCCTGTTAGATATGCTGTGGGCAGGGTCACGAGCGTACTCTTCGAATGATGA
TGGGATACCGATTCGAGGAATTCACTTCTTCTGGCCACATGGTACGGGTATGTTTCCAAATTT
AACCGCGATTTCACCATTGACTGAACTGTCTACCTCCTACTCTCAGAACTTGACCTCTGGCCC
TTGTGCAGCGTGTAACCAGTCCTTCTATGGTATCACCTGAACTTCTGTGCATGCTGCCGCTTC
GGTAGTTTTCCTTTGCGGCGGAAGCAGGCTGTAGTTCAAAGGG**TATAAA**TGGTATGTTGTCG
CAGGTCTAATTCATTGTGAGCCCGTCTCATATTCCATCCTCTCCTAGGTCCTCTAGCAGCTTC
ACTATGC

Figure 3.9: *C. cinerea* genomic DNA (1kb) upstream from the CC1G_10592 gene. A TATA box and CAAT regions are highlighted in bold and underlined. The forward primer is highlighted in red and the reverse primer is highlighted in blue.

3.5.2 PCR amplification of the SPR 10592 promoter sequence

The primers for the PCR amplification of the putative SPR promoter region were next designed from the sequence shown in Figure 3.9. Primers with appropriate restriction enzyme sites can be used to facilitate directional cloning. Analysis of the cloning vector, peGFPi004, revealed that the plasmid contained the restriction sites *Eco*RI, *Sac*I and *Sac*II upstream of the *gpd*II promoter and a *Hind*III restriction site at the 3' end of the promoter. Restriction digestion of this plasmid with either *Sac*I-*Hind*III, *Sac*II-*Hind*III or *Eco*RI-*Hind*III would, therefore, result in the excision of the *gpd*II promoter, leaving the intron-eGFP-terminator cassette intact. Examination of the promoter sequence using the online tool, webcutter, determined that both *Eco*RI and *Sac*I cut within the SPR 10592 promoter, while *Sac*II and *Hind*III do not. Based on this analysis, a *Sac*II site was included on the forward primer and a *Hind*III site was included on the reverse primer (see Section Materials and Methods, 2.8.1, Table 2.2) to allow directional cloning.

These primers were used in a PCR with *C. cinerea* genomic DNA, employing an annealing step at 70°C for 90 seconds and an extension step at 72°C for 2 minutes (as per Materials and Methods, Section 2.8.3). Following PCR, 5µL of the product was analysed by agarose gel electrophoresis to check for migration of the fragment at the expected size of 814bps and the results can be seen in Figure 3.10. The putative promoter was successfully amplified as indicated by the band at the expected size of approximately 814bps as seen in lane 1.

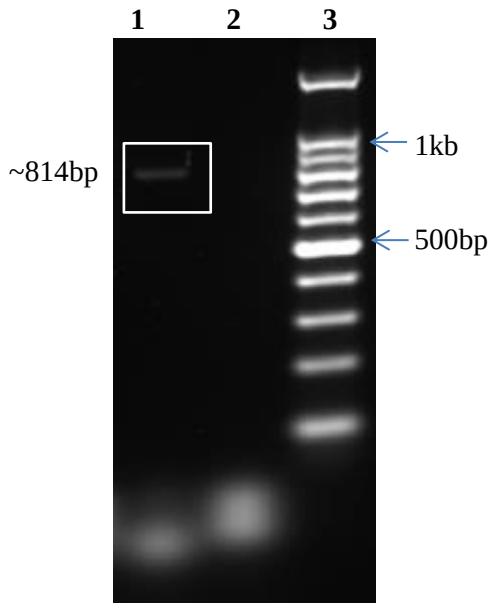


Figure 3.10: The putative SPR 10592 promoter DNA electrophoresed on a 1% agarose gel at 120V for 45 minutes. Lane 1: 5µL SPR 10592 promoter; lane 2: negative control; and lane 3: 100bp NEB ladder. The white box indicates the location of the promoter DNA.

Following confirmation that the PCR product resolved at approximately 814bps, a purification step was performed. The SPR 10592 promoter product was excised from the gel and purified using the Omega bio-tek gel extraction kit (as per Materials and Methods, Section 2.6.2). 3µL of the purified SPR 10592 promoter was electrophoresed on a 1% agarose gel, as shown below in Figure 3.11. This analysis confirms that the promoter was successfully purified as can be seen in lane 2 below. The white box highlights the location of the purified DNA.

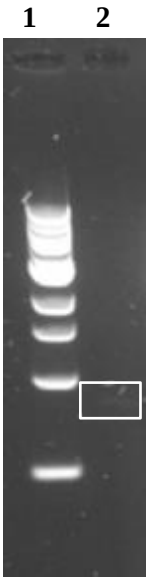


Figure 3.11: Purified *C. cinerea* SPR 10592 promoter electrophoresed on a 1% agarose gel at 100V for 1 hour. Lane 1: 1kb NEB ladder, lane 2: 3µL purified SPR 10592 promoter DNA. The white box indicates the location of the promoter DNA.

3.5.3 Cloning of SPR 10592 promoter into pCR®2.1-TOPO

The purified promoter was next cloned into pCR®2.1-TOPO (Materials and Methods, Section 2.3) to produce the plasmid p10592TOPO. The vector pCR®2.1-TOPO is a commercial vector (Invitrogen) that is specifically designed for cloning of PCR products. The DNA fragment was cloned into pCR®2.1-TOPO following the manufacturer's instructions and subsequently transformed into *E.coli* JM109 (as per Materials and Methods, Section 2.5). Single colonies were used to inoculate cultures from which DNA mini preparations were performed. Restriction digestions using *Kpn*I were performed on the DNA to monitor for the presence of the insert.

The restriction enzyme *Kpn*I is expected to cut the plasmid once. Following digestion, 3µL of the linearized p10592TOPO was analysed by agarose gel electrophoresis, as shown in Figure 3.12. 3µL of undigested p10592TOPO was included on the gel as a control. In Figure 3.12, lane 2, the DNA fragment appeared at approximately 6kb and not at the expected size of approximately 4745bps, (the TOPO plasmid backbone is 3931bps, so an expected plasmid size of 4745bps was predicted after the insertion of the 814bps promoter). It has been previously suggested that high affinity nucleic acid binding dyes, such as gel red can affect DNA migration during electrophoresis. Therefore, it is possible that the banding patterns observed here have been affected by their interaction

with gel red during migration. In order to address this concern, it was next decided to perform additional analysis of all of the recombinant plasmids using PCR and sequencing.

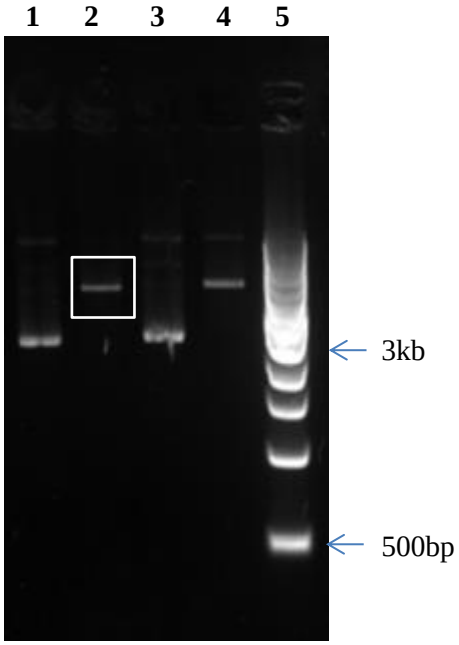


Figure 3.12: Restriction digestion of p10592TOPO electrophoresed on a 1% gel at 120V for 45 minutes. Lane 1: 3µL undigested p10592TOPO; lane 2: 3µL p10592TOPO digested with *Kpn*I; lane 5: 1kb NEB ladder, (lane 3 and 4 are not relevant to this experiment and will be discussed later in the document). The white box indicates the location of the digested DNA fragment.

In order to further confirm the integration of the promoter sequence into the pCR®2.1-TOPO vector, a PCR amplification using M13 primers was next undertaken. The M13 primers hybridise either side of the multicloning (MCS) on pCR®2.1-TOPO and are designed to amplify the inserted DNA and 500bps of the vector. PCR was performed using an annealing step of 55°C for 90 seconds and an extension step of 72°C for 2 minutes (as per Materials and Method, Section 2.8.3). 5µL of the PCR products were analysed as before using agarose gel electrophoresis and analysis indicated that a product at the approximate estimated size of 1314bps (SPR promoter 814bps + 500bps of TOPO vector) as seen in Figure 3.13 had been amplified.

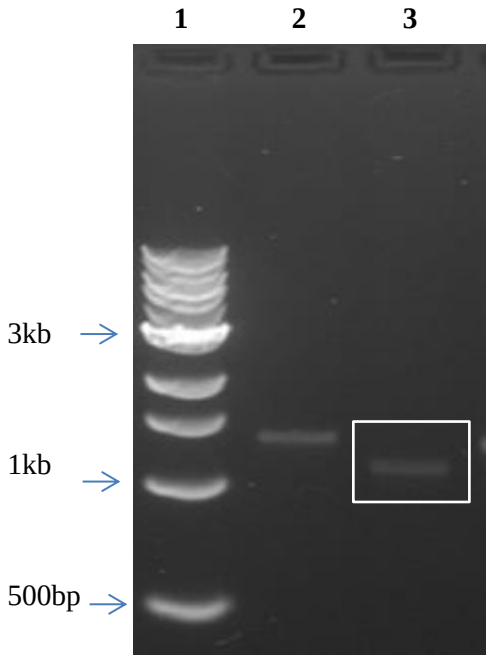


Figure 3.13: PCR using M13 primers to amplify the SPR 10592 promoter from p10592TOPO, electrophoresed on a 1% agarose gel at 100V for 1 hour. Lane 1: 1kb NEB ladder; (lane 2 is not relevant to this experiment and will be discussed later in the document); lane 3: 5µL PCR amplified SPR 10592 promoter insert. The white box indicates the location of the PCR product.

Once this analysis was complete, the plasmid p10592TOPO was sequenced using M13 forward and reverse primers (as per Materials and Methods Section 2.10.1). Forward and reverse reads were undertaken to ensure higher quality sequence data. The sequence results were predicted to include the promoter region and 500bps of the TOPO vector. The results were obtained electronically as linear nucleotide sequences and are seen in Figure 3.14, labelled as 10592topoM13Fwd and 10592topoM13Rev. The original SPR 10592 promoter sequence was obtained from Genbank, (genbankSPRprom10592) (Figure 3.14) and was aligned with the above sequences using the MUSCLE analysis tool (Edgar 2004). The 10592topoFwd sequence was seen to share 98% nucleotide identity when compared to the original SPR 10592 promoter sequence. The 10592topoM13Rev sequence was 99% identical over the length of two fragments analysed (Table 3.1; Figure 3.14).

Table 3.1: The Percent Identity Matrix created by Clustal2.1, shows the nucleotide alignment and the similarity of sequencing results for vector p10592TOPO.

Percent Identity Matrix - created by Clustal2.1				
Sequence	**Primers**			
Originalpromoter10592		100.00	98.65	99.17
10592topoM13Fwd	M13F	98.65	100.00	100.00
10592topoM13Rev	M13R	99.17	100.00	100.00

```
10592topoM13Fwd          CGATATCTAATGTTCGAGATAACAGGCGAGTGGTGACGCTTGCCATACCAGCTTCTTCTG
10592topoM13Rev          CGATATCTAATGTTCGAGATAACAGGCGAGTGGTGACGCTTGCCATACCAGCTTCTTCTG
genbankSPRprom10592      CGATATCTAATGTTCGAGATAACAGGCGAGTGGTGACGCTTGCCATACCAACTTCTTCTG
                         ************************************************* *********

10592topoM13Fwd          TTCTAACGGGTGTTCTTCTCCACTATCAAGGCCAACGCACACGTTCGCTTGACAATATTC
10592topoM13Rev          TTCTAACGGGTGTTCTTCTCCACTATCAAGGCCAACGCACACGTTCGCTTGACAATATTC
genbankSPRprom10592      TTCTAACGGGTGTTCTTCTCCACTATCAAGGCCAACGCACACGTTCACTTGACAATATTC
                         ********************************************** *************

10592topoM13Fwd          ATGTGGTGTCGCTTGGATATAGCACCCAGGCCTAACGCGCGATTGGTAGTATCCATGTCG
10592topoM13Rev          ATGTGGTGTCGCTTGGATATAGCACCCAGGCCTAACGCGCGATTGGTAGTATCCATGTCG
genbankSPRprom10592      ATGTGGTGGCGCTTGGATATAGCACCCAGGCCCAACGCACGATTGGTAGTATCCATGTCG
                         ******** ********************** ***** *********************

10592topoM13Fwd          AGCTCGTCCTGCTCGACTACACCTGACTCCATCGCAGTGCTTCTCTGCGTCCCAATTGGT
10592topoM13Rev          AGCTCGTCCTGCTCGACTACACCTGACTCCATCGCAGTGCTTCTCTGCGTCCCAATTGGT
genbankSPRprom10592      AGCTCGTCCTGTTCGACTACACCTGACTCCATCGCAGTGCTTCTCTGCGTCCCAATTGGT
                         *********** ************************************************

10592topoM13Fwd          AATAAGCACCACAGGAACCCCACAAACACCCGTCTCCAATCAGGATATGGCGTTGTGAGC
10592topoM13Rev          AATAAGCACCACAGGAACCCCACAAACACCCGTCTCCAATCAGGATATGGCGTTGTGAGC
genbankSPRprom10592      AATAAACACCACAGGAACCCCACAAACACCCGTCTCCAATCAGGATATGGCGTTGTGAGC
                         ***** ************************************************

10592topoM13Fwd          AGCGACGGTACAGACCGGCCCATCGTTTCCCGCGACCCGCGTTAGTTATCCTATGCAACG
10592topoM13Rev          AGCGACGGTACAGACCGGCCCATCGTTTCCCGCGACCCGCGTTAGTTATCCTATGCAACG
genbankSPRprom10592      AGCGACGGTACAGACCGGCCCATCGTTTCCCGCGACCCGCGTTAGTTATCCTATGCAACG
                         ************************************************************

10592topoM13Fwd          CACGAGCCTAGACACTTGACGCCCAGTTCTCTTGATGAAGGCCGCAACAGATAGGTTGTG
10592topoM13Rev          CACGAGCCTAGACACTTGACGCCCAGTTCTCTTGATGAAGGCCGCAACAGATAGGTTGTG
genbankSPRprom10592      CACGAGCCTAGACACTTGACGCCCAGTTCTCTTGATGAAGGCCGCAACAGATAGGTTGTC
                         ************************************************************

10592topoM13Fwd          AAACAGTGAAACGCGCGATCTCCTGTTCGATATGCAGTGGGCAGGGTCACGAGCGTACTC
10592topoM13Rev          AAACAGTGAAACGCGCGATCTCCTGTTCGATATGCAGTGGGCAGGGTCACGAGCGTACTC
genbankSPRprom10592      AAACAGTGAAACGCGCGATCTCCTGTTAGATATGCTGTGGGCAGGGTCACGAGCGTACTC
                         *************************** ******* ************************

10592topoM13Fwd          TTCGAATGATGATGGGATACCGATTCGAGAAATTCACTTCTTCTGGCCACATGGTACGGG
10592topoM13Rev          TTCGAATGATGATGGGATACCGATTCGAGAAATTCACTTCTTCTGGCCACATGGTACGGG
genbankSPRprom10592      TTCGAATGATGATGGGATACCGATTCGAGGAATTCACTTCTTCTGGCCACATGGTACGGG
                         ***************************** ******************************

10592topoM13Fwd          TATGTTTCCAAATTTAACCGCGATTTCACCATTGACTGAACTGTCTACCTCCTACTCTCA
10592topoM13Rev          TATGTTTCCAAATTTAACCGCGATTTCACCATTGACTGAACTGTCTACCTCCTACTCTCA
genbankSPRprom10592      TATGTTTCCAAATTTAACCGCGATTTCACCATTGACTGAACTGTCTACCTCCTACTCTCA
                         ************************************************************

10592topoM13Fwd          GAACTTGACCTCTGGCCCTTGTGCAGCGTGTAACCAGTCCTTCTATGGTATCACCTGAAC
10592topoM13Rev          GAACTTGACCTCTGGCCCTTGTGCAGCGTGTAACCAGTCCTTCTATGGTATCACCTGAAC
genbankSPRprom10592      GAACTTGACCTCTGGCCCTTGTGCAGCGTGTAACCAGTCCTTCTATGGTATCACCTGAAC
                         ************************************************************

10592topoM13Fwd          TTCTGTGCATGCTGCCGCTTCGGTAGTTTTCCTTTGCGGCGGAAGCAGGCTGTAGTTCAA
10592topoM13Rev          TTCTGTGCATGCTGCCGCTTCGGTAGTTTTCCTTTGCGGCGGAAGCAGGCTGTAGTTCAA
genbankSPRprom10592      TTCTGTGCATGCTGCCGCTTCGGTAGTTTTCCTTTGCGGCGGAAGCAGGCTGTAGTTCAA
                         ************************************************************

10592topoM13Fwd          AGGGTATAAATGGTATGTTGTCGCAGGTCTAATTCATTGTGAGCCCGTCTCATATTCCAT
10592topoM13Rev          AGGGTATAAATGGTATGTTGTCGCAGGTCTAATTCATTGTGAGCCCGTCTCATATTCCAT
```

```
genbankSPRprom10592      AGGGTATAAATGGTATGTTGTCGCAGGTCTAATTCATTGTGAGCCCGTCTCATATTCCAT
                         ************************************************************

10592topoM13Fwd          CCTCTCCTAGGTCCTCTAGCAGCTTCACTATGC
10592topoM13Rev          CCTCTCCTAGGTCCTCTAGCAGCTTCACTATGC
genbankSPRprom10592      CCTCTCCTAGGTCCTCTAGCAGCTTCACTATGC
                         ********************************
```

Figure 3.14: A MUSCLE nucleotide alignment of p10592TOPO (sequenced using M13 forward and reverse primers) and the original SPR 10592 promoter sequence. The 10592topoFwd sequence was seen to share 98% nucleotide identity when compared to the original SPR 10592 promoter sequence. The 10592topoM13Rev sequence was 99% identical over the length of the two fragments analysed.

This analysis using restriction digestion, PCR and sequencing verified the successful integration of the SPR promoter fragment into the TOPO vector, confirming the integrity of the p10592TOPO.

3.6 Preparation of the peGFPi10592 expression plasmid backbone

Having successfully cloned the SPR 10592 promoter into the commercial pCR®2.1TOPO vector, the next step in this process was the preparation of peGFPi004 to host the SPR 10592 promoter sequence. Based on the earlier bioinformatics analysis of the predicted restriction digestion patterns of the SPR 10592 promoter sequence (Section 3.5.2) it was decided to digest the vector backbone using *Sac*II and *Hind*III to create *Sac*II-*Hind*III sticky ends. A restriction digest was performed on peGFPi004 using *Sac*II and *Hind*III to remove the 300bps *gpd*II promoter. 10µL of the digested DNA was then analysed by agarose gel electrophoresis, as shown in Figure 3.15. As can be seen in Figure 3.15, the backbone was successfully digested, as indicated by the DNA band at the expected size of approximately 4417bps.

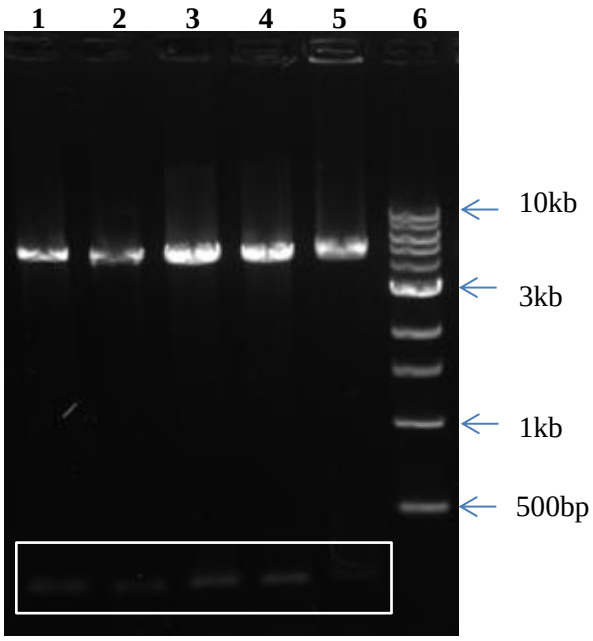


Figure 3.15: The vector peGFPi004 digested using the restriction enzymes *Sac*II and *Hind*III electrophoresed on a 1% agarose gel at 100V for 1 hour. Lanes 1-5: 10µL peGFPi004 plasmid digested with *Sac*II and *Hind*III; lane 6: 1kb NEB ladder. The white box indicates the location of the 300bp *gpd*II promoter.

Once the removal of the *gpd*II promoter was confirmed, the vector backbone was then extracted and purified using a gel extraction kit (as per Materials and Methods, Section 2.6.2). 10µL of the purified vector backbone was analysed and is presented in Figure 3.16. From this analysis, the backbone was successfully purified as indicated by the bands seen in lane 1-4 on the gel.

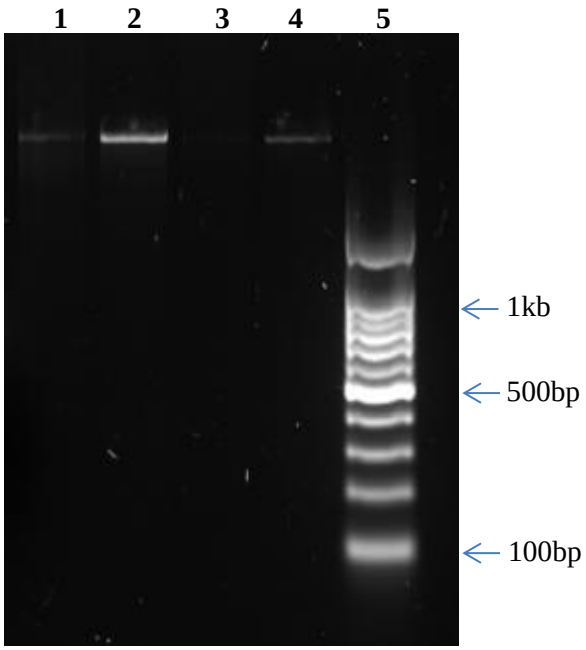


Figure 3.16: The purified backbone DNA electrophoresed on a 1% agarose gel at 100V for 1 hour. Lanes 1-4: 10µL backbone; lane 5: 100bp ladder.

3.7 Isolation of the SPR 10592 promoter from vector pCR®2.1-TOPO

In order to prepare the SPR 10592 promoter for ligation into the expression plasmid backbone, p10592TOPO was digested with *Sac*II and *Hind*III to remove the putative promoter fragment from the TOPO vector. As can be seen from Figure 3.17, p10592TOPO was successfully digested with *Sac*II and *Hind*III to remove the SPR 10592 promoter from the TOPO vector, as shown by the band of the expected size of approximately 814bps. The SPR 10592 promoter was then purified from the gel (as per Materials and Methods, Section 2.6.2) to prepare for cloning into the expression plasmid backbone.

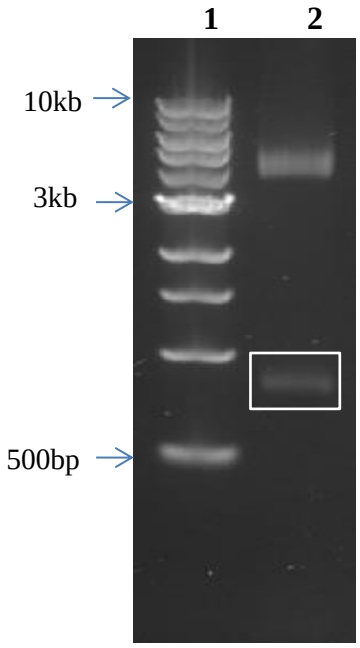


Figure 3.17: The vector p10592TOPO digested using the restriction enzymes *Sac*II and *Hind*III electrophoresed on a 1% agarose gel at 90V for 1 hour. Lane 1: 1kb NEB ladder and lane 2: p10592TOPO digested with *Sac*II and *Hind*III. The white box indicates the location of the promoter.

Following confirmation that the promoter excised from TOPO was the expected size of approximately 814bps, a purification step was performed. The SPR 10592 promoter was extracted from the agarose gel and purified using the Omega bio-tek gel extraction kit (as per Materials and Methods, Section 2.6.2). 5µL of the purified SPR 10592 promoter was then analysed by agarose gel electrophoresis as depicted in Figure 3.18. This analysis confirmed that the SPR gene 10592 was successfully purified as indicated by the approximately 814bps band seen in lane 2.

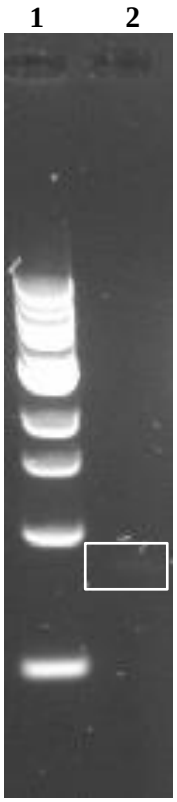


Figure 3.18: Purified *C. cinerea* SPR 10592 promoter electrophoresed on a 1% agarose gel at 100V for 1 hour. Lane 1: 1kb NEB ladder and lane 2: purified SPR promoter 10592. The white box indicates the location of the promoter DNA.

3.8 Ligation of the SPR 10592 promoter into the fungal expression plasmid backbone

Using the Thermo Scientific InsTAclone PCR Cloning Kit, the SPR 10592 promoter fragment was next cloned into the backbone following the manufacturer's instructions and subsequently transformed into *E.coli* JM109 (as per Materials and Methods, Section 2.5). A miniprep was performed to isolate the recombinant plasmid DNA from the *E.coli* cells (as per Materials and Methods, Section 2.6) and 5µL of three separate mini-preparations of the plasmid DNA were analysed by agarose gel electrophoresis, as seen in Figure 3.19. As can be seen, the minipreps were successful and the plasmid DNA was extracted from *E.coli* as indicated by the bands seen in lane 2-4.

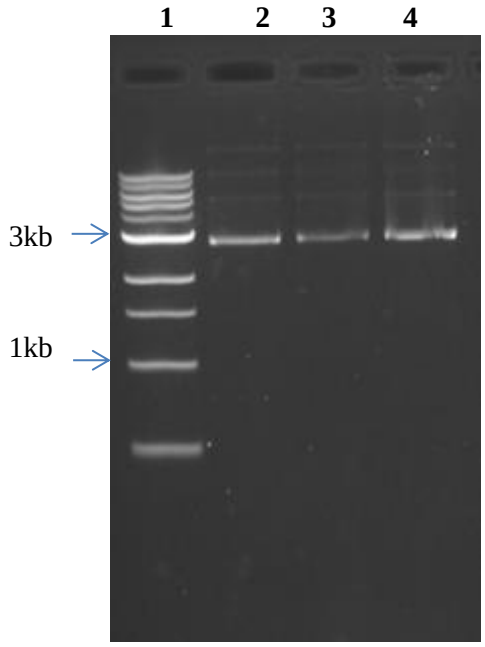


Figure 3.19: Three separate minipreps of peGFPi10592 plasmid DNA electrophoresed on a 1% agarose gel at 100V for 1 hour. Lane 1: 1kb NEB ladder: lanes 2-4: 5µL peGFPi10592 (from three separate mini preparations).

3.9 Molecular validation of the peGFPi10592 fungal expression plasmid

3.9.1 PCR analysis of peGFPi10592

PCR analysis was employed to confirm the identity of the recombinant plasmid. The schematic below (Figure 3.20 A) illustrates the primers used in the PCR validation experiments and also indicates the expected product sizes. The primer pair SPRprom10592fwd and SPRprom10592rev were used in a PCR to amplify the promoter region of 814bps. 5µL of DNA was electrophoresed on a 1% agarose gel and can be seen in Figure 3.20 B, lane 2-4. The primer pair SPRprom10592fwd and GFPrev were also used in a PCR to amplify the promoter, intron and the eGFP region as one fragment. Of this reaction, 5µL was again electrophoresed on a 1% agarose gel as depicted in Figure 3.20 C, lane 2. A product at the predicted size of approximately 1581bps was successfully amplified. This PCR analysis indicates that the recombinant peGFPi10592 vector was structurally intact.

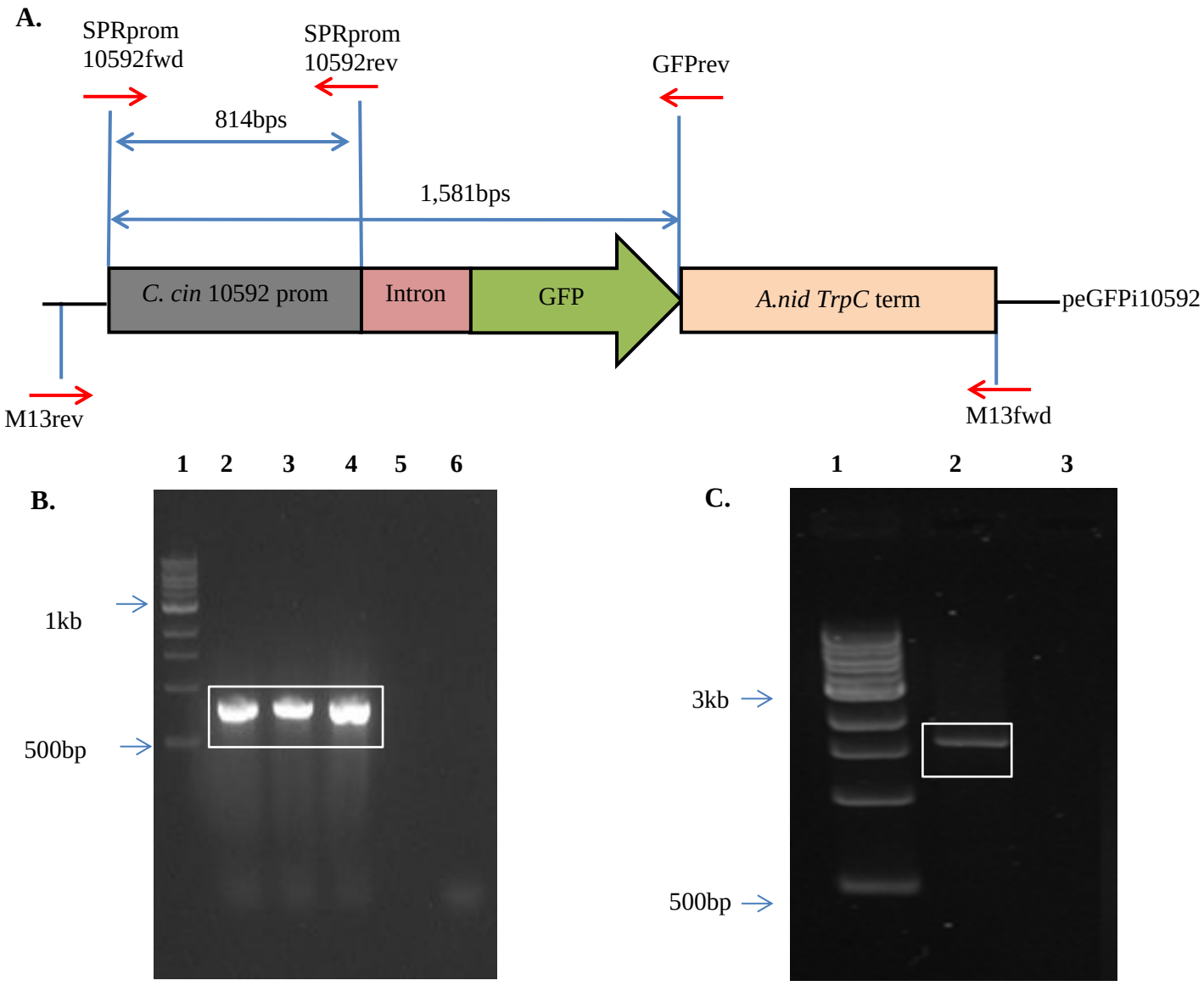


Figure 3.20: PCR analysis of the recombinant peGFPi10592 vector. A: A schematic of peGFPi10592 and the primers designed to amplify regions of the vector. Arrows indicate the directionality of the primers and predicted product sizes are displayed. B: PCR analysis of the SPR 10592 promoter fragment electrophoresed on a 1% agarose gel at 100V for 1 hour. Lane 1: 1kb NEB ladder; lane 2-4: 5µL SPR 10592 promoter DNA; lane 5: blank; lane 6: negative control. C: PCR analysis of the amplified fragment that incorporates the SPR 10592 promoter, intron and the eGFP region of peGFPi10592, electrophoresed on a 1% agarose gel at 100V for 1 hour. Lane 1: 1kb NEB ladder; lane 2: 5µL DNA fragment (SPR 10592 promoter, intron and eGFP region); lane 3: negative control. The white boxes indicate the location of the DNA.

3.9.2 Restriction analysis of peGFPi10592

Restriction analysis was performed in order to further confirm the presence of the SPR 10592 promoter in peGFPi10592. The recombinant sequence was first analysed using webcutter software. From this analysis, the restriction enzyme *Mfe*I was predicted to only cut within the SPR 10592 promoter and not within peGFPi004. Following digestion, 5µL digested DNA was analysed on 1% agarose gel as seen in Figure 3.21. Both peGFPi10592 and the cloning vector peGFPi004 were digested using *Mfe*I. Linearized peGFPi10592 was the predicted size of 5.2kb and can be seen in Figure 3.21, lane 4 below. As was expected, *Mfe*I did not digest the peGFPi004 vector as seen in lane 6.

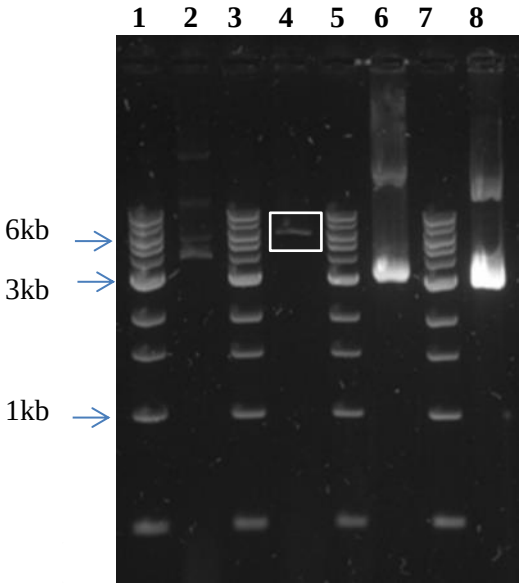


Figure 3.21: A restriction digest of the vectors peGFPi10592 and peGFPi004 electrophoresed on a 1% agarose gel at 100V for 1 hour. Lane 1, 3, 5, 7: 1kb NEB ladder; lane 2: 3µL undigested peGFPi10592; lane 4: 5µL peGFPi10592 digested with *Mfe*I; lane 6: 5µL peGFPi004 digested with *Mfe*I and lane 8: 3µL undigested peGFPi004. The white box indicates the location of peGFPi10592 digested with *Mfe*I.

3.9.3 Sequence analysis of peGFPi10592

The vector peGFPi10592 was next sequenced using the primers M13 forward, M13 reverse and GFP reverse (as per Materials and Methods, Section 2.10.1). The resulting sequences were labelled as 10592topoM13Fwd, 10592topoM13Rev and peGFPi10592GFPRev, and were aligned with the original SPR 10592 promoter sequence (genbankSPRprom10592) using the online software MUSCLE. A number of independent sequencing reactions were carried out to analyse the plasmid from the SPR

10592 promoter to the end of the eGFP region, thus ensuring high-quality sequence data. The results of the Clustal analysis are seen in Table 3.2 and Figure 3.22.

Table 3.2: The Percent Identity Matrix created by Clustal 2.1, shows the nucleotide alignment and the similarity of sequencing results for vector peGFPi10592.

Percent Identity Matrix - created by Clustal 2.1					
Sequence	**Primers**				
GenbankSPRprom10592		98.65	98.65	98.65	100.00
peGFPi10592GFPRev	GFPRev	100.00	100.00	100.00	98.65
10592TopoM13Fwd	M13Fwd	100.00	100.00	100.00	98.65
10592TopoM13Rev	M13Rev	100.00	100.00	100.00	98.65

```
peGFPi10592GFPRev       CGATATCTAATGTTCGAGATAACAGGCGAGTGGTGACGCTTGCCATACCAGCTTCTTCTG
10592topoM13Fwd         CGATATCTAATGTTCGAGATAACAGGCGAGTGGTGACGCTTGCCATACCAGCTTCTTCTG
10592topoM13Rev         CGATATCTAATGTTCGAGATAACAGGCGAGTGGTGACGCTTGCCATACCAGCTTCTTCTG
genbankSPRprom10592     CGATATCTAATGTTCGAGATAACAGGCGAGTGGTGACGCTTGCCATACCAACTTCTTCTG
                        ************************************************** *********

peGFPi10592GFPRev       TTCTAACGGGTGTTCTTCTCCACTATCAAGGCCAACGCACACGTTCGCTTGACAATATTC
10592topoM13Fwd         TTCTAACGGGTGTTCTTCTCCACTATCAAGGCCAACGCACACGTTCGCTTGACAATATTC
10592topoM13Rev         TTCTAACGGGTGTTCTTCTCCACTATCAAGGCCAACGCACACGTTCGCTTGACAATATTC
genbankSPRprom10592     TTCTAACGGGTGTTCTTCTCCACTATCAAGGCCAACGCACACGTTCACTTGACAATATTC
                        ******************************************** *************

peGFPi10592GFPRev       ATGTGGTGTCGCTTGGATATAGCACCCAGGCCTAACGCGCGATTGGTAGTATCCATGTCG
10592topoM13Fwd         ATGTGGTGTCGCTTGGATATAGCACCCAGGCCTAACGCGCGATTGGTAGTATCCATGTCG
10592topoM13Rev         ATGTGGTGTCGCTTGGATATAGCACCCAGGCCTAACGCGCGATTGGTAGTATCCATGTCG
genbankSPRprom10592     ATGTGGTGGCGCTTGGATATAGCACCCAGGCCCAACGCACGATTGGTAGTATCCATGTCG
                        ******** ********************** ***** ********************

peGFPi10592GFPRev       AGCTCGTCCTGCTCGACTACACCTGACTCCATCGCAGTGCTTCTCTGCGTCCCAATTGGT
10592topoM13Fwd         AGCTCGTCCTGCTCGACTACACCTGACTCCATCGCAGTGCTTCTCTGCGTCCCAATTGGT
10592topoM13Rev         AGCTCGTCCTGCTCGACTACACCTGACTCCATCGCAGTGCTTCTCTGCGTCCCAATTGGT
genbankSPRprom10592     AGCTCGTCCTGTTCGACTACACCTGACTCCATCGCAGTGCTTCTCTGCGTCCCAATTGGT
                        *********** ************************************************

peGFPi10592GFPRev       AATAAGCACCACAGGAACCCCACAAACACCCGTCTCCAATCAGGATATGGCGTTGTGAGC
10592topoM13Fwd         AATAAGCACCACAGGAACCCCACAAACACCCGTCTCCAATCAGGATATGGCGTTGTGAGC
10592topoM13Rev         AATAAGCACCACAGGAACCCCACAAACACCCGTCTCCAATCAGGATATGGCGTTGTGAGC
genbankSPRprom10592     AATAAACACCACAGGAACCCCACAAACACCCGTCTCCAATCAGGATATGGCGTTGTGAGC
                        ***** ********************************************************

peGFPi10592GFPRev       AGCGACGGTACAGACCGGCCCATCGTTTCCCGCGACCCGCGTTAGTTATCCTATGCAACG
10592topoM13Fwd         AGCGACGGTACAGACCGGCCCATCGTTTCCCGCGACCCGCGTTAGTTATCCTATGCAACG
10592topoM13Rev         AGCGACGGTACAGACCGGCCCATCGTTTCCCGCGACCCGCGTTAGTTATCCTATGCAACG
genbankSPRprom10592     AGCGACGGTACAGACCGGCCCATCGTTTCCCGCGACCCGCGTTAGTTATCCTATGCAACG
                        ************************************************************

peGFPi10592GFPRev       CACGAGCCTAGACACTTGACGCCCAGTTCTCTTGATGAAGGCCGCAACAGATAGGTTGTG
10592topoM13Fwd         CACGAGCCTAGACACTTGACGCCCAGTTCTCTTGATGAAGGCCGCAACAGATAGGTTGTG
10592topoM13Rev         CACGAGCCTAGACACTTGACGCCCAGTTCTCTTGATGAAGGCCGCAACAGATAGGTTGTG
genbankSPRprom10592     CACGAGCCTAGACACTTGACGCCCAGTTCTCTTGATGAAGGCCGCAACAGATAGGTTGTC
                        ************************************************************

peGFPi10592GFPRev       AAACAGTGAAACGCGCGATCTCCTGTTCGATATGCAGTGGGCAGGGTCACGAGCGTACTC
10592topoM13Fwd         AAACAGTGAAACGCGCGATCTCCTGTTCGATATGCAGTGGGCAGGGTCACGAGCGTACTC
10592topoM13Rev         AAACAGTGAAACGCGCGATCTCCTGTTCGATATGCAGTGGGCAGGGTCACGAGCGTACTC
genbankSPRprom10592     AAACAGTGAAACGCGCGATCTCCTGTTAGATATGCTGTGGGCAGGGTCACGAGCGTACTC
                        *************************** ******* ************************

peGFPi10592GFPRev       TTCGAATGATGATGGGATACCGATTCGAGAAATTCACTTCTTCTGGCCACATGGTACGGG
10592topoM13Fwd         TTCGAATGATGATGGGATACCGATTCGAGAAATTCACTTCTTCTGGCCACATGGTACGGG
10592topoM13Rev         TTCGAATGATGATGGGATACCGATTCGAGAAATTCACTTCTTCTGGCCACATGGTACGGG
genbankSPRprom10592     TTCGAATGATGATGGGATACCGATTCGAGGAATTCACTTCTTCTGGCCACATGGTACGGG
                        ***************************** ******************************
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peGFPi10592GFPRev        TATGTTTCCAAATTTAACCGCGATTTCACCATTGACTGAACTGTCTACCTCCTACTCTCA
10592topoM13Fwd          TATGTTTCCAAATTTAACCGCGATTTCACCATTGACTGAACTGTCTACCTCCTACTCTCA
10592topoM13Rev          TATGTTTCCAAATTTAACCGCGATTTCACCATTGACTGAACTGTCTACCTCCTACTCTCA
genbankSPRprom10592      TATGTTTCCAAATTTAACCGCGATTTCACCATTGACTGAACTGTCTACCTCCTACTCTCA
                         ************************************************************

peGFPi10592GFPRev        GAACTTGACCTCTGGCCCTTGTGCAGCGTGTAACCAGTCCTTCTATGGTATCACCTGAAC
10592topoM13Fwd          GAACTTGACCTCTGGCCCTTGTGCAGCGTGTAACCAGTCCTTCTATGGTATCACCTGAAC
10592topoM13Rev          GAACTTGACCTCTGGCCCTTGTGCAGCGTGTAACCAGTCCTTCTATGGTATCACCTGAAC
genbankSPRprom10592      GAACTTGACCTCTGGCCCTTGTGCAGCGTGTAACCAGTCCTTCTATGGTATCACCTGAAC
                         ************************************************************

peGFPi10592GFPRev        TTCTGTGCATGCTGCCGCTTCGGTAGTTTTCCTTTGCGGCGGAAGCAGGCTGTAGTTCAA
10592topoM13Fwd          TTCTGTGCATGCTGCCGCTTCGGTAGTTTTCCTTTGCGGCGGAAGCAGGCTGTAGTTCAA
10592topoM13Rev          TTCTGTGCATGCTGCCGCTTCGGTAGTTTTCCTTTGCGGCGGAAGCAGGCTGTAGTTCAA
genbankSPRprom10592      TTCTGTGCATGCTGCCGCTTCGGTAGTTTTCCTTTGCGGCGGAAGCAGGCTGTAGTTCAA
                         ************************************************************

peGFPi10592GFPRev        AGGGTATAAATGGTATGTTGTCGCAGGTCTAATTCATTGTGAGCCCGTCTCATATTCCAT
10592topoM13Fwd          AGGGTATAAATGGTATGTTGTCGCAGGTCTAATTCATTGTGAGCCCGTCTCATATTCCAT
10592topoM13Rev          AGGGTATAAATGGTATGTTGTCGCAGGTCTAATTCATTGTGAGCCCGTCTCATATTCCAT
genbankSPRprom10592      AGGGTATAAATGGTATGTTGTCGCAGGTCTAATTCATTGTGAGCCCGTCTCATATTCCAT
                         ************************************************************

peGFPi10592GFPRev        CCTCTCCTAGGTCCTCTAGCAGCTTCACTATGC
10592topoM13Fwd          CCTCTCCTAGGTCCTCTAGCAGCTTCACTATGC
10592topoM13Rev          CCTCTCCTAGGTCCTCTAGCAGCTTCACTATGC
genbankSPRprom10592      CCTCTCCTAGGTCCTCTAGCAGCTTCACTATGC
                         ********************************
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Figure 3.22: A MUSCLE nucleotide alignment of peGFPi10592 sequences (sequenced with primers M13 reverse, M13 forward and GFP reverse) and the original 10592 promoter sequence (genbankSPRprom10592). The peGFPi10592GFPRev sequence shared 98% nucleotide identity when compared to the original SPR 10592 promoter sequence. The 10592topoM13Fwd sequence shared 98% identity to the original 10592 promoter sequence. The 10592topoM13Rev sequence shared 98% identity to the original 10592 promoter sequence.

This analysis using restriction digests, PCR and sequencing verified the successful integration of the SPR promoter fragment into the expression plasmid peGFPi004, resulting in the recombinant vector peGFPi10592.

This plasmid is now ready for transformation studies in *C. cinerea* facilitating the investigation of the SPR 10592 in this fungus. Following transformation, the transformants can be fruited and analysed at different stages of development for the expression of eGFP.

3.10 Construction of the *Coprinopsis cinerea* serine protease 10592 antisense plasmid (pAS10592)

Gene silencing is a powerful technique that can be used to study gene functionality. Previous studies (Heneghan *et al.* 2007, Heneghan *et al.* 2016) have demonstrated that silencing in *A. bisporus* and *C. cinerea* results in the production of a range of transformants that display different levels of silencing. Antisense technology is a method used to interfere with gene expression by producing single-stranded RNA (ssRNA) molecules that bind to the target messenger RNA (mRNA), triggering degradation of the mRNA molecule. This interruption of gene expression can be accomplished using short pieces of the coding sequence which bind to the target mRNA thus interfering with translation into a functioning protein.

Gene silencing will be used here to evaluate the function of the *C. cinerea* SPR 10592 gene and help establish what role it plays in the life cycle of the fungus. This research requires the construction of an antisense vector to silence the *C. cinerea* SPR gene. The peGFPi004 plasmid was again employed here (Section 3.5.2), as the backbone for the vector construct.

The first step in this process was to design the plasmid pAS10592. Figure 3.23 represents a summary of this design. The intact peGFPi004 plasmid including the *A. bisporus gpd*II promoter, intron, eGFP and *A. nidulans trpC* terminator was employed in this construction (Figure 3.23 A). This plasmid was digested with restriction enzymes *Hind*III and *Bam*HI to remove the intron and eGFP region (Figure 3.23 B). The SPR gene was PCR amplified to include the restriction sites *Bam*HI and *Hind*III for use in directional cloning (Figure 3.23 C). The intron and eGFP gene would then be replaced by the SPR 10592 gene in the antisense orientation resulting in the construction of the pAS10592 plasmid (Figure 3.23D).

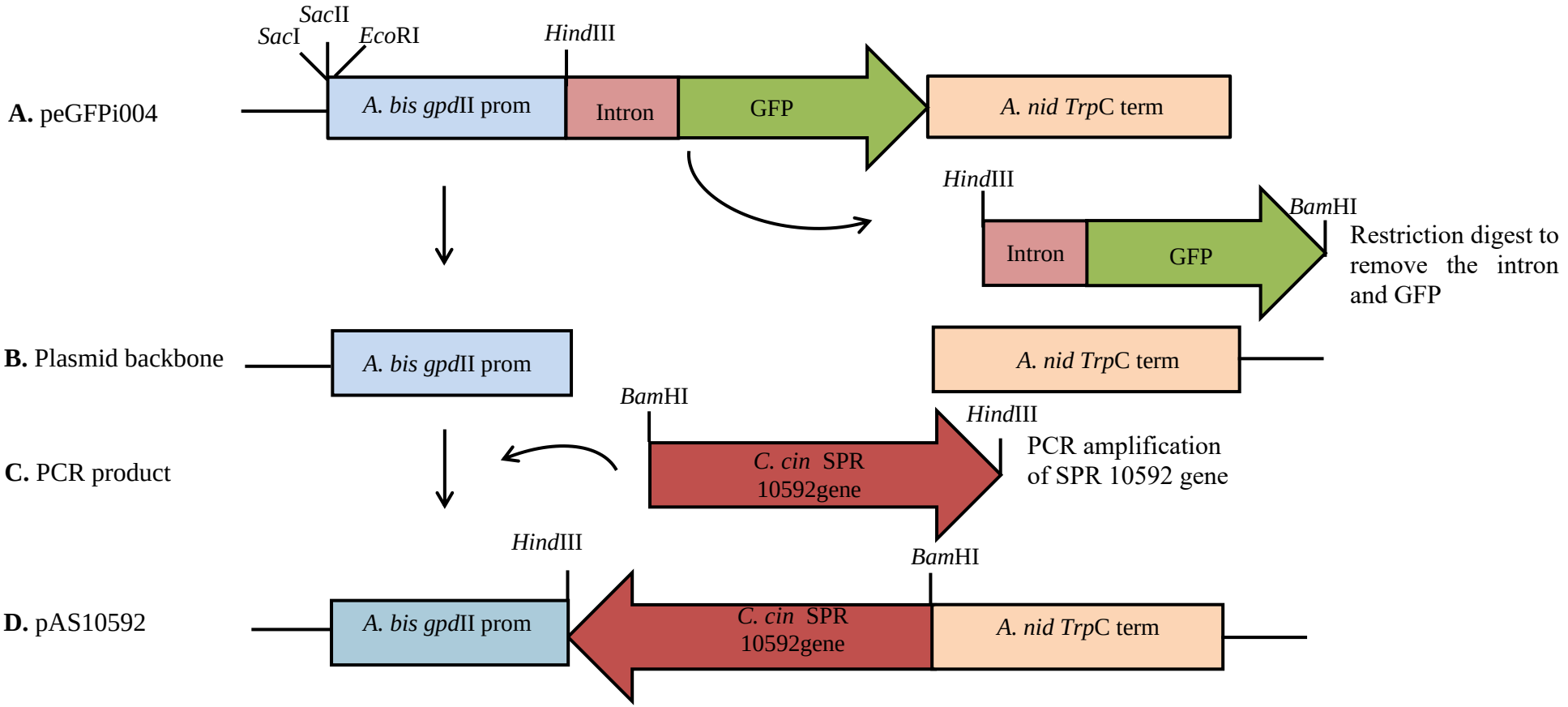

Figure 3.23: A schematic representation of the design and construction of the antisense cassette pAS10592. A: The peGFPi004 plasmid containing the *A. bisporus gpd*II promoter (*A. bis gpd*II prom), intron, eGFP and the *A. nidulans TrpC* terminator (*A. nid TrpC* term). B: The plasmid digested with *Hind*III and *Bam*HI to remove the intron and eGFP. C: The amplified 10592 gene flanked with restriction sites *Bam*HI and *Hind*III ready for directional cloning. D: The constructed plasmid pAS10592 contains the *A. bisporus gpd*II promoter, *C. cinerea* SPR 10592 gene (in the antisense orientation) and the *A.nidulans TrpC* terminator.

3.11 Preparation of the SPR 10592 gene

3.11.1 Sequence retrieval and analysis

The SPR 10592 gene sequence (1151bps product) was next identified using the Genbank database and primers were designed to amplify this sequence. Initially, the gene sequence was analysed for restriction enzyme recognition sites using the online tool, webcutter. The restriction enzymes *Aat*II and *Kpn*I were found to cut within the gene sequence, while *Hind*III and *Bam*HI do not. Based on this analysis, it was decided to include a *Bam*HI site on the forward primer and a *Hind*III site on the reverse primer to allow directional cloning of the gene in the antisense orientation into the peGFPi004 plasmid.

3.11.2 PCR amplification of the SPR 10592 gene fragment

These primers were then used in a PCR with *C. cinerea* gDNA, employing an annealing step at 60ºC for 40 seconds and an extension step at 72ºC for 45 seconds (as per Materials and Methods, Section 2.8.3). Following PCR, 5µL of the product was analysed by agarose gel electrophoresis to check the migration of the gene at the expected size. The result can be seen in Figure 3.24, lane 3, which confirms the successful amplification of the SPR 10592 gene, at approximately 1151bps.

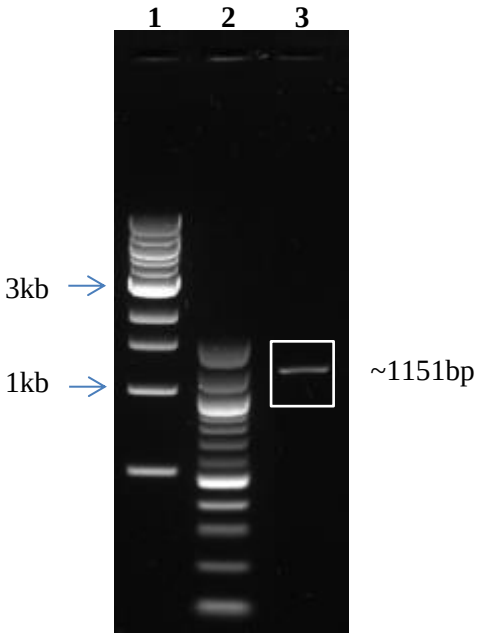

Figure 3.24: The SPR 10592 gene DNA fragment electrophoresed on a 1% agarose gel at 100V for 1 hour. Lane 1: 1kb NEB ladder; lane 2: 100bp NEB ladder; lane 3: 5µL SPR 10592 gene. The white box indicates the location of the DNA.

Following confirmation that the PCR product was the expected size of 1151bps, a purification step was performed. The SPR 10592 gene was extracted from the agarose gel and purified using the Omega bio-tek gel extraction kit (as per Materials and Methods, Section 2.6.2). 5µL of the purified SPR 10592 gene was then analysed by agarose gel electrophoresis as depicted in Figure 3.25. This analysis confirmed that the SPR 10592 gene was successfully purified as indicated by the approximate size of 1151bps, seen in lane 1.

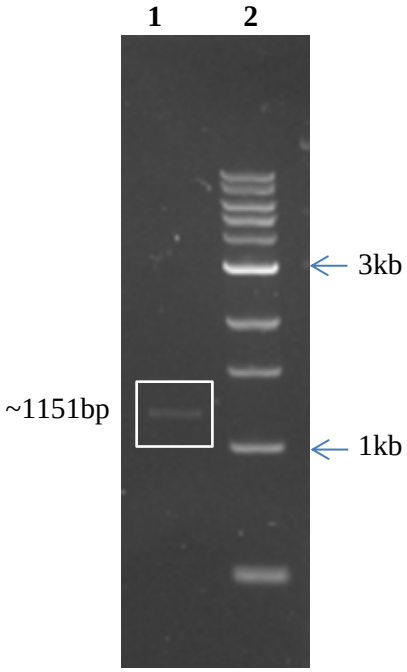

Figure 3.25: Purified SPR 10592 gene electrophoresed on a 1% agarose gel at 100V for 1 hour. Lane 1: 5µL purified SPR 10592 gene fragment; lane 2: 1kb NEB ladder. The white box indicates the location of the SPR gene fragment.

3.11.3 Cloning of the SPR 10592 gene into pTZ57R/T

Having successfully isolated the SPR 10592 gene, it was next cloned into pTZ57R/T to produce the plasmid pTZAS10592. The vector pTZ57R/T is a commercial vector that is specifically designed for cloning of PCR products. The DNA fragment was cloned into pTZ57R/T following the manufacturer's instructions and subsequently transformed into *E.coli* JM109 (as per Materials and Methods, Section 2.5). Single colonies were used to inoculate cultures from which DNA mini preparations were performed. Restriction digestions using *Kpn*I were performed on this DNA to monitor for the presence of the insert.

The restriction enzyme *Kpn*I was expected to cut the plasmid once. Following digestion, 3µL of the linearized plasmid pTZAS10592 was analysed by agarose gel electrophoresis, as shown in Figure 3.26. The plasmid pTZ57R/T is 2886bps and an expected plasmid size of 4037bps was anticipated after the insertion of the 1151bps SPR gene (Figure 3.26, lane 1). The digestion of pTZAS10592 was successful as indicated by the band at the expected size of approximately size of 4037bp as seen in lane 1.

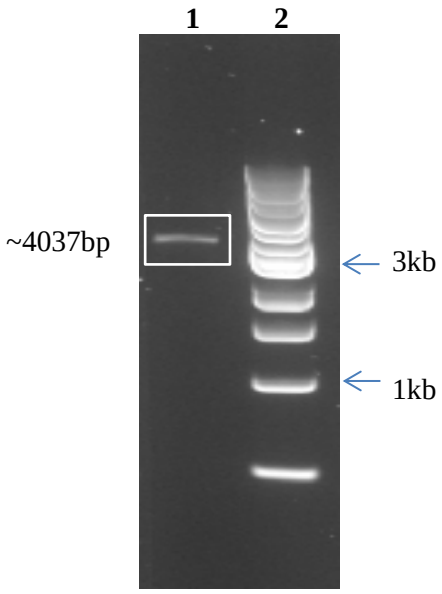

Figure 3.26: Restriction digestion of plasmid pTZAS10592 electrophoresed on a 1% agarose gel at 100V for 1 hour. Lane 1: 3µL pTZAS10592 digested with *Kpn*I and lane 2: 1kb NEB ladder. The white box indicates the location of the linearized DNA fragment.

To further confirm the integration of the gene sequence into the pTZ57R/T vector, a PCR amplification using M13 primers was next performed (as per Materials and Methods, Section 2.8.1). The M13 primers hybridise either side of the multicloning site (MCS) on pTZ57R/T and are designed to amplify the inserted DNA and 138bps of the vector sequence. PCR was performed employing an annealing step of 55°C for 90 seconds and an extension step of 72°C for 2 minutes (as per Materials and Methods, Section 2.8.3). Following PCR, 5µL of the reaction was analysed as before using agarose gel electrophoresis indicating the successful amplification of the product at the approximate size of 1289bps (SPR gene 1151bps + 138bps of pTZ57R/T vector) as seen in Figure 3.27.

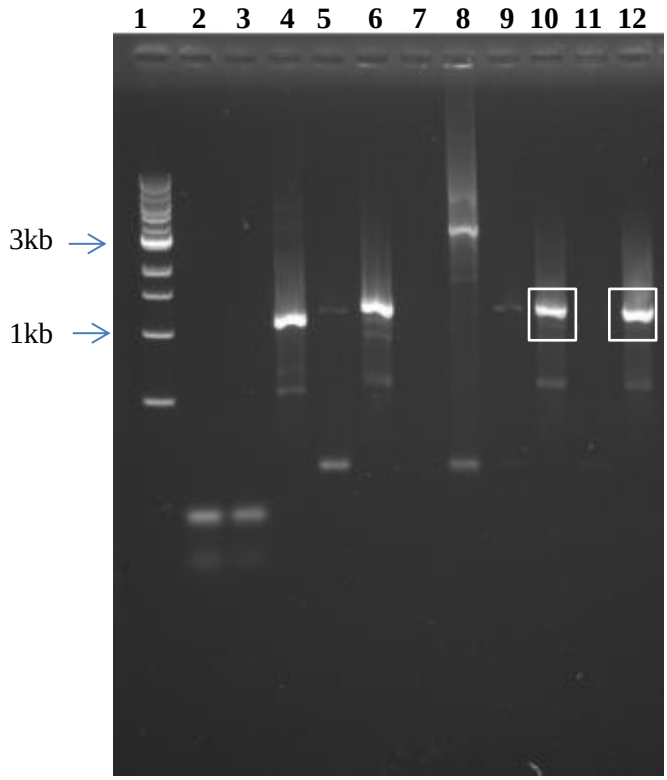

Figure 3.27: PCR using M13 primers to amplify the SPR 10592 gene from pTZAS10592, electrophoresed on a 1% agarose gel at 100V for 1 hour. Lane 1: 1kb NEB ladder; (lane 2-9: are not relevant to this experiment and will be discussed later in the document); lane 10 and 12: 5µL PCR amplified DNA containing the SPR 10592 gene; lane 11: negative control. The white boxes indicate the location of the DNA.

Once this analysis was complete, the vector pTZAS10592 was sequenced using an M13 forward primer (pTZAS10592) (as per Materials and Methods, Section 2.10.1). The resulting nucleotide sequence was aligned with the original SPR 10592 gene sequence (original10592gene) using the online MUSCLE software. As can be seen from the analysis, the pTZAS10592 sequence was seen to share 98% nucleotide identity with the original SPR 10592 gene (Table 3.3; Figure 3.28).

Table 3.3: The Percent Identity Matrix created by Clustal2.1, shows the nucleotide alignment and the similarity of sequencing results for vector pTZAS10592.

Percent Identity Matrix- created by Clustal2.1			
Sequence	**Primer**		
pTZAS10592	M13F	100.00	98.51
original10592gene		98.51	100.00

```
pTZAS10592M13F          CGCGTAACGAGAATCTTACCGATATTTACAGATCCAACGCCCCTTGGGGTTTGGGCCGT
original10592gene       CGCGTAACGAGAATCTTACCGATATTTACAGATCCAACGCCCCTTGGGGTTTGGGCCGT
                        ************************************************************

pTZAS10592M13F          CTCAACCAAGCTGGTCGCTTGGCTAACCAGGCCACTGGGTACGTCCCATGATCTCGTTTC
original10592gene       CTCAACCAAGCTGGCCGCTTGGCTAACCAGGCCACTGGGTACGTCCCATGATCTCGTTTC
                        ************* ************************************************

pTZAS10592M13F          GTGATACCTTGCTGAAATACTGGTCTAGCTCCTTGGCCTTCAACTTCACCTACGACGACT
original10592gene       GTGATACCTTGCTGAAATACTGGTCTAGCTCCTTGGCCTTCAACTTCACCTACGACGACT
                        ************************************************************

pTZAS10592M13F          CTGCCGGCGCTGGGGTCGATATCTATGTTCTTGGTAAGTGGCGACGTTGTTCCCAGTTGC
original10592gene       CTGCCGGCGCTGGGGTCGATATCTATGTTCTTGGTAAGTGGCGACGATGTTCCCAGTTGC
                        ********************************************* ************

pTZAS10592M13F          TGCCGTACTCACTATTTCTTGTAGACACTGGTAAGAGTGCCGCCCTGCCAGCACGCACCA
original10592gene       TGCCGTACTCACTATTTCTTGTAGACACTGGTAAGAGTGCCGCCCTGCCAGCACGCACCA
                        ************************************************************

pTZAS10592M13F          CTTCGAACTGACCCATCCACCAACAGGTGTTCGCGTGACCCACGTAAGTATCCCTGGTCA
original10592gene       CTTCGAACTGACCCATCCACCAACAGGTGTTCGCGTGACCCACGTAAGTATCCCTGGTCA
                        ************************************************************

pTZAS10592M13F          TGCGAGATCGCTATCGAGATTCAAACTGACACCACTTGCAGAGCCAATTTGGTGGTCGTG
original10592gene       TGCGAGATCGCTATCGAGATTCAAACTGACACCACTTGCAGAGCCAATTTGGTGGTCGTG
                        ************************************************************

pTZAS10592M13F          CTCGCTGGGGCACCTCCTTCGTCGGCGCATCCACCGATGGACATGGCCACGGCACCCACT
original10592gene       CTCGCTGGGGCACCTCCTTCGTCGGCGCATCCACCGATGGACATGGCCACGGCACCCACT
                        ************************************************************

pTZAS10592M13F          GCGCGTAAGCATTCTGTCTCTTAAACGTTTTCGCTCCCCGGCTGACCAGTGGGATTAAAG
original10592gene       GCGCGTAAGCATTCTGTCTCTTAAACGTTTTCGCTCCCCGGCTGACCAGTGGGATTAAAG
                        ************************************************************

pTZAS10592M13F          GGGCACTGCTGCTGGCTCCCAATTCGGTGTTGCCAAGGTTGGTGGTCTTCTCGCCTTCTT
original10592gene       GGGCACTGCTGCTGGCTCCCAATTCGGTGTTGCCAAGGTTGGTGGTCTTCTCGTCTTCTT
                        ********************************************************* ******

pTZAS10592M13F          CACCTTCGAACTTGATGGCTAATATCCGCTATCCCCTTACTACCTTAAGCGGGCCAACAT
original10592gene       CACCTTCGAACTTGATGGCTAATATCCGCTATCCCCTTACTACCTTAAGCGGGCCAACAT
                        ************************************************************

pTZAS10592M13F          CATTGCCGTCCAGGTTCTCAACTCCAGCGGGTACGTATTCTCGGTACCCTTTCGGGGAAA
original10592gene       CATTGCCGTCCAGGTTCTCAACTCCAGCGGGTACGTATTCTCGGTACCCTTTCGGGGAAA
                        ************************************************************

pTZAS10592M13F          TCCTACAATTAACGCGGTTCTCGTTTCAGCTCTGGCGCGACCTCCGGAATGTAAGCGTCG
original10592gene       TCCTTCAATTAACGCGGTTCTCGTTACAGCTCTGGCGCGACCTCCGGAATGTAAGTGTCG
                        **** ******************** **************************** ****

pTZAS10592M13F          ATGCCAATTCTCCTCCACAGCCTTGAAAAGGTACTGACGAACCGATACCAGTGTCTCCGG
original10592gene       ATGCCAATTCTCCTCCACAGCATTGGAAAGGTATTCACGACCCGACCAGTGTCTCCGG
                        ********************* *** ******* * **** **** **************

pTZAS10592M13F          CCTCAACTGGGTCTTGACCCAGGCCCGCAACTCTGGCCGCCCTTCCGTCGTCTCCATGTC
original10592gene       TCTCAACTGGGTGTTGACCCAGGCCCGCAACTCTGGCCGTCCCTCCGTCGTCTCCATGTC
                         *********** ************************** ** *****************

pTZAS10592M13F          GCTCGGCGGTAGTGCTTCGACCGCCCTCGACAACGCTGTTGCCCAGCTCACCTCTGCCGG
original10592gene       GCTCGGCGGTAGTGCTTCGACCGCCCTCGACAACGCTGTTGCCCAGCTCACCTCTGCCGG
                        ************************************************************
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pTZAS10592M13F          TGTCCACGTCGTCGTTGCTGCCGGAAACTCCAACACCAACGCTGCCAACACCTCGCCCGC
original10592gene       TGTCCACGTCGTCGTTGCTGCCGGAAACTCCAACACCAACGCTGCCAACACCTCGCCCGC
                        ************************************************************

pTZAS10592M13F          CCGTGCACCCTCTGCTATCACCGTCGCCGCCTCCACCATCGCTGACGCCAAGGCCTCGTA
original10592gene       CCGTGCACCCTCTGCTATCACCGTCGCCGCCTCCACCATCGCTGACGCCAAGGCCTCGTA
                        ************************************************************

pTZAS10592M13F          CTCTAACTACGGCGCCATCGTCGACGTCTGGGCTCCTGGTTCCAACGTCATCTCTGCC
original10592gene       CTCTAACTACGGCGCCATCGTCGACGTCTGGGCTCCTGGTTCCAACGTCATCTCTGCC
                        ************************************************************
```

Figure 3.28: A MUSCLE nucleotide alignment of pTZAS10592 (sequenced using a M13 forward primer) and the original SPR 10592 gene sequence. The pTZAS10592 shared 98% nucleotide identity when compared to the original SPR 10592 gene sequence.

This analysis using restriction digestion, PCR and sequencing verified the successful integration of the SPR promoter fragment into the pTZ57R/T vector, confirming the integrity of the pTZAS10592 plasmid.

3.12 Preparation of the pAS10592 fungal expression plasmid backbone

Having successfully cloned the SPR 10592 gene into the commercial pTZ57R/T plasmid, the next step in this research was the preparation of peGFPi004 to host the SPR 10592 gene sequence in the reverse orientation. Based on the earlier bioinformatic analysis of the predicted restriction digestion patterns of the SPR 10592 antisense gene sequence, it was decided to digest the vector backbone using *Hind*III and *Bam*HI to produce *Hind*III-*Bam*HI sticky ends. First, a restriction digest was performed on peGFPi004 using *Hind*III-*Bam*HI to remove the 70bps intron and 717bps eGFP region. 10µL of the digested DNA was then analysed by agarose gel electrophoresis, as shown in Figure 3.29. Once the removal of the intron and eGFP region (787bps) was confirmed, the reaction was repeated on a larger scale to provide enough DNA for subsequent cloning experiments.

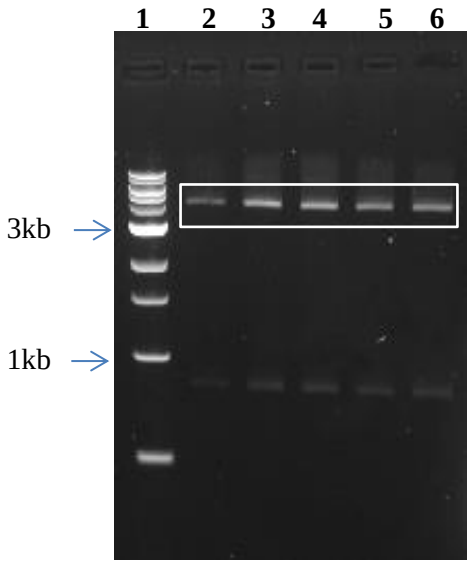

Figure 3.29: The peGFPi004 vector digested using restriction enzymes *Hind*III and *Bam*HI, electrophoresed on a 1% agarose gel at 100V for 1 hour. Lane 1: 1kb NEB ladder, lane 2-6: 10µL of peGFPi004 digested with restriction enzymes *Hind*III and *Bam*HI. The white box indicates the location of the plasmid backbone.

Once the removal of the intron and eGFP gene was confirmed (Figure 3.29), the vector backbone was then extracted and purified using a gel extraction kit (as per Materials and Methods, Section 2.6.2). 10µL of the purified vector backbone was analysed by agarose gel electrophoresis. There was no band of DNA at the expected size present in the gel. This indicated that the gel extraction had not been successful. In order to continue this work, the restriction digest of peGFPi004 using *Hind*III-*Bam*HI should be repeated and the subsequent experiment to purify the vector backbone fragment would again be performed. Following this, the construction of pAS10592 would involve the removal of the 10592 gene from the pTZ57R/T construct and subcloning in the reverse orientation into the prepared peGFPi004 backbone. The next step would involve using the Thermo Scientific InsTAclone PCR Cloning Kit and subsequent transformation of the plasmid pAS10592 into *E.coli* JM109 (as per Materials and Methods, Section 2.5). Following this, a miniprep would be employed to isolate the recombinant plasmid DNA from *E.coli* cells and molecular validation of the pAS10592 fungal expression plasmid using restriction digests, PCR and sequencing techniques would be carried out to verify the identity of the recombinant vector. This plasmid would then be ready for transformation into *C. cinerea* to facilitate the study of the effects silencing the SPR 10592 gene would have on mushroom development.

Chapter 4: Construction of peGFPi04562, pAS04562 and peGFPi10615

4.1 Introduction

Heneghan *et al*., 2009 previously identified seven *C. cinerea* SPR genes (CC1G_10592, CC1G_04562, CC1G_10615, CC1G_07792, CC1G_10606, CC1G_03122 and CC1G_4470) (Heneghan *et al.* 2009). This chapter presents the preliminary results for the construction of SPR-promoter and SPR-silencing plasmids that will facilitate the study of the 04562 and 10615 genes.

4.2 Construction of the *C. cinerea* SPR 04562 promoter fungal expression plasmid

The second *C. cinerea* SPR selected for study was SPR 04562. This *C. cinerea* gene also shows significant homology to the *A. bisporus* SPRs, with an amino acid sequence identity value of 61% when aligned with *A. bisporus* SPR1 and 55% identity when aligned with the *A. bisporus* SPR2 gene (Heneghan *et al.* 2009).

The first step in this process was to design the peGFPi04562 plasmid. Figure 4.1 represents a schematic overview of this design. The plasmid peGFPi004 (as described in Section 3.5.2) was again employed here (Figure 4.1 A). Restriction enzymes *Sac*I and *Hind*III were identified to remove the 300bps *A. bisporus gpd*II promoter leaving the rest of the plasmid intact (Figure 4.1 B). The SPR promoter was PCR amplified to include the restriction sites *Sac*I and *Hind*III for directional cloning (Figure 4.1 C). To complete this construct, the *gpd*II promoter would next be replaced by the *C. cinerea* SPR 04562 promoter forming the SPR promoter plasmid peGFPi04562 (Figure 4.1 D).

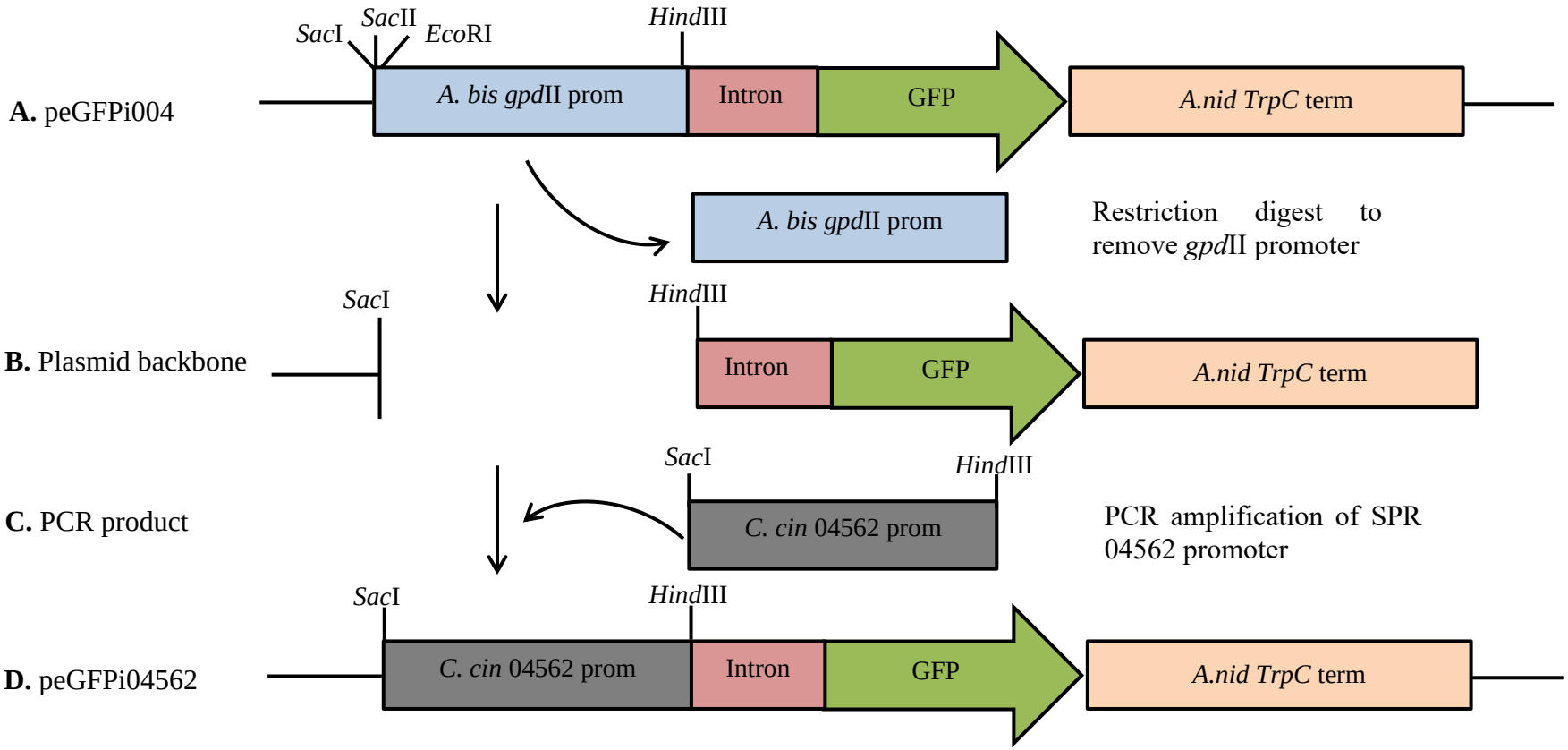

Figure 4.1: A schematic representation of the design of recombinant vector peGFPi04562. A: The intact peGFPi004 plasmid containing the original *A. bisporus gpd*II promoter (*A.bis gpd*II prom), intron, eGFP and *A.nidulans trpC* terminator (*A.nid TrpC* term). B: The plasmid digested with restriction enzymes *Sac*I and *Hind*III to remove the *gpd*II promoter leaving the plasmid backbone. C: The amplified *C. cinerea* SPR 04562 promoter flanked with *Sac*I and *Hind*III restriction sites. D: The intact peGFPi04562 contains the *C. cinerea* SPR 04562 promoter ligated into the peGFPi004 backbone.

4.3. Preparation of the SPR 04562 promoter fragment

4.3.1 Sequence retrieval and analysis

The *C. cinerea* SPR 04562 gene was first identified using the Genbank database and a 1kb stretch of DNA representing a putative promoter sequence upstream of the ATG start site was selected. This putative SPR 04562 promoter sequence was analysed for the presence of CAAT regions and TATA boxes and the motifs identified are highlighted in bold and underlined in Figure 4.2 below.

GGAGGAGCGCTTTCGAGAGTCTCGAGAATGTGGGCCAACCATGACCCATTTAGGTGAGGTTT
TCGAAGTCCACTAGGTCCTAACTGGCCCACCACCATAAGCGGACTGCCGCTTTCGTCTCTCG
AGAAGGCACCGGCTTGAAAGGTTATAGTTAAGCGGTTCAGATCGGATAATGCTATTTGGCAA
CCCCCCTGCCGCGAGCAACCCCGGTTGTTTTCAAAGGTGTTATTCTGCTACCATACTCTACTT
TTCAGTTAGAGGCACAGAAGCACGTAGCCTGATACTGCTAGTCTTCAAGCCTGGTCTTCTCT
CACATCGCTCCTGTCGTCGACTTTCCGAATTTGTGCCTATCGCTTCCATTGACTGGATGCTAT
TTTTAGGCCAACTAGACCAAAACTAGACACTAGACGAGGAGGTCTGGAGAGTAGGATGACA
AATAGAGTAGCTTTCTGATATGTTGGGGTGGATTAGGTGCGTGCCGGACTTTATTCTTGATAT
TCACTTTCACTTTCCACACGAATCAACACTTCTCAGACGTCGCGGATGTGGTTCTTTTCGAAT
GCCCTTGTATGTTCTTTCTCTTCTTACTCACAGCTATCACAGGATATTAGTCCTATGGGATAA
AACATGCAGCATAACGGCAGATGAATGTGGATTAGGCAAAGACTTCATTCATTCTGAAACCC
CGTAAGGTTACGACCATCACCTTTTTAGTTCTCGAACGCGTTGCCGCGATTGCCAGTGGTCCC
AGC**CAAT**CGCAAGTTGCATGTGGTGCAGACG**CAAT**GTTGGCTACCTGCAGCCATAAAGGCG
ACTTCATTGCGGGAAACGATTGACTTCCGGCAAGCGGCGCATTGGTTGAGGAAGCATATGCA
CAGGCTTTCAGTAGTGTGTTCTGAAGG**TATAAA**AGCGCTTCGACATGTCCTCTGTTCACTAC
ACTGGAACCTTTTCCAAGTGACCTCTCTCTCCTTCCTGACCTGCCCGTCGCTTCCCTGTCTCTG
CCATG

Figure 4.2: *C. cinerea* genomic DNA (1kb) upstream from the CC1G_04562 gene. TATA box and CAAT regions are highlighted in bold and underlined. The forward primer is highlighted in red and the reverse primer is highlighted in blue.

4.3.2 PCR amplification of the SPR 04562 promoter sequence

The primers for the PCR amplification of the putative SPR 04562 promoter region were next designed from the sequence shown in Figure 4.2. Analysis of the cloning vector, peGFPi004 had been undertaken previously in the preparation of the SPR 10592 promoter (Section 3.5.2) and the results of this analysis were used here. This analysis revealed that the plasmid contained the restriction sites *Eco*RI, *Sac*I and *Sac*II upstream of the *gpd*II promoter and a *Hind*III restriction site at the 3' end. Therefore restriction digestion of this plasmid with either *Sac*I-*Hind*III, *Sac*II-*Hind*III or *Eco*RI-*Hind*III would, result in the excision of the *gpd*II promoter, leaving the intron-eGFP-terminator cassette intact. Analysis of the SPR 04562 promoter sequence was performed and determined that both *Eco*RI and *Sac*II cut within the SPR 04562 promoter, while *Sac*I and *Hind*III do not. Based on this information, a *Sac*I site was included on the forward primer and a *Hind*III

site on the reverse primer (Materials and Methods, 2.8.1, Table 2.3) to allow directional cloning.

These primers were then used in a PCR with *C. cinerea* genomic DNA, employing an annealing step at 70°C for 90 seconds and an extension step at 72°C for 2 minutes (as per Materials and Methods, Section 2.8.3). Following PCR, 5µL of the product was analysed by agarose gel electrophoresis to check for migration of the fragment at the expected size of 1003bps and the results can be seen in Figure 4.3. The putative promoter was successfully amplified as indicated by the band at approximately 1003bps, as seen in lane 1.

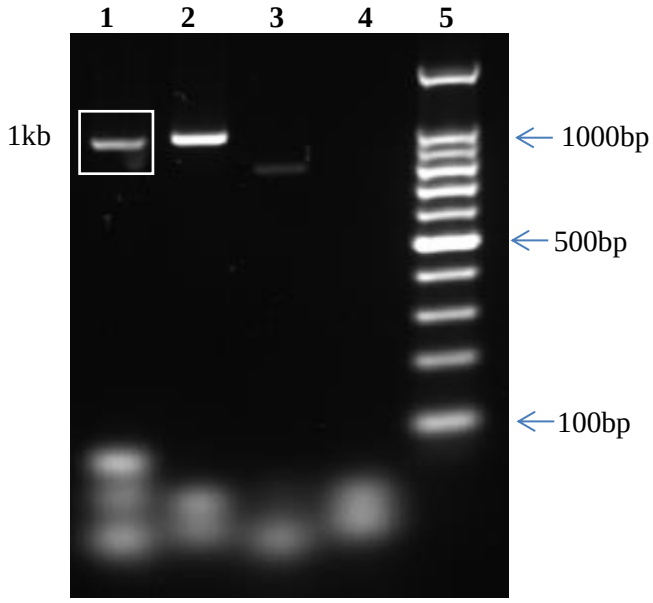

Figure 4.3: The putative SPR 04562 promoter DNA electrophoresed on a 1% agarose gel at 120V for 45 minutes. Lane 1: 5µL SPR 04562 promoter (lane 2-4: are not relevant here and are discussed elsewhere in the document) and lane 5: 100bp ladder. The white box indicates the location of the DNA fragment.

Following confirmation that the PCR product appeared to be at the approximate size of 1003bps as expected, a purification step was next performed. The SPR 04562 promoter product was excised from the gel and purified using the Omega bio-tek gel extraction kit (as per Materials and Methods, Section 2.6.2). Following this, 3µL of the purified SPR 04562 promoter was electrophoresed on a 1% agarose gel to confirm that the promoter was successfully purified (results not shown).

4.3.3 Cloning of the SPR 04562 promoter into pCR®2.1-TOPO

The purified PCR product was next cloned into pCR®2.1-TOPO following manufacturer's instructions to produce the plasmid p04562TOPO and this plasmid was subsequently transformed into *E.coli* JM109 (as per Material and Methods, Section 2.5). Single colonies were used to inoculate cultures from which DNA mini preparations were performed.

Restriction digestion using *Kpn*I was then performed on the DNA to monitor for the presence of the insert. 3µL of the linearized p04562TOPO was analysed by agarose gel electrophoresis, as shown in Figure 4.4. 3µL of undigested p04562TOPO was included on the gel as a control. The TOPO plasmid is 3931bps, so an expected plasmid size of 4934bps was predicted after insertion of the 1003bps promoter. The restriction enzyme *Kpn*I was expected to cut the plasmid once. The digestion of p04562TOPO is indicated by a single band in lane 2 of Figure 4.4; however, this band appears at approximately 6kb in size. This is not at the predicted size of approximately 4934bps. It is possible that the banding patterns observed have been affected by their interaction with gel red during migration again here as in Section 3.5.3. In order to address this concern, it was next decided to perform additional analysis using PCR and sequencing.

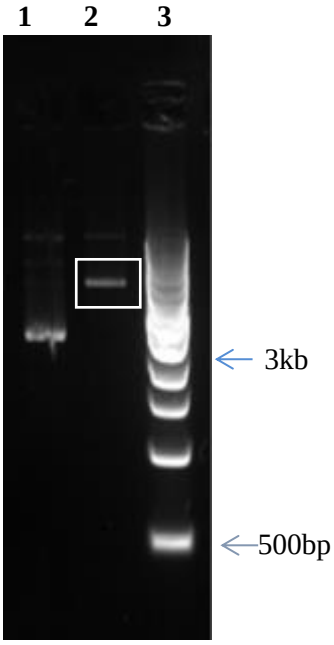

Figure 4.4: Restriction digest of p04562TOPO electrophoresed on a 1% agarose gel at 100V for 45 minutes. Lane 1: 3µL undigested p04562TOPO; lane 2: 3µL p04562TOPO digested with *Kpn*I; lane 3: 1kb NEB ladder. The white box indicates the location of the DNA fragment.

In order to further confirm the integration of the promoter sequence into the pCR®2.1-TOPO vector, a PCR amplification using M13 primers was next undertaken. PCR was performed employing an annealing step of 55°C for 90 seconds and an extension step of 72°C for 2 minutes (as per Material and Method, Section 2.8.3). 5µL of the PCR product was analysed as before using agarose gel electrophoresis confirming the amplification of the product at the estimated size of 1503bps: (SPR gene 1003bps + 500bps of TOPO vector) as seen in lane 2 of Figure 4.5.

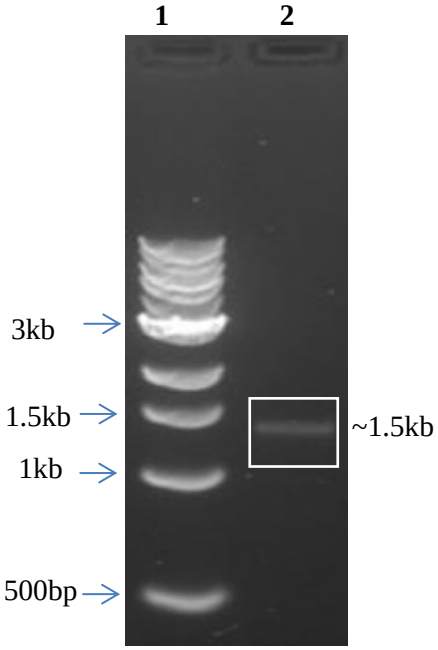

Figure 4.5: PCR using M13 primers to amplify the SPR 04562 promoter from p04562TOPO, electrophoresed on a 1% agarose gel at 100V for 1 hour. Lane 1:1kb NEB ladder; lane 2: 5µL PCR amplified SPR 04562 promoter DNA. The white box indicates the location of the PCR product.

Once this analysis was complete, the plasmid p04562TOPO was sequenced using forward and reverse M13 primers (as per Materials and Methods, Section 2.10.1), and the corresponding sequencing results were labelled as 04562M13F and 04562M13R. The original SPR 04562 promoter sequence (original04562) was obtained from Genbank and was aligned with the above sequences using the MUSCLE analysis tool. The 04562M13F sequence was seen to share 99% nucleotide identity when compared to the original SPR 04562 promoter sequence. The 04562M13R was also 99% identical when compared to the original SPR 04562 promoter sequence (Table 4.1; Figure 4.6).

Table 4.1: The Percent Identity Matrix created by Clustal2.1, shows the nucleotide alignment and the similarity of sequencing results for p04562TOPO.

Percent Identity Matrix- created by Clustal2.1				
Sequence	**Primers**			
original04562		99.57	99.90	100.00
04562M13F	M13F	99.71	100.00	99.90
04562M13R	M13R	100.00	99.71	99.57

```
04562M13R          ----------------TACCATACTCNACTTTTCAGTTANAGGCACAGAAGCACGTAGC
04562M13F          CAAAGGTGTTATTCTGCTACCATACTCTACTTTTCAGTTAGAGGCACAGAAGCACGTAGC
original04562      CAAAGGTGTTATTCTGCTACCATACTCTACTTTTCAGTTAGAGGCACAGAAGCACGTAGC
                               **********.**********.**********

04562M13R          CTGATACTGCTAGTCTTCAAGCCTGGTCTTCTCTCACATCGCTCCTGTCGTCGACTTTCC
04562M13F          CTGATACTGCTAGTCTTCAAGCCTGGTCTTCTCTCACATCGCTCCTGTCGTCGACTTTCC
original04562      CTGATACTGCTAGTCTTCAAGCCTGGTCTTCTCTCACATCGCTCCTGTCGTCGACTTTCC
                   ************************************************************

04562M13R          GAATTTGTGCCTATCGCTTCCATTGACTGGATGCTATTTTTAGGCC-----------AAC
04562M13F          GAATTTGTGCCTATCGCTTCCATTGACTGGATGCTATTTTTAGGCC-----------AAC
original04562      GAATTTGTGCCTATCGCTTCCATTGACTGGATGCTATTTTTAGGCCAACTAGACCAAAAC
                   *********************************************           ***

04562M13R          TAGACACTAGACGAGGAGGTCTGGAGAGTAGGATGACAAATAGAGTAGCTTTCTGATATG
04562M13F          TAGACACTAGACGAGGAGGTCTGGAGAGTAGGATGACAAATAGAGTAGCTTTCTGATATG
original04562      TAGACACTAGACGAGGAGGTCTGGAGAGTAGGATGACAAATAGAGTAGCTTTCTGATATG
                   ************************************************************

04562M13R          TTGGGGTGGATTAGGTGCGTGCCGGACTTTATTCTTGATATTCACTTTCACTTTCCACAC
04562M13F          TTGGGGTGGATTAGGTGCGTGCCGGACTTTATTCTTGATATTCACTTTCACTTTCCACAC
original04562      TTGGGGTGGATTAGGTGCGTGCCGGACTTTATTCTTGATATTCACTTTCACTTTCCACAC
                   ************************************************************

04562M13R          GAATCAACACTTCTCAGACGTCGCGGATGTGGTTCTTTTCGAATGCCCTTGTATGTTCTT
04562M13F          GAATCAACACTTCTCAGACGTCGCGGATGTGGTTCTTTTCGAATGCCCTTGTATGTTCTT
original04562      GAATCAACACTTCTCAGACGTCGCGGATGTGGTTCTTTTCGAATGCCCTTGTATGTTCTT
                   ************************************************************

04562M13R          TCTCTTCTTACTCACAGCTATCACAGGATATTAGTCCTATGGGATAAAACATGCAGCATA
04562M13F          TCTCTTCTTACTCACAGCTATCACAGGATATTAGTCCTATGGGATAAAACATGCAGCATA
original04562      TCTCTTCTTACTCACAGCTATCACAGGATATTAGTCCTATGGGATAAAACATGCAGCATA
                   ************************************************************

04562M13R          ACGGCAGATGAATGTGGATTAGGCAAAGACTTCATTCATTCTGAAACCCCGTAAGGTTAC
04562M13F          ACGGCAGATGAATGTGGATTAGGCAAAGACTTCATTCATTCTGAAACCCCGTAAGGTTAC
original04562      ACGGCAGATGAATGTGGATTAGGCAAAGACTTCATTCATTCTGAAACCCCGTAAGGTTAC
                   ************************************************************

04562M13R          GACCATCACCCTTTTAGTTCTCGAACGCGTTGCCGCGATTGCCAGTGGTCCCAGCCAATC
04562M13F          GACCATCACCCTTTTAGTTCTCGAACGCGTTGCCGCGATTGCCAGTGGTCCCAGCCAATC
original04562      GACCATCACCTTTTTAGTTCTCGAACGCGTTGCCGCGATTGCCAGTGGTCCCAGCCAATC
                   **********.*************************************************

04562M13R          GCAAGTTGCATGTGGTGCAGACGCAATGTTGGCTACCTGCAGCCATAAAGGCGACTTCAT
04562M13F          GCAAGTTGCATGTGGTGCAGACGCAATGTTGGCTACCTGCAGCCATAAAGGCGACTTCAT
original04562      GCAAGTTGCATGTGGTGCAGACGCAATGTTGGCTACCTGCAGCCATAAAGGCGACTTCAT
                   ************************************************************

04562M13R          TGCGGGAAACGATTGACTTCCGGCAAGCGGCGCATTGGTTGAGGAAGCATATGCACAGGC
04562M13F          TGCGGGAAACGATTGACTTCCGGCAAGCGGCGCATTGGTTGAGGAAGCATATGCACAGGC
original04562      TGCGGGAAACGATTGACTTCCGGCAAGCGGCGCATTGGTTGAGGAAGCATATGCACAGGC
                   ************************************************************

04562M13R          TTTCAGTAGTGTGTTCTGAAGGTATAAAAGCGCTTCGACATGTCCTCTGTTCACTACACT
04562M13F          TTTCAGTAGTGTGTTCTGAAGGTATAAAAGCGCTTCGACATGTCCTCTGTTCACTACACT
original04562      TTTCAGTAGTGTGTTCTGAAGGTATAAAAGCGCTTCGACATGTCCTCTGTTCACTACACT
                   ************************************************************

04562M13R          GGAACC
04562M13F          GGAACC
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```
original04562       GGAACC
                    ******
```

Figure 4.6: A MUSCLE nucleotide alignment of p04562TOPO (sequenced with M13 forward and reverse primers) and the original SPR 04562 promoter sequence. Both the 04562M13F and 04562M13R sequences shared 99% nucleotide identity when compared to the original SPR 04562 promoter sequence.

This analysis using restriction digestion, PCR and sequencing verified the successful integration of the SPR 04562 promoter into p04652TOPO, confirming the integrity of the p04562TOPO vector.

4.4 Preparation of the peGFPi04562 fungal expression plasmid backbone

The vector backbone fragment was next prepared from the peGFPi004 plasmid to host the SPR 04562 promoter sequence. Based on the earlier bioinformatics analysis of the predicted restriction digestion patterns of the SPR 04562 promoter sequence (Section 2.8.1) it was decided to digest the vector using *Sac*I and *Hind*III to generate *Sac*I-*Hind*III sticky ends. First, a restriction digest was performed on peGFPi004 using *Sac*I and *Hind*III to remove the 300bps *gpd*II promoter. 10µL of the digested DNA was then analysed by agarose gel electrophoresis, as shown in Figure 4.7. As can be seen, the backbone was successfully digested as indicated by the DNA bands at the expected sizes of approximately 4425bps (peGFPi004 minus *gpd*II promoter) and 300bps (*gpd*II promoter).

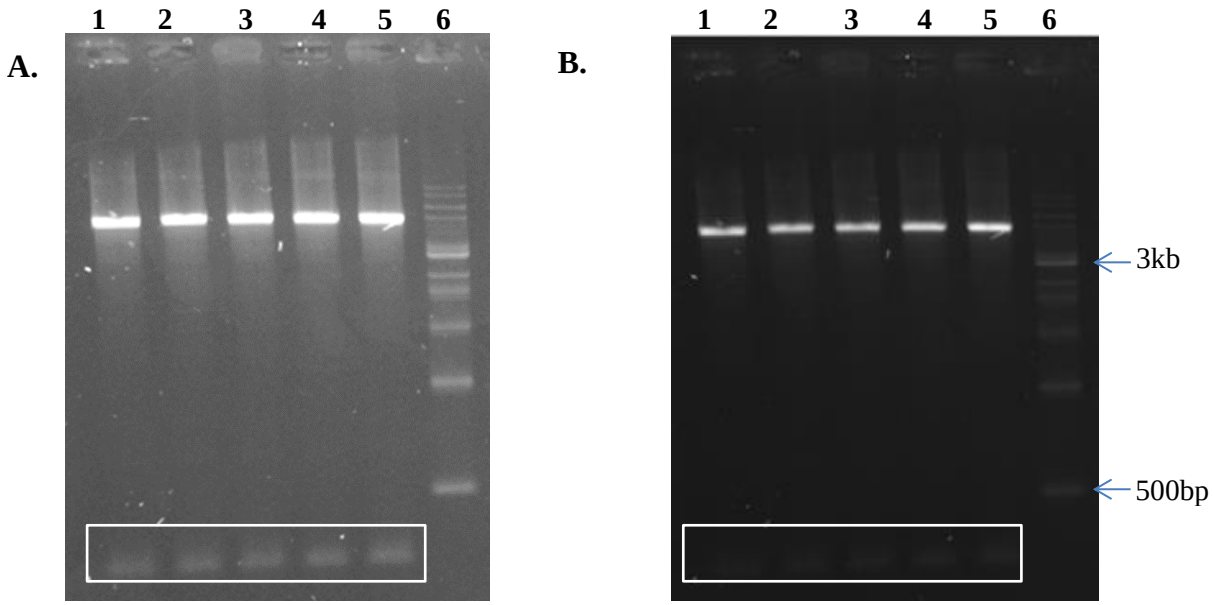

Figure 4.7: The peGFPi004 vector digested using restriction enzymes, *Sac*I and *Hind*III electrophoresed on a 1% agarose gel at 100V for 1 hour. Lanes 1-5: 10µL of peGFPi004 digested with *Sac*I and *Hind*III; lane 6: 1kb NEB ladder. The white boxes indicate the location of the 300bps *gpd*II promoter. Images A and B are the same experiment. Gel A has been brightened to visualise the location of the 300bps *gpd*II promoter.

Once the removal of the *gpd*II promoter had been confirmed, the vector backbone was then extracted and purified using a gel extraction kit (as per Materials and Methods, Section 2.6.2). 5µL of the purified vector backbone was analysed and is presented in Figure 4.8. As can be seen, the backbone was successfully purified as indicated by the single band at approximately 4425bps seen in lane 2 on the gel.

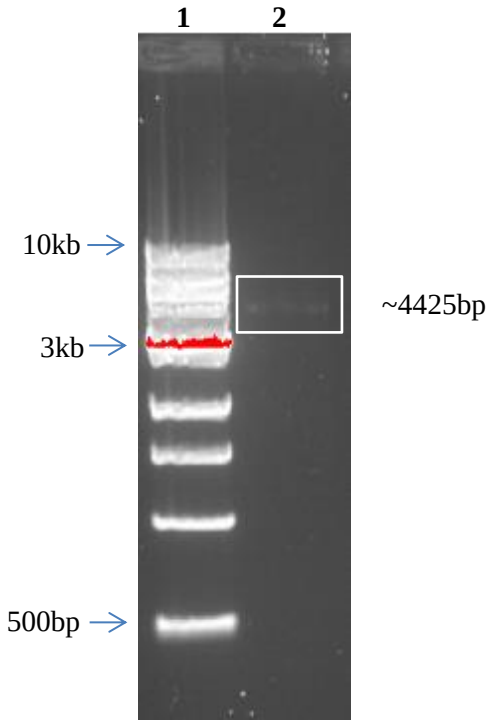

Figure 4.8: The purified backbone DNA electrophoresed on a 1% agarose gel at 100V for 1 hour. Lane 1: 1kb ladder and lane 2: 10µL backbone. The white box indicates the location of the DNA.

4.5 Isolation of the SPR 04562 promoter from vector pCR®2.1-TOPO

In order to prepare the SPR 04562 promoter for ligation into the peGFPi004 backbone, p04562TOPO was digested with *Sac*I and *Hind*III to remove the putative promoter fragment from the TOPO vector. As can be seen from Figure 4.9, p04562TOPO was successfully digested with *Sac*I and *Hind*III to remove the SPR 04562 promoter from TOPO, with a band resolving at the expected size of approximately 1003bps. The SPR 04562 promoter was then purified from the gel (Materials and Methods, Section 2.6.2) to prepare for cloning into the plasmid backbone.

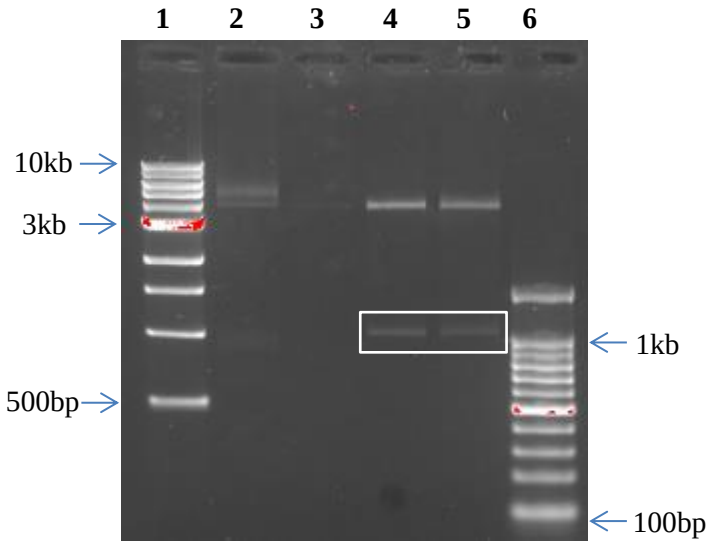

Figure 4.9: The vector p04562TOPO digested with *Sac*I and *Hind*III. Lane 1: 1kb ladder; (lane 2-3: are not relevant to this experiment and are discussed elsewhere in the document), lane 4-5: p04562TOPO digested with *Sac*I and *Hind*III and lane 6: 100bp ladder. The white box indicates the location of the SPR 04562 promoter DNA.

3µL of the purified SPR 04562 promoter was electrophoresed on a 1% agarose gel as shown below in Figure 4.10, confirming successful purification of the promoter fragment. A band at the approximate size of 1003bps can be seen in lane 1 below. The white box highlights the location of the purified DNA.

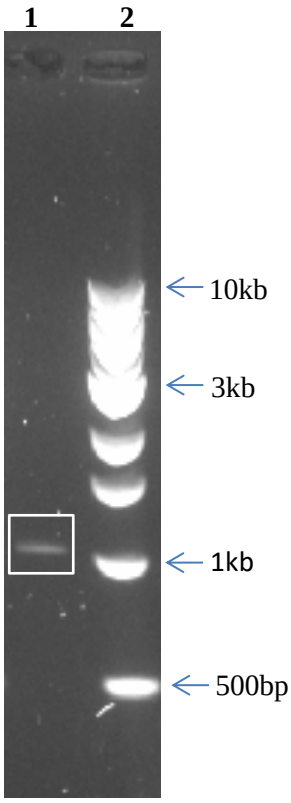

Figure 4.10: Purified *C. cinerea* SPR 04562 promoter DNA electrophoresed on a 1% agarose gel at 100V for 1 hour. Lane 1: 3µL purified SPR 04562 promoter DNA and lane 2: 1kb NEB ladder. The white box indicates the location of the promoter DNA.

The next step in the construction of peGFPi04562 would include ligation of the SPR 04562 promoter into the fungal expression plasmid backbone, peGFPi004 (prepared in Section 4.5) using the Thermo Scientific InsTAclone PCR Cloning Kit and subsequent transformation of the plasmid into *E.coli* JM109 (as per Materials and Methods, Section 2.5). Following this, a miniprep would be performed to isolate the recombinant plasmid DNA from *E.coli* cells and molecular validation of the peGFPi04562 fungal expression plasmid using restriction digests, PCR and sequencing techniques would be carried out to verify the identity of the recombinant vector.

This plasmid would then be ready for transformation studies in *C. cinerea* facilitating the investigation of the SPR 04562 in this fungus. Following transformation, the transformants could be fruited and analysed at different stages of development for the expression of eGFP.

4.6 Construction of the *C. cinerea* serine protease 04562 antisense plasmid

Gene silencing is proposed to evaluate the function of the SPR 04562 gene in *C. cinerea*, as described previously for the SPR 10592 gene. The first step in this process was to design the pAS04562 plasmid. Figure 4.11 represents a summary of this design. The pMCS004 plasmid containing the *A. bisporus gpd*II promoter, a multiple cloning site and *A. nidulans trp*C terminator was employed in this construction (Figure 4.11 A). This plasmid was digested with restriction enzymes *Xma*I *and Aat*II to remove the multiple cloning site region (Figure 4.11 B). The SPR gene was PCR amplified to include the restriction sites *Xma*I and *Aat*II for use in directional cloning (Figure 4.11 C). The multiple cloning site would then be replaced by the SPR 04562 gene in the antisense orientation resulting in the construction of the pAS04562 plasmid (Figure 4.11 D).

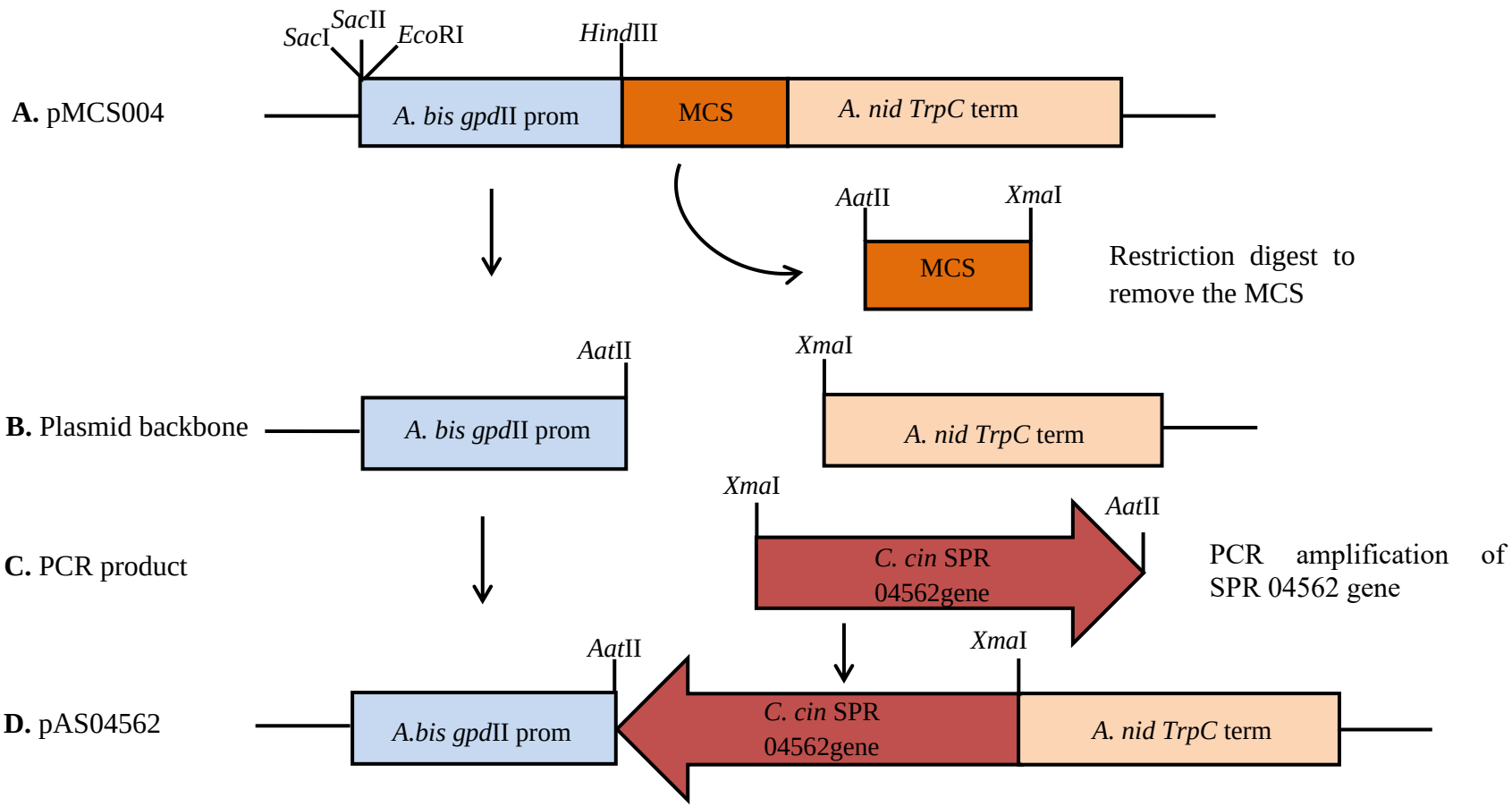

Figure 4.11: A schematic representation of the design and construction of the antisense cassette pAS04562. A: The pMCS004 plasmid containing the *A. bisporus gpd*II promoter (*A. bis gpd*II prom), multiple cloning site (MCS) and the *A. nidulans TrpC* terminator (*A. nid TrpC* term). B: The plasmid digested with *Aat*II and *Xma*I to remove the MCS. C: The amplified SPR 04562 gene flanked with restriction sites *Aat*II and *Xma*I ready for directional cloning. D: The constructed plasmid pAS04562 contains the *A. bisporus gpd*II promoter, *C. cinerea* SPR 04562 gene (in the antisense orientation) and the *A.nidulans TrpC* terminator.

4.7 Preparation of the SPR 04562 gene

4.7.1 Sequence retrieval and analysis

The SPR 04562 gene sequence was identified from the Genbank database and primers were then designed to amplify this gene. The first step in this process was to analyse the gene for restriction enzyme recognition sites using the online tool, webcutter. The restriction enzymes *Aat*II and *Xma*I were shown not to cut within the gene sequence. Based on this analysis, it was decided to include *Xma*I site on the forward primer and include an *Aat*II site on the reverse primer in order to allow cloning of the gene fragment in the antisense orientation in pMCS004.

4.7.2 PCR amplification of the SPR 04562 gene

These primers were then used in a PCR with *C. cinerea* gDNA, employing an annealing step at 60ºC for 50 seconds and extension at 72ºC for 45 seconds (as per Materials and Methods, Section 2.8.3). Following PCR, 5µL of the product was analysed by agarose gel electrophoresis to check migration of the gene at the expected size of 1139bps. The results can be seen in Figure 4.12, lane 1, which confirms the successful amplification of the SPR 04562 gene.

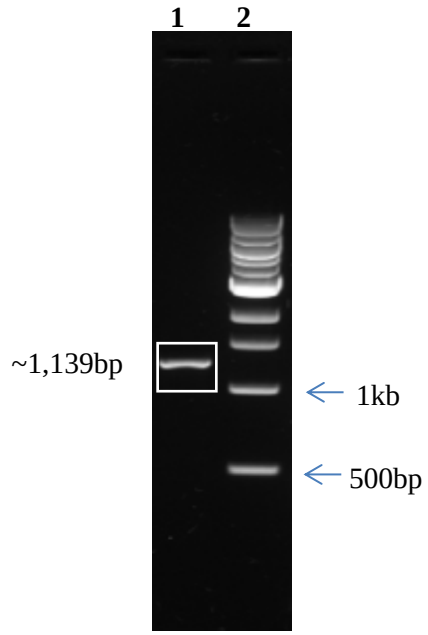

Figure 4.12: PCR amplification of the SPR 04562 gene fragment electrophoresed on a 1% agarose gel at 100V for 1 hour. Lane 1: 5µL SPR 04562 gene; lane 2: 1kb NEB ladder. The white box indicates the location of the DNA.

Following confirmation that the PCR product was the expected size of approximately 1139bps, a purification step was performed. The SPR 04562 gene was extracted from the agarose gel and purified using the Omega bio-tek gel extraction kit (as per Materials and Methods, Section 2.6.2). 5µL of the purified SPR 04562 gene was then analysed by agarose gel electrophoresis as seen in Figure 4.13. However, it is not at the predicted size of approximately 1139bps and seems to be resolving at higher than the expected size. It is possible that the banding patterns observed here have been affected by their interaction with gel red during migration again, as in Section 3.5.3. In order to address this concern, it was next decided to perform additional analysis using PCR and sequencing.

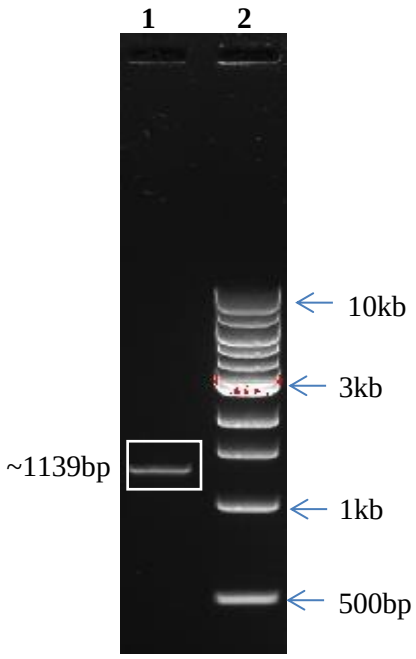

Figure 4.13: Purified SPR 04562 gene fragment electrophoresed on a 1% agarose gel at 100V for 1 hour. Lane 1: 5µL purified SPR 04562 gene DNA; lane 2: 1kb NEB ladder. The white box indicates the location of the DNA.

4.7.3 Cloning of the SPR 04562 gene into pCR®2.1-TOPO

The purified SPR 04562 gene was next cloned into pCR®2.1-TOPO following manufacturer's instructions to produce the plasmid pAS04562TOPO and this plasmid was subsequently transformed into *E. coli* JM109 (as per Materials and Methods, Section 2.5). Single colonies were used to inoculate cultures from which DNA mini preparations were performed. 4µL of this DNA was then analysed by agarose gel electrophoresis, as shown in Figure 4.14.

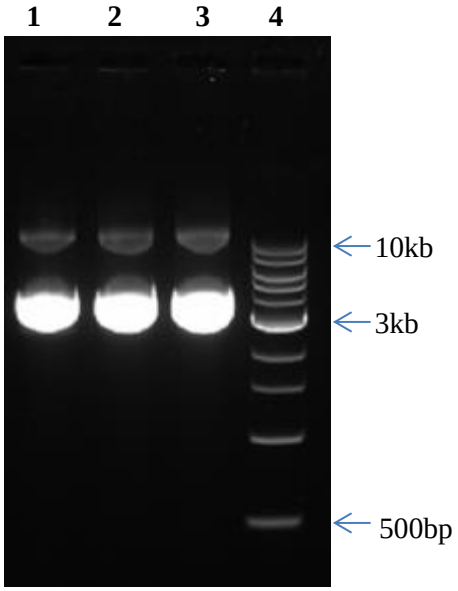

Figure 4.14: The vector pAS04562TOPO electrophoresed on a 1% agarose gel at 100V for 1 hour. Lane 1-3:4µL pAS04562TOPO and lane 4: 1kb NEB ladder.

To verify the integrity of this plasmid, a double digest using both *Nco*I *and Xho*I was next performed. As the pCR®2.1-TOPO plasmid is 3931bps an expected plasmid size of 5067bps was predicted after insertion of the 1136bps SPR gene. The restriction enzyme *Nco*I was expected to cut the vector twice; once within the SPR 04562 gene and once in the TOPO vector, while *Xho*I was expected to cut the TOPO plasmid once. The double digest reaction was predicted to produce three separate DNA fragments at approximately 3kb, 1.5kb and 567bps. 3µL of digested plasmid pAS04562TOPO was analysed by agarose gel electrophoresis, as shown in Figure 4.15. The digestion of the pAS04562TOPO was successful as indicated by the bands at the expected sizes as seen in lane 3.

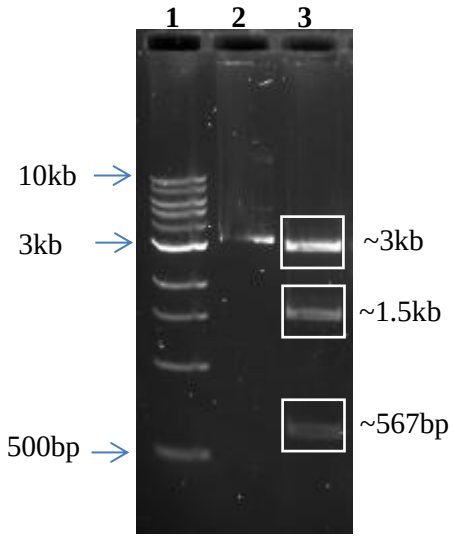

Figure 4.15: Restriction digestion of plasmid pAS04562TOPO electrophoresed on a 1% agarose gel at 100V for 1 hour. Lane 1: 1kb NEB ladder; lane 2: undigested pAS04562TOPO and lane 3: 3µL pAS04562TOPO digested with *Nco*I and *Xho*I.

To further confirm the integration of the gene sequence into the pCR®2.1-TOPO vector, a PCR amplification using M13 primers was next performed (as per Materials and Methods, Section 2.8.1). PCR was performed employing an annealing step at 61°C for 90 seconds and an extension step of 72°C for 2 minutes (as per Materials and Methods, Section 2.8.3). 10µL of PCR product was analysed by agarose gel electrophoresis confirming the amplification of the product at the predicted approximate size of 1636bps (1136bps SPR gene + 500bps TOPO) as seen in Figure 4.16.

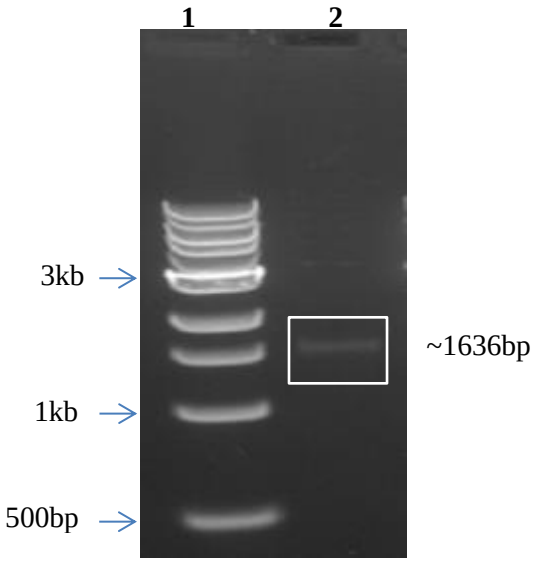

Figure 4.16: PCR using M13 primers to amplify the SPR 04562 gene from pAS04562TOPO, electrophoresed on a 1% agarose gel at 100V for 1 hour. Lane 1: 1kb NEB ladder and lane 2: PCR amplified SPR 04562 gene. The white box indicates the location of the DNA.

Once this analysis was complete, the vector pAS04562TOPO was sequenced using a primer that was complementary to the start of the SPR 04562 gene and the corresponding sequence result was labelled anti04562. The resulting nucleotide sequence was aligned with the original SPR 04562 gene sequence (original04562gene) obtained from Genbank, using the online MUSCLE software. As can be seen from the analysis, the pAS04562TOPO sequence was seen to share 99% nucleotide identity with the original SPR 04562 gene (Table 4.2; Figure 4.17).

Table 4.2: The Percent Identity Matrix created by Clustal2.1, shows the nucleotide alignment and the similarity of sequencing results for vector pAS04562TOPO.

Percent Identity Matrix - created by Clustal2.1			
Sequence	**Primer**		
original04562gene		100.00	99.46
anti04562	04562fwd	99.46	100.00

```
original04562gene       GTACACCATGTCCCCTGTCACCCAGTAGGACATCCTTTCACGTTCGGTGTGGTTGGGTGT
anti04562               ------------------------------CTCCTTTC-CGTTCGGTGTGGTTGGGTGT
                                                       ******* ********************

original04562gene       ACTTACTCCCCTGGATCAGGTCCGATGCTCCTTGGGGTCTTCAAAGGGTCAGCCAGGCCG
anti04562               ACTTACTCCCCTGGATCAGGTCCGATGCTCCTTGGGGTCTTCAAAGGGTCAGCCAGGCCG
                        ************************************************************

original04562gene       CGAGGCTCAGCAACACGGATGTCGCGTACGATTAGCCTCCATTACGCCTCGTCTGCGCAT
anti04562               CGAGGCTCAGCAACACGGATGTCGCGTACGATTAGCCTCCATTACGCCTCGTCTGCGCAT
                        ************************************************************
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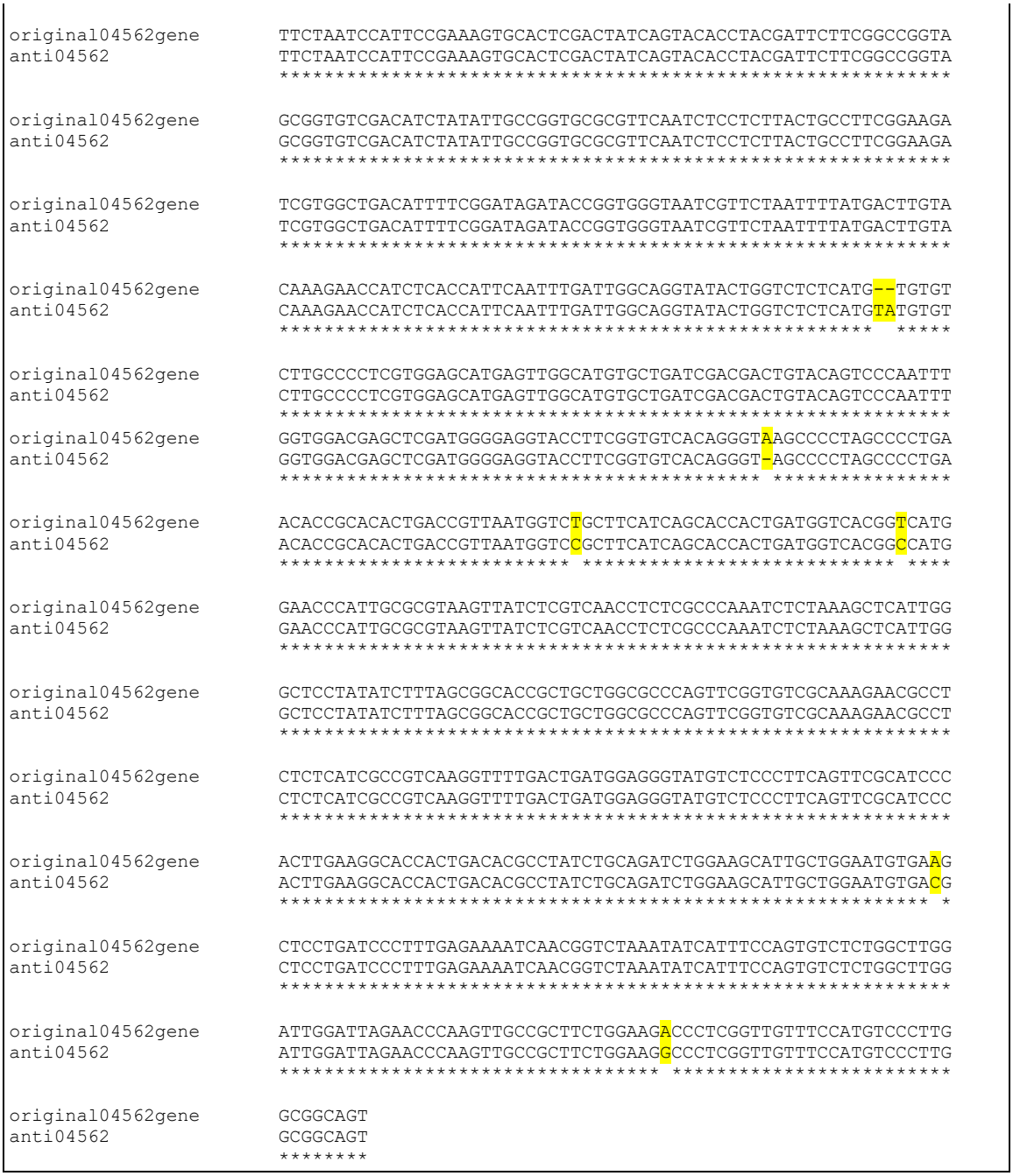

Figure 4.17: A MUSCLE nucleotide alignment of pAS04562TOPO (anti04562) and the original SPR 04562 gene sequence (original04562gene). The pAS04562TOPO vector shared 99% nucleotide identity when compared to the original SPR 04562 gene.

This analysis using restriction digestion, PCR and sequencing verify the successful integration of the SPR 04562 gene into the TOPO vector.

The next step in the construction of pAS04562 would include the preparation of the pMCS004 vector backbone to facilitate directional cloning. This involves digestion of the plasmid with *AatII* and *Xma*I restriction enzymes and purification using a gel

extraction kit (as per Materials and Methods, Section 2.6.2). Following this, the SPR 04562 gene would be ligated into the fungal expression plasmid backbone using the Thermo Scientific InsTAclone PCR Cloning Kit. The recombinant vector, pAS04562, would then be transformed into *E.coli* JM109 (as per Materials and Methods, Section 2.5). A miniprep would next be performed to isolate the recombinant plasmid DNA from *E.coli* cells and molecular validation of the pAS04562 fungal expression plasmid using restriction digests, PCR and sequencing techniques would be carried out to verify the identity of the recombinant vector. This plasmid would then be ready for transformation into *C. cinerea* to facilitate analysis of the effect silencing the SPR 04562 gene would have on mushroom development.

4.8 Construction of the *C. cinerea* serine protease 10615 promoter fungal expression plasmid

The *C. cinerea* SPR 10615 was next selected for study. The SPR 10615 gene has also shown significant homology to the *A. bisporus* SPRs. Sequence analysis of *C. cinerea* SPR 10615 revealed similarity to the value of 55% when aligned with *A. bisporus* SPR1 and 53% when aligned with *A. bisporus* SPR2 (Heneghan *et al.* 2009). Again, the construction of a promoter-reporter construct will facilitate the investigation of the role this SPR plays in *C. cinerea.*

The first step in this process was to design the plasmid peGFPi10615. Figure 4.18 represents a schematic overview of this design. The plasmid peGFPi004 (as described in Section 3.5.2) was again employed here (Figure 4.18 A). The restriction enzymes *Sac*II and *Hind*III were identified to remove the 300bps *A. bisporus gpd*II promoter leaving the rest of the plasmid intact (Figure 4.18 B). The SPR promoter was PCR amplified to include the restriction sites *Sac*II and *Hind*III for directional cloning (Figure 4.18 C). In order to complete this vector, the *gpd*II promoter would be replaced by the *C. cinerea* SPR 10615 promoter forming the SPR promoter plasmid peGFPi10615 (Figure 4.18 D).

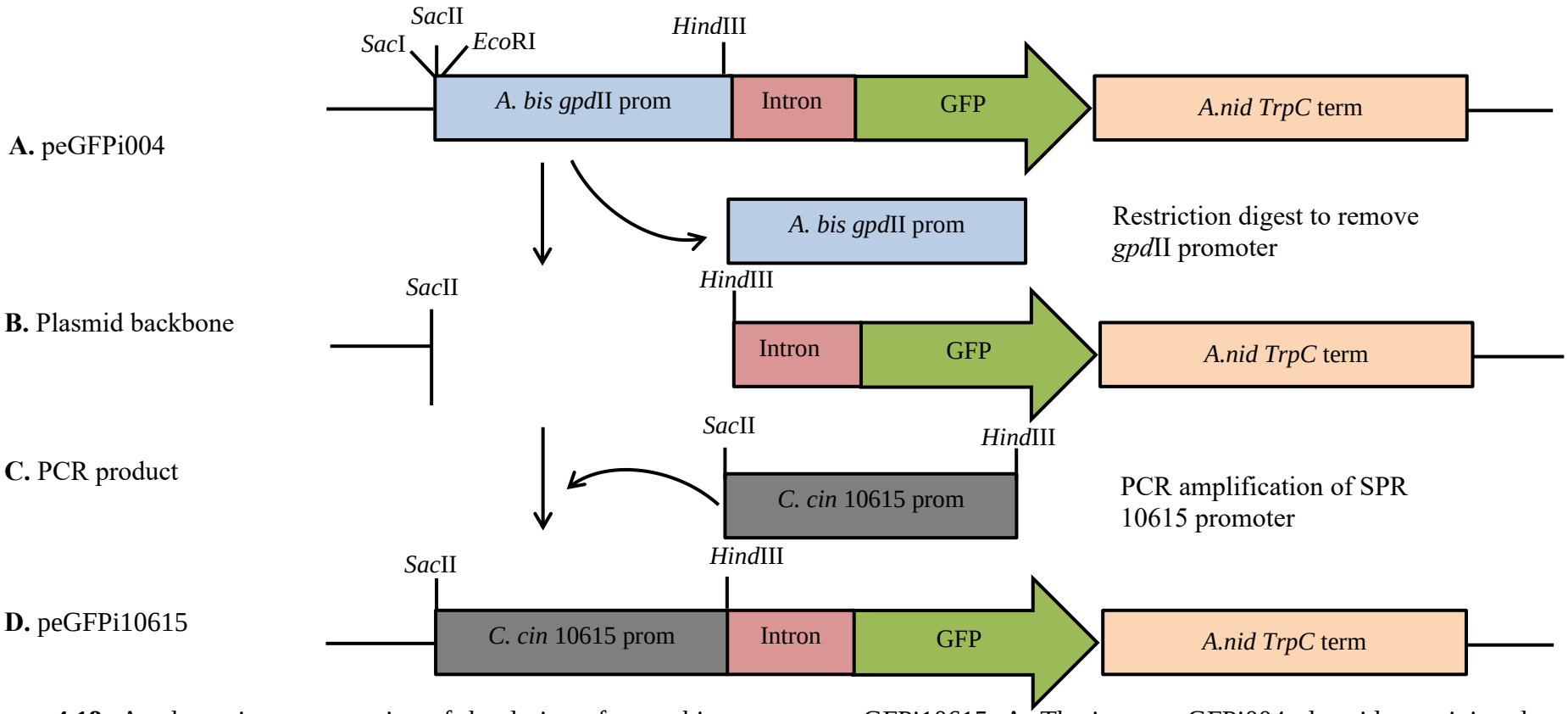

Figure 4.18: A schematic representation of the design of recombinant vector peGFPi10615. A: The intact peGFPi004 plasmid containing the original *A. bisporus gpd*II promoter (*A.bis gpd*II prom), intron, eGFP and *A.nidulans trpC* terminator (*A.nid TrpC* term). B: The plasmid digested with restriction enzymes *Sac*II and *Hind*III to remove the *gpd*II promoter leaving the plasmid backbone. C: The amplified *C. cinerea* SPR 10615 promoter flanked with *Sac*II and *Hind*III restriction sites. D: The intact peGFPi10615 contains the *C. cinerea* SPR 10615 promoter ligated into the peGFPi004 backbone.

4.9 Preparation of SPR 10615 promoter fragment

4.9.1 Sequence retrieval and analysis

The *C. cinerea* SPR 10615 gene was identified using the Genbank database and a 1kb stretch of DNA representing a putative promoter upstream of the ATG start site was selected. This putative 10615 promoter sequence was analysed for the presence of CAAT regions and TATA boxes and the motifs identified are highlighted in bold and underlined in Figure 4.19 below.

ACCCTATGTTATGAAAACATATGTCGCGTGGATGGATAGGCACTCTTGGTCTCCCTGATCGACGAA
GCACATGGTTCTCGTTCAGGTTTATGCCCCCGG**CAAT**GGACTAATTCAACGCGAATACGGGACTGC
GAGGGGCTTTGGCTCACGCGACCATGATGGTTGAGTTCGCGCTGTCGGGAACGCATGTCAACGGG
GTGACCTCTAAACACTTGGACTAGGGCGACTCAAGTGACCGTCTAATCTTGTGATATAGCTCTCAA
CCCGCCTTCTTGGCAAGGGCGATTTAAGGGCCACTTCACCTTTATCGACTCTGTCGCCCCACACACT
CCCACACGGCCGTTATGCTTATCTTGGTCACTAGGCATCCGCTGACTCCCTATGTCGTATGGAGTTA
CCACCTTCCTCGGTGCCTTTGTGAGGTCTCTATGCCCCCGACCCGAGCCCGTAGTTATCCCTTTCTG
GGCATAACCAGCCCATTGGGGCGAAGGTCAGGATAT**CAAT**CTCCTG**CAAT**TGGTTTGCTCCCCATT
CAATGACCATATCCCGCGCTTAACCTCAAGTTCCAGTCGAATATTTTCATTAGCTTTCACTTCTGGA
CTATGTATCGCCGTTCCGTCCGTATCTCATCGTGGAATGCAGGTTGCCTGTTCACCGCATTGAACAT
CAGCTTCTTCCACGCGTGCCATATGGCTTTCGACTC**CAAT**GTGCACTACCCCAAGGCAAAGGGGCC
TTGTGTCAACGCGACCGTCCTCATGTTTCAACTACTTCTCTGGGTATCAGGTGAGATACGCGCATGT
TGTTCACCAACGTCGAGTGTTGCGTCTTTGAACTACGTTTTCAGTCGCGGTGACGACCTGTATCTTC
CTTTTCGAGGATACAGCGATACGTGAAAACA**TATAAA**AAGGTTCGTGTTCAGTGCAGT**CAAT**TCC
CCGAGCTAGTTCCAACTTCT**CAAT**CCCCAGGTCCTTTTCCGAGTTTCTTCTCGTCGCCCCTTCTCTTC
ACGATGA

Figure 4.19: *C. cinerea* genomic DNA (1kb) upstream from the CC1G_10615 gene. A TATA box and CAAT regions are highlighted in bold and underlined. The forward primer is highlighted in red and the reverse primer is highlighted in blue.

4.9.2 PCR amplification of the SPR 10615 promoter sequence

The primers for the PCR amplification of the putative SPR 10615 promoter region were next designed from the sequence shown in Figure 4.19. Analysis of the promoter sequence using webcutter, determined that both *Eco*RI and *Sac*I cut within the 10615 promoter, while *Sac*II and *Hind*III do not. Based on this analysis, it was decided to include a *Sac*II site on the forward primer and a *Hind*III site on the reverse primer (see Material and Methods Section 2.8.1, Table 2.2) to allow directional cloning.

These primers were then used in a PCR with *C. cinerea* gDNA, employing an annealing step at 70°C for 90 seconds and an extension step at 72°C for 2 minutes (as per Materials and Methods, Section 2.8.3). Following PCR, 5µL of the product was analysed by agarose gel electrophoresis to check for migration of the fragment at the expected size of 1004bps and the results can be seen in Figure 4.20. As can be seen, in lane 1, the putative promoter was successfully amplified as indicated by the band at approximately 1004bps, as expected.

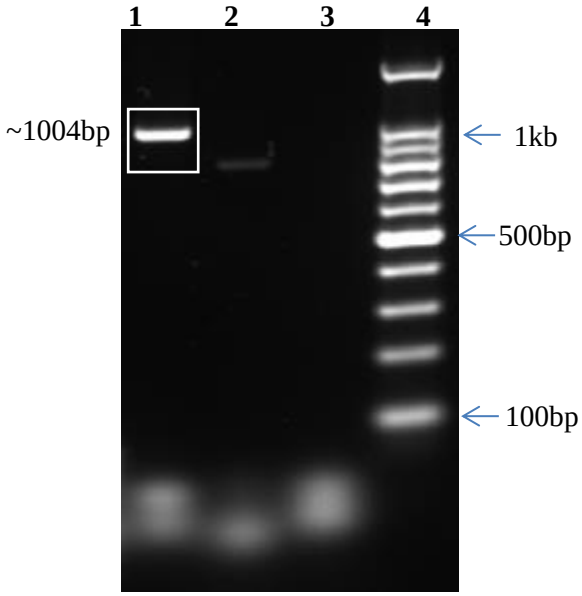

Figure 4.20: The putative SPR 10615 promoter DNA electrophoresed on a 1% agarose gel at 100V for 45 minutes. Lane 1: 5µL SPR 10615 promoter; lane 4: 100bp ladder. (Lane 2-3: is not relevant to this experiment and will be discussed elsewhere in the document). The white box indicates the location of the DNA.

Following confirmation that the PCR product was the expected size of approximately 1004bps, a purification step was next performed. The SPR 10615 promoter was excised from the gel and purified using the Omega bio-tek gel extraction kit (as per Materials and Methods, Section 2.6.2). 3µL of the purified SPR 10615 promoter was electrophoresed on a 1% agarose gel and analysis confirmed that the promoter fragment was successfully purified (results not shown).

4.9.3 Cloning of the SPR 10615 promoter into pCR®2.1-TOPO

The purified SPR 10615 promoter was next cloned into pCR®2.1-TOPO following the manufacturer's instructions in order to construct the recombinant plasmid p10615TOPO. This vector was subsequently transformed into *E. coli* JM109 (as per Material and Methods, Section 2.5). Single colonies were used to inoculate cultures from which DNA mini preparations were performed.

Restriction digestions using *Kpn*I were performed on p10615TOPO to monitor for the presence of the insert. 3µL of linearized p10615TOPO was analysed by agarose gel electrophoresis, as shown in lane 2 of Figure 4.21. 3µL of undigested p10615TOPO was also used for comparison and is shown in lane 1 of Figure 4.21. The TOPO plasmid is 3931bps, and an expected plasmid size of 4935bps was predicted after the insertion of the 1004bps promoter. The restriction

enzyme *Kpn*I was expected to cut the plasmid once. The digestion of p10615TOPO plasmid was indicated by the band in lane 2 of Figure 4.21 below; however, it is not at the predicted size of approximately 4935bps. It is possible that the banding patterns observed have been affected by their interaction with gel red during migration, again here as in Section 3.5.3. In order to address this concern, additional analysis of p10615TOPO using PCR and sequencing was perfomed.

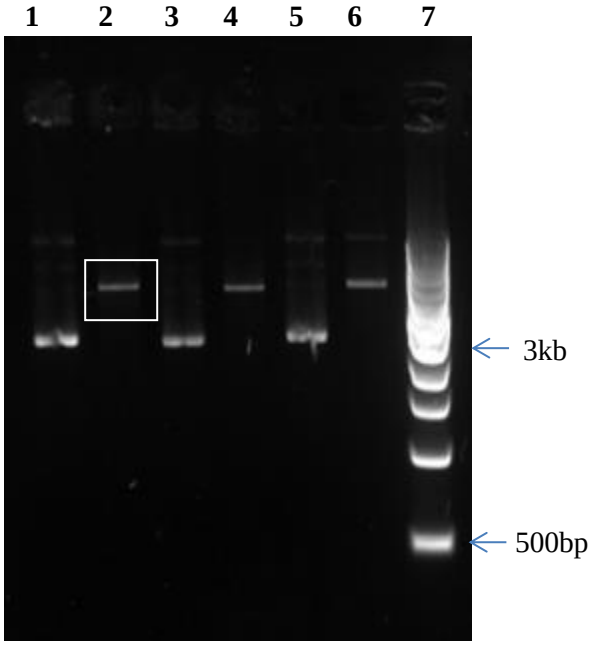

Figure 4.21: Restriction digestion of p10615TOPO electrophoresed on a 1% agarose gel at 100V for 45 minutes. Lane 1: p10615TOPO undigested; lane 2: p10615TOPO digested with *Kpn*I and lane 7: 1kb ladder. (Lane 3-6: are not relevant in this experiment and will be discussed elsewhere in the document). The white box indicates the location of the digested DNA fragment.

In order to further confirm the integration of the promoter sequence into the pCR®2.1-TOPO vector, a PCR amplification using M13 primers was next undertaken. PCR was performed employing an annealing step of 55°C for 90 seconds and an extension step of 72°C for 2 minutes (as per Material and Method, Section 2.8.3). 5µL of PCR product was analysed as before using agarose gel electrophoresis, confirming the amplification of a product at the correct approximate size of 1504bps, (SPR promoter 1004bps + 500bps from the TOPO vector) as seen in Figure 4.22.

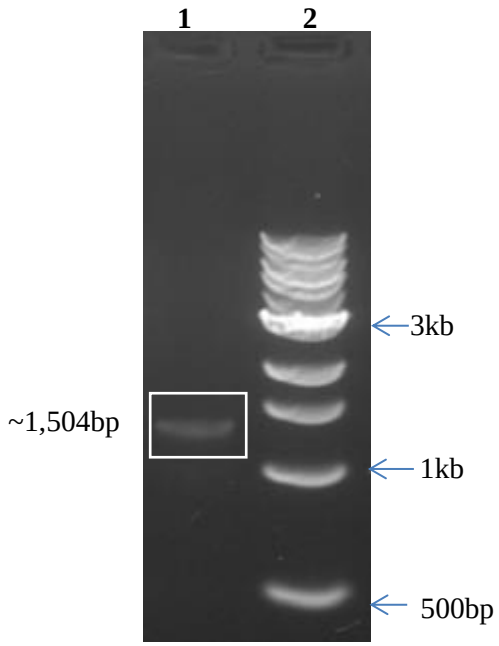

Figure 4.22: PCR using M13 primers to amplify the SPR 10615 promoter from p10615TOPO, electrophoresed on a 1% agarose gel at 100V for 45 minutes. Lane 1: p10615TOPO, lane 2: 1kb ladder. The white box indicates the location of the DNA.

Once this analysis was complete, the plasmid p10615TOPO was sequenced using forward and reverse M13 primers, and the corresponding sequences were labelled prom10615M13F and prom10615R. The original published SPR 10615 promoter sequence was obtained from Genbank (named original10615) and was aligned with the above sequences using the MUSCLE analysis tool. The prom10615M13F sequence shared 98% nucleotide identity when compared to the original SPR 10615 promoter. The prom10615R sequence was 99% identical over the length of the two fragments analysed (Table 4.3, Figure 4.23).

Table 4.3: The Percent Identity Matrix created by Clustal2.1, shows the nucleotide alignment and the similarity of sequencing result for vector p10615TOPO.

Percent Identity Matrix - created by Clustal2.1				
Sequence	**Primers**			
original10615		100.00	98.65	99.00
prom10615M13F	M13F	98.74	100.00	100.00
prom10615R	M13R	99.00	100.00	100.00

```
prom10615R          CAGGCAACCTGCATTCCACGATGAGATACGAACGGAACGGCGATACATAGTCCAGAAGTG
prom10615M13F       CAGGCAACCTGCATTCCACGATGAGATACGAACGGAACGGCGATACATAGTCCAGAAGTG
original10615       CAGGCAACCTGCATTCCACGATGAGATACGGACGGAACGGCGATACATAGTCCAGAAGTG
                    ******************************.*****************************

prom10615R          AAAGCTAATGAAAATATTCGACTGGAACTTGAGGTTAAGCGCGGGATATGGTCATTGAAT
prom10615M13F       AAAGCTAATGAAAATATTCGACTGGAACTTGAGGTTAAGCGCGGGATATGGTCATTGAAT
original10615       AAAGCTAATGAAAATATTCGACTGGAACTTGAGGTTAAGCGCGGGATATGGTCATTGAAT
                    ************************************************************

prom10615R          GGGGAGCAAACCAATTGCAGGAGATTGATATCCTGACCTTCGCCCCAATGGGCTGGTTAT
prom10615M13F       GGGGAGCAAACCAATTGCAGGAGATTGATATCCTGACCTTCGCCCCAATGGGCTGGTTAT
original10615       GGGGAGCAAACCAATTGCAGGAGATTGATATCCTGACCTTCGCCCCAATGGGCTGGTTAT
                    ************************************************************

prom10615R          GCCCAGAAAGGGATAACTACGGGCTCGGGTCGGGGGCATAGAGACCTCACAAAGGCACCG
prom10615M13F       GCCCAGAAAGGGATAACTACGGGCTCGGGTCGGGGGCATAGAGACCTCACAAAGGCACCG
original10615       GCCCAGAAAGGGATAACTACGGGCTCGGGTCGGGGGCATAGAGACCTCACAAAGGCACCG
                    ************************************************************

prom10615R          AGGAAGGTGGTAACTCCATACGACATAGGGAGTCAGCGGATGCCTAGTGACCAAGATAAG
prom10615M13F       AGGAAGGTGGTAACTCCATACGACATAGGGAGTCAGCGGATGCCTAGTGACCAAGATAAG
original10615       AGGAAGGTGGTAACTCCATACGACATAGGGAGTCAGCGGATGCCTAGTGACCAAGATAAG
                    ************************************************************

prom10615R          CATAACGGCCGTGTGGGAGTGTGTGGGGCGACAGAGTCGATAAAGGTGAAGTGGCCCTTA
prom10615M13F       CATAACGGCCGTGTGGGAGTGTGTGGGGCGACAGAGTCGATAAAGGTGAAGTGGCCCTTA
original10615       CATAACGGCCGTGTGGGAGTGTGTGGGGCGACAGAGTCGATAAAGGTGAAGTGGCCCTTA
                    ************************************************************

prom10615R          AATCGCCCTTGCCAAGAAGGCGGGTTGAGAGCTATATCACAAGATTAGACGGTCACTTGA
prom10615M13F       AATCGCCCTTGCCAAGAAGGCGGGTTGAGAGCTATATCACAAGATTAGACGGTCACTTGA
original10615       AATCGCCCTTGCCAAGAAGGCGGGTTGAGAGCTATATCACAAGATTAGACGGTCACTTGA
                    ************************************************************

prom10615R          GTCGCCCTAGTCCAAGTGTTTAGAGGTCACCCCGTTGACATGCGTTCCCGACAGCGCGAA
prom10615M13F       GTCGCCCTAGTCCAAGTGTTTAGAGGTCACCCCGTTGACATGCGTTCCCGACAGCGCGAA
original10615       GTCGCCCTAGTCCAAGTGTTTAGAGGTCACCCCGTTGACATGCGTTCCCGACAGCGCGAA
                    ************************************************************

prom10615R          CTCAACCATCATGGTCGCGTGAGCCAAAGCCCCTCGCAGTCCCGTATTCGCGTTGAATTA
prom10615M13F       CTCAACCATCATGGTCGCGTGAGCCAAAGCCCCTCGCAGTCCCGTATTCGCGTTGAATTA
original10615       CTCAACCATCATGGTCGCGTGAGCCAAAGCCCCTCGCAGTCCCGTATTCGCGTTGAATTA
                    ************************************************************

prom10615R          GTCCATTGCCGGGGGCATAAACCTGAACGAGAACCATGTGCTTCGTCGATCAGGGAGACC
prom10615M13F       GTCCATTGCCGGGGGCATAAACCTGAACGAGAACCATGTGCTTCGTCGATCAGGGAGACC
original10615       GTCCATTGCCGGGGGCATAAACCTGAACGAGAACCATGTGCTTCGTCGATCAGGGAGACC
                    ************************************************************

prom10615R          AAGAGTGCCTATCCATCCACGCGACATATGTTTTCATAACATAGGGT
prom10615M13F       AAGAGTGCCTATCCATCCACGCGACATATGTTTTCATAACATAGGGT
original10615       AAGAGTGCCTATCCATCCACGCGACATATGTTTTCATAACATAGGGT
                    *********************************************
```

Figure 4.23: A MUSCLE nucleotide alignment of p10615TOPO (sequenced using M13 forward and reverse primers) and the original SPR 10615 promoter sequence. The prom10615M13F sequence shared 98% nucleotide identity when compared to the original SPR

10615 promoter sequence. The prom10615R sequence shares 99% identity with the original 10615 promoter sequence.

This analysis using restriction digestion, PCR and sequencing verified the successful integration of the SPR promoter fragment into TOPO vector, confirming the integrity of p10615TOPO.

The next step in the construction of peGFPi10615 would include the preparation of the peGFPi004 vector backbone (which is outlined in Section 3.6; using restriction enzymes *Sac*II and *Hind*III to remove the *gpd*II promoter). This would then be followed by ligation of the SPR 10615 promoter into the fungal expression plasmid backbone, using the Thermo Scientific InsTAclone PCR Cloning Kit. The construct would then be transformed into *E.coli* JM109 (as per Materials and Methods, Section 2.5). Following this, a miniprep would be employed to isolate the recombinant plasmid DNA from *E.coli* cells and molecular validation of the pAS04562 fungal expression plasmid using restriction digests, PCR and sequencing techniques would be carried out to verify the identity of the recombinant vector. This plasmid would then be ready for transformation studies in *C. cinerea* facilitating the investigation of the SPR 04562 in this fungus. Following transformation, the transformants could be fruited and analysed at different stages of development for the expression of eGFP.

Chapter 5 :Design and development *C. cinerea* plasmids to facilitate the analysis of fruiting body enzymes

5.1 Introduction

Fruiting body formation in basidiomycetes is a complex developmental process. The research presented here reports preliminary results obtained for the construction of a set of promoter-eGFP fusion plasmids designed to facilitate the study of selected fruiting body enzymes. The enzymes selected for study were the *C. cinerea* ornithine aminotransferase (*OAT*), galectin I (*CGL*1), galectin II (*CGL*2) and *A. bisporus lectin* (*ABL*).

The first step in this process was to design each plasmid construct. Figure 5.1-5.3 represents schematic overviews of this design. The plasmid peGFPi004 (as described in Section 3.5.2) was again employed here for all four fruiting body promoter constructs (Figure 5.1-5.3 A). Several sets of restriction enzymes (depending on the promoter sequence) were identified to remove the 300bps *A. bisporus gpd*II promoter leaving the rest of the plasmid intact (Figure 5.1-5.3 B). The promoter sequence was then PCR amplified to include the appropriate restriction sites for directional cloning: The *oat* promoter was engineered to include restriction sites for *Sac*II-*Hind*III (Figure 5.1 C); while the *cgl*1 and *abl* promoters included restriction sites for *Sac*I-*Cla*I (Figure 5.2 C). The *cgl*2 promoter was designed to include restriction sites for *Sac*II-*Cla*I (Figure 5.3 C). To complete each construct, the *gpd*II promoter in peGFPi004 would next be replaced by either the *oat, cgl*1, *cgl*2 or *abl* promoters, forming the promoter-reporter plasmids peGFPi*OAT*, peGFPi*CGL1*, peGFPi*CGL2* and peGFPi*ABL* (Figure 5.1- 5.3 D).

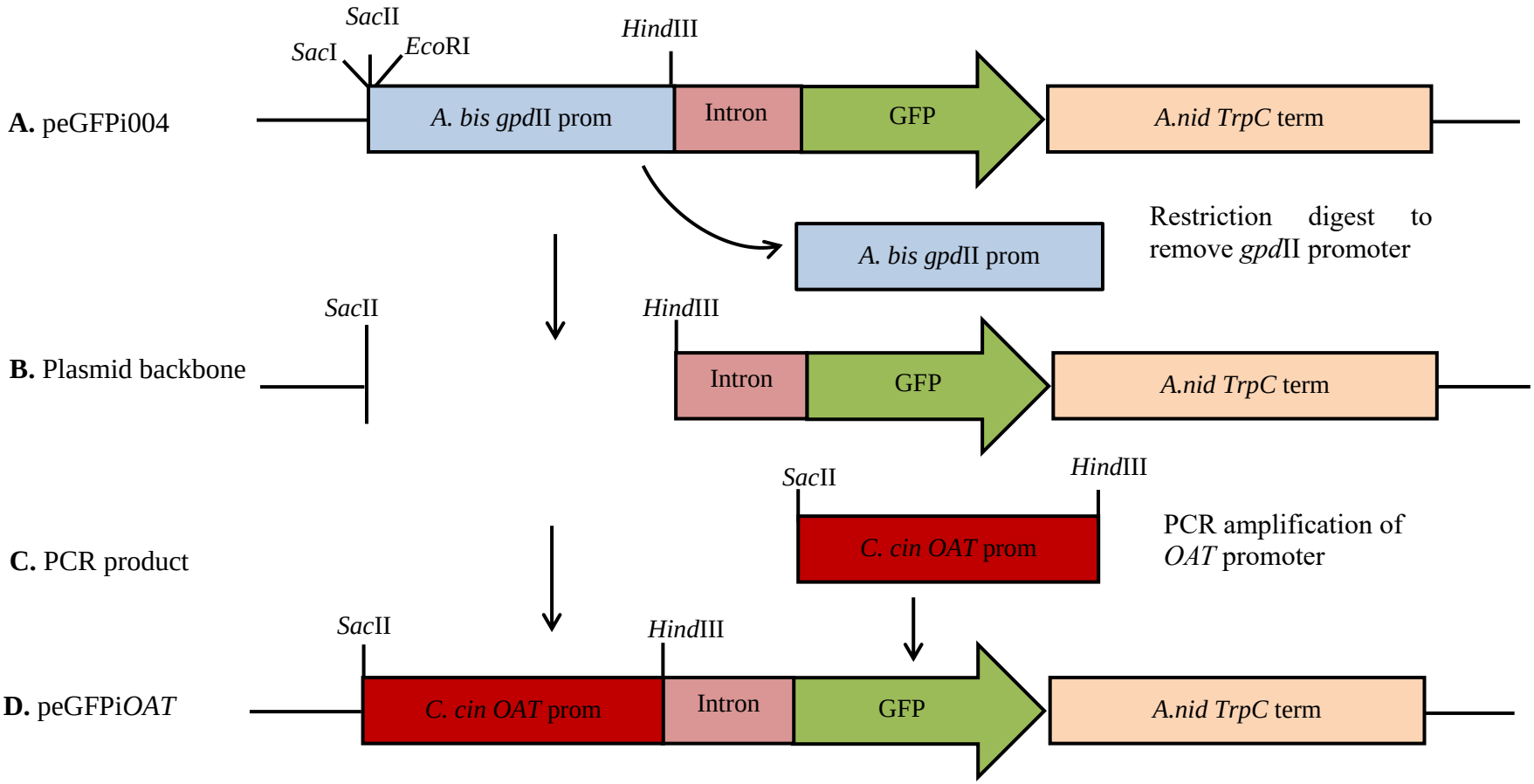

Figure 5.1: A schematic representation of the design of recombinant vector peGFPi*OAT*. A: The intact peGFPi004 plasmid containing the original *A. bisporus gpd*II promoter (*A.bis gpd*II prom), an intron, eGFP and *A.nidulans TrpC* terminator (*A.nid TrpC* term). B: The peGFPi004 plasmid digested with restriction enzymes *Sac*II and *Hind*III to remove the *gpd*II promoter leaving the plasmid backbone. C: The amplified *C. cinerea OAT* promoter flanked with *Sac*II and *Hind*III restriction sites. D: The intact peGFPi*OAT* contains the *C. cinerea OAT* promoter ligated to the peGFPi004 backbone.

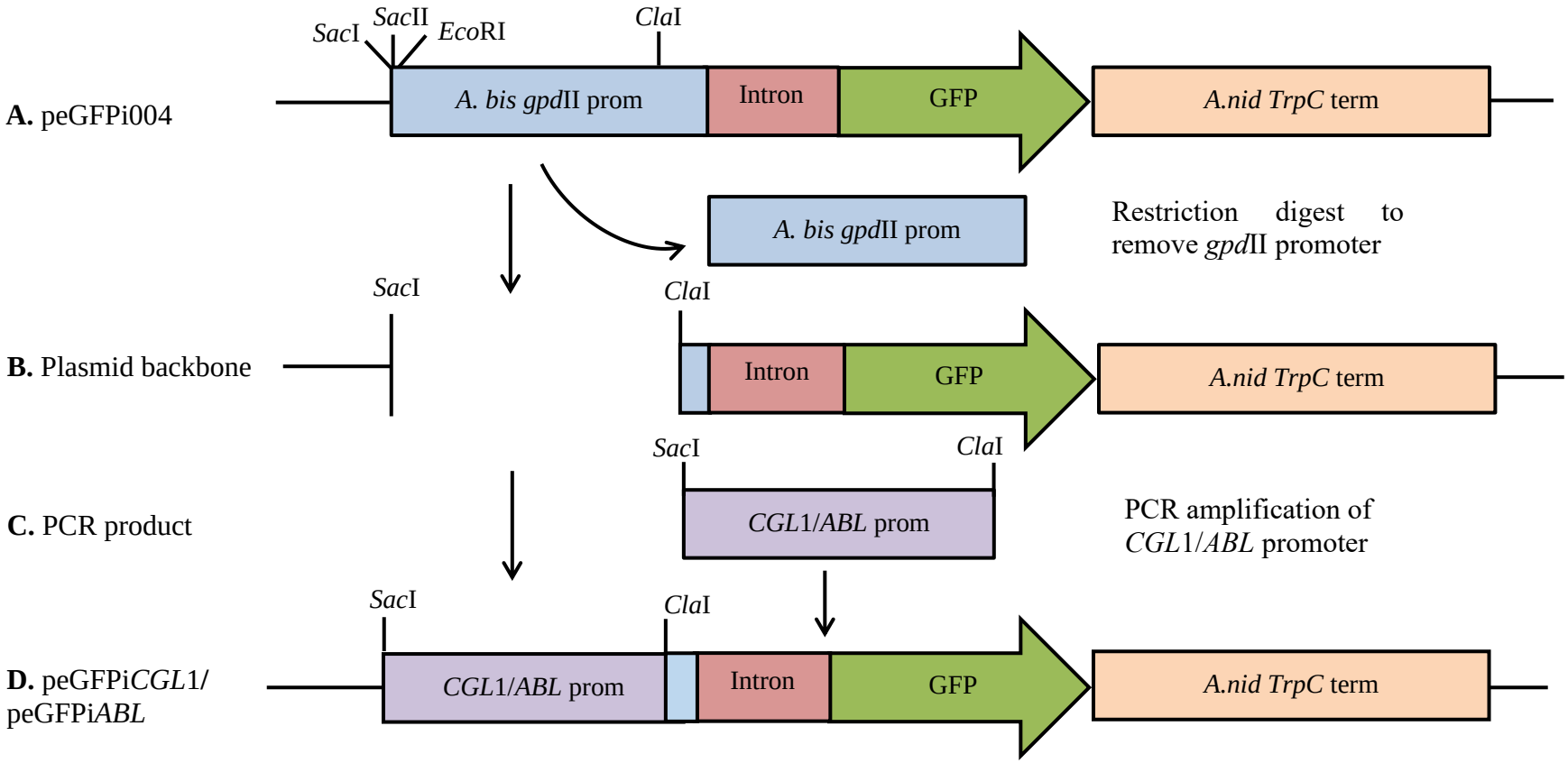

Figure 5.2: A schematic representation of the design of recombinant vectors peGFPi*CGL*1 and peGFPi*ABL*. A: The intact peGFPi004 plasmid containing the original *A. bisporus gpd*II promoter (*A.bis gpd*II prom), an intron, eGFP and *A.nidulans TrpC* terminator (*A.nid TrpC* term). B: The plasmid digested with restriction enzymes *Sac*I and *Cla*I to remove 211bps of the *gpd*II promoter leaving the plasmid backbone. C: The amplified promoter flanked with *Sac*I and *Cla*I restriction sites. D: The intact peGFPi*CGL*1 and peGFPi*ABL* contains the *C. cinerea CGL*1 or *A. bisporus ABL* promoter ligated to the peGFPi004 backbone.

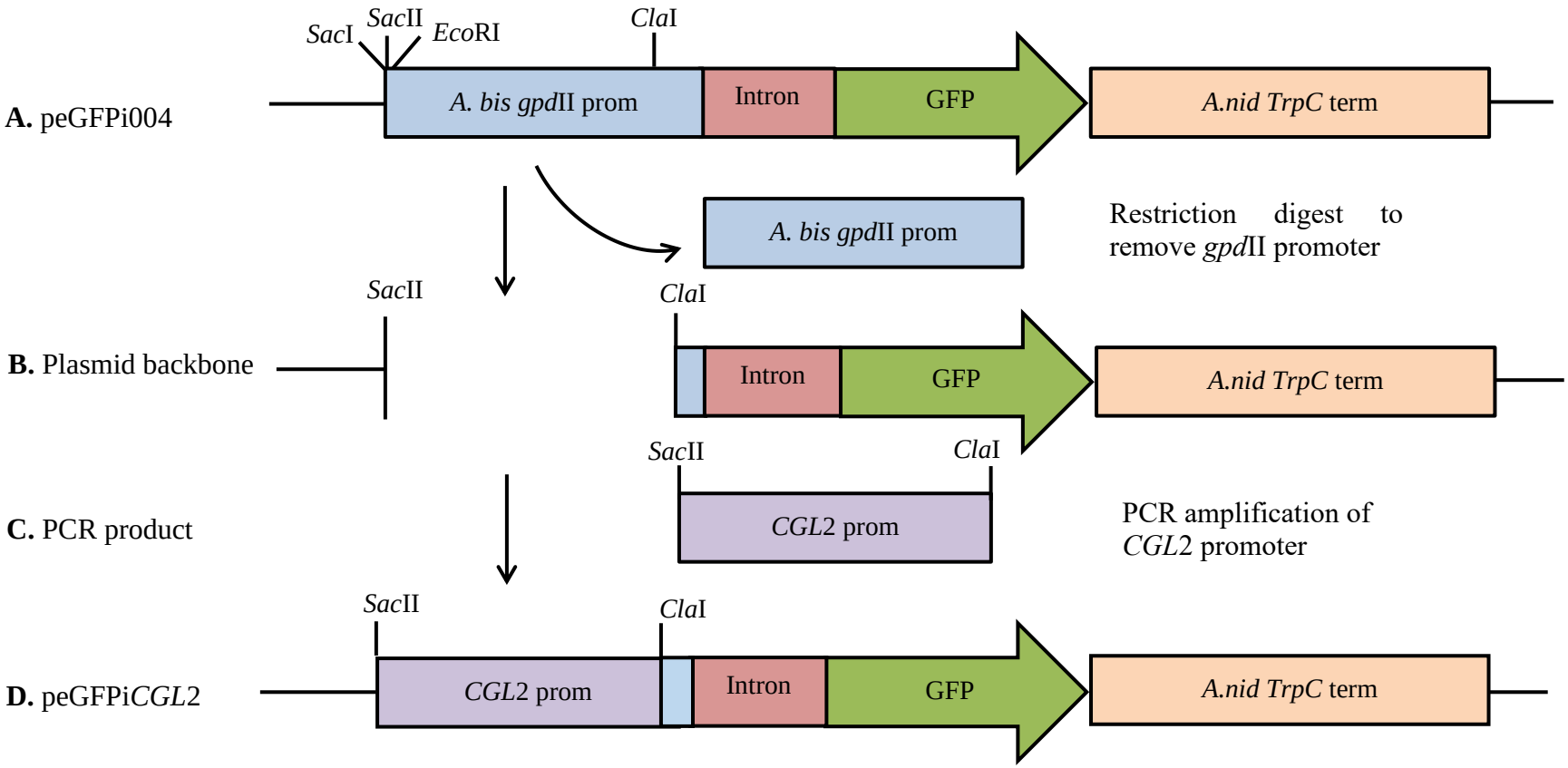

Figure 5.3: A schematic representation of the design of recombinant vector peGFPi*CGL2*. A: The intact peGFPi004 plasmid containing the original *A. bisporus gpd*II promoter (*A.bis gpd*II prom), an intron, eGFP and *A.nidulans TrpC* terminator (*A.nid TrpC* term). B: The plasmid digested with restriction enzymes *Sac*II and *Cla*I to remove 203bps of the *gpd*II promoter leaving the plasmid backbone. C: The amplified promoter flanked with *Sac*I and *Cla*I restriction sites. D: The intact peGFPi*CGL2* contains the *C. cinerea CGL2* promoter ligated to the peGFPi004 backbone.

5.2 Preparation of promoter fragment

5.2.1 Sequence retrieval and analysis

The *C. cinerea oat*, *cgl*1, *cgl*2 and the *A. bisporus abl* gene sequences were first identified using the Genbank database, and a 1kb stretch of DNA representing a putative promoter sequence upstream of the ATG start site was selected for each. These putative promoters were analysed for the presence of CAAT regions and TATA boxes; the motifs identified are highlighted in bold and are underlined in Figures 5.4- 5.7 below.

GGAGTCGTGTCGGGACAACGGCCAACGTTTCCTTTGTGTTCGCGGGAGTGGAGTCGGTGTGT
TGTATCCCGAGTCATCCCTATCGCACTCCGTCCCCTGTTATGGGTACCTCGCGCTTGGCAAAG
GGCCCTCGCTATTCCAGTCCTTGGTCACACGACTTCGCCTCTCCATGAAGTATGCACCGACTT
CCCAGCTCCCTTCCTCCTCCAAGAATAGAACACCCTCCTTGTCAAGTCCCCGCGTCTTCGGCC
CTCCGGCTCGCTCAGCCCGTCCCCTCCCCTCCCTCCGGAAACGCCATTACACCATAGATG**CA**
ATCCATGTCACTCCGTCGTCGCACGAGCCATAGTCCATGCTGTACCCTTCGCCTCCCCCTATC
TCCAAAGTCAGACAACCTTCCCCACCTTCAGTACTGTGTGTGTCTGCACTGGAAAGAGCCAC
CGTCGACGCCACTGCTGCCCCCCTCCCTGCACCAGACTTCCAGAAGTCGACGGCAGCCTGCG
TTTGAGCGATCACACCTGCCACCAAGTACACCCCGAGGAGAAGGATGAGGAGGG**CAAT**AGC
AAGGACCGACTCGCCCATACCGCGTTCGGCTCAAGGGTTTCTTATGTTGTCGTCGAGGCCTA
CAGGCGTCGGCCTTTGGCTAGTCGACCATTTCCTCTTGTTCTATATACGAGGATAGACAGGG
GCTTCAGCTAGCAGGACAGTTCATCATCGCTCGATAGCCAGTGGCGGACATAATCGAGGTGA
CAGGGGGAAGAGGGATATCAACCAGAGATAGGGATTACTTCCAGTCGAAAGAGGCTTTTCA
AACCCTCGCAGCAGCTGCGTTGGTTTGGCTGTTCCCATTTTGATCCTTATTGCCTCTCGGCTC
CTTTTGAGGTTCACGAGGGCTCTGGCTTTCGCAGGAAGAGAGGTTTATCATCTGAGGCTTTTT
TGCCTTTCCGCTGACGGGAGGTGGATGGTTGGGCTAAATATGTAACTGGACCTCGTACAGGC
ATTGGTG

Figure 5.4: *C. cinerea* genomic DNA (1kb) upstream from the *oat* gene. CAAT regions are highlighted in bold and underlined. The forward primer is highlighted in red and the reverse primer is highlighted in blue.

TACACGTTTCAGCTTTAGCTACCGTTCTTCCTTCTTGCTCGATAAGGTTGCGGTTTGTCTGTTG
TTCATCGTGGTTGTTTTGCAGAGGAAGACGGGTTGGGTATTCAAGAGTAAGACTTGTGTTTT
CATAACCCATGCGGGCCGCAGCCAAGAAAGGGATTTGCCGAGAGGCTTGTTGGTCAAGCAG
TGCCAAACCTTGGACGTATTTTGCTCTCGACCACCATTATCTTTGGCGCTGAAGGCTTTGCTT
ACTGTTACTGGATGCGAAGGGGCCTAAGGCTGGATCACTGGAAACTTCTGTGGCTGTCTCTC
CTTGTACTGTGGCACTGTGAAGACTCTCGCCAGCCCCGCGTTCGGGTGTCAGTCGGGCGTCA
GTGTGTCTCCGCTCCAGGTCGACGCCTAGCTAGAATTGTGCCACGACTGTAACTCTGGATCT
CATCAGTGGAAGAACGAA**CAAT**ATCCCGGAAGCTTCAGTCTGGAAGGATTGGAAATTGGGA
GATCTATTCTTAGGAAAATGAACCGGCGTTTGCAAACTTCTTCAAACCTATGAATGGTTCTCT
TGAGGGCCTGATGGTCTATTCTTAGAGTGTGTGATTGGGATACTGGAAACCTCGAGCCTCGT
ATTCGCCTCGCGATGGACTTTGAACGGCATTGTGGCGATTTGGGACC**CAAT**AGTCATCCCGT
TGATGCTATGATTCGGCTCTTCCGACTTCATCTCCGTCATTGGGGATGCCGATCTATCAAGCT
AACTCTGGTCATATCTGGCCCAAG**CAAT**ATTCAGCGATCTCCGGTGGTCAGGTGGCTCAGGG
ATG**CAAT**GAAATTGCGAATTTTCTGTGTTTTCTGGTG**TATAAA**AGGCTCCCGGAGATTCGGG
GTCGTGAACTCTCCAGAAC**CAAT**TCCTCAGTTCTACAAACCCAAGCCAAGTAAGTACTCGGC
CTCCCTCTCACTTTCCTGTATCGCTAACCACCCTACCGGTAGTTCAACATCATGCTCTACC

Figure 5.5: *C. cinerea* genomic DNA (1kb) upstream from the *cgl*1 gene. A TATA box and CAAT regions are highlighted in bold and underlined. The forward primer is highlighted in red and the reverse primer is highlighted in blue.

GCTGCTATAGCGAGATTTGTAGTACAAGACTAGAGTAGAGAGTGGGATACACATTGCGCTCT
TTCTTTACTTCCGAGAAGCATCCGATGGGGCTGAGGAGCTTGACGCTTGACCTATTGTGCAG
GCAACCAAGGAAGGGATTTGCGGAAAGAGAGTCAGCTTGTCGGGGAGGGGGTCCTCGCGCC
CGTTATCATCTTCGGCGGTGAAGCGGCCCAAATCTCTGTCCGCCTTACTCGGTACGCGAAGG
GGCCCAAGCCTGACG**CAAT**GAGGAAATCCATGGCTGTCCCTCCTTGTACTGTGGCGCTCTGA
AGATTCTTGTTATGGCGTTTGGATTCGGGCGTCACAGTCTCCGGACCTCTCTTCTTCAGTCGA
CACCTTTCCAGGACTGCGTCACGACTATAACACCGGACTCCCTCATCCCTTGGATGAATGCG
AACGACGTCTTGGAAGTTTCAAGTGTGGAAGGATTCTCAGGAAAATGAACCGGCGTTTGCA
AAAGTGTCGAACCTCTAAATGGCCCTCTTGGAGACCTGTGACCAAGGGTTCTCTATTCTTAG
AGCTTATGATTGGCATAGTGGAGGTCTCGAAGTTTCTATTCGCCTCG**CAAT**GGACTTTGAGC
GGCATTCTGTGCGATTTGAGCCCAGGATTACCTTATTTGTTCCGGGTATCAGGTCTTCCGACT
TCATCTCCGTCATTGGGGATGACGATCTGAGTTTAACTCTAGTGGTCTGACAGTTATGGACA
GTGGAGAAGCACCCCCAGGAGTATGCTGTGTCCGGGGAGAAGTGATATCCGGTGGTCAGCT
GGCTCTGGGATG**CAAT**GAAATTC**CAAT**TTTCTTGTCGATTTCCTTGCGCTTTTTGACG**TATAA**
AAGGGTGGATTTGAGGTCGTGAACTCTCCAGAACCACTTCCTCAGTTCTACAGCTCAAAGCC
AAGTAGGCATTCGACTTTCCTCTCATTATCCTACAGTGCTAACTACTGTATCACCAGTCTAAC
ATCATGCTCTACCAC

Figure 5.6: *C. cinerea* genomic DNA (1kb) upstream from the *cgl*2 gene. A TATA box and several CAAT regions are highlighted in bold and underlined. The forward primer is highlighted in red and the reverse primer is highlighted in blue.

CTCAACGGATTAACATGGATTGGTGTACTTAGTGAGGCTAGCTTGATGCAAGCAGGTACAAG
TTCCGCCCGGTCATATGTACTGAAGTCATGAATGGAAGCAGGAGCAGAGAAGGTGGCGGAC
GGTCGGGTGATCGAGTTCCCTAGTAGTGGTTTTAATAACACTGGCAAAAACGCGGACTTTAG
TAATTATCCCGGCATATTTTGAGGTCAGATTTGTATTTTAGAAGATTCGTACAAAGCACTGA
GAGCTACCATTTTCTT**CAAT**GTTGTTCATCATACTTTTCCTGAGCCCTTGACCGGTTTTGCCG
ATCAACGAATACAGAAGTACTATCTACTATACCCTTGATTTGCGATTGAAGTAGATAAGATT
AGTAGTAGGGTGTTCGGCTTTCATAGCTTAATGAGTCCGTGACTGGTCTCCATCACTTAAACT
TTGGTCTGCCACGGTTTTAATACCAGCCTGGCACCTTGACAGAATCTAGAATACGATGATCG
CACCCTCGATTTCCATGAGCTAAAAATATCTGGTCCTGGCACTAGTCACAGTGGTCACCGAC
TCGAAAATTTCCCCGTCCATAGTTGACTTTTTTCAACCACAAGTACT**CAAT**CAAATCTACTAG
CTTGTAGAAACATTGTAGCGCTTGATGCTGAATAAATTCAATG**CAAT**CATATAGCATACTAC
AGATCAACAGGTGCTCAAGCTCAAGCGGGACACTCGATCTATAATTAGCACATACCCCAAA
GTGCGGGGGTTAATGGCGCTGGACACTTCGTCTTCATAGATATGGCCAAGCATCCTTCATGC
CATGCTCGAATTATGCTACTTGTAGATAAGTTTATCTACACCCGCACGGACCAACTAACGTA
GATATATAACGATGTC**CAAT**ACTTGTGAATACCTCAGTGGATTATATCCTCTTGACTGCGATT
CACCTGCTCCACTAACACAGTAACCATCTTTCTCTACCACTGTTTCAACTAACTCATAACGTT
GAGCAG

Figure 5.7: *A. bisporus* genomic DNA (1kb) upstream from the *abl* gene. CAAT regions are highlighted in bold and underlined. The forward primer is highlighted in red and the reverse primer is highlighted in blue.

5.2.2 PCR amplification of the promoter sequences

Primers for the PCR amplification of the putative promoter regions were designed from the sequences shown in Figures 5.4-5.7. Restriction analysis of the cloning vector plasmid peGFPi004 had been undertaken previously during the preparation of the SPR promoter constructs and was again employed here (Section 3.5.2). This analysis revealed

that the plasmid contained a *Sac*II restriction site upstream of the *gpd*II promoter and *Hind*III restriction site at the 3' end of the promoter. Therefore, restriction digestion of this plasmid with *Sac*II-*Hind*III would result in the excision of the *gpd*II promoter, leaving the intron-eGFP-terminator cassette intact. Analysis of the *oat* promoter sequence was performed and it was determined that a *Sac*II site should be included on the forward primer and a *Hind*III site should be incorporated into the reverse primer (see Materials and Methods 2.8.1, Table 2.4) to allow directional cloning.

PCR primers for the amplification of the putative *cgl*1, *cgl*2 and *abl* promoter region were designed from the sequences shown in Figures 5.5, 5.6 and 5.7. Again, the restriction analysis of the cloning vector, peGFPi004 was used here, revealing that the plasmid harbours restriction sites for *Sac*I and *Sac*II upstream of the *gpd*II promoter and *Cla*I and *Hind*III restriction sites at the 3' end. Examination of the promoter sequences determined that *Hind*III cuts within the *cgl*1, *cgl*2 and *abl* promoters, while *Cla*I does not. Therefore, restriction digestion of the peGFPi004 plasmid with either *Sac*I-*Cla*I (in the case of *cgl*1 and *abl*) or *Sac*II-*Cla*I (in the case of *cgl*2) would result in the excision of 203bps or 211bps of the *gpd*II promoter, leaving the intron-eGFP-terminator cassette intact.

These primers were then used in a PCR with *C. cinerea* gDNA (or *A. bisporus* gDNA in the case of *abl*). Amplification of the *oat* promoter required an annealing step at 68°C for 30 seconds and an extension step at 72°C for 1 minute whereas an annealing step at 70°C for 90 seconds and an extension step at 72°C for 2 minutes was employed in the case of *cgl*1, *cgl*2 and *abl* (as per Materials and Methods, Section 2.8.3). Following PCR, 5µL of the products were analysed by agarose gel electrophoresis to check for migration of the fragments at the expected size (*oat* 1003bps; *cgl*1 1006bps; *cgl*2 1008bps and *abl* 1001bps). The results of this analysis can be seen in Figures 5.8 A, B, C and D, confirming that the putative promoters were successfully amplified as indicated by the bands at the expected sizes.

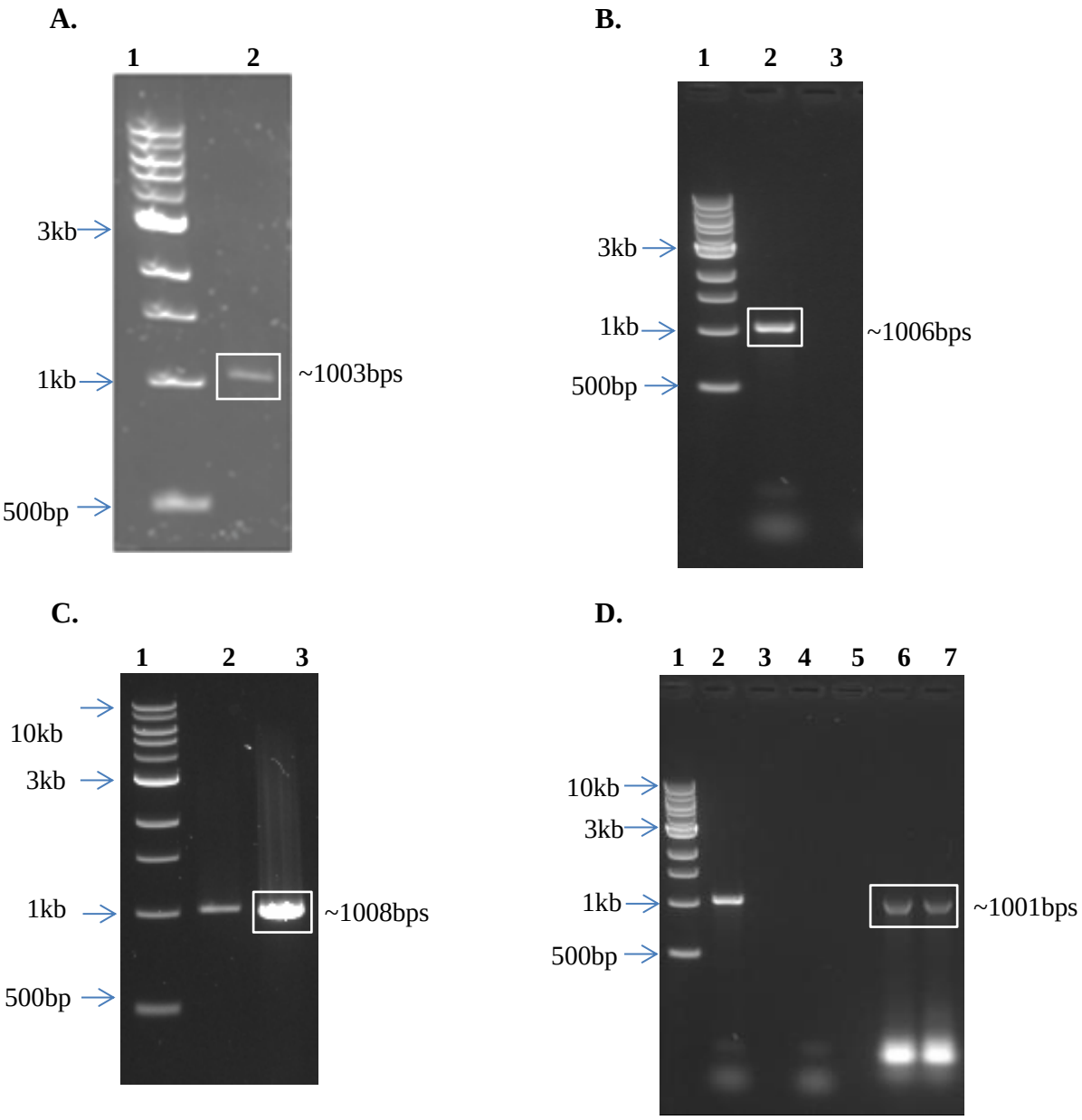

Figure 5.8: PCR amplification of the fruiting body promoter sequences. A: The putative *oat* promoter electrophoresed on a 1% agarose gel at 100V for 45 minutes. Lane 1: 1 kb ladder and lane 2: *oat* promoter DNA. B: The putative *cgl*1 promoter DNA electrophoresed on a 1% agarose gel at 120V for 45 minutes. Lane 1: 1kb NEB ladder, lane 2: *cgl*1 promoter, lane 3 negative control. C: The *cgl*2 promoter DNA electrophoresed on a 1% agarose gel at 100V for 1 hour. Lane 1: 1kb ladder and lane 3: *cgl*2 promoter. (Lane 2: is not relevant here and is discussed elsewhere in the document). D: The *abl* promoter DNA electrophoresed on a 1% agarose gel at 100V for 1 hour. Lane 1: 1kb ladder; lanes 3 and 5: blank; lane 4: negative control; and lanes 6-7: *abl* promoter. (Lane 2 is not relevant here and is discussed elsewhere in the document). The white boxes indicate the location of the DNA fragments.

Following confirmation that the PCR products were at the expected sizes of approximately 1003bps (*oat*), 1006bps (*cgl*1), 1008bps *(cgl*2) and 1001bps (*abl*), a purification step was next performed. The individual promoter fragments were excised from the gel and purified separately using the Omega bio-tek gel extraction kit (as per Materials and Methods, Section 2.6.2). The purified promoters were then electrophoresed on a 1% agarose gel, as shown in Figure 5.9 below, confirming the successful purification of the various fragments (results for *cgl*1 promoter are not shown).

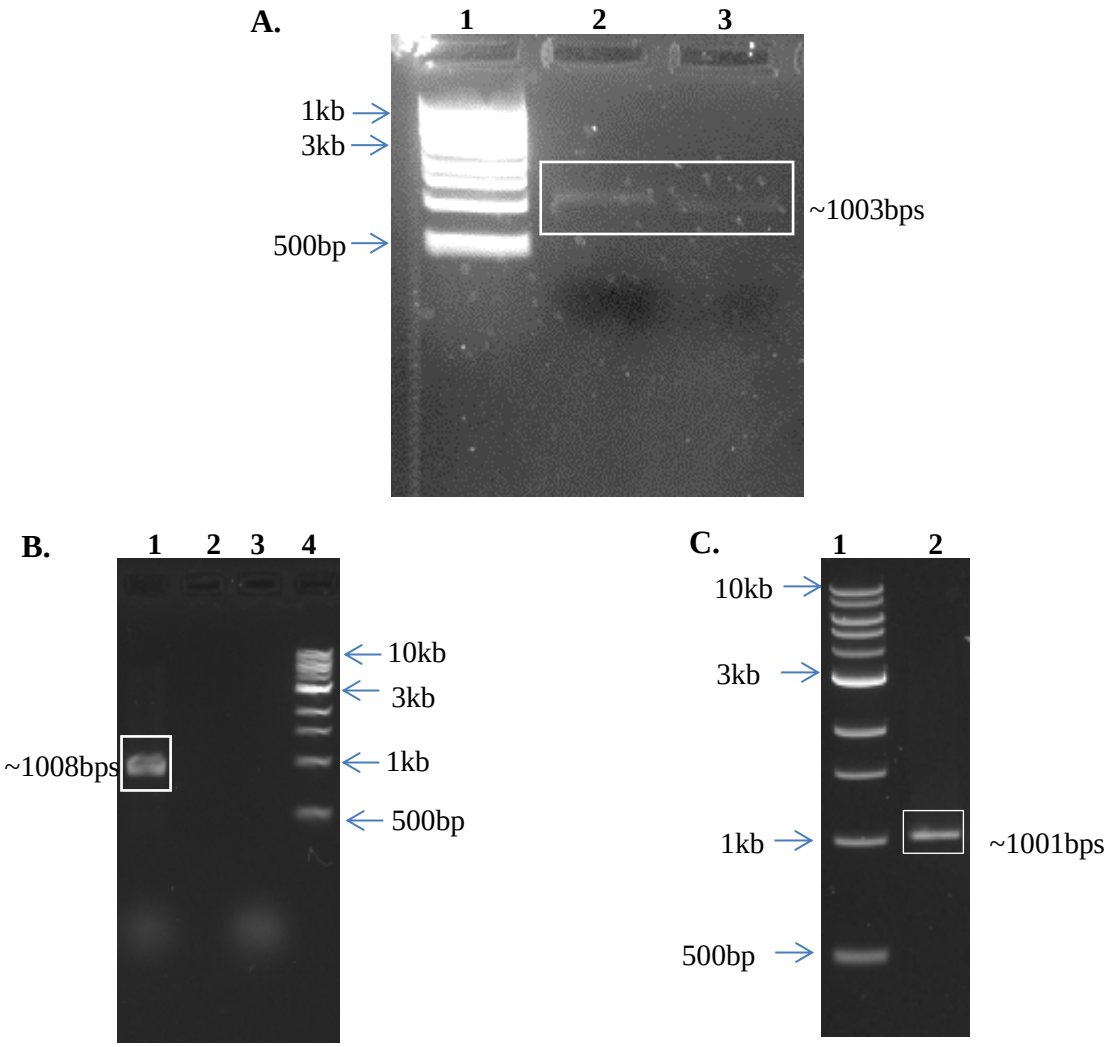

Figure 5.9: Analysis of the purified fruiting body gene promoter fragments. A: Purified *oat* promoter electrophoresed on a 1% agarose gel at 100V for 30 minutes. Lanes 1: 1kb NEB ladder and lane 2-3: purified *oat* promoter. B: Purified *cgl*2 promoter electrophoresed on a 1% agarose gel at 100V for 90 minutes. Lane 1: *cgl*2 promoter, lane 2: blank and lane 4: 1kb NEB ladder. (Lane 3: is not relevant here and is discussed elsewhere in the document). C: Purified *abl* promoter electrophoresed on a 1% agarose gel at 100V for 1 hour. Lane 1: 1kb NEB ladder and lane 2: purified *abl* promoter. The white boxes indicate the location of the promoter DNA.

5.2.3 Cloning of fruiting body promoters into pTZ57R/T

These purified PCR products were next cloned into pTZ57R/T (Materials and Methods, Section 2.3) to produce the plasmids pTZ*OAT*, pTZ*CGL*1, pTZ*CGL*2 and pTZ*ABL*. The pTZ57R/T is a commercial vector that is designed for the cloning of PCR products. Each DNA fragment was cloned into pTZ57R/T following the manufacturer's instructions and these plasmids were subsequently transformed into *E.coli* JM109 (as per Material and Methods, Section 2.5). Single colonies were used to inoculate cultures from which DNA mini preparations were then performed.

Initial analysis of the pTZ*OAT* and pTZ*CGL*1 involved restriction digestion of the plasmids using *Sac*I and *Sma*I (in the case of pTZ*OAT*) and *Kpn*I (in the case of pTZ*CGL*I). 3µL of digested plasmid pTZ*OAT* was analysed by agarose gel electrophoresis, as shown in Figure 5.10 A. The pTZ*OAT* was estimated at a size of 3889bps (pTZ57R/T 2886bps + 1003bps *oat* promoter). The double digest for pTZ*OAT* produced two separate DNA fragments at approximately 3kb and 889bps. The digestion of the pTZ*OAT* was successful as indicated by the bands at the expected sizes as seen in lane 1-6.

The digestion of pTZ*CGL*1 resulted in a single band as seen in lane 1-2; resolving at approximately 4.5kb (Figure 5.10 B). However, the predicted size of pTZ*CGL*1 was 3892bp (pTZ57R/T 2886bps + *cgl*1 promoter 1006bps). It is possible that the banding patterns observed here have been affected by their interaction with gel red during migration, again here as in Section 3.5.3. In order to address this concern, additional analysis of all of pTZ*CGL*1 was perfomed using PCR and sequencing.

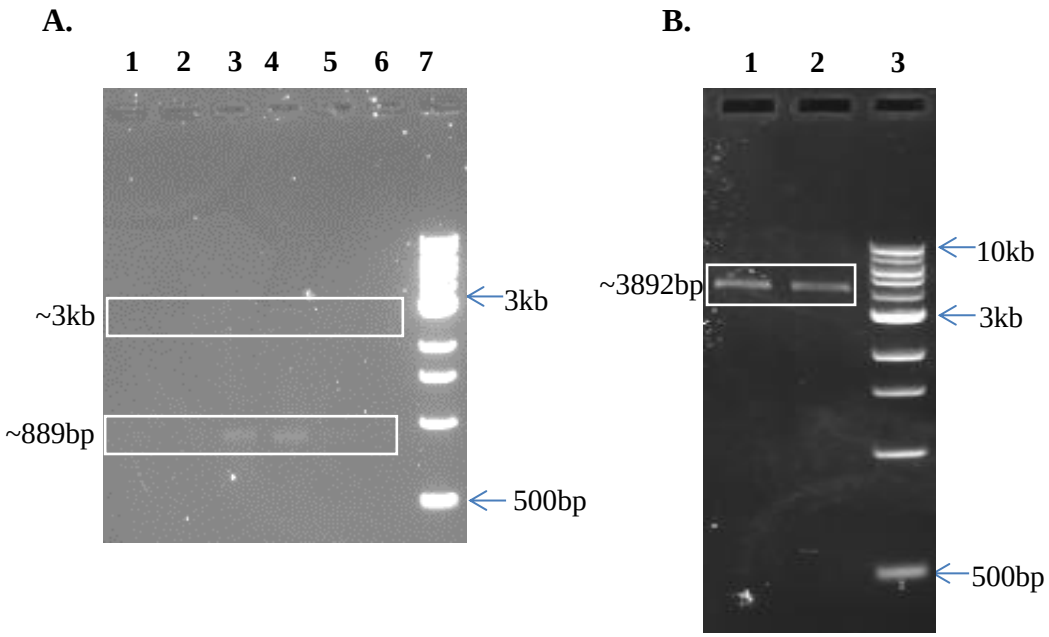

Figure 5.10: Restriction digestion of pTZ*OAT* and pTZ*CGL*1. A: pTZ*OAT* was electrophoresed on a 1% agarose gel at 120V for 45 minutes. Lane 1-6: pTZ*OAT* digested with *Sac*I and *Sma*I and lane 7: 1kb ladder. B: Restriction digestion of pTZ*CGL*1 electrophoresed on 1% agarose gel at 120V for 45 minutes. Lane 1 and 2: pTZ*CGL*1 digested with *Kpn*I and lane 3: 1kb ladder. The white boxes indicate the location of the digested DNA fragments.

PCR was performed on all four recombinant constructs using M13 primers. An annealing step of 61°C for 90 seconds and an extension step of 72°C for 2 minutes was employed in the amplification of pTZ*OAT*, while an annealing step at 55°C for 90 seconds and an extension step 72°C for 2 minutes was used in the amplification of pTZ*CGL*1, pTZ*CGL*2 and pTZ*ABL* (as per Material and Method, Section 2.8.3). Subsequently, 5µL of PCR product was analysed as before using agarose gel electrophoresis to check migration of the products at the estimated sizes. The PCR product pTZ*OAT* was estimated at 1141bps which contains the *oat* promoter at 1003bps and 138bps of the pTZ57R/T vector (Figure 5.11 A). The pTZ*CGL*1 PCR product was estimated at 1144bps which included the *cgl*1 promoter of 1006bps and 138bps of the pTZ57R/T vector as seen in Figure 5.11 B. The pTZ*CGL*2 PCR product was estimated at 1146bps which comprised of the *cgl*2 promoter at 1008bps and 138bps of the pTZ57R/T vector (Figure 5.11 C). The PCR product pTZ*ABL* was estimated at 1139bps which is the amplification of the *abl* promoter of 1001bps and 138bps of the pTZ57R/T vector as seen in Figure 5.11 D. These PCRs

suggested the successful integration of the fruiting body promoters into the pTZ57R/T vector as indicated by the individual bands at the approximate predicted sizes as seen in the Figure 5.11 below.

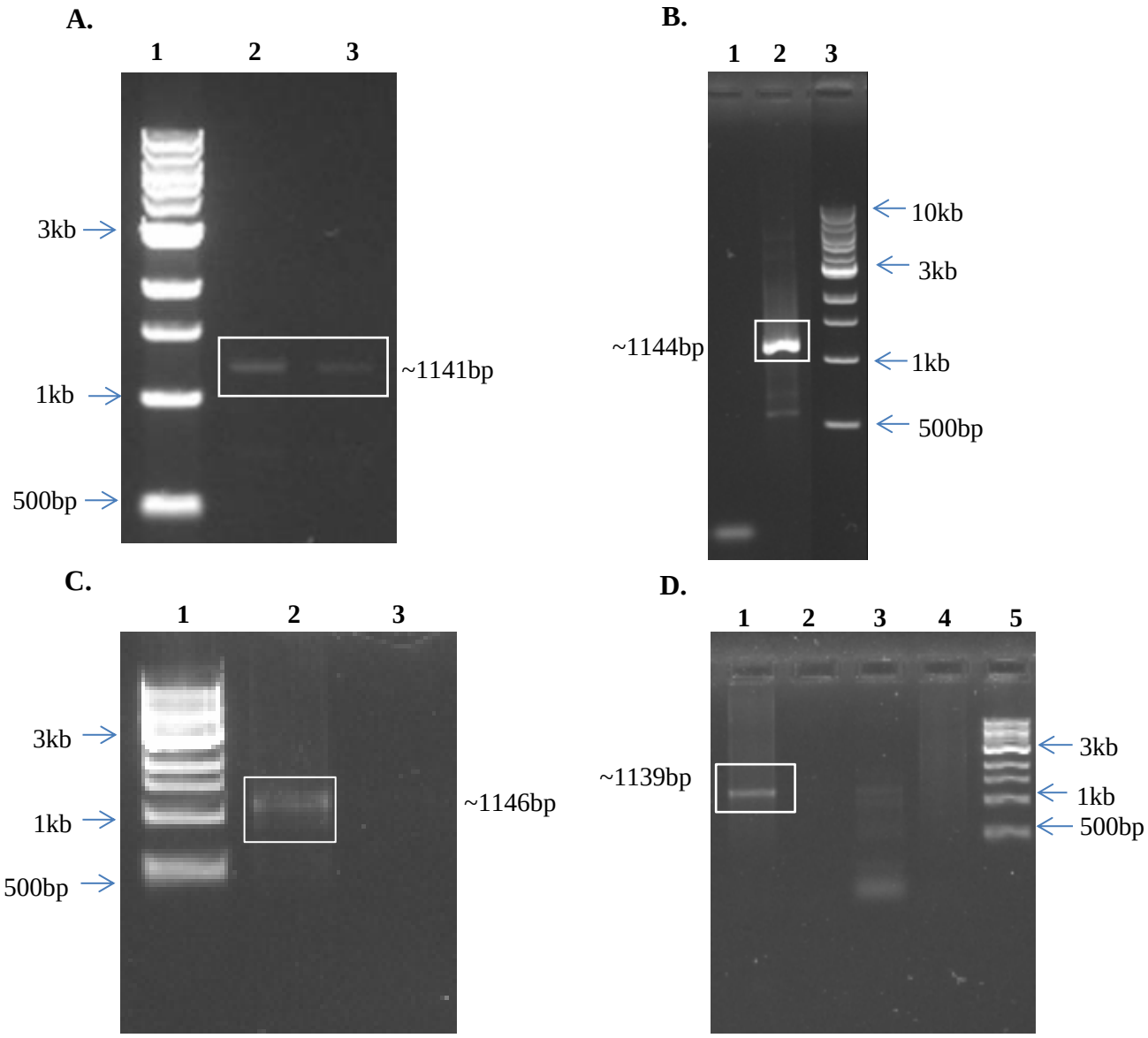

Figure 5.11: PCR analysis on fruiting body promoters in pTZ57R/T using M13 primers. All samples were electrophoresed on a 1% agarose gel at 100V for 1 hour. A: The *oat* promoter amplified from pTZ*OAT*. Lane 1: 1kb ladder, lane 2 and 3: PCR amplified *oat* promoter. B: The *cgl*1 promoter amplified from pTZ*CGL*1. Lane 1: negative control; lane 2: PCR amplified *cgl*1 promoter and lane 3: 1kb ladder. C: The *cgl*2 promoter amplified from pTZ*CGL*2. Lane 1: 1kb ladder, lane 2: *cgl*2 promoter and lane 3: negative control. D: The *abl* promoter amplified from pTZ*ABL*. Lane 1: PCR amplified *abl* promoter; lane 2: blank; lane 3: negative control and lane 5: 1kb ladder (Lane 4: is not relevant here and is discussed elsewhere in the document). The white boxes indicate the location of the DNA fragments.

5.2.4 Sequence analysis of fruiting body plasmids

Sequence analysis was performed on pTZ*OAT* using the primers M13 forward and M13 reverse, pTZ*CGL*1 using the primers *cgl*1 forward and M13 forward, pTZ*CGL*2 using the primers *cgl*2 forward and *cgl*2 reverse and pTZ*ABL* using the primers *abl* forward and *abl* reverse. The original promoter sequences were obtained from Genbank and were aligned with the sequence results using the MUSCLE analysis tool. As can be seen from this analysis, both the pTZ*OAT* sequence and the pTZOATm13R sequences showed 99% nucleotide identity when compared to the published *oat* sequence (Table 5.1; Figure 5.12). Similarly, the *cgl*1fwd and the *cgl*1M13F sequences were seen to be 99% identical when compared to the published *cgl*1 promoter sequence (Table 5.2: Figure 5.13). C*gl*2F and *cgl*2R sequences shared 97% nucleotide identity when compared to the published *cgl*2 promoter sequence (Table 5.3; Figure 5.14). The *abl*F sequence showed 98% nucleotide identity when compared to the original *abl* promoter sequence while the *abl*R sequence was 99% identical over the length of the two fragments (Table 5.4; Figure 5.15).

Table 5.1: The Percent Identity Matrix created by Clustal2.1, shows the nucleotide alignment and the similarity of sequencing results for vector pTZ*OAT*.

Percent Identity Matrix - created by Clustal2.1				
Sequence	**Primers**			
originalOAT		100.00	99.07	99.10
pTZoat	M13F	99.07	100.00	100.00
pTZOATm13R	M13R	99.10	100.00	100.00

```
originalOAT       CGGGAGTGGAGTCGGTGTGTTGTATCCCGAGTCATCCCTATCGCACTCCGTCCCCTGTTA
pTZoat            CGGGAGTGGAGTCGGTGTGTTGTATCCCGAGTCATCCCTATCGCACTCCGTCCCCTGTTA
pTZOATm13R        CGGGAGTGGAGTCGGTGTGTTGTATCCCGAGTCATCCCTATCGCACTCCGTCCCCTGTTA
                  ************************************************************

originalOAT       TGGGTACCTCGCGCTTGGCAAAGGGCCCTCGCTATTCCAGTCCTTGGTCACACGACTTCG
pTZoat            TGGGTACCTCGCGCTTGGCAAAGGGCCCTCGCTATTCCAGTCCTTGGTCACACGACTTCG
pTZOATm13R        TGGGTACCTCGCGCTTGGCAAAGGGCCCTCGCTATTCCAGTCCTTGGTCACACGACTTCG
                  ************************************************************

originalOAT       CCTCTCCATGAAGTATGCACCGACTTCCCAGCTCCCTTCCTCCTCCAAGAATAGAACACC
pTZoat            CCTCTCCATGAAGTATGCACCGACTTCCCGGCTCCCTTCCTCCTCCAAGAATAGAACACC
pTZOATm13R        CCTCTCCATGAAGTATGCACCGACTTCCCGGCTCCCTTCCTCCTCCAAGAATAGAACACC
                  ***************************** ******************************

originalOAT       CTCCTTGTCAAGTCCCCGCGTCTTCGGCCCTCCGGCTCGCTCAGCCCGTCCCCTCCCCTC
pTZoat            CTCCTTGTCAAGTCCCCGCATCTTCGGCCCTCCGGCTCGCTCAGCCCGTCCCCTCCCCTC
pTZOATm13R        CTCCTTGTCAAGTCCCCGCATCTTCGGCCCTCCGGCTCGCTCAGCCCGTCCCCTCCCCTC
                  ****************** ****************************************

originalOAT       CCTCCGGAAACGCCATTACACCATAGATGCAATCCATGTCACTCCGTCGTCGCACGAGCC
pTZoat            CCTCCGGAAACGCCATTACACCATAGATGCAATCCATGTCACTCCGTCGTCGCACGAGCC
pTZOATm13R        CCTCCGGAAACGCCATTACACCATAGATGCAATCCATGTCACTCCGTCGTCGCACGAGCC
                  ************************************************************

originalOAT       ATAGTCCATGCTGTACCCTTCGCCTCCCCCTATCTCCAAAGTCAGACAACCTTCCCCACC
pTZoat            ATAGTCCATGCTGTACCCTTCGCCTCCCCCTATCTCCAAAGTCAGACAACCTTCCCCACC
pTZOATm13R        ATAGTCCATGCTGTACCCTTCGCCTCCCCCTATCTCCAAAGTCAGACAACCTTCCCCACC
                  ************************************************************
```

Figure 5.12: A MUSCLE nucleotide alignment of pTZ*OAT* sequenced using M13 forward and reverse primers which were labelled as pTZoat and pTZOATm13R, respectively and the published *oat* promoter sequence (originalOAT) that was obtained from Genbank. The pTZOAT and pTZOATm13R sequences share 99% nucleotide identity when compared to the original *oat* sequence.

Table 5.2: The Percent Identity Matrix created by Clustal2.1, shows the nucleotide alignment and the similarity of sequencing results for vector pTZ*CGL*1.

Percent Identity Matrix - created by Clustal2.1				
Sequence	**Primers**			
***Cgl*1fwd**	*Cgl*1F	100.00	100.00	99.26
***Cgl*1M13F**	M13F	100.00	100.00	99.26
original*cgl*1		99.26	99.26	100.00

```
Cgl1fwd           ----------------------------------TGCTCGAT-AGGTTGCGGTTTGTCT
Cgl1M13F             ---------------------------------TGCTCGAT-AGGTTGCGGTTTGTCT
originalcgl1      TACACGTTTCAGCTTTAGCTACCGTTCTTCCTTCTTGCTCGATAAGGTTGCGGTTTGTCT
                                                    ******** ***************

Cgl1fwd           GTTGTTC-TCGTGGTTGTTTTGCAGAGGAAGACGGGTTGGGTATTCAAGAGTAAGACTTG
Cgl1M13F          GTTGTTC-TCGTGGTTGTTTTGCAGAGGAAGACGGGTTGGGTATTCAAGAGTAAGACTTG
originalcgl1      GTTGTTCATCGTGGTTGTTTTGCAGAGGAAGACGGGTTGGGTATTCAAGAGTAAGACTTG
                  ******* **************************************************

Cgl1fwd           TGTTTTCATAACCCATGCGGGCCGCAGCCAAGAAGGGGATTTGCCGAGAGGCTTGTTGGT
Cgl1M13F          TGTTTTCATAACCCATGCGGGCCGCAGCCAAGAAGGGGATTTGCCGAGAGGCTTGTTGGT
originalcgl1      TGTTTTCATAACCCATGCGGGCCGCAGCCAAGAAAGGGATTTGCCGAGAGGCTTGTTGGT
                  ********************************* ************************

Cgl1fwd           CAAGCAGTGCCAAACCTTGGACGTATTTTGCTCTCGACCACCATTATCTTTGGCGCTGAA
Cgl1M13F          CAAGCAGTGCCAAACCTTGGACGTATTTTGCTCTCGACCACCATTATCTTTGGCGCTGAA
originalcgl1      CAAGCAGTGCCAAACCTTGGACGTATTTTGCTCTCGACCACCATTATCTTTGGCGCTGAA
                  ************************************************************

Cgl1fwd           GGCTTTGCTTACTGTTACTGGATGCGAAGGGGCCTAAGGCTGGATCACTGGAAACTTCTG
Cgl1M13F          GGCTTTGCTTACTGTTACTGGATGCGAAGGGGCCTAAGGCTGGATCACTGGAAACTTCTG
originalcgl1      GGCTTTGCTTACTGTTACTGGATGCGAAGGGGCCTAAGGCTGGATCACTGGAAACTTCTG
                  ************************************************************

Cgl1fwd           TGGCTGTCTCTCCTTGTACTGTGGCACTGTGAAGACTCTCGCCAGTCCCGCGTTCGGGTG
Cgl1M13F          TGGCTGTCTCTCCTTGTACTGTGGCACTGTGAAGACTCTCGCCAGTCCCGCGTTCGGGTG
originalcgl1      TGGCTGTCTCTCCTTGTACTGTGGCACTGTGAAGACTCTCGCCAGCCCCGCGTTCGGGTG
                  ******************************************** ***************

Cgl1fwd           TCAGTCGGGCGTCAGTGTGTCTCCGCTCCAGGTCGACGCCTAGCTAGAATTGTGCCACGA
Cgl1M13F          TCAGTCGGGCGTCAGTGTGTCTCCGCTCCAGGTCGACGCCTAGCTAGAATTGTGCCACGA
originalcgl1      TCAGTCGGGCGTCAGTGTGTCTCCGCTCCAGGTCGACGCCTAGCTAGAATTGTGCCACGA
                  ************************************************************

Cgl1fwd           CTGTAACTCTGGATCTCATCCTTGGAAGAACGAACAATATCCCGGAAGCTTCAGTCTGGA
Cgl1M13F          CTGTAACTCTGGATCTCATCCTTGGAAGAACGAACAATATCCCGGAAGCTTCAGTCTGGA
originalcgl1      CTGTAACTCTGGATCTCATCAGTGGAAGAACGAACAATATCCCGGAAGCTTCAGTCTGGA
                  ******************  ************************************

Cgl1fwd           AGGATTAGAAATTGGGAGATCTATTCTTAGGAAAATGAACCGGCGTTTGCAAACTTCTTC
Cgl1M13F          AGGATTAGAAATTGGGAGATCTATTCTTAGGAAAATGAACCGGCGTTTGCAAACTTCTTC
originalcgl1      AGGATTGGAAATTGGGAGATCTATTCTTAGGAAAATGAACCGGCGTTTGCAAACTTCTTC
                  ****** ******************************************************

Cgl1fwd           AAACCTATGAATGGTTCTCTTGAGGGCCTGATGGTCTATTCTTAGAGTGTGTGATTGGGA
Cgl1M13F          AAACCTATGAATGGTTCTCTTGAGGGCCTGATGGTCTATTCTTAGAGTGTGTGATTGGGA
originalcgl1      AAACCTATGAATGGTTCTCTTGAGGGCCTGATGGTCTATTCTTAGAGTGTGTGATTGGGA
                  ************************************************************

Cgl1fwd           TACTGGAAACCTCGAGCCTCGTAT----CTCGCGATGGACTTTGAACGGCATTGTGGCGA
Cgl1M13F          TACTGGAAACCTCGAGCCTCGTAT----CTCGCGATGGACTTTGAACGGCATTGTGGCGA
originalcgl1      TACTGGAAACCTCGAGCCTCGTATTCGCCTCGCGATGGACTTTGAACGGCATTGTGGCGA
                  ************************    ********************************

Cgl1fwd           TTTGGGACCCAATAGTCATCCCGTTGATGCTATGATTTGGCTCTTCCGACTTCATCTCCG
Cgl1M13F          TTTGGGACCCAATAGTCATCCCGTTGATGCTATGATTTGGCTCTTCCGACTTCATCTCCG
originalcgl1      TTTGGGACCCAATAGTCATCCCGTTGATGCTATGATTCGGCTCTTCCGACTTCATCTCCG
                  ************************************ ********************

Cgl1fwd           TCATTGGGGATGCCGATCTATCAAGCTAACTCTGGTCATATCTGGCCCAAGCAATATTCA
Cgl1M13F          TCATTGGGGATGCCGATCTATCAAGCTAACTCTGGTCATATCTGGCCCAAGCAATATTCA
originalcgl1      TCATTGGGGATGCCGATCTATCAAGCTAACTCTGGTCATATCTGGCCCAAGCAATATTCA
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                  ************************************************************

Cgl1fwd           GCGTCCGGGGAGAAGTGATCTCCGGTGGTCAGGTGGCTCAGGGATGCAATGAAATTGCGA
Cgl1M13F          GCGTCCGGGGAGAAGTGATCTCCGGTGGTCAGGTGGCTCAGGGATGCAATGAAATTGCGA
originalcgl1      GC--------------GATCTCCGGTGGTCAGGTGGCTCAGGGATGCAATGAAATTGCGA
                  **              ******************************************

Cgl1fwd           ATTTTCTGTGTTTTCTGGTGTATAAAAGG-----GGAGATTCGGGGTCGTGAACTCTCCA
Cgl1M13F          ATTTTCTGTGTTTTCTGGTGTATAAAAGG-----GGAGATTCGGGGTCGTGAACTCTCCA
originalcgl1      ATTTTCTGTGTTTTCTGGTGTATAAAAGGCTCCCGGAGATTCGGGGTCGTGAACTCTCCA
                  ****************************     *************************

Cgl1fwd           GAACCAATTCCTCAGTTCTACAAACCCAAGCCAAGTAAGTACTCGGCCTCCCTCTCACTT
Cgl1M13F          GAACCAATTCCTCAGTTCTACAAACCCAAGCCAAGTAAGTACTCGGCCTCCCTCTCACTT
originalcgl1      GAACCAATTCCTCAGTTCTACAAACCCAAGCCAAGTAAGTACTCGGCCTCCCTCTCACTT
                  ************************************************************

Cgl1fwd           TCCTGTATCGCTAACCACCCTACCGGTAGTTCAACATCATGCTCTACC
Cgl1M13F          TCCTGTATCGCTAACCACCCTACCGGTAGTTCAACATCATGCTCTACC
originalcgl1      TCCTGTATCGCTAACCACCCTACCGGTAGTTCAACATCATGCTCTACC
                  *********************************************
```

Figure 5.13: A MUSCLE nucleotide alignment of pTZ*CGL*1 sequenced using *cgl*1 promoter forward primer (*cgl*1fwd) and M13 forward primer (*cgl*1M13F). The published *cgl*1 promoter sequence (original*cgl*1) was obtained from Genbank. The *cgl*1fwd and *cgl*1M13F sequences share 99% nucleotide identity when compared to the original *cgl*1 promoter sequence.

Table 5.3: The Percent Identity Matrix, created by Clustal 2.1, shows the nucleotide alignment and the similarity of sequencing results for vector pTZ*CGL*2.

Percent Identity Matrix - created by Clustal2.1				
Sequence	**Primers**			
original*cgl*2		100.00	97.14	97.66
***cgl*2F**	*Cgl*2F	97.14	100.00	97.66
***Cgl*2R**	*Cgl*2R	97.66	97.14	100.00

```
originalcgl2      GCTGCTATAGCGAGATTTGTAGTACAAGACTAGAGTAGAGAGTGGGATACACATTGCGCT
cgl2F             ----------------------------------------------------ACACATTGCACT
Cgl2R             ----------------------------------------------------ACACATTGCACT
                                                                       ********* **

originalcgl2      CTTTCTTTACTTCCGAGAAGCATCCGATGGGGCTGAGGAGCTTGACGCTTGACCTATTGT
cgl2F             CTTTCTATACTTCCGAGAAGCATCCGATGGGGCTGAGGAGCTTGACGCT-----TATTGT
Cgl2R             CTTTCTATACTTCCGAGAAGCATCCGATGGGGCTGAGGAGCTTGACGCT-----TATTGT
                  ****** ****************************************     ******

originalcgl2      GCAGGCAACCAAGGAAGGGATTTGCGGAAAGAGAGTCAGCTTGTCGGGGAGGGGGTCCTC
cgl2F             GCAGGCAACCAAGGAAGGGATTTGCGGAAAGAGAGTCAGCTTGTCGGGGAGGGGGTCCTC
Cgl2R             GCAGGCAACCAAGGAAGGGATTTGCGGAAAGAGAGTCAGCTTGTCGGGGAGGGGGTCCTC
                  ************************************************************

originalcgl2      GCGCCCGTTATCATCTTCGGCGGTGAAGCGGCCCAAATCTCTGTCCGCCTTACTCGGTAC
cgl2F             GCGCCCGTTATCATCTTCGGCGGTGAAGCGGCCCAAATCTCTGTCCGCCTTACTCGGTAC
Cgl2R             GCGCCCGTTATCATCTTCGGCGGTGAAGCGGCCCAAATCTCTGTCCGCCTTACTCGGTAC
                  ************************************************************

originalcgl2      GCGAAGGGGCCCAAGCCTGACGCAATGAGGAAATCCATGGCTGTCCCTCCTTGTACTGTG
cgl2F             GCGAAGGGGCCCAAGCCTGACGCAATGAGGAATTCCATGGCTGTCCCTGCTTGTACTGTG
Cgl2R             GCGAAGGGGCCCAAGCCTGACGCAATGAGGAATTCCATGGCTGTCCCTGCTTGTACTGTG
                  ******************************** *************** ***********

originalcgl2      GCGCTCTGAAGATTCTTGTTATGGCGTTTGGATTCGGGCGTCACAGTCTCCGGACCTCTC
cgl2F             GAGCTCTGAAGCTTCTTGTTATCGCGTTTGGATTCGGGCGTCACAGTCTCCGGGCCTCTC
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Cgl2R             GAGCTCTGAAGCTTCTTGTTATCGCGTTTGGATTCGGGCGTCACAGTCTCCGGGCCTCTC
                  * ********* ********** ****************************** ******

originalcgl2      TTCTTCAGTCGACACCTTTCCAGGACTGCGTCACGACTATAACACCGGACTCCCTCATCC
cgl2F             TTCTTCAGTCGACACCTTTTCAGGGCTGTGTCACGACTATAACACCGGACTCCCTCATCC
Cgl2R             TTCTTCAGTCGACACCTTTTCAGGGCTGTGTCACGACTATAACACCGGACTCCCTCATCC
                  ******************* **** *** ******************************

originalcgl2      CTTGGATGAATGCGAACGACGTCTTGGAAGTTTCAAGTGTGGAAGGATTCTCAGGAAAAT
cgl2F             CTTGGATGGATGCGAACGACGTCTTGGAAGTTTCAAGTGTGGAAGGATTCACAGGAAAAT
Cgl2R             CTTGGATGGATGCGAACGACGTCTTGGAAGTTTCAAGTGTGGAAGGATTCACAGGAAAAT
                  ******** ***************************************** *********

originalcgl2      GAACCGGCGTTTGCAAAAGTGTCGAACCTCTAAATGGCCCTCTTGGAGACCTGTGACCAA
cgl2F             GAACCGGCGTTTGCAAAAGTGTCGAACCTCTAAATGGCCCTCTTGGAGACCTGTGACCAA
Cgl2R             GAACCGGCGTTTGCAAAAGTGTCGAACCTCTAAATGGCCCTCTTGGAGACCTGTGACCAA
                  ************************************************************

originalcgl2      GGG
cgl2F             GGT
Cgl2R             GGG
                  **
```

Figure 5.14: A MUSCLE nucleotide alignment of pTZ*CGL*2 using *cgl*2 promoter forward primer (*cgl*2F) and *cgl*2 promoter reverse primer *(cgl*2R). The published *cgl*2 sequence (originalcgl2) was obtained from Genbank. The *cgl*2F and *cgl*2R sequence shares 97% nucleotide identity when compared to the original *cgl*2 promoter sequence. The sequence was 97% identical over the length of the two fragments analysed.

Table 5.4: The Percent Identity Matrix, created by Clustal 2.1, shows the nucleotide alignment and the similarity of sequencing results for vector pTZ*ABL*.

Percent Identity Matrix - created by Clustal2.1				
Sequence	**Primers**			
OrignalABL		100.00	98.77	99.18
ablF	ABLF	98.77	100.00	100.00
ABLR	ABLR	99.18	99.38	100.00

```
orignalABL        AGTTCCGCCCGGTCATATGTACTGAAGTCATGAATGGAAGCAGGAGCAGAGAAGGTGGCG
ablF              AGTTCCGCCCGGTCATATGTACTGAANTCATGAATGGAAGCAGGAGCAGAGAAGGNGGCG
ABLR              AGTTCCGCCCGGTCATATGTACTGAAGTCATGAATGGAAGCAGGAGCAGAGAAGGTGGCG
                  ************************* *************************** ****

orignalABL        GACGGTCGGGTGATCGAGTTCCCTAGTAGTGGTTTTAATAACACTGGCAAAAACGCGGAC
ablF              GACGGTCGGGTGATCGAGTTCCCTAGTAGTGGTTTTAATAACACTGGCAAAAANGCGGAC
ABLR              GACGGTCGGGTGATCGAGTTCCCTAGTAGTGGTTTTAATAACACTGGCAAAAANGCGGAC
                  ************************************************** ******

orignalABL        TTTAGTAATTATCCCGGCATATTTTGAGGTCAGATTTGTATTTTAGAAGATTCGTACAAA
ablF              TTTAGTAATTATCCCGGCATATTTTGAGGTCAGATTTGTATTTCAGAAGATTCGTACAAA
ABLR              TTTAGTAATTATCCCGGCATATTTTGAGGTCAGATTTGTATTTNAGAAGATTCGTACAAA
                  ******************************************* ****************

orignalABL        GCACTGAGAGCTACCATTTTCTTCAATGTTGTTCATCATACTTTTCCTGAGCCCTTGACC
ablF              GCACTGAGAGCTACCATTTTCTTCAATGTTGTTCATCATACTTTTCCTGAGCCCTTGACC
ABLR              GCACTGAGAGCTACCATTTTCTTCAATGTTGTTCATCATACTTTTCCTGAGCCCTTGACC
                  ************************************************************

orignalABL        GGTTTTGCCGATCAACGAATACAGAAGTACTATCTACTATACCCTTGATTTGCGATTGAA
ablF              GGTTTTGCCGATCAACGAATACAGAAGTACTATCTACTATACCCTTGATTTGCGATNGAA
ABLR              GGTTTTGCCGATCAACGAATACAGAAGTACTATCTACTATACCCTTGATTTGCGATNGAA
                  ************************************************************

orignalABL        GTAGATAAGATTAGTAGTAGGGTGTTCGGCTTTCATAGCTTAATGAGTCCGTGACTGGTC
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ablF            GTAGATAAGATTAGTAGTAGGGTGTTCGGCTTTCATAGCTTAATGAGTCCGTGACTGGTC
ABLR            GTAGATAAGATTAGTAGTAGGGTGTTCGGCTTTCATAGCTTAATGAGTCCGTGACTGGTC
                ************************************************************

orignalABL      TCCATCACTTAAACTTTGGTCTGCCACGGTTTTAATACCAGCCTGGCACCTTGACAGAAT
ablF            TCCATCACTTAAACTTTGGTCTGCCACGGTTTTAATACCAGCCTGGCACCTTGACAGAAT
ABLR            TCCATCACTTAAACTTTGGTCTGCCACGGTTTTAATACCAGCCTGGCACCTTGACAGAAT
                ************************************************************

orignalABL      CTAGAATACGATGATCGCACCCTCGATTTCCATGAGCTAAAAATATCTGGTCCTGGCACT
ablF            CTAGAATACGATGATCGCACCCTCGATTTCCATGAGCTAAAAATATCTGGTCCTGGCACT
ABLR            CTAGAATACGATGATCGCACCCTCGATTTCCATGAGCTAAAAATATCTGGTCCTGGCACT
                ************************************************************

orignalABL      AG
ablF            GG
ABLR            GG
                 *
```

Figure 5.15: A MUSCLE nucleotide alignment of pTZ*ABL*, sequenced using an *abl* promoter forward primer (*abl*F) and an *abl* promoter reverse primer (*abl*R). The published *abl* promoter sequence was obtained from Genbank. The *abl*F sequence shares 98% nucleotide identity when compared to the original *abl* promoter sequence. The *abl*R sequence was 99% identical over the length of the two fragments.

This analysis verified the successful integration of the various promoter fragments into pTZ57R/T vector, thus confirming the integrity of the pTZ*OAT*, pTZ*CGL*1, p*CGL*2 and pTZ*ABL* plasmids.

5.3 Preparation of the fungal expression plasmid backbones

The vector backbone fragments were prepared from the peGFPi004 plasmid to host each of the fruiting body promoter sequences (*oat*, *cgl*1, c*gl*2 and *abl*). Based on the earlier analysis (see Section 5.2.2), vector backbone fragments were prepared to generate either *Sac*I-*Cla*I or *Sac*II-*Cla*I sticky ends. Initially, a restriction digest was performed on the peGFPi004 plasmid using either *Sac*I-*Cla*I or *Sac*II-*Cla*I to remove the approximately 203bps or 211bps *gpd*II promoter region. 10µL of the digested DNA was then analysed using agarose gel electrophoresis as shown in Figure 5.16. As can be seen, the backbone was successfully digested as indicated by the DNA bands resolving at approximately 4607bps (*Sac*I-*Cla*I) or 4599bps (*Sac*II-*Cla*I).

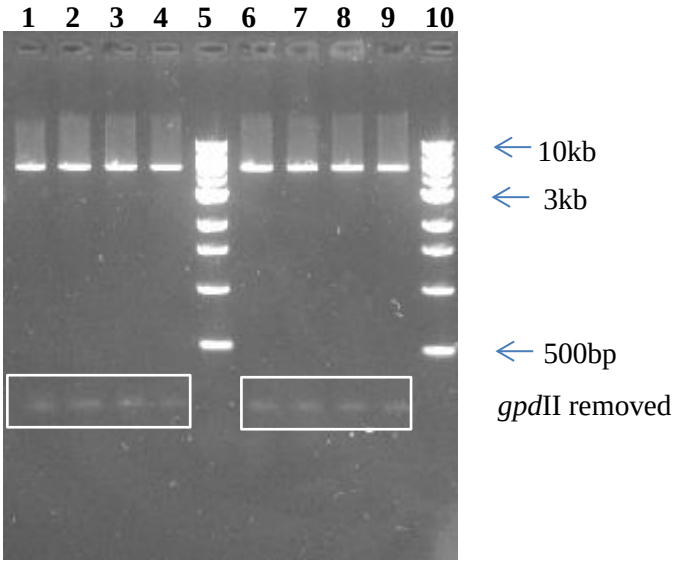

Figure 5.16: The vector peGFPi004 digested using the restriction enzymes *Sac*I-*Cla*I or *Sac*II-*Cla*I. Lanes 1-4: 10µL peGFPi004 digested with *Sac*I and *Cla*I; lane 5 and 10: 1kb ladder and lanes 6-9: peGFPi004 digested with *Sac*II and *Cla*I. The white boxes indicate the location of the *gpd*II promoter.

Once the removal of the *gpd*II promoter was confirmed (Figure 5.16), the vector backbones were then extracted from the gel and purified using a gel extraction kit (as per Materials and Methods, Section 2.6.2). 10µL of the purified vector backbones were subsequently analysed by agarose gel electrophoresis. However, no DNA was visualised on the gel. In order to proceed with this work, the restriction digest of peGFPi004 with both *Sac*I-*Cla*I and *Sac*II-*Cla*I, followed by a gel extraction to purify the backbones, would again be performed.

Once the vector backbones are isolated, the construction of pTZ*OAT*, pTZ*CGL*1, pTZ*CGL*2 and pTZ*ABL* can then take place. This would involve removal of each individual promoter from the pTZ57R/T vector and subsequent subcloning of each fragment into the fungal expression plasmid backbone peGFPi004 (which is outlined in Section 5.3) using the Thermo Scientific InsTAclone PCR Cloning Kit. The constructs would then be transformed into *E.coli* JM109 (as per Materials and Methods, Section 2.5). Following this, a miniprep would be performed to isolate each of the recombinant

plasmids and molecular validation employing restriction digests, PCR and sequence analysis would be carried out to verify the identity of each vector.

These plasmid vectors would then be ready for transformation studies in *C. cinerea* facilitating investigation of the OAT, CGL1, CGL2 and ABL enzymes in this fungus. Following transformation, the transformants could be fruited and analysed at different stages of development for the expression of eGFP.

Chapter 6: Discussion

The aim of this research was to develop fungal expression plasmids to facilitate promoter profiling and gene silencing studies in C*oprinopsis cinerea.* This inkcap mushroom is found naturally in heaps of mixed dung, rotten straw or vegetable refuse and can complete its full life cycle in 14-21 days (Moore and Pukkila 1985). The genome has been fully sequenced and has been made available since 2003 (https://www.ncbi.nlm.nih.gov/bioproject/1447). This makes it the perfect model mushroom for studying gene expression in the life cycle stages of fruiting body development, somatic recombination and mating type determination.

Serine protease (SPR) enzymes have been shown to be involved in postharvest senescence and nutrient acquisition in mushrooms (Burton *et al.* 1993a, Burton *et al.* 1994, Burton *et al.* 1997, Burton 1997, Heneghan *et al.* 2009, Heneghan *et al.* 2016). Previous work has primarily focused on the *Agaricus bisporus* SPRs. Burton studied these enzymes extensively and first discovered the *A. bisporus* serine proteases in 1993 and reported the purification of serine protease 1 (SPR1) from senescent sporophore tissue (Burton *et al*. 1993a). SPRs are produced in abundance by *A. bisporus* in sporophores during senescence (Burton *et al.* 1997) and extracellular to mycelium in colonized compost, where nitrogen is largely in the form of protein thus suggesting a nutritional role for this enzyme (Burton *et al.* 1997b). Sequencing of the *A. bisporus* genome in 2012 confirmed the presence of four serine protease genes (Morin *et al*. 2012).

Global climate change has now increased the interest in an ecosystems carbon sequestration potential (Strickland and Rousk 2010) and SPRs have been shown to play a role in both carbon and nitrogen cycling from humic environments (Morin *et al.* 2012, Heneghan *et al*. 2016). This was demonstrated by Heneghan *et al.*, (2016) who silenced SPR1 in *A. bisporus* to examine the effect it has on nutrient acquisition and adaption of the mushroom to its humic-rich ecological niche. This silencing produced a range of transformants each displaying different levels of silencing. Some transformants failed to produce mushrooms, and some failed to colonise the compost while others had a reduction in mushroom yield when compared to the wildtype *A. bisporus*. This demonstrated that SPR1 is an essential enzyme for growth and development of the mushroom and plays an important role in nutrient acquisition from humic associated material in compost. Thus, SPR1 plays a significant role in *A. bisporus* accessing nitrogen

and carbon sources during degradation of plant material in the soil, thus contributing to the nitrogen and carbon cycles (Heneghan *et al*. 2016).

Blast analysis of the *A. bisporus* SPR genes against the *C. cinerea* genome revealed the presence of seven putative SPR genes in this basidiomycete (Heneghan *et al*. 2009). An expression profile confirmed the presence of extracellular serine proteases, while in fruiting body development serine protease activity was low in the primordium, karyogamy, meiosis and immature stages but rapidly increased at the mature stage. In both the *A. bisporus* and *C. cinerea* SPR regulatory sequences at least one CreA and several Nit2/AreA repression transcription factor-binding sites (TFBS) were identified (Heneghan *et al*. 2009). CreA regulates the expression of several catabolic pathway genes (Mäkelä *et al.* 2018) and is involved in carbon repression in fungi (Souza *et al.* 2011). Nit2/AreA transcription factors belong to the GATA transcription factor family and play a fundamental role in the regulation of nitrogen metabolism (Bernardes *et al.* 2017). Sequencing data revealed that there is 98% amino acid identity between Nit2 and AreA proteins (Daniel-Vedele and Caboche 1993). The presence of these TFBS in the regulatory sequences indicates that these SPR enzymes have a potential role in carbon and nitrogen sequestration. Previous experimental work reports evidence of *A. bisporus* SPR1 expression in response to available nitrogen sources. This research involved the cultivation of *A. bisporus* and *C. cinerea* SPR 1 promoter-eGFP transformants on media containing different nitrogen sources. eGFP expression was observed in *A. bisporus* and *C. cinerea* transformants grown in ammonia free media supplemented with humic fraction, milk or glutamate as a nitrogen source (Burton 1997, Heneghan *et al*. 2009). These experiments demonstrated that in response to available nitrogen, both *C. cinerea* and *A. bisporus* produce serine proteases (Heneghan *et al.* 2009).

A combinatorial approach, utilising promoter profiling and gene silencing to study these endogenous SPR's would provide valuable insight into the role these enzymes play in fruiting body development and nutrient acquisition. Promoter profiling of the *A. bisporus* SPR1 using an SPR::GFP fusion construct has been reported in both *A. bisporus* and *C. cinerea* (Heneghan *et al*. 2009). GFP is an extremely useful tool for molecular analysis, (Chiu *et al.* 1996, Cormack 1998, Hunt *et al.* 1999, Lorang *et al.* 2001, Hakkila *et al.* 2002) and facilitates the study of various biological systems including monitoring protein

locations in living fungal cells or to follow protein secretion in filamentous fungi (Lorang *et al.* 2001, Jensen and Schulz 2003, Poggeler *et al.* 2003). Linking a promoter to a reporter gene such as GFP enables evaluation of the promoters' activity by monitoring GFP fluorescence. One of the advantages of using GFP is that it enables visual analysis of the fungus to establish when and where particular genes are active (turned on) and its detection does not require the addition of any substrates. It can provide an insight into what environmental factors trigger a particular genes expression.

Employing antisense studies to complement the promoter profiling would provide valuable insight into the functionality of the genes. Gene silencing in basidiomycetes has been well documented (de Jong *et al.* 2006, Walti *et al.* 2006, Heneghan *et al.* 2007). Antisense silencing involves the production of single-stranded mRNA in the antisense orientation that corresponds to the target mRNA. These bind together forming double-stranded RNA, which is perceived by the cell as nonsense RNA and targeted for degradation. This inhibits translation of the target mRNA into a functioning protein. The rationale behind using antisense technology as the optimal construct design for gene silencing are multifaceted. Antisense technology has successfully downregulated gene expression in many fungal strains (Hamada and Spanu 1998, Zheng *et al.* 1998; Kitamoto *et al.* 1999; De Backer *et al.* 2001; Gorlach *et al.* 2002; Liu *et al.* 2018), including *A. bisporus* (Heneghan *et al*. 2016) and *C. cinerea* (Walti *et al.* 2006; Heneghan *et al.* 2007). The availability of the *C. cinerea* molecular toolkit facilitates a cloning method for the rapid construction of antisense cassettes. Furthermore, gene inactivation by knockout technology is difficult to achieve in basidiomycetes, which requires inactivation of the target gene in both nuclei of the dikaryon (de Jong *et al.* 2006). Antisense silencing provides a range of transformants exhibiting differing levels of gene suppression and down-regulation, resulting in a range of phenotypes (Walti *et al*. 2006). A range of phenotypes is beneficial as it can be informative of the levels of gene inhibition, from total inactivation of the gene to a small reduction in gene expression (Senior 1998; Heneghan *et al.* 2007; Kilaru *et al.* 2009).

The construction of expression plasmid peGFPi10592 was performed in order to investigate the role of the *C. cinerea* SPR 10592 promoter. This promoter was selected due to the similarity of the 10592 gene to the *A. bisporus* serine proteases SPR1 and SPR2.

An alignment of *C. cinerea* 10592 with *A. bisporus* SPR1 resulted in a sequence homology of 57%, while an alignment of *C. cinerea* SPR 10592 with *A. bisporus* SPR2 resulted in 54% homology. In parallel to this, the SPR 10592 gene was selected for silencing studies to determine if down-regulation of the gene would have a detrimental effect on basidiomycete development. Silencing studies on the *A. bisporus* SPR1 gene were detrimental to the development of the mushroom and suggested that *A. bisporus* SPR1 is a key enzyme for this basidiomycete (Heneghan *et al.* 2016). Based on these results, it is likely that SPR 10592 is a key enzyme for the growth and development of *C. cinerea*.

6.1 DNA Isolation

The first step in the construction of the SPR 10592 promoter and antisense plasmids was the extraction of *C. cinerea* genomic DNA. Based on the published method of Zolan and Pukkila (1986), a large-scale DNA extraction was performed on *C. cinerea* liquid cultures. To generate enough biomass for this large-scale extraction, 100mL liquid cultures of *C. cinerea* were prepared. This involved culturing of the fungus on solid media for 3 days before inoculating into liquid media and growing for a further 5 days. Following harvesting, the first step in the extraction was grinding of the mycelia to release the contents of the cell. This grinding step is needed due to the architecture of the fungal cell wall, which renders many fungi resistant to standard DNA extraction procedures employed for yeast and bacteria. However, grinding using a pestle and mortar with the addition of liquid nitrogen can be time-consuming, taking up to 30 minutes per sample depending on the fungal species and amount of biomass. This extraction resulted in the isolation of *C. cinerea* gDNA but was time-consuming and generated a poor yield (Figure 3.2). Given that this is quite a labour intensive method involving a pre-grinding step of 30 minutes followed by 8 hours to complete the extraction, the yield obtained was not sufficient quantity to utilise this protocol for multiple samples. Therefore, for high throughput screening and analysis, it was essential to establish a rapid DNA extraction method for fungal samples.

There are many commercial kits available for the extraction of fungal DNA; they are rapid and the extraction can be completed within 1 hour. The E.N.Z.A SP Fungal DNA Mini kit was selected for this project. To eliminate the 30-minute grinding step (discussed above), an alternative cell disruption method was investigated. The Minilys® personal homogenizer is a compact instrument designed to lyse and homogenize samples.

Following some research, the homogenizer was selected for the cell disruption step due to the range of speeds (3,000 to 5,000rpm) and cycle times (varying from 5 to 240 seconds) possible on the instrument. The addition of Precellys® beads is recommended to improve the success of penetrating the cell wall of the mycelia and they were employed in this study. The DNA extraction was performed on *C. cinerea* mycelia harvested from liquid culture, as described above. 1g of mycelia sample was placed into the Precellys® vial containing ceramic/glass beads, using a sterile pipette tip to help push the mycelia into contact with the contents of the tube. Samples were homogenized for 60 seconds in the Minilys® bead mill homogenizer at the low/medium setting until the contents were seen to be blitzed. 600µL extraction buffer containing RNase A was added to the vial and vortexed to ensure that samples were suspended and that no clumps remained. The extraction was then carried out from step 3 of the Mini Kit protocol. This resulted in a similar yield to the Zolan and Pukkila method as can be seen in Figure 3.3. However, the overall time of the extraction was reduced from 8 hours to 1 hour. Furthermore, the pre-grinding step was reduced from 30 minutes to 60 seconds, making this a much more efficient method.

This rapid method was then tested on *C. cinerea* and *A. bisporus* mycelia scraped from solid culture. This decision was made to investigate if the liquid culture step could be eliminated and therefore reduce the protocol by the number of days it takes for liquid cultivation. Approximately 0.5g of mycelia was aseptically removed from solid cultures, and the extraction performed as described above. This resulted in the successful isolation of gDNA from both *C. cinerea* and *A. bisporus,* as can be seen in Figures 3.4 and 3.5. Liquid cultivation of *C. cinerea* took approximately 8 days to obtain sufficient biomass for the Zolan and Pukkila method, whereas solid-state cultivation employed for the homogenizer-kit method required only 3 days' incubation.

Quantitative analysis was not performed on the different extraction methods as the overall yield obtained would not be comparable. This is due to the large variation in the quantity of starting material (fungal biomass) i.e. 100mL cultures versus 0.5g scraped mycelia. The focus on DNA isolation for this MSc was to identify a method that would ensure rapid extraction of gDNA that could be used in PCR and transformant screening.

In summary, the application of the rapid method for the gDNA extractions compared to the Zolan and Pukkila method (1986) resulted in a significant time reduction, including

the culturing, grinding and protocol steps. The overall culturing time was reduced by 5 days as culturing took just 3 days, compared to the previous 8 days. Grinding was 60 seconds reduced from 30 minutes, and the extraction protocol took just 1 hour compared to the previous 8 hours. This was a welcomed research outcome as this reduced the time and labour intensive method of the Zolan and Pukkila (1986) method. A technical report was produced for this protocol as this was the first time the Minilys® personal homogenizer had been used to lyse filamentous fungi cells. This was published online and is available at https://homogenizers.net/pages/p-dna-extraction-from-fungal-organisms. A copy of this report can also be seen in Appendix A.

As discussed above, the previous DNA extraction methods were quite time-consuming, ranging from 3-8 days. Walch *et al.*, (2016) described a method to perform direct colony PCR on fungal samples, thus eliminating the need for DNA extraction. This method was tested on three basidiomycetes; *C. cinerea* FA2222, *Hypholoma fascicular* (Kampichler *et al.* 2004, Jafari *et al.* 2018) and *Tyromyces stipticus* (Liaud *et al.* 2014), using ITS (Internal Transcribed Spacer) primers. Research has shown that the ITS regions of fungal ribosomal DNA (rDNA) is used as a DNA barcode for fungi. The ITS region includes the 5.8S-coding sequence which is situated between the Small SubUnit-coding sequence (SSU) and the Large SubUnit-coding sequence (LSU) of the ribosomal operon (Martin and Rygiewicz 1999, Schoch *et al.* 2012). Due to its PCR amplification success rate and large number of ITS copies per cell, this makes it a reliable target for PCR analysis. The ITS region is highly variable among morphologically distinct fungal species (Gardes *et al.* 1991, Baura *et al.* 1992, Chen 1992, Lee and Taylor 1992) and ranges between 650bps and 750bps (White *et al.* 1990). The primers employed in this study were designed to amplify a fragment of 600bps. PCR fragments of the expected size (approximately 600bps) were successfully amplified for the three fungal strains, as can be seen in Figure 3.6. The fragments from *C. cinerea* and *H. fascicular* were sequenced using ITS primers to confirm their identity. BLAST analysis performed on the sequences indicated that the *C. cinerea* ITS sequence has a percentage identity matrix of 99% (Figure 3.7) and *H. fascicular* ITS sequence had a percentage matrix identity of 99% (Figure 3.8). This sequence analysis confirmed the successful isolation of ITS fragments using the Walch method (Walch *et al.* 2016). The convenience of this screening method is the use of material directly from a solid culture and straight into a PCR, thus eliminating the need

for liquid cultivation and DNA extraction. This method was subsequently used throughout the course of this work.

6.2 Construction of the *Coprinopsis cinerea* serine protease 10592 promoter (peGFPi10592)and antisense plasmids (pAS10592)

To investigate the SPR 10592 promoter, the approach taken was to produce an SPR::GFP fusion construct. The fungal expression plasmid peGFPi004 formed the backbone of this expression plasmid. This plasmid is based on the commercial pBluescript vector and contains the *A. bisporus* glyceraldehyde-3-phosphate dehydrogenase (*gpd*II) promoter, an intron, eGFP, and the *A. nidulans TrpC* terminator. It also contains the bacterial selection marker ampicillin and sequences that allow shuttling of the plasmid through *E. coli*. The vector peGFPi004 was engineered to contain an intron as it has previously been reported that efficient GFP expression in *C. cinerea* requires a 5' intron (Burns *et al*. 2005). This plasmid was selected as the backbone as it has previously been successfully transformed into *C. cinerea* resulting in a number of transformants exhibiting eGFP fluorescence (Heneghan *et al*. 2007). To prepare the plasmid for ligation of the SPR 10592 promoter, the *gpd*II promoter had to be first excised. The bioinformatic analysis of peGFPi004 revealed that the plasmid contained the restriction sites *Eco*RI, *Sac*I and *Sac*II upstream of the *gpd*II promoter and a *Hind*III restriction site at the 3' end of the promoter. Restriction digestion of this plasmid with either *Sac*I-*Hind*III or *Sac*II-*Hind*III would, therefore, result in the excision of the *gpd*II promoter, leaving the intron-eGFP-terminator cassette intact.

To identify the putative SPR 10592 promoter, genome mining was employed using Genbank. Once the ATG start site of the gene was identified, a 1000bps sequence upstream was extracted. This sequence contains regulatory sites including specific motifs such as TATA boxes and CAAT regions, which indicate that a gene's promoter element is located within this sequence. The 1000bps putative promoter sequence was analysed using the online tool, webcutter. This tool indicates where in the DNA sequence, particular restriction sites are located, and also what restriction enzymes do not cut within the sequence. This analysis was carried out in order to evaluate which restriction enzyme sites should be incorporated into primers to facilitate directional cloning of the promoter region into the final vector backbone. This sequence analysis revealed that cleavage sites for the restriction enzymes *Sac*II and *Hind*III proved suitable for directional cloning of the SPR 10592 promoter as they did not cut within the target 1000bps sequence. Primers

were designed to incorporate a cleavage site for *Sac*II on the forward primer and a cleavage site for *Hind*III was included on the reverse primer to allow directional cloning.

PCR performed on *C. cinerea* genomic DNA using SPR10592fwd and SPR10592rev primers resulted in the amplification of an 814bps fragment. This putative promoter fragment was then excised and purified from the agarose gel (Figure 3.11) and subsequently, sub cloned into the commercial vector pCR®2.1-TOPO producing the plasmid p10592TOPO.

To confirm the identity of p10592TOPO, a restriction digest was performed using the enzyme *Kpn*I. This enzyme cuts once in the TOPO vector and should result in the linearization of the plasmid and a band resolving at 4745bps (814bps promoter + 3933bps TOPO vector). However, the DNA fragment resolved on the gel at approximately 6kb and not at the expected size of 4745bps. It has been previously suggested that high affinity nucleic acid binding dyes such as gel red can affect DNA migration during electrophoresis. Research has shown that GelRed is a bisintercalator, which can add to the DNA length and weight thus affecting movement of the DNA molecule through the gel (Crisafuli *et al*. 2015). Therefore, it is possible that the banding patterns observed here have been affected by their interaction with gel red during migration. It would have proved useful to test a different DNA staining dye to verify the reproducibility of the pattern of the linearised plasmid. Unfortunately, alternative staining dyes were not available in the laboratory at the time of this work. In order to address this concern, it was next decided to perform additional analysis of the plasmid using PCR and sequencing.

PCR analysis was performed on p10592TOPO using M13 forward and M13 reverse primers as these primers hybridise either side of the MCS in TOPO. This should result in the amplification of the 814bps insert (SPR 10592 promoter) and 500bps of TOPO vector producing a fragment size of 1314bps. As can be seen in Figure 3.13, the DNA fragment separated at approximately 1314bps, further indicating the successful cloning of the promoter into the TOPO vector. p10592TOPO was then sequenced with M13 forward and M13 reverse primers resulting in two sequence reads, namely 10592topoM13Fwd and 10592topoM13Rev. These sequences were aligned with the published Genbank SPR 10592 promoter using a MUSCLE alignment which generates a

Percent Identity Matrix. The Percent Identity Matrix is a calculation of similarity and homology between aligned sequences. The 10592topoM13Fwd sequence, when aligned with the original SPR 10592 promoter sequence, resulted in a 98% Percent Identity Matrix. The 10592TOPOM13Rev sequence, when aligned with the original SPR 10592 promoter, calculated a 99% Percent Identity Matrix, as seen in Figure 3.14.

Based on the predicted restriction digestion patterns of the SPR 10592 promoter sequence, a vector backbone was constructed from peGFPi004 to generate *Sac*II-*Hind*III sticky ends. peGFPi004 was digested with *Sac*II and *Hind*III to remove the *gpd*II promoter (Figure 3.15). The backbone fragment (approximately 4417bps) was excised from the agarose gel and subsequently purified (Figure 3.16).

The putative SPR 10592 promoter was next digested out of the TOPO vector using *Sac*II and *Hind*III restriction enzymes as can be seen in Figure 3.17. The promoter was then ligated into the peGFPi004 backbone as a *Sac*II-*Hind*III fragment (Figure 3.18). To confirm the identity of peGFPi10592, two PCR amplifications were performed. The primer pair spr10592fwd and spr10592rev amplify the 814bps 10592 promoter fragment and the resulting product can be seen in Figure 3.19 B. A second PCR was performed using the primers SPR10592fwd and GFPrev. SPR10592fwd anneals at the 5′ end of the promoter while GFPrev anneals at the 3′ end of the eGFP gene. This resulted in the amplification of a fragment of DNA that spans the SPR 10592 promoter, intron and eGFP. A product of the expected 1581bps can be seen in Figure 3.19 C. These PCRs indicate that the peGFPi10592 expression construct is intact. A restriction digest using *Mfe*I which cuts once within the SPR 10592 promoter resulted in a single linearized fragment of approximately 5.2kb. This is the expected construct size as the peGFPi10592 plasmid is composed of the peGFPi004 backbone, which is approximately 4417bps and the 814bps promoter (Figure 3.20). Sequencing was also performed using M13 forward, M13 reverse and GFP reverse primers. The sequencing reads were aligned with the published SPR 10592 promoter as portrayed in Figure 3.21. The percent identity matrix was 98% for all three sequence reads. This combined analysis (PCRs, digests and sequencing) confirmed the identity of peGFPi10592.

Currently, the recombinant plasmid peGFPi10592 has been constructed and can be used in future experiments to elucidate the role of this SPR in *C. cinerea* fruiting body

development and nutrient acquisition from humic- rich environments. The construction of this construct is a significant addition to the basidiomycete molecular toolkit (Burns *et al*. 2005). Future work will involve transforming this plasmid into the basidiomycete *C. cinerea*. The procedure described by Binninger *et al.,* (1987) would require protoplasts co-transformed with plasmid pCcl001 which contains the *C. cinerea* tryptophan synthetase gene (*trp*1) gene cloned into the plasmid pUC9 (Vieira and Messing 1982). The *trp*1 gene can be used as a selectable marker for efficient transformation in *C. cinerea.*

Following transformation, mycelia would be plated onto regeneration media and the resulting transformants would be isolated for PCR analysis. Primers could be designed to amplify the *trp*1 gene on the pCc1001 plasmid. This PCR would confirm that the putative transformants were true transformants and that selection was successful. To confirm that the putative transformants contain an intact peGFPi10592 plasmid, verification primers could be designed. These primers would target specific regions on the plasmid such as the SPR promoter, intron, GFP, and *trpC* terminator. A combination of PCR's amplifying each region would confirm the presence of the intact construct. Intact plasmids are required to study gene expression and previous studies have shown that during transformation disintegration of the vector can occur and only part of the constructed has been inserted into *C. cinerea* genome (Namekawa *et al.* 2005).

Transformants containing the intact plasmid peGFPi10592 would then be selected and analysed for quantification of GFP activity. Transformants could be inoculated onto a range of media containing different nitrogen and carbon sources. Visual analysis of fluorescence would be performed while western blot analysis could be employed for quantification of GFP protein. This would provide valuable insight into what carbon and nitrogen sources trigger expression of SPR 10592. Fruiting studies could be performed under standard fruiting conditions (Granado *et al.* 1997, Liu *et al.* 2006), and GFP activity measured in the different developmental stages of the fruiting body (primordium, karyogamy, meiosis, immature, mature and autolysis stages). The activity of the SPR 10592 enzyme throughout the life cycle of the basidiomycete would be indicated by GFP expression. Monitoring transformants through mushroom sporophore development would help to fully elucidate the role of the SPR 10592 protein.

In parallel to the construction of peGFPi10592, an antisense cassette was designed to silence the *C. cinerea* SPR 10592 gene, in order to study the effects that downregulation of this gene would have on fruiting body development.

The first step in the construction of the antisense cassette was extracting the SPR 10592 gene sequence from the Genbank database. Sequence analysis of the gene was performed using webcutter to evaluate which restriction enzyme sites should be incorporated into primers to facilitate antisense cloning into the final expression plasmid backbone. This sequence analysis revealed that the restriction enzymes *Bam*HI and *Hind*III proved suitable for directional cloning of the SPR 10592 gene as they did not cut within the target sequence. A cleavage site for the restriction enzyme *Bam*HI was included in the forward primer and a cleavage site for *Hind*III was included in the reverse primer. This would result in a *Bam*HI site being included at the 5′ end of the SPR 10592 gene fragment and the *Hind*III site included at the 3′ end. The silencing vector was designed based on the fungal expression plasmid peGFPi004, which contains the *A. bisporus gpd*II promoter, intron, eGFP gene and *A. nidulans trp*C terminator. The restriction enzymes *Bam*HI (which cleaves at the 3′ end of the GFP gene) and *Hind*III (which cleave at the 5′ end of the intron) were used to remove the intron and GFP gene region, leaving the rest of the plasmid intact and ready for the ligation of the SPR 10592 gene. This would facilitate the cloning of the gene fragment in the antisense (3′-5′) orientation as a *Hind*III-*Bam*HI fragment.

PCR of *C. cinerea* genomic DNA using Antisense10592forward and Antisense10592reverse primers resulted in the amplification of the SPR 10592 gene at the expected size of approximately 1151bps (Figure 3.23). This gene fragment was purified from the agarose gel and subcloned into the commercial vector pTZ57R/T producing pTZAS10592.

To confirm the identity of pTZAS10592, a restriction digest was performed on the plasmid using *Kpn*I, which should cut once in the vector. This should result in the linearization of the plasmid and a band resolving at 4037bps composed of 1151bps SPR 10592 gene and 2886bps of the pTZ57R/T vector. Following electrophoresis, a band was obtained at the expected size of approximately 4037bps (Figure 3.25) indicating the successful cloning of the SPR 10592 gene into the pTZ57R/T vector. PCR analysis was performed on pTZAS10592 using M13 forward and reverse primers as these primers

hybridise either side of the MCS of pTZ57R/T. This resulted in the amplification of the 1151bps insert (SPR 10592 gene) and 138bps of the pTZ57R/T vector producing a fragment size of 1289bps (Figure 3.26). This further indicated the successful cloning of the SPR 10592 gene into the pTZ57R/T vector. pTZAS10592 was then sequenced with M13 forward primer and the antisense fragment displayed 98% homology to the published Genbank SPR 10592 gene sequence (Figure 3.27). This combined analysis of PCR, digest and sequencing confirmed the identity of pTZAS10592.

The fungal expression plasmid peGFPi004 will form the backbone of the antisense cassette (Heneghan *et al*. 2007). To prepare the plasmid for cloning of the antisense fragment, the intron and GFP gene need to be removed and subsequently replaced with the antisense fragment (see schematic in Chapter 3, Section 3.10, and Figure 3.23). The presence of a 5′ intron has previously been shown to be essential for gene expression in basidiomycetes and it has been proposed that this intron provides stability to the mRNA. The removal of the intron from the expression plasmid should lead to the production of unstable mRNA and thus will trigger silencing pathways (Heneghan *et al*. 2007). Using intronless plasmids for gene silencing has previously been performed in *C. cinerea* (Heneghan *et al*. 2007, Heneghan *et al*. 2016). Previous bioinformatic analysis of peGFPi004 revealed a *Hind*III restriction site is located at the 3′ end of the *A. bisporus gpd*II promoter just before the start of the intron, while a *Bam*HI site is at the 3′ end of the GFP region, just before the start of the *A. nidulans trpC* terminator. Digestion of this plasmid with *Hind*III-*Bam*HI would, therefore, result in the excision of the intron and GFP region, leaving the promoter and terminator regions intact in the plasmid backbone. The SPR 10592 gene will be under the control of the *A. bisporus gpd*II promoter, which has proved to be successful in driving gene expression in *C. cinerea* (Burns *et al.* 2005). It is a constitutive promoter and will be switched on all the time, thus triggering consistent silencing of the gene. The intron and GFP were digested out of peGFPi004 to form the backbone as indicated by bands at the expected size of 3930bps, Figure 3.28.

Once the removal of the intron and eGFP genes were confirmed (Figure 3.29), the vector backbone was then extracted and purified using a gel extraction kit. However, no purified DNA was recovered. This may have been due to too low a concentration of digested DNA prior to purification. The next step would be to repeat a restriction digest of peGFPi004 with a higher starting concentration of DNA, using *Hind*III and *Bam*HI to remove the 70bps intron and 717bps GFP region.

Silencing of the SPR 10592 gene would provide a functional analysis of the SPR 10592 protein and identify whether it is critical in the development of the basidiomycete fruiting body. Future experimental work leading on from this project would include, the isolation of the SPR 10592 gene from pTZAS10592 using the restriction enzymes *Bam*HI and *Hind*III and ligation of the purified gene into the backbone to produce the fungal expression plasmid pAS10592. Following this, molecular validation of the pAS10592 plasmid would be carried out to confirm the integrity of the vector. PCR analysis of pAS10592 could be performed using a forward primer specific to the *A. bisporus gpd*II promoter and a reverse primer specific to the *A. nidulans trp*C terminator. This would amplify a product that contains the *gpd*II promoter, 10592 gene and *trpC* terminator, confirming an intact pAS10592 expression construct. Restriction analysis would establish the presence of a band at the expected size of the SPR 10592 gene in pAS10592. Sequence analysis of pAS10592 would confirm the identity of the vector. This plasmid could then be transformed into the basidiomycete *C. cinerea* and positive transformants recovered and analysed.

PCR analysis would be performed on the putative transformants, using primers that would target specific regions on the plasmid such as the *gpd*II promoter, SPR 10592 gene and *trpC* terminator. A combination of PCRs successful in amplifying these regions would confirm whether the transformants contained an intact construct.

Biochemical analysis on transformants containing the intact pAS10592 vector and wild-type *C. cinerea* (to use as a control) would be performed to evaluate protease activity. Transformants could be grown in media supplemented with humic acid as this has previously been shown to induce SPR activity (Burton *et al.* 1997, Heneghan *et al.* 2016). SPR 10592 mRNA transcript levels in transformants would be quantified by RT-qPCR, while protein quantification could be confirmed by western blot analysis. Fruiting trials could be commenced to evaluate if transformants could colonize compost and mushroom yields compared to the wildtype. The results of these experiments could provide detailed knowledge of the exact role of the SPR 10592 enzyme in the development of the basidiocarp.

6.3 Construction of peGFPi04562, pAS04562 and peGFPi10615

The *C. cinerea* SPR 04562 and 10615 promoters were next selected for study. These *C. cinerea* genes (SPRs 04562 and 10615) also show significant homology to the *A. bisporus* SPRs. BLAST analysis of the SPR 04562 gene displayed an amino acid sequence identity

value of 61% when aligned with *A. bisporus* SPR1 and 55% when aligned with the *A. bisporus* SPR2 gene (Heneghan *et al.* 2009). The SPR 10615 gene analysis revealed amino acid sequence identity values of 55% when aligned with *A. bisporus* SPR1 and 54% when aligned with the *A. bisporus* SPR2 gene (Heneghan *et al*. 2009).

Genome mining was employed to extract from Genbank a 1kb stretch of sequence upstream of the 04562 and 10615 ATG start sites, thus representing the putative promoters. These sequences were analyzed using webcutter to evaluate which restriction enzyme sites should be incorporated into primers to facilitate directional cloning of the promoter regions into the final vector backbone.

Sequence analysis of the putative promoter regions revealed that the restriction enzymes *Sac*I and *Hind*III proved suitable for directional cloning of the SPR 04562 promoter, while the restriction enzymes *Sac*II and *Hind*III were suitable for directional cloning of the SPR 10615 promoter. Primers were designed to amplify the SPR 04562 promoter by incorporating a cleavage site for the restriction enzyme *Sac*I into the forward primer and a cleavage site for *Hind*III into the reverse primer. To amplify the SPR 10615 promoter, a cleavage site for *Sac*II was incorporated into the forward primer and a cleavage site for *Hind*III into the reverse primer.

PCR performed on *C. cinerea* genomic DNA using SPR04562fwd and SPR04562rev primers resulted in the amplification of the putative SPR 04562 promoter at the expected size of 1003bps (Figure 4.3). The amplification of the putative SPR 10615 promoter using SPR10615fwd and SPR10615rev primers resulted in a fragment at the expected size of 1004bps (Figure 4.20). These promoter fragments were purified from the agarose gel and subsequently subcloned into the commercial vector pCR2.1-TOPO producing the plasmids p04562TOPO and p10615TOPO.

To confirm the identity of p04562TOPO and p10615TOPO, a restriction digest was performed using the enzyme *Kpn*I. This enzyme cuts once in the vector backbone resulting in the linearization of the plasmids and bands resolving at approximately 4934bp for p04562TOPO and 4935bp for 10615TOPO. However, the bands were significantly larger than expected, resolving at approximately 6kb. As previously observed with p10592TOPO, it is possible that the banding patterns here have been affected by their interactions with gel red during migration. In order to address this concern, it was next decided to perform additional analysis using PCR and sequencing.

PCR analysis was performed on p04562TOPO and p10615TOPO using M13 forward and reverse primers. This resulted in the amplification of the SPR promoter and 500bp of TOPO vector. p04562TOPO resulted in a band resolving at 1503bps and the DNA fragment representing p10615TOPO separated at approximately 1504bp. These results further indicated the successful cloning of the promoters into the TOPO vector.

p04562TOPO and p10615TOPO were sequenced using M13 forward and reverse primers. The sequence analysis of p04562TOPO resulted in 99% homology to the published Genbank SPR 04562 promoter (Table 4.1 and Figure 4.6). p10615TOPO had 98% homology when aligned with the published SPR 10615 promoter (Table 4.3 and Figure 4.22).

The SPR 04562 promoter was digested out of the TOPO vector using restriction enzymes *Sac*I and *Hind*III and was subsequently purified. Based on the predicted restriction digestion patterns of peGFPi004 (as discussed previously), the peGFPi04562 linear backbone was designed to contain *Sac*I-*Hind*III sticky ends. peGFPi004 was digested with *Sac*I and *Hind*III to remove the *gpd*II promoter , resulting in a fragment at the expected size of approximately 4425bps (Figure 4.8).

Future work that could lead on from this project could include digestion of the SPR 04562 and 10615 promoters from TOPO. The promoters would then be ligated into the appropriate expression plasmid backbones producing peGFPi04562 and peGFPi10615. PCR analysis could be performed by targeting regions of peGFPi04562 and peGFPi10615, such as the promoter, intron, GFP and *trpC* terminator to confirm that the plasmids are intact. Restriction digest analysis would confirm the size of the plasmids. These vectors could be sequenced, and the resulting reads would determine the homology values when aligned with the published sequence of the SPR promoters. All these molecular techniques of PCR, restriction digest and sequencing would indicate if the plasmids are suitable for further transformation experiments.

The fungal expression plasmid constructs peGFPi04562 and peGFPi10615, could be involved in further experiments to elucidate the role of these SPR proteins in *C. cinerea* fruiting body development. Experimental work using these constructs could include transformation into the basidiomycete *C. cinerea*. PCR would be employed to confirm positive transformants and biochemical analysis would evaluate protease activity. Transformants could be inoculated onto a range of media containing different nitrogen

and carbon sources. This would investigate whether changes in nutrient availability would alter the expression of the proteases. Visual analysis would be performed using a fluorescent microscope, while western blot analysis could be used for quantification of GFP. *C. cinerea* fruiting studies would allow GFP activity to be monitored throughout the development stages of the fruiting body (primordium, karyogamy, meiosis, immature, mature and autolysis stages). The observation of transformants through mushroom sporophore development may fully elucidate the role of the SPR 04562 and SPR 10615 proteins.

In parallel to the construction of peGFPi04562, the *C. cinerea* SPR 04562 gene was also chosen for silencing studies. The rationale for the investigation of the SPR 04562 gene is the significant homology it has to the *A. bisporus* SPRs, as previously discussed. This research could establish if silencing this SPR would have a detrimental effect on the development of the basidiomycete.

The first step in the construction of the antisense cassette was to extract the gene sequence from the Genbank database. Sequence analysis of the SPR 04562 gene was performed using webcutter to evaluate which restriction enzyme sites should be incorporated into primers to facilitate cloning of the gene in the antisense orientation into the final expression plasmid backbone. This sequence analysis revealed that the restriction enzymes *Aat*II and *Xma*I proved suitable for directional cloning of the SPR 04562 gene as they did not cut within the target sequence. A cleavage site for the restriction enzyme *AatI*I site was included in the reverse primer and a cleavage site for *Xma*I was included in the forward primer. pMCS004 was selected as the backbone for this silencing vector as this plasmid had previously been employed to construct antisense cassettes (Heneghan *et al*. 2007). Furthermore, this vector lacks the 5′ intron required for gene expression in *C. cinerea* (Burns *et al*. 2005), and is therefore more likely to trigger silencing pathways. The restriction enzymes *Xma*I and *Aat*II would be used to remove the multiple cloning site leaving the rest of the plasmid intact and ready for the ligation of the SPR 04562 gene. This would facilitate the SPR gene fragment to be cloned in the antisense (3′-5′) direction as an *Xma*I-*Aat*II fragment.

PCR of the *C. cinerea* genomic DNA using Antisense04562forward and Antisense04562reverse primers resulted in amplification of the SPR 04562 gene at approximately 1500bps (Figure 4.12). However, it is not at the predicted size of

approximately 1139bp and seems to be at a larger size than expected. It is possible that the banding patterns observed here have been affected by their interaction with gel red during migration (as discussed previously). However, given the previous issues observed with gel red and the increased DNA fragment sizes, it was decided to isolate the fragment for further analysis. The purified SPR 04562 gene was subcloned into the commercial vector pCR2.1-TOPO producing pAS04562TOPO.

To confirm the identity of pAS0462TOPO, a double digest was performed using *Nco*I and *Xho*I, which should result in the vector being cut into three separate fragments. The restriction enzyme *Nco*I was predicted to cut the plasmid twice, once in the SPR 04562 gene region and once in the TOPO plasmid, while *Xho*I cuts the vector once in the TOPO plasmid. The resulting agarose gel depicts the bands resolving at the approximate expected sizes of 3kb, 1.5kb and 567bps (Figure 4.15). PCR analysis was performed on pAS04562TOPO using M13 forward and reverse primers. A band of approximately 1636bps was amplified, which accounts for 1136bps of the SPR 04562 gene and 500bps of TOPO vector.

pAS04562TOPO was then sequenced using the SPR 04562 forward primer and the resulting sequence read had 99% homology when aligned with the original Genbank SPR 04562 gene sequence. This combined analysis of PCR, digest and sequencing confirmed the identity of pAS04562TOPO.

Future work on the antisense silencing of SPR 04562 gene would provide a practical, functional analysis of this gene and determine if its critical in the development of the basidiomycete fruiting structures. Experimental work would include the isolation of the SPR 04562 gene from pAS04562TOPO and ligation into the fungal expression plasmid backbone producing pAS04562. Following on from this, molecular validation of the expression plasmid would be carried out. PCR analysis of pAS04562 would be employed to confirm if the expression construct is intact. Restriction analysis could be performed in order to further confirm the presence of the SPR 04562 gene in pAS04562 and determine the size of the plasmid. Sequence analysis of pAS04562 would confirm the identity of the vector. The analysis using PCR, restriction digests and sequencing would verify the successful integration of the 04562 gene into pMCS004, resulting in the recombinant vector pAS04562.

pAS04562 would then be transformed into *C. cinerea* and putative transformants recovered for molecular analysis. PCR would confirm positive transformants and biochemical assays would be performed to measure protease activity. mRNA transcript levels could be quantified by RT-qPCR, while protein quantification could be confirmed by western blot analysis. Fruiting studies could be commenced to evaluate if transformants could colonize the compost and mushrooms yields determined. The observation of transformants through mushroom sporophore development would help to fully elucidate the effects of silencing the SPR 04562 gene.

6.4 Design and development of *Coprinopsis cinerea* plasmids to facilitate the analysis of fruiting body enzymes

Further insight into the molecular pathways underpinning fruiting body formation would give a better understanding of the mechanisms regulating mushroom development (Cheng *et al.* 2013, Pelkmans *et al.* 2016, Ewaze *et al.* 1978). To this end, a number of candidate genes were identified that play a role in fruiting body development; *C. cinerea* ornithine aminotransferase gene (*oat*) (Ewaze *et al.* 1978), *C. cinerea* galectin gene I (*cgl*1), *C. cinerea* galectin gene II (*cgl*2) (Boulianne *et al*. 2000) and *A. bisporus* lectin gene (*abl*) (Presant and Kornfeld 1972). Expression studies using promoter-reporter constructs of these candidate genes may help elucidate their role in the development of fruiting structures. Little research has been conducted on the *C. cinerea oat* gene and it was chosen for study as it has been previously shown to be involved in the metabolism of the developing fruiting body. OAT is highly active in the developing cap and to a lesser extent in the stipe (Ewaze *et al.* 1978, Moore 2013, Moore and Pukkila 1985) and it has been hypothesized that OAT may play an essential role in fruiting body formation in the mushroom. The *C. cinerea cgl*1 and *cgl*2 genes have previously been shown to be involved in fruiting body development. CGL1 is induced by light while CGL2 is repressed by light and these enzymes were chosen for study due to their role in the light and dark stages that a mushroom requires to fruit. CGL1 and CGL2 are located throughout most tissues, but predominantly within the veil and the lipsanoblem (the external tissue of the stipe). The role of CGL1 and CGL2 may be important as they are specific to fruiting body development (Boulianne *et al*. 2000). The *A. bisporus abl* gene was chosen for study as previous research demonstrated that lectin expression is closely correlated with and could be a significant feature in regulating fruiting body development (Lakkireddy *et al.* 2011, De Groot *et al*. 1997, Crenshaw *et al*. 1995, Presant and Kornfield 1972). In basidiomycetes, lectins are present in the cap, stipe, spores, and

mycelia (Zhang *et al*. 2009, Eastwood *et al.* 2008, Ng 2004). The *A. bisporus abl* gene was chosen as there is very little research regarding the role of the *abl* gene in fruiting body development. Investigating these promoters may help to elucidate their role in fruiting body formation.

Similar to the experiments described for the SPR promoters, these fruiting body promoters will be cloned into peGFPi004 to produce a promoter-reporter construct. By linking these promoters to eGFP and transforming them into *C. cinerea*, this would give fundamental data about what genes are expressed at each stage of fruiting body development.

Genome mining was employed to identify the promoters using Genbank. A 1kb stretch of sequence upstream of the start site of each gene was extracted. These promoter sequences were analyzed using webcutter to evaluate which restriction enzyme sites should be incorporated into primers to facilitate directional cloning of the promoter into the final expression backbone. Cleavage sites for the restriction enzymes *Sac*II and *Hind*III were incorporated into the *oat* primers. For *cgl*1 and *abl*, cleavage sites for the restriction enzymes *Sac*I and *Cla*I were included into the primers, while cleavage sites for the restriction enzymes *Sac*II and *Cla*I were incorporated into the primers for *cgl*2. Previous analysis of the peGFPi004 backbone revealed that the *gpd*II promoter could be removed as either an *Eco*RI-*Hind*III, *Sac*I-*Hind*III or a *Sac*II-*Hind*III fragment, with *Hind*III cleaving at the 3' end of the promoter. Analysis of the *cgl*1, *cgl*2 and *abl* promoters revealed that *Hind*III cuts within them, rendering it unsuitable for directional cloning. *Cla*I was selected as an alternative as it cleaves near the 3' end of the *gpd*II promoter. However approximately 80bps of the *gpd*II promoter would remain in the recombinant plasmids and could potentially interfere with gene expression. In order to address this, bioinformatic analysis of the sequence was performed to check for putative regulatory regions within the 80bps. The sequence data revealed a CAAT region within the 80bps *gpd*II promoter region which could have a potential role in driving transcription. It would, therefore, be critical that the promoter responsible for driving expression of GFP could be determined in any promoter-reporter studies undertaken. In contrast to *cgl*1, *cgl*2 and *abl*, the *gpd*II promoter is constitutively expressed and should drive expression of GFP continuously once transformed. Expression from the *cgl*1, *cgl*2 and *abl* promoters could potentially be regulated in response to environmental conditions such as restriction of nutritional requirements. The ability to control GFP expression

could therefore be used as a method to confirm which promoter is driving expression. However, further studies would be required to determine which specific conditions regulate each promoter. Having established a method to elucidate which promoter was responsible for expression, it was decided to continue to include a cleavage site for the restriction enzyme *Cla*I on the reverse primers (see Material and Methods, 2.8.1, Table 2.4) to facilitate directional cloning.

PCR was performed on *C. cinerea* genomic DNA using the primers designed above. The amplification of the putative promoters resulted in fragments at the expected sizes of approximately 1003bps (*oat*), 1006bps (*cgl*1), 1008bps (*cgl*2) and 1001bps (*abl*) (Figure 5.8). These promoter fragments were purified from the agarose gel and subcloned into vector pTZ57R/T producing pTZ*OAT*, pTZ*CGL*1, pTZ*CGL*2 and pTZ*ABL*. Initial analysis of pTZ*OAT* and pTZ*CGL*1 was performed using restriction digestion. A double digest was performed on pTZ*OAT* using the restriction enzymes *Sac*I and *Sma*I. Both enzymes are predicted to cut once within the vector, so the double digest should result in two DNA fragments. Following electrophoresis, bands were obtained at the expected sizes of 3kb and 889bps which accounts for 1003bps *oat* promoter and 2886bps pTZ57R/T vector (Figure 5.10A). A restriction digest was performed on pTZ*CGL*1 using *Kpn*I, which should cut once in the vector and should result in the linearization of the plasmid. The digestion of pTZ*CGL*1 resulted in a single band. However, the plasmid size of pTZ*CGL*1 was approximately 4.5kb and the predicted expected size was 3892bps (which accounts for 2886bps of the pTZ57R/T vector and 1006bps of the *cgl*1 promoter). It is possible as previously discussed that the banding patterns observed here have been affected by their interaction with gel red during migration. In order the address this concern, it was next decided to perform additional analysis of the recombinant plasmids using PCR and sequencing.

PCR analysis was performed on pTZ*OAT*, pTZ*CGL*1, pTZ*CGL*2 and pTZ*ABL* using M13 forward and M13 reverse primers. This resulted in the amplification of a 1141bp fragment for pTZ*OAT* (1003bps *oat* promoter and 138bps of the pTZ57R/T vector), a 1144bps fragment for pTZ*CGL*1 (1006bps *cgl*1 promoter and 138bps of the pTZ57R/T vector), a 1146bps fragment for pTZ*CGL*2 (1008bps *cgl*2 promoter and 138bps of the pTZ57R/T vector), and a 1139bps fragment for pTZ*ABL* (1001bps *abl* promoter and 138bps of the pTZ57R/T vector). This amplification of the DNA fragments at the expected approximate sizes indicates the successful cloning of these promoters into pTZ57R/T (Figure 5.11).

The promoter plasmids pTZ*OAT*, pTZ*CGL*1, pTZ*CGL*2 and pTZ*ABL* were subsequently sequenced to verify the identity of these plasmids. pTZ*OAT* was sequenced with M13 forward and M13 reverse primers, and the resulting sequence was 99% homologous when aligned with the published *oat* promoter sequence. pTZC*GL*1 was sequenced with an M13 forward primer and a *cgl*1 forward primer. The resulting sequences were 99% homologous when aligned with the published *cgl*2 promoter sequence. pTZ*CGL*2 was sequenced with a *cgl*2 forward primer and *cgl*2 reverse primer, and the resulting sequences were 99% homologous when aligned with the published *cgl*2 promoter sequence. pTZ*ABL* was sequenced with an *abl* forward primer and an *abl* reverse primer. The resulting sequence reads were 98% and 99% homologous respectively, when aligned with the published *abl* promoter sequence. This sequence analysis confirmed the identity of pTZ*OAT*, pTZ*CGL*1, pTZ*CGL*2 and pTZ*ABL*.

The fungal expression plasmid peGFPi004 was selected as the backbone for the promoter-GFP fusion plasmids as previously described in the construction of peGFPi10592. The peGFPi004 vector was digested with *Sac*I and *Cla*I or *Sac*II and *Cla*I to generate sticky ends. These restriction enzymes were used to remove the *gpd*II promoter and purified using a gel extraction kit. However, no purified DNA was recovered. This may have been due to using too low a concentration of plasmid DNA for the initial digestion. The next step would be to repeat the restriction digest with a higher concentration of DNA.

Future work that could lead from this project regarding these promoters would involve ligation of the promoters into the expression plasmid backbones and subsequently transforming the recombinant plasmids into *C. cinerea.* PCR analysis would then be performed on the putative transformants using primers that would target specific regions on the plasmids including the intron, GFP gene and *trpC* terminator. The results from these PCRs would confirm intact constructs and the transformants would be analysed for GFP activity. Visual analysis would be performed using fluorescent microscopy, and western blot analysis could be used to quantify protein levels. Fruiting studies could be performed and GFP fluorescence monitored throughout the life cycle of the basidiomycete. These experiments could provide essential knowledge regarding what genes are expressed at each stage of the fruiting body development process.

The knowledge of enzymes involved in fruiting body development provided by these studies could lead to the design of silencing experiments to down regulate genes at specific fruiting body development stages. Previous research of silencing the SPR1 under the constitutive *gpd*II promoter resulted in one transformant (AS5) that was unable to colonize the compost, and the transformants that could colonize the compost demonstrated a 60% reduction in mushroom yield. The role of fruiting body development is still not understood. Silencing studies under fruiting specific promoters would switch off protein expression at specific developmental stages and this would provide a further insight into the roles of these enzymes. This methodology could also be applied to analyse each of the endogenous *C. cinerea* SPRs. The results of these experiments could provide detailed knowledge of the exact role of these enzymes in development of the basidiomycetes, and as *C. cinerea* is the model organism for *A. bisporus* studies, the outcomes of this research could contribute to improvements in mushroom production.

Chapter 7: Conclusion

The aim of this MSc research was to develop fungal expression plasmids to facilitate promoter profiling and gene silencing studies in *Coprinopsis cinerea*. These plasmids could potentially be employed in future research to elucidate the role of the *C. cinerea* endogenous serine proteases in fruiting body development and nutrient acquisition. The development of a rapid DNA extraction method for fungal samples was central to this work. A protocol was successfully developed and a technical report was produced and published online. This was a welcomed research outcome as this reduced the time and labour intensive method of the Zolan and Pukkila (1986) method. This was the first time the Precellys bead mill homogeniser had been used to lyse filamentous fungi cells. A combination of the rapid gDNA extract method and PCR screening method was used throughout the MSc.

The *Coprinopsis cinerea* serine protease 10592 plasmid was constructed. The plasmid was designed and constructed to link the promoter to efficient Green Fluorescent Protein (eGFP) producing the peGFPi10592 construct. The construction of this plasmid is a significant advancement in the development of a molecular toolkit for transformation of *C. cinerea.* The results of these experiments could provide essential data, advancing the knowledge of the role the SPR 10592 enzyme has in the development of the basidiocarp.

An antisense cassette was designed to silence the *C. cinerea* SPR 10592 gene, in order to study the effects that downregulation of this gene would have on fruiting body development. The use of antisense constructs for rapid and high-throughput silencing could provide essential data regarding the functionality of this *C. cinerea* enzyme. The construction of the serine protease 10592 gene antisense plasmid (pAS10592) resulted in successful isolation of this gene and the generation of the corresponding plasmid backbone ready for future cloning experiments.

GFP fusion cassettes were also designed for the SPR promoters 04562 and 10615. Work was undertaken to identify and isolate the *C. cinerea* SPR promoter regions of 04562 and 10615 and the preparation of plasmid backbones suitable for cloning these fragments.

An antisense cassette was designed for the SPR 04562 gene. This work resulted in successful isolation of the gene and the generation of the corresponding plasmid

backbone, which are now ready for cloning experiments. The availability of these plasmids will enhance the existing *C. cinerea* molecular toolkit which facilitates molecular analysis of genes in this basidiomycete. Future experiments employing these plasmids will provide further knowledge to enhance our understanding of these SPR enzymes.

The design and development of *C. cinerea* plasmids to facilitate analysis of the fruiting body genes *Agaricus bisporus* lectin (ABL), *C. cinerea* ornithine transferase (OAT), and *C. cinerea* galectin genes (CGL1 and CGL2) was conducted. This work included the identification, isolation and sequence analysis of the promoters of the fruiting body development genes. These genes could provide essential information regarding what enzymes are expressed at each stage of the fruiting process.

This research will form a foundation for future experiments to study *C. cinerea* SPRs and fruiting body development genes in the fungal life cycle. This work contributes to elucidating the role of these enzymes in development of the basidiomycete and the outcomes of this research could lead to improvements in commercial mushroom production.

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Appendix A

Fungal DNA extraction protocol using the Precellys Minilys® personal homogenizer. This technical report was produced for Precellys and published online. It is available at https://homogenizers.net/pages/p-dna-extraction-from-fungal-organisms.

MICROBIOLOGY

Precellys

DNA EXTRACTION FROM THE FUNGAL ORGANISMS *COPRINOPSIS CINEREA* AND *TALAROMYCES EMERSONII*.

Institute of Technology, Sligo, Ireland

/ CONTEXT

DNA extraction is an essential tool in the molecular analysis of fungi. The architecture of the fungal cell wall renders many fungi resistant to standard DNA extraction procedures employed for yeast and bacteria. Fungal DNA extraction protocols tend to be time consuming and/or result in a poor yield of DNA. While commercial kits are available for the extraction of fungal DNA, they do require a pre step of grinding (with or without liquid N_2) for the initial breaking up of mycelia. Grinding can be consuming, taking up to 30 minutes per sample depending on the fungal species. For high throughput screening and analysis, a rapid DNA extraction method is required for fungal samples. This method was tested on two different classes of fungal species; an ascomycete (*T. emersonii*) and a basidiomycete (*C. cinerea*).

/ MATERIALS

- Sample: *C. cinerea* and *T. emersonii* were grown in both solid and liquid state cultures following standard protocols (Binninger *et al.*, 1987; Gupta *et al.*, 2012). 0.5g of mycelia scrapped from solid culture and 0.4g of mycelia harvested from liquid culture were used for DNA extractions.
- Precellys® Lysing Kit, VK05 Cat n° KT03961-1-004.2
- Minilys® personal homogenizer
- Omega bio-tek E.N.Z.A SP Fungal DNA Mini kit D5542-01

/ PROTOCOL

Mycelia samples were placed into the Precellys® vial containing ceramic/glass beads, using a sterile pipette tip to help push mycelia into contact with the tubes contents. Samples were homogenize for 60 seconds in the Minilys® bead mill homogenizer at the low/medium setting until the contents were seen to be blitzed. 600 µL SFG1 Buffer and 4 µL RNase A (E.N.Z.A SP Fungal DNA Mini kit D5542-01) were added to the vial, and vortexed to ensure that samples were suspended and that no clumps remained. The extraction was carried out from step 3 of the E.N.Z.A SP Fungal DNA Mini kit, according to manufactures instructions.

/ RESULTS

DNA extractions were performed on both *T. emersonii* and *C. cinerea* mycelia harvested from liquid culture. The combination of the Minilys® personal homogenizer with the E.N.Z.A fungal DNA extraction kit resulted in a successful and rapid DNA extraction from both organisms. In order to increase efficiency for high throughput screening, the method was then tested on mycelia scraped from solid state cultures. DNA was successfully extracted from both organisms thus eliminating the need for liquid cultivation.

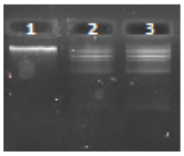

Figure 1: DNA extraction from plate mycelia performed using Minilys® bead mill, Precellys® lysing kit tubes and E.N.Z.A. fungal extraction kit on *Coprinopsis cinerea* FA2222. 1% agarose gel with gel red, lane 1: *C. cinerea* DNA, lane 2&3: 1kb molecular weight ladder.

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/ CONCLUSION

Minilys® personal homogenizer along with the appropriate lysing kit is a suitable, simple and convenient homogenization system to break open the cell wall of fungal species. Minilys® personal homogenizer combined with a suitable fungal extraction kit provides a rapid method for DNA extraction when compared with previous published protocols.

SPPP-810-126

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