Non-ribosomal peptide synthetases from the genomes of selected *Chrysoporthe* species

by
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DECLARATION

I, undersigned, hereby declare that the dissertation submitted herewith for the degree Magister Scientiae to the University of Pretoria, contains my own independent work and has not been submitted hitherto for any other degree at this or any other University.

Signature.....

Date.....

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Contents

Preface

Secondary metabolites are small molecules of low molecular weight. Fungal secondary metabolites are very diverse and play a crucial role in pathogenicity and virulence. *Chrysoporthe* species are pathogens that cause damage to various economically important trees, including *Eucalyptus*. In this dissertation, nonribosomal peptide synthatases (NRPS), one of the major classes of secondary metabolites, are studied in three *Chrysoporthe* species, namely *C. austroafricana, C. cubensis* and *C. deuterocubensis.*

A review of the main classes of secondary metabolites and the gene clusters in which they occur, as well as their role in fungi are presented in Chapter One. Strategies employed to induce gene clusters and those used to mine gene clusters from a range of fungal genomes are highlighted, and their importance in discovering and studying new secondary metabolites are also discussed.

The experimental chapter of this dissertation is directed at characterizing three *Chrysoporthe* species based on their secondary metabolites gene clusters, but specifically focusing on NRPS clusters. Using AntiSMASH, CLC Genomics Workbench, and other software, NRPS genes and gene clusters were mined and located within the individual genomes. Differential expression analyses of selected NRPS genes present in the three *Chrysoporthe* species was carried out using RT-qPCR. No significant variation were observed between the three *Chrysoporthe* species based on their secondary metabolites. NRPS genes putatively involved in siderophore production were also present in three *Chrysoporthe* species. A fraction of NRPS gene were found to be upregulated when exposed to near Ultraviolet light.

Chapter one

Literature Review

Secondary metabolite gene clusters in ascomycetes

Introduction

Fungi produce primary and secondary metabolites, which are both essential for fungal survival (Brakhage and Schroech, 2011). Primary metabolites are those compounds that are essential for metabolic functions and are required for growth and development (Brakhage and Schroech, 2011). Secondary metabolites are low molecular weight compounds that are needed for secondary activities, such as communication, increase in fitness, as well as decrease in the fitness of surrounding organisms (Brakhage, 2013; Netzker *et al*., 2015). These compounds are produced by a limited number of living organisms and, in addition to the functions of primary metabolites, they protect against herbivores and/or ultraviolet radiation (Frisvad *et al*, 2008). Secondary metabolite producing organisms range from bacteria, fungi, viruses, plants and algae to protozoa and metazoans (Brakhage and Schroech, 2011). Since fungi live in complex ecosystems, it is unsurprising that they communicate and compete for available resources with other organisms (Kusari *et al*., 2009). For this reason, secondary metabolites evolved as chemical signals for communication and defense in fungi (Brakhage and Schroech, 2011).

Although secondary metabolites are not important for growth and development, they provide other environmentally important benefits, for example, by supporting plant survival. They are also primary targets for the development of a variety of medicines such as antibiotics (e.g. penicillin and cephalosporin), immunosuppressants (e.g. cyclosporins), cholesterol-lowering drugs (e.g. statins), angiogenesis inhibitors (e.g. fumagillin and pseurotin derivatives), anti-osteoporosis agents (e.g. orsellinic acid derivatives), anti-migraine (e.g. ergot alkaloids) and hypertension-lowering medications (e.g. gibberellins), as well as plant growth hormones and food additives (e.g. carotenoids) (Keller *et al*., 2005; Wiemann and Keller, 2014). Many of the chemicals that are employed in the agricultural sector are derived from natural products (Asolkar *et al*., 2013; Rimando and Duke, 2006).

Primary and secondary fungal metabolites have been studied since 1922 (Raistrick, 1950). However, secondary metabolites were not extensively studied until the discovery of penicillin from *Penicillium chrysogenum* in 1929 by Alexander Fleming (Keller *et al*., 2005). Hadacidin (N-formyl hydroxyaminoacetic acid) is one of the compounds found in *Penicillium* spp., including *Penicillium frequentans*, and has been regarded as the simplest fungal secondary metabolite (Dulaney and Gray, 1962). Studies have estimated that, from more than 1.5 million fungal species, only 7% have been investigated for bioactive

compounds (Hawksworth, 2004; Yeh and Kirschner, 2019; Bandaranayake, 1998). However, it is very difficult to estimate given that there are far more species that may have been studied but not reported and the percentage is only based on the described work (Miyamoto *et al*., 2014).

Recent studies showed that some secondary metabolites of fungi play significant roles in pathogenicity and/or virulence (Collemare *et al*., 2008; Haas *et al*., 2008). Siderophores and melanin are some of the examples of secondary metabolites that play a role in fungal pathogenicity and virulence. They sequestrate iron from host (siderophores) and produce molecules that are toxic to the host (melanin) (Scharf *et al*., 2014).

Natural products produced by bacteria and fungi are rich in novel bioactive compounds (Medema *et al*., 2011; Andersen *et al*., 2013). To date, about 500 000 natural products have been documented. From these, about 15,600 are of fungal origin (Bérdy, 2012; Nett *et al*., 2009). In fungi, these natural products are produced by genes that are located in the same genome region, i.e. they are clustered and co-expressed. These clustered genes are expressed conditionally, and some are silent under laboratory conditions (Khaldi *et al*., 2008). Several methods have been developed to aid in the activation of silent genes in a cluster, while computer software had been developed to aid in their discovery and annotation (Brakhage and Schroeckh, 2011; Chiang *et al*., 2011).

Secondary metabolites are classified according to which precursor molecules from primary metabolism are used for their biosynthesis, and which specific enzyme class synthesizes their basic chemical structure (Hoffmeister and Keller, 2007). There are four major classes of secondary metabolites, which are the polyketides, non-ribosomal peptides, terpenes, and prenylated tryptophan derivatives. Polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs), terpene cyclases/ synthase (TCs) and dimethylallyltryptophan synthases (DMATSs) are the enzymes involved in the biosynthesis of these secondary metabolites, respectively. These enzymes catalyze the initiation step of secondary metabolite biosynthesis. The enzyme that catalyzes the initiation step determines the class of the secondary metabolites produced (Hoffmeister and Keller, 2007; Khaldi *et al*., 2010; Wiemann and Keller, 2014).

This review will focus on the fungal secondary metabolite gene clusters and their products that contribute to pathogenicity and virulence. Different classes of secondary metabolites are described together with mechanisms employed in producing one, with a special focus on

NRPSs. In addition to this, the review also describes current methods that aid in the activation of secondary metabolite gene clusters.

Polyketides

Polyketides are a class of secondary metabolites that are abundant in fungi (Staunton and Weissman, 2001). They are structurally diverse, ranging from small molecules like aflatoxin to large, complex structures like melanins. They are made from short chain carboxylic molecules, such as acyl coenzyme A (CoA). This process resembles that of fatty acid biosynthesis (Keller *et al* 2005; Xue *et al*., 1999). Thus far, roughly 7000 polyketides have been identified (Keller *et al*., 2005).

There are three types of polyketides based on their method of assembly, namely modular (type I PKSs), iterative (type II PKSs) and type III PKSs (Kennedy *et al*., 1999). Bacterial polyketides consist of both the modular and iterative systems. Modular system consists of a series of domains from which each domain carries out one cycle of polyketide chain elongation. In contrast to Modular system, Iterative system consist of fewer domains that each carries out multiple cycles of chain elongation (Crawford and Townsend, 2010). The majority of polyketides in fungi are synthesized by the type I system, which consists of multi-domain proteins of up to thousands of amino acids that are related to eukaryotic fatty-acid synthases and contain similar domain structures (Keller *et al*., 2005; Cox, 2007). They are widely distributed in fungi, with *Saccharomycetes* and *Schizosaccharomycetes* being the exceptions in ascomycetes, while *Zygomycetes* and *Chytridiomycetes* also do not possess these proteins (Kroken *et al*., 2003).

In both plants and fungi, PKS III enzymes are much smaller than type I PKSs and consist of a single multifunctional protein domain. The number of genes encoding the type III proteins in the fungal genome is also lower than those encoding for type I PKSs. In filamentous ascomycetes there is usually a single type III PKS gene per genome, while such genes are absent in basidiomycetes, non-filamentous ascomycetes, chytrids and zygomycetes (Teichert and Nowrousian, 2011). Type II PKSs enzymes consists of maximum of two domains, of which each domain is used multiple times (iterative) in the synthesis of PKS product (Hertweck *et al*., 2007)

In fungal PKSs, the acyltransferase (AT), acyl carrier (ACP), and ketoacyl CoA synthase (KS) domains are crucial for polyketide synthesis, whereas the dehydratase (DH), enoyl

reductase (ER) and ketoreductase (KR) domains that are required for ketone reduction in fatty acids are not present in all fungal PKS enzymes. Like fungi, bacteria also have multi-domain type I polyketide synthases. An example is the antibiotic erythromycin, which is produced by the enzyme 6-deoxyerythronolide B synthase (DEBS) (Keller *et al*., 2005).

Each domain in the module has a specific function in the production of polyketides. The process of loading of starter, extender and intermediate acyl units is facilitated by the enzyme AT. Thereafter, the ACP holds the growing macrolide as a thiol ester while extension is facilitated by the KS (also called the b-keto-acyl synthases). KR is responsible for the first reduction of ketone groups to an alcohol (hydroxyl groups). DH eliminates a water molecule to yield an unsaturated thioester, final reduction to full saturation is facilitated by the ER, and the release of the macrolide and its cyclization is catalyzed by TE (Cox, 2007).

The structural diversity of fungal polyketides results from the iteration reaction number, *i.e.*, the reduction reaction number. During iterative reaction, the extender unit is used repeatedly followed by cyclization of the nascent polyketide chain in aromatic polyketides. PKS diversity is also brought about by the introduction of different post-polyketide synthesis steps (Keller *et al*., 2005).

Polyketides can act as virulence or pathogenicity factors of plant pathogenic fungi (Van der Does and Rep, 2007; Rose *et al*., 2002). An example is T-toxin produced by *Cochliobolus heterostrophus*, which is the causal agent of southern corn leaf blight (Kono *et al*., 1980). Yang *et al* (1996) has found that, disruption of *PKS1* gene which encodes a PKS causes a loss of ability to produce T-toxins. The reduced virulence to T-cytoplasm maize accompanies *PKS1* desruption undicates that T-toxin is an essential factor in pathogenesis of *C. heterostrophus* race T. Additionally, Vining (1990) suggested that soil-dwelling fungi produce polyketides and other secondary metabolites to protect against predators such as arthropods or nematodes, and to inhibit competing microorganisms. Although there is little evidence for this hypothesis, a recent comparative study by Rohlfs *et al*., (2007), during which the fitness of mutant strains was compared with that of wild-type strains, showed that a *LaeA* mutant of *Aspergillus nidulans* is preferentially consumed by the fungivorous springtail *Folsomia candida* (Rohlfs *et al*., 2007).

Melanin is a large group of diverse substances with similar properties that can be synthesized using various pathways (Wheeler and Bell, 1988), including oxidative polymerization of indolic or phenolic compounds (Langfelder *et al*. 2003). Fungal melanin has a range of

functions, such as protection from various environmental stresses and a role in pathogenicity (Teichert and Nowrousian, 2011). 1,8-dyhydroxynaphthalene(DHN) has been reported as a virulence secondary metabolite in fungi. It is required by plant pathogenic fungi for the penetration of the host, which is a process mediated by appressoria. Appressorium is a specialized cell of many plant pathogenic fungi used to infect host plants (Griffiths *et al*., 2018). *Magnaporthe grisea* is one of example of fungi that causes rice blast through the involvement of DHN melanin (Howard and Valent, 1996)

Biosynthesis of melanin in fungi can be achieved via at least four different pathways, including from tyrosine via L-3,4-dihydroxyphenylalanine (DOPA), from catechol, from g-glutaminyl-3,4-dihydroxybenzene (GDHB), or from 1.8-dihydroxynaphthalene (DHN) (Bell and Wheeler, 1986). In several basidiomycetes, GDHB and catechol-derived melanin has been described (Wang *et al*., 1996). Most fungi, however, either produce DOPA or DHN-derived melanin (Teichert and Nowrousian, 2011). A study by Yu *et al*. (2015) identified and demonstrated one of the sixteen PKS genes (*pks1*) to be responsible for the biosynthesis of melanin in the conidia of *Pestalotiopsis microspora* NK17.

Fungal melanin is thought to protect fungi from various environmental stresses such as UV radiation, host immune system, desiccation, enzymatic lysis, predators, or even radioactivity (Bell and Wheeler, 1986; Gómez and Nosanchuk, 2003). In melanin deficient *A. alternata* and *A. rabiei* strains, conidia and pycnidia were more sensitive to light than wild-type structures (Kawamura *et al*., 1999; Akamatsu *et al*., 2010). Thus, the high diversity of fungi and their ability to exploit extreme environments can also be linked to melanin production (Kawamura *et al*., 1999). Fungal melanin can also facilitate growth during ionizing radiation (Dadachova *et al*., 2007), which changes the electronic properties of melanin to enhance growth by energy capturing and utilization.

Non-ribosomal peptide synthetases (NRPSs)

Non-ribosomal peptide synthetases (NRPSs) are multimodular megasynthetases involved in the biosynthesis of small bioactive peptides by a mechanism that is independent of ribosomes. Such synthesis is similar to polyketide synthesis in that it involves multidomain enzymes, but different in the sense that protein synthesis takes place in the absence of ribosomes. This mechanism involves a thiotemplate (Bushley and Turgeon, 2010), which is produced when amino acid residues from adenylates are transferred to thiol groups of a peptide synthetase. The amino acid residues on a phosphopantetheine remains bound during

the synthesis and this process continues until the mature peptide is cleaved off with the involvement of a cyclizing transpeptidation reaction (Bushley and Turgeon, 2010).

NRPS proteins are encoded by the *NRPS* genes in the genomes of both fungi and bacteria (Bushley and Turgeon, 2010), but not animals or plants. These genes code for enzymes involved in the biosynthesis of a large diversity of chemical products. In ribosomal peptide synthesis, only 20 different proteinogenic amino acids are involved during translation. In contrast to proteinogenic amino acid, nonproteinogenic amino acids include thousands of amino acids that are not involved in ribosomal translation (Caboche *et al*., 2010). In NRPS biosynthesis, both proteinogenic and nonproteinogenic amino acids are used, and this is one of the reasons for the structural diversity in non-ribosomal peptides (NRPs) (Caboche *et al*., 2010). Synthesis of peptides in this fashion is possible because of the availability of both L and D forms of the 20 amino acids as substrates used in ribosomal protein synthesis, as well as non-proteinogenic amino acids such as ornithine, and hydroxyl acids such as α-butyric acids and α-aminoadipic acid (Finking and Marahiel, 2004).

The first fungal NRPS to be identified is δ-(l-α-aminoadipyl)-l-cysteinyl-d-valine synthetase (ACVS). This NRPS catalyzes the first step involved in the biosynthesis of cephalosporin and penicillin (Keller *et al*., 2005). However, it is only recently that NRPSs were found to play a fundamental role in fungal pathogenic and reproductive development, stress management, cell surface properties, morphology and prominent nutrient production (Bushley and Turgeon, 2010). These functions can be harvested in pharmaceuticals such as antibiotics, anticancer agents, and immunosuppressants (Oide *et al*., 2006).

For peptide synthesis to be complete, NRPS proteins use a set of core domains organized into modules (Finking and Marahiel, 2004; Hoffmeister and Keller, 2007). Each module minimally consists of three core domains, namely adenylation, thiolation, and condensation domains. The adenylation domain recognizes and activates the substrate through adenylation with ATP. A peptidyl carrier protein (PCP) domain binds the activated substrate to the 4-phosphopantetheine (PP) cofactor. Subsequently, the condensation process occurs at the megasynthase complex, with the help of thioester which transfers the substrate to a domain that catalyzes the peptide bond formation between substrate domains (Hoffmeister and Keller 2007). The number and the order of the module determines the length and the type of the product produced. Figure 1 shows an example of NRPS module order which contains a series of domains within them.

A recent phylogenetic study revealed that fungal NRPSs fall into two major groups, and these groups can be further subdivided into distinct subfamilies (Bushley and Turgeon, 2010). The first group of NRPSs are mono/bi-modular and these have more ancient origins and more conserved domain architecture than the second group, which consists of multimodular NRPSs. The first group is comprised of bacterial NRPSs that are clustered with fungal NRPSs, whereas the second group consists only of fungal NRPSs (Bushley and Turgeon, 2010).

Similar to the PKS genes, there is evidence for horizontal gene transfer of several *NRPS* genes from bacteria to fungi (Bushley and Turgeon, 2010). An example involves well-studied genes encoding the ACV synthetases involved in the biosynthesis of beta-lactam antibiotics in *Aspergillus nidulans*, *Acremonium chrysogenum*, and *Penicillium chrysogenum* (Landan *et al*., 1990; Penalva *et al*., 1990; Buades and Moya, 1996; Bushley and Turgeon 2010).

The functions of NRPS range from the producer's cellular metabolism to roles in stress response, pathogenicity and development (Oide *et al*., 2006, 2007). The more conserved mono-modular and bi-modular NRPSs are often involved in more basic cellular processes. Conversely, the multimodular NRPSs from the second group of fungal NRPSs are thought to play a role in species-specific or even strain-specific processes, which is consistent with the classical definition of secondary metabolites (Bushley and Turgeon, 2010).

Siderophores are iron chelators of low molecular mass that can either be used for intracellular iron storage or secreted for acquisition of extracellular iron. The biosynthesis of siderophores is catalyzed by the enzymes that play a role in basic metabolism, which are the second group of NRPSs (Haas *et al*., 2008). In fungi, siderophores also play roles in their biology, such as in homeostasis (Haas *et al*., 2012).

Several NRPSs are pathogenicity factors in plant pathogenic fungi (Scharf *et al*., 2014). These peptides are produced mostly by multimodular NRPSs with restricted phylogenetic distribution and often act as host-specific toxins. Examples of such secondary metabolite toxins include HC-toxin from *Cochliobolus carbonum* (Panaccione *et al*., 1992), AM-toxin from *Alternaria alternata* (Johnson *et al*., 2000), enniatin from *Fusarium avenaceum* (Herrmann *et al*., 1996), and sirodesmin from *Leptosphaeria maculans* (Gardiner *et al*., 2004; Elliott *et al*., 2007).

Terpenes

Terpenes represent a class of secondary metabolites that includes the carotenoids, gibberellins, indole-diterpenes, trichothecenes and aristolochene from fungi, and well-known metabolites such as turpentine and camphor synthesized by plants (Hoffmeister and Keller, 2007). They are composed of activated isoprene units that can be linear or cyclic, and that can be modified in various ways. Isoprene is a hydrocarbon molecule of five carbon atoms, thus a typical terpene is usually a multiple of five-carbon structures. In contrast to terpenes, terpenoids are modified class of terpenes which consists of different functional groups and oxidized methyl groups moved or removed from various positions. An example of modified terpenes is terpenoids or isoprenoids, which are formed from the oxidation of terpenes. Terpene molecules like sterols, *e.g.* carotenoids, are important for basic cellular functions in many organisms and they also serve as communication molecules in fungi (Gershenzon and Dudareva, 2007). Many terpenes are aromatic and thus function they play a role in protecting the producing organism (Pichersky *et al*., 2018). Another example of terpene includes carotene-derivative, which has been found to function as communication molecules (Wöstemeyer *et al*., 2016). The defining enzyme in terpene synthesis is TC, which is essential for the production of different terpenes from diphosphates. Apart from TC, there are many other enzymes which play a role in chain elongation, cyclization, and functionalization. TC is highly variable in sequence and difficult to detect by bioinformatics methods (Keller *et al*., 2005; Townsend, 1997).

Analyzing the fungal genomes for the genes involved in trichothecenes biosynthesis (Hohn and Beremand, 1989; Brown *et al*., 2004), gibberllin biosynthesis (Bömke and Tudzynski, 2009), and indole-diterpenes biosynthesis (Zhang *et al*., 2004) has revealed that the terpenes encoding genes, like most other secondary metabolites, were clustered in the genomes of fungi.

Indole alkaloids

Indole alkaloids also form part of the natural products synthesized by fungi. The first step in the synthesis of indole-alkaloids is prenylation of tryptophan by DMATS (Tsai *et al*. 1995). Following methylation of dimethylallyl tryptophan, a series of oxidation steps proceed through agroclavine to lysergic acid. Lysergic acid is then activated by a single-module NRPS, condensed with a tripeptide that is produced by a second NRPS, and released as ergotamine (Keller *et al*., 2005). Since DMATS genes are present in the genomes of many

fungi (Tsai *et al*. 1995), it is likely that the production of ergot alkaloids or similar molecules is an ancestral trait.

The biosynthesis of alkaloids is not dependent on a specific enzyme, which is in contrast with PKS biosynthesis. Like NRPS, alkaloids show a large degree of chemical diversity. The loline and ergot alkaloids are the best studied alkaloids, with the latter being known for their pharmacological properties (Hoffmeister and Keller, 2007).

Gene clusters

Secondary metabolites are produced by genes that are co-located in a genome, and usually co-expressed. Such groupings of genes are known as gene clusters, also referred to as biosynthesis gene cluster (Keller *et al*., 2005). In fungi, the evolutionary mechanism that led to the clustering of these genes is still unclear, although natural selection can be implicated (Khaldi *et al*., 2008). Additionally, only a small proportion of known gene clusters are conserved between different species (Khaldi *et al*., 2008; Brakhage and Schroeckh, 2011).

The clustering of these genes is often near the end of chromosomes, such as in the sub-telomeres, and clusters can be structurally highly diverse (McDonagh *et al*., 2008; Palmer and Keller, 2010; Perrin *et al*., 2007) and often species-specific (Teichert and Nowrousian, 2011). These chromosomal regions are where transcription is impacted by chromatin modifiers (Palmer and Keller, 2010). In addition to location, the order of biosynthetic genes is crucial for their regulation (Palmer and Keller, 2010). There are several types of secondary metabolite gene cluster variants. These gene cluster variants are based on the genetic variability of the secondary metabolite gene cluster. Examples of the variants are those that were observed in *Aspergillus fumigatus*, and they include single-nucleotide polymorphisms and indels, gene content polymorphisms, whole gene cluster polymorphisms, cluster idiomorphs, and mobile clusters (Lind, 2017).

Distantly related fungal species can have one or more secondary metabolite in common. For example, Cytochalasin D, which prevents cytoplasmic cleavage in fungi, is produced by *Coriolus vernicipes* (Basidiomycetes), *Zygosporium masonii* (Zygomycetes), and *Metarrhizium anisopliae*, *Engleromyces goetzii*, and *Hypoxylon terricola* (Ascomycetes) (Cole *et al*., 2003; Frisvad *et al*., 2008). This can be due to horizontal gene transfer (HGT) of gene clusters between species. However, other examples include arabenoic acid (also known as verrucolone) produced by *Penicillium* subgenus *Penicillium* series *Verrucosa*, and

penicillic acid produced by all species in *Aspergillus* section *Circumdati,* which are thought to have arisen independently (Frisvad *et al*., 2008). In addition, similar products can be produced by both plants and animals, although via different enzymatic pathways. Gibberellins is one of the examples, which is a product of both plants (diterpernoid plant hormones) and fungi (Bömke and Tudzynski, 2009)

One core biosynthetic gene is generally contained within a gene cluster (Brown *et al*., 2015). This core gene codes for PKS, NRPS, TC, or DMATS. Modifications of the chemical scaffold of a secondary metabolite is catalyzed by enzymes encoded by other genes in a cluster. Such modifications include acylation, cyclization, or oxidation. At least one transcription factor is usually also encoded by most gene clusters (Keller *et al*., 2005; Brown *et al*., 2015). This transcription factor regulates gene expression within the cluster, as well as transcription of a transporter protein that facilitates the movement of secondary metabolites out of the cell (Brown *et al*., 2015).

Recombinant DNA technology has aided in the discovery of novel secondary metabolite biosynthesis gene clusters. These discoveries led to the development of genetic engineering approaches to produce new bioactive compounds (Niehaus *et al*., 2014). However, most of these potential secondary metabolites are only predicted by bioinformatics analyses of putative gene clusters (Niehaus *et al*., 2014; Weber *et al*., 2015). These secondary metabolites are mostly not produced under laboratory conditions, or they are present at levels that are too low to detect by standard methods (Breitling *et al*., 2013). Nonetheless, Lim *et al*. (2012) showed that some secondary metabolites can be induced *via* genetic manipulation.

Two challenges limit access to these potentially important natural products. The first is the inability to cultivate new strains of fungi that are potential producers of useful natural products, while the second challenge is the observation that most secondary metabolite gene clusters are silent under laboratory conditions. However, numerous methods have been developed to activate such silent gene clusters (Lee, 2010; Brakhage and schroeckh, 2011).

Activation of secondary metabolite gene clusters

The general lack of characterized secondary metabolite gene clusters can be ascribed to the fact that most of these clusters are transcriptionally silent under laboratory conditions (Brakhage and Schroeckh, 2011). However, some of these clusters can be transcriptionally induced (Brakhage and schroeckh, 2011; Brakhage *et al*., 2008; Netzker *et al*., 2015). For

example, treatment with epigenetic modifiers (Cichewicz *et al*, 2010; Nützmann *et al*., 2011; Brakhage, 2013), genetic engineering (Bergmann *et al*., 2007), mutagenesis by one strain-many compound (OSMAC) (Bode *et al*., 2002), and co-cultivation (Bertrand *et al*., 2014) can be used to activate gene clusters.

Epigenetic modification is the stable genetic modification that leads to changes in gene expression without changes in the genetic sequence. Activation of fungal secondary metabolite gene clusters using epigenetic modifying agents has only recently been explored. One example is the increased production of carotenoids from *N. crassa* using 5-azacytidine (Cichewicz *et al*., 2010). Roles of carotenoids is to provide pigmentation to the producer and thus protecting against UV light (Goodwin., 1952).

Genetic engineering of secondary metabolite gene clusters involves mining for available cryptic gene clusters that might code for biosynthesis of secondary metabolite products (Brakhage, 2013). One example was the study by Bergmann *et al*. (2007) which involved the induction of a silent metabolite in *Aspergillus nidulans*, which led to the discovery of a novel PKS-NRPS hybrid.

Mutagenesis by one-strain many compounds is one of the methods used to activate silent secondary metabolites. The concept came about due to the idea that one strain has the potential to produce many compounds, despite a subset of these compounds being produced only under specific conditions (Romano *et al*., 2018). Variation of different parameters can achieve these results. These parameters include salinity, temperature, aeration and the shape of the flasks in which strains are cultured. One example is fungus ochraceous which produced 15 compounds when cultivation conditions are changed. Only a subset of these metabolites are produced under normal growth conditions (Bode *et al*., 2002; Romano *et al*., 2018).

Co-cultivation of fungi with bacteria or other fungi mimics the physiological conditions that occur in a particular habitat, which triggers communication and competition, leading to the production of secondary metabolites (Netzker *et al*., 2015). For example, the interaction between *Streptomyces rapamycinicus* and *A. nidulans* leads to the activation of a silent fungal gene cluster (Schroeckh *et al*., 2009; Nützmann *et al*., 2011), while there are several examples in fungus-fungus co-cultures (Zhu *et al*., 2011; Degenkolb *et al*., 2002; Li *et al*., 2014).

Favorable conditions are required for fungi to reproduce sexually or asexually through sporulation. One of the factors that promotes sporulation is light exposure, to which light-sensitive fungi respond. These light-sensitive fungi respond to light wavelengths between 350 nm and 510 nm, while some fungi can respond to ultraviolet radiation of less than 370 nm (Honda and Yunoki, 1978; Barnett and Binder, 1973). For example, *Botrytis cinerea* produces asexual spores (conidia) only when irradiated with blue-violet light (short wave ligh), and under no other wavelengths (Canessa *et al*., 2013). Also, during sexual sporulation of fungi, the mating-type genes are expressed (Moore-Landecker, 1983; Honda and Yunoki, 1978). Thus, these genes and gene clusters can be induced by exposing the fungi to light (stress) of the appropriate wavelength. In addition to light, other conditions that can promote sporulation include nutrition, humidity, pH, temperature, gravity and aeration (Moore-Landecker, 1983).

Secretion of secondary metabolites

To fulfil their functions in communication, protection and competition, most secondary metabolite products are secreted. The role of transmembrane proteins in secondary metabolite biosynthesis and secretion is thus becoming clearer. These proteins include an efflux system for excretion of industrially important compounds (Martin *et al*., 2005). These compounds are usually detoxified by the producer strains to avoid producers' suicide. Thus, fungal strains that produce these compounds have evolved a resistance mechanism for protecting themselves against their own products (Martin *et al* 2005). These mechanisms include the development of membrane permeability which is coupled with efficient efflux mechanisms that then remove the toxic molecules from the cell. They also include the sequestration or inactivation of intracellular toxic molecules and their biologically active precursors. Lastly, they replace and manipulate specific drugs target sites (Cundliffe, 1991).

In fungi, the mechanism employed to secrete secondary metabolites has been well documented and has been extensively studied in fungi that produce antibiotics and mycotoxins as a secondary metabolite product (Martin *et al*., 2005). In addition to classical biochemical and genetic studies, genomic sequence data have revealed that fungi have multidrug transporters, which facilitate the secretion of secondary metabolites. These transporter (membrane) proteins recognize and secrete a wide range of structurally different compounds from the cytoplasm to the outer medium (Martin *et al*., 2005; Goossens *et al*., 2003).

Thioesterase is an enzyme that catalyzes the release of synthesized secondary metabolite products (Kohli *et al*., 2002). The thioesterase domain is fused at the end of an appropriate module of the megasynthase, together with other optional domains including epimerization (E) and N-methylation domains (Keller *et al*., 2005). The enzyme has been extensively studied in polyketide synthesis, where the mechanism involves the transfer of the linear peptide from the terminal PCP domain to the active site serine in the TE domain to form a peptide-O-TE intermediate and then later the diacylation process (Grünewald and Marahiel, 2006). The diacylation process can either be through hydrolysis to release the linear peptide or cyclization in the case of cyclic products. The domain has gained much attention in NRPS research because it controls the last step of NRPS product biosynthesis (Kohli, 2002). Therefore, if the product is destined to be released, transporter proteins are crucial for the transport of the product out of the cell.

Transporter proteins are structurally diverse and include the Major Facilitator Superfamily (MFS), ATP-binding cassette (ABC), Small drug resistance (SMR), and resistance nodulation determinants (RNDs) (Pao *et al*., 1998; Georgiev, 2000). Multidrug transporters are further divided into two major classes, namely primary active transporters and secondary multidrug transporters. The primary class of transporter protein includes the ABC transporter, which requires ATP for transportation. Conversely, secondary transporter proteins include multidrug transporters, which utilize transmembrane electrochemical drive for the expulsion of the product out of the cell and includes MFS, SMR, and RND transporters (Martin *et al*., 2005).

Model organisms for the study of secondary metabolites

Model organisms provide a framework to develop and optimize methods for standardized analysis (Karathia *et al*., 2011). They are also useful to serve as a reference (Müller and Grossniklaus., 2010). These organisms are chosen because of their size, generation time, manipulation characteristics, genetics, conservation of mechanisms, and potential economic benefits (Karathia *et al*., 2011).

Secondary metabolites have been well studied in *Aspergillus* species. This species enabled the discovery that secondary metabolite biosynthesis gene clusters contain specific transcription factors within the cluster. A study by Brown *et al*. (1996) also found that transcription factor *aflR* regulates aflatoxin clusters in *Aspergillus flavus* and *A. parasiticus* and the *sterigmatocystin* cluster of *A. nidulans* (Brown *et al*., 1996). *LaeA*, which is a nuclear

protein in aspergilli, is also a master regulator of secondary metabolite biosynthesis. It was also shown that disruption of the *LaeA* gene resulted in strains producing lower levels of secondary metabolites (Bok *et al*., 2004). To date, there a number of *Aspergillus* genomes that have been sequenced. *A. fumigatus, A. niger, A. flavus*, *A. oryzae*, *A. fumigatus*, and N. *fischeri* are few of the *Aspergillus* species with available genomes (Rokas *et al*., 2007).

Saccharomyces cerevisiae is one of the fungi that had been studied for centuries and is a model organism in molecular and cell biology, much like *Escherichia coli* is the model bacterium. The organism's genome was fully sequenced in 1996 (Goffeau *et al*., 1996; Duina *et al*., 2014) whereafter it was found that *S. cerevisiae* can produce a plethora of secondary metabolites that includes all principal classes of secondary metabolites found in nature (Huang *et al*., 2008).

Another well-studied fungal species is *Neurospora crassa*. In the twentieth century, *N. crassa* has been a central organism for the study of genetics, biochemistry and molecular biology. Draft sequence has revealed that the *N. crassa* genome is much larger than that of *S. pombe* and that of *S. cerevisiae*, having more than 40 megabases (mb; with both yeast species having about 12mb). It was also predicted to contain 10 082 protein coding genes, which is twice as many as *S. cerevisiae* (Galagan *et al*. 2003). Recent studies on secondary metabolites of *N. crassa* involved the investigation of their biological activity against bacteria and other fungi (Kumar *et al*., 2015).

Chrysoporthe

Chrysoporthe species are important canker pathogens of economically important *Eucalyptus* species, which are grown in plantations in both tropical and subtropical areas worldwide (Nakabonge *et al*., 2006). *Chrysoporthe* canker was previously reported to be caused by *Cryphonectria cubensis* (Gryzenhout *et al*., 2006). The genus was established in 2004 to include phylogenetically related pathogens that cause disease of *Myrtales*. To date, eight *Chrysoporthe* species have been documented and they include *C. austroafricana* (Gryzenhout *et al*., 2004), *C. cubensis* (Myburg *et al*., 2002), *C. deuterocubensis* (Van der Merwe *et al*., 2010), *C. doradensis* (Gryzenhout *et al*., 2005), *C. hodgesiana* (Gryzenhout *et al*., 2006), *C. inopina* (Gryzenhout *et al*., 2004), *C. syzygiicola* (Chungu *et al*., 2010), *and C. zambiensis* (Chungu *et al*., 2010). This review will only focus on three closely related species, namely, *C austroafricana, C. cubensis* and *C. deuterocubensis.*

Chrysoporthe austroafricana

This fungus is the causal agent of stem cankers found in *Malestomataceae* and *Myrtaceae* family, for example the *Tibocina spp*. and *Eucalyptus spp*., respectively. It was first isolated on *Eucalyptus grandis* during the late 1980s in northern Kwazulu-Natal. There is substantial evidence that the species is native to Africa, which is based on its widespread presence and pathogenicity on native *S. cordatum* and *S. guineense* in southern African countries, with *S. cordatum* being more tolerant to the infection by this pathogen than non-native *Eucalyptus* clones (Vermeulen *et al*., 2013, 2011). It has been subsequently reported in Mozambique, Malawi, Zambia and Namibia (Nakabonge, 2006; Roux *et al*., 2006). Despite extensive collection from other *Eucalyptus* growing region of the world, the specie has not been discovered elsewhere (Vermeulen *et al*., 2013; Nakabonge *et al.*, 2007). One other evidence that supports the origin of the species being South African was the high population diversity that was observed for *C. austroafricana*, which was higher in the countries (Vermeulen *et al*., 2013).

Chrysoporthe Cubensis

Chrysoporthe Cubensis is the pathogen for *eucalyptus* species. Disease induced by this species is considered to be the most destructive among the *Eucalyptus* trees and the cankers caused by this species is found at the base of the tree (Van Heerden and Wingfield, 2001; Nakabonge *et al*., 2007). Bruner first described this species as *Diaporthe cubensis* Bruner in 1917, which was then transferred to genus *Cryphonectria* by Hodge in 1980 as *C. Cubensis* (Nakabonge *et al*., 2007). This fungus species was isolated at countries such as South America, central and North America, Asia and Africa, also in Australia, where it was observed to be expressing different symptoms of the disease which gave the first clue of the difference between pathogen between the countries (South Africa from other countries) (Gryzenhout *et al*., 2004; Nakabonge *et al*., 2007).

Both *Eucalyptus spp*. and *S. aromaticum*, the first known hosts of *C. cubensis*, belong to the family *Myrtaceae*, order *Myrtales* ((Nakabonge, 2006). The order *Myrtales* includes a relatively large number of families, namely the *Alzateaceae, Combretaceae, Crypteroniaceae, Heteropyxidaceae, Lythraceae, Melastomataceae, Memecylaceae, Myrtaceae, Oliniaceae, Onagraceae, Penaeaceae, Psiloxylaceae, Rhynchocalycaceae* and *Vochysiaceae* (Nakabonge, 2006).

Chrysoporthe deuterocubensis

Chrysoporthe deuterocubensis is primarily found in Southeast Asia and it has been suggested to be introduced to Australia, China, Hawaii and parts of east Africa (Van der Merwe *et al*., 2010). *C. deuterocubensis* were also treated as *C. cubensis* previously. It was then found that they cause significantly different lesion when tested with seven different *Eucalyptus spp*. (Chen *et al*., 2010).

To date, the genome sequences of *C. cubensis*, *C. deuterocubensis*, and *C. austroafricana* have been determined (Wingfield *et al*., 2015). However, although several studies have been conducted to determine the pathogenicity of especially *C. austroafricana* towards *Eucalyptus* species and their hybrids (Chen *et al*., 2010; Nakabonge *et al*., 2006), no research has been done to determine the potential involvement of secondary metabolites.

Future prospects

The are several classes of secondary metabolites that are produced by filamentous fungi. Most classes have been documented to play a role in pathogenicity or to act as virulence factors in fungi. Within the secondary metabolite classes, NRPS has recently gained a lot of attention in fungal research. This is because it has been implicated in pathogenicity or virulence.

Despite species of *Chrysoporthe* causing serious disease in various hosts, information pertaining to secondary metabolites of these species is lacking. In order to better understand the pathogenicity mechanisms that these fungi employ, studies on their secondary metabolite gene clusters are required. Thus, in this study, we aimed to characterize secondary metabolite gene clusters in three *Chrysoporthe* species, namely *C. austroafricana*, *C. cubensis* and *C. deuterocubensis*. The study will also profile the expression level of minimal genes from selected NRPS gene clusters. In addition to the available genomes, transcriptome data for *C. austroafricana* under different growth conditions, as well as *in planta*, was also available to be leveraged for expression analyses (Mangwanda *et al*., 2015).

Tables and figures

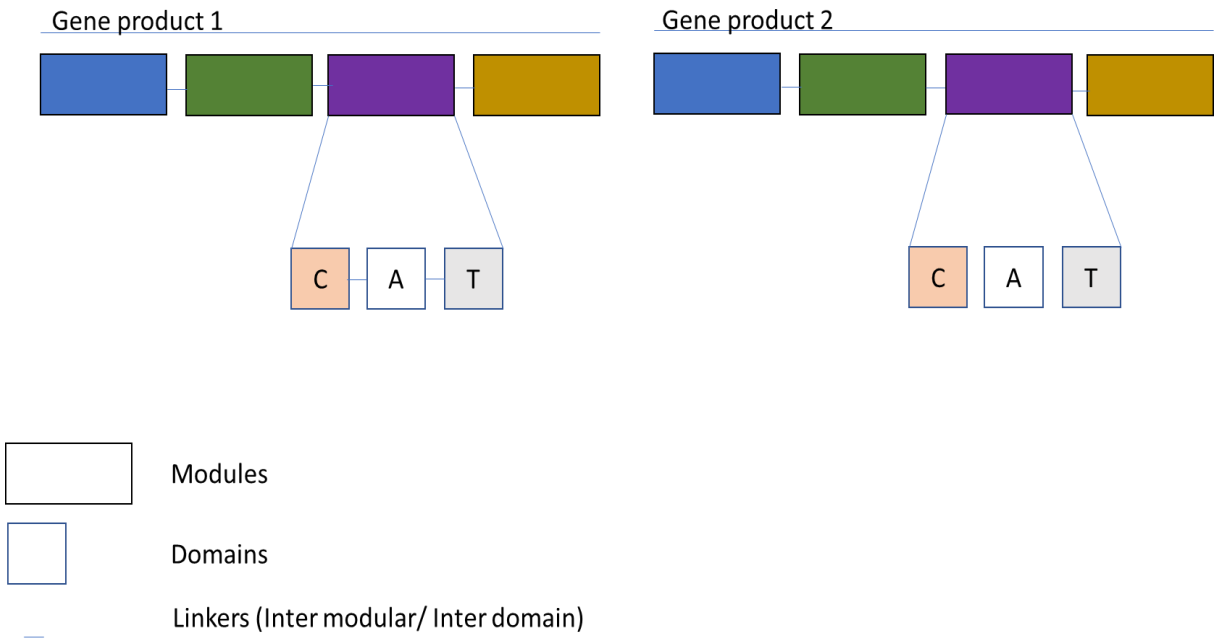

Figure 1 Modular organization of NRPS containing three critical domains, condensation (C), adenylation (A) and thiolation (T).

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Chapter two

Non-ribosomal peptide synthetase gene clusters in *Chrysoporthe* species

Abstract

Fungi produce different classes of secondary metabolites that are either beneficial or detrimental to themselves or other microorganisms in the environment. These metabolites have received great attention from researchers due to their potential for pharmaceutical drug development, and their potential applications in agriculture. Despite their benefits, secondary metabolites can also have a negative effect on other living organisms such as plants. Recent studies have suggested that secondary metabolites, especially non-ribosomal peptide synthetase (NRPS) products, play a significant role in pathogenicity and virulence of fungi. However, the secondary metabolites of the important *Eucalyptus* pathogens in the genus *Chrysoporthe* have not yet been characterized. Thus, the aim of this study was to characterize *NRPS* secondary metabolite gene clusters from selected *Chrysoporthe* species, and to profile their expression levels when exposed to ultraviolet light, which mimics stress conditions. The genomes of three *Chrysoporthe* species, namely *C. austroafricana*, *C. cubensis* and *C. deuterocubensis*, were mined for the presence of *NRPS* gene clusters using AntiSMASH software and CLC Genomics Workbench. DNA was extracted from isolates cultured in normal growth conditions and total RNA was extracted from ultraviolet exposed isolates, followed by cDNA synthesis. DNA amplification and RT-qPCR assays were carried out, for confirmation of *NRPS* genes mined and gene expression level profiling, respectively. The results report the first characterization of *NRPS* gene clusters in *Chrysoporthe* species. There was no significant variation between the three species based on their secondary metabolite gene clusters. All three species possess *NRPS* gene clusters that are putatively involved in the production of siderophores. A fraction of *NRPS* genes were upregulated when exposed to ultraviolet light. However, functional studies are needed to elucidate the functions of these genes during ultraviolet exposure.

Introduction

Filamentous fungi produce a wide range of low-molecular-mass compounds known as secondary metabolites. These molecules have a variety of functions, including communication, increasing fitness, as well as pathogenicity factors (Yoder & Turgeon, 2001). They are produced by large megasynthase enzymes (Weber *et al*., 2015). There are four major classes of secondary metabolites, namely nonribosomal peptide synthetases (NRPS), polyketides (PKS), indole alkaloids and terpenes. They are classified based on the main enzyme that catalyzes their synthesis (Keller *et al*., 2005).

NRPSs form one of the major classes of secondary metabolites that synthesize non-ribosomal peptides (NRPs). The process involves multimodular enzymes that synthesize peptides through thiotemplate, which is a mechanism that assembles amino acid chains without the involvement of ribosomes (Turgeon *et al*., 2008). This modular protein synthesizes peptides by selectively binding, activating (Keller *et al*., 2005), and condensing amino acids in an orderly manner (Marahiel *et al*., 2009). An NRPS is composed of several modules that play a role in the addition of an individual peptide. Each module consists of a number of domains, each of which codes for an enzyme that co-catalyzes the process. Minimally, NRPS contains three domains that are crucial for NRP synthesis, including adenylation (A), condensation (C) and thiolation (T). The latter is also known as a peptidyl carrier protein domain (Ansari *et al*., 2008; Rausch *et al*., 2007). The length and structure of the peptide to be released from the NRPS is generally determined by the number and the order of modules present in the NRPS (Keller *et al*., 2005). In some NRPSs, a thioesterase domain is also found at the C-terminal of the protein. This domain is thought to be involved in the release of the synthesized NRP from the NRPS (Brown *et al*., 1996; Walsh, 2004).

NRPSs catalyze the synthesis of a variety of products in fungi that confer a variety of benefits. Because of this, NRPSs also play a role in the pathogenicity of fungi (Yoder & Turgeon, 2001). Although several secondary metabolites have been found to contribute to pathogenicity, melanins and siderophores have received the most attention recently. A functional study of NRPSs revealed that these enzymes are involved in fungal virulence and pathogenicity (Lee *et al*., 2005) as several NRPS products can act as virulence factors (Oide *et al*., 2006). For example, HC toxin is required for the virulence of *Cochliobolus carbonum* race 1 on corn, enniatin for *Fusarium avenaceum* on potato, and AM-toxin for *Alternaria alternata* on apple (Castell-Miller *et al*., 2016; Scharf *et al*, 2014).

Siderophores are iron chelating molecules (Haas *et al*., 2008). Although iron is abundant in the earth's crust, its bioavailability is very low in fungi (Miethke & Marahiel, 2007). Because of the crucial role iron plays, fungi have evolved a way to sequestrate iron from their host by producing siderophore molecules upon invasion. Siderophore synthesis has been confirmed by researchers as one of the fungal virulence determinants as the sequestration of iron from the fungal host causes host stress (Lee *et al*., 2005). Siderophores are NRPs that are synthesized by an NRPS, specifically the one coded for by the Nonribosomal Peptide 6 gene (*NPS6*). Knocking down or deletion of the *NPS6* gene results in the reduction of asexual sporulation together with a reduction in virulence of *Cochliobolus heterostrophus* to the host (Oide *et al*., 2006). Additionally, *NPS6* is functionally conserved among species of filamentous fungi (Lee *et al*., 2005).

Previous studies (Chiang *et al*., 2011; Keller *et al*., 2005) showed that some of the genes in gene clusters are silent under laboratory conditions. This is one of the limiting factors to studying secondary metabolite gene clusters and their products. However, several methods have been developed to aid in the activation of the silent genes (Brakhage & Schroeckh, 2011). The key method in the activation of silent genes is variation of growth conditions to induce stress, which encompasses methods including the one-strain many compounds (OSMAC) approach, composition of media, aeration rate, vessel type and addition of enzyme inhibitors (Bode *et al*, 2002). UV light has been found to induce stress in fungi, which subsequently leads to fungal sporulation and upregulation of genes (Tisch and Schmoll, 2010; Sommer *et al*., 1989).

The genus *Chrysoporthe* accommodates eight species, namely *C. austroafricana* (Gryzenhout *et al*., 2004), *C. cubensis* (Myburg *et al*., 2002), *C. deuterocubensis* (Van der Merwe *et al*., 2010), *C. doradensis* (Gryzenhout *et al*., 2005), *C. hodgesiana* (Gryzenhout *et al*., 2006), *C. inopina* (Gryzenhout *et al*., 2004), *C. syzygiicola* (Chungu *et al*., 2010), *and C. zambiensis* (Chungu *et al*., 2010). All these species have been reported from hosts in the *Myrtales*, including *Eucalyptus*. Most of these species are well known canker pathogens of trees, and they are thus of great concern to forestry industries because they can ultimately result in the death of trees.

Despite the attention secondary metabolites have received in recent years, these compounds and the genes that produce them have not yet been elucidated in *Chrysoporthe* species. Therefore, the aim of this study was to identify and characterize all *NRPS* genes from the

three selected *Chrysoporthe* species, namely *C. austroafricana, C. cubensis, and C. deuterocubensis*. From the *Chrysoporthe* genus, the above-mentioned species have been found and documented to be closely related (Wingfield *et al*., 2015).

Materials and Methods

Fungal cultures and DNA extraction

Fungal isolates were obtained from the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria. These were *C. austroafricana* CMW2113, *C. cubensis* CMW10028 and *C. deuterocubensis* CMW8650 (Table 1). Only the isolates of the three species mentioned above have their genomes sequenced. Thus, the genome data came from single isolate per species. Isolates were grown on 2% w/v MEA plates (2% malt extract, 2% agar, Biolab, Germiston, South Africa) at 25 °C in the dark for 14 d. Mycelia were scraped from agar plates, and total genomic DNA was extracted from each isolate using the cetyltrimethylammonium bromide (CTAB)/phenol-chloroform method described by Steenkamp *et al*. (1999).

Identification and confirmation of secondary metabolite gene clusters

Secondary metabolite gene clusters in the genomes of *C. austroafricana* (Wingfield *et al*., 2015), *C. cubensis*, and *C. deuterocubensis* (Wingfield *et al*., 2015) were identified using the online prediction tool antiSMASH (http://www.secondarymetabolites.org/) (Medema *et al*. 2011; Weber *et al*., 2015). The website identifies putative gene clusters in the genome using data from NCBI. For the confirmation of gene arrangements within clusters, contigs containing gene clusters were further annotated using AUGUSTUS (http://augustus.gobics.de/). The PKS/NRPS analysis website (http://nrps.igs.umaryland.edu/nrps) and InterPro database (https://www.ebi.ac.uk/interpro/) were used to determine and confirm the organization of modules. To confirm the functions of domains, modules were submitted to BLASTP searches against the NCBI database to find known modules. The whole gene cluster as well as the individual genes were manually identified for each genome using CLC Genomics Workbench (CLC Bio-Qiagen, Aarhus, Denmark).

Reciprocal nucleotide BLAST

To investigate the presence or absence of genes and/or gene clusters in the three *Chrysoporthe* genomes, reciprocal BLAST searches in all possible pairwise combinations was performed using CLC Genomics Workbench. This was done for individual genes in

clusters, as well as for entire clusters. The genomes from two distantly related species, namely *Diaporthe ampelina* and *Valsa mali*, were also used for comparison and to investigate the possibility of horizontal gene transfers (HGT). Gene or cluster BLAST hit results greater than 80% sequence identity was deemed to be present in the target genome, and any hits below 80% were classified as nonsignificant.

Gene presence confirmation using PCR

A PCR assay was used to confirm the presence or absence of selected genes within gene clusters. From each gene cluster, at most three functionally different genes were selected, namely the core biosynthesis gene, an additional biosynthesis gene, and the transport related gene. All the three genes are crucial for the biosynthesis of secondary metabolites. These primers were tested against genomic DNA from *C. austroafricana* CMW2113, *C. cubensis* CMW10028 and *C. deuterocubensis* CMW8650.

A set of 52 primers (Table 2) were designed for standard PCR analysis using CLC Genomics Workbench based on the three selected genes. PCR amplifications were carried out using the Applied Biosystems Veriti Thermal Cycler (Applied Biosystems). Each PCR reaction consisted of 10 × PCR reaction buffer with $MgCl_2$, 1 U Kapa *Taq* polymerase (Kapa Biosystems, South Africa), 200 mM of dNTPs, 100 ng genomic DNA and 0.25 µM of each primer. Reaction volumes were adjusted to 25 µl using sterile distilled water. PCR conditions were the same for all gene regions, and they included an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 35 s, primer annealing at a specific temperature (Table 2) for 30 s, and chain elongation at 72 °C for 30 s. Reactions were concluded with a final elongation step at 72 °C for 7 min. Amplicons were separated using agarose gel electrophoresis (1% w/v agarose) and visualized under ultraviolet light using GelRed.

RNA-Seq data analysis

Expression levels of genes in gene clusters of *C. austroafricana* were investigated from already available RNA-Seq data obtained from two different experiments that were previously performed (Mangwanda *et al*., 2015). The first RNA-Seq data set was generated from RNA extracted from *C. austroafricana* (CMW 2113) cultures grown on minimal and complete media, respectively. Minimal media generally have minimum nutrients while complete media contains all the nutrients required for colony growth. *C. austroafricana* (CMW2113) was cultured on complete and minimal media until the

mycelium covered the nylon membrane. The second RNA-Seq data set was generated from two different *Eucalyptus* clones inoculated with *C. austroafricana* (CMW 2113), namely TAG5 (tolerant) and ZG14 (susceptible). *Eucalyptus grandis* genotypes TAG5 and ZG14 were inoculated with *C. austroafricana* CMW2113 (Mangwanda *et al*., 2015). The aim of the experiments was to investigate *Eucalyptus grandis-C. austroafricana* interaction and to test the hypothesis that the pathogen possesses mechanisms to modulate the tree phytohormone-mediated defenses. FASTQC v0.5 (Andrews., 2010) and FASTQ groomer v1.0.4 software packages (Blankenberg *et al*., 2010) were used to assess the quality of reads. TopHat v2.0.4 (Kim *et al*., 2013) was subsequently used to map the reads against the assembled *C. austroafricana* genome (Wingfield *et al*, 2015). Quantification of transcript abundance was performed using Cufflinks v1.03 (Trapnell *et al*., 2010) and analyzed and organized using the RStudio v1.1.456 software (Team, 2014).

Expression profiling of *NRPS* genes exposed to UV light

Four replicates of liquid malt extract (20% w/v) cultures were prepared for each of the three *Chrysoporthe* isolates and incubated at 25 °C in the dark to allow growth and acclimatization. For each isolate, two flasks were moved to a room at 25 °C flooded with near-UV light (350-510 nm, Blue light), while the remaining two remained in the dark. On the 12th day, the set of flasks incubated under near-UV conditions were exposed to brief UV (302 nm) for 2 min using an ultraviolet light source, and then returned to near-UV for 1 more day to allow recovery.

On the 14th day, mycelia were harvested from all 12 liquid cultures and frozen. RNA was extracted from frozen mycelia using a RNeasy extraction kit (Qiagen, Inc., Valencia, CA, USA) and the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from 100 ng of total RNA using the SuperScript IV Reverse Transcriptase Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), and the synthesized cDNA was treated with RNase H to remove residual RNA. To obtain the final cDNA product, a 1:2 dilution was prepared using RNase free water. Thus, there were 12 preparations of cDNA, each representing an individual liquid culture.

To profile the expression levels of individual genes of gene clusters, a quantitative reverse transcription PCR (RT-qPCR) assay was performed using a QuantStudio™ 12K Flex thermocycler (Applied Biosystems). Only expression for the genes that were common amongst the three species we profiled. Primers for 14 genes (Table 3) were designed as

previously described. For each sample, three technical replicates were used. Actin, Elongation Factor 1α, Beta-tubulin 1 and 2, 18S and 28S rDNA genes were used as internal reference genes (Table 4) to determine the expression levels of the subset of gene clusters, using the comparative CT (threshold cycles) method (Schmittgen & Livak, 2008). Differential expression was calculated to compare between expression levels of samples under standard and near ultraviolet light conditions. Relative expression levels were calculated for the combined samples using fold change calculation formula.

Results

Identification and confirmation of secondary metabolite gene clusters

The antiSMASH results showed that all genomes contained varying numbers and types of secondary metabolite gene clusters. These gene clusters included PKS, NRPS, and those for the synthesis of indole alkaloids and terpenes. In addition to these four major classes of secondary metabolite synthesis genes, phosphonates and hybrid clusters were also detected. Such hybrid clusters included combinations of PKS and NRPS, indole and PKS, and lantipeptide and PKS (Table 5). AntiSMASH predicted at least 60 putative gene clusters from the three *Chrysoporthe* species. There were more PKS gene clusters compared to the other gene clusters, with at least 20 clusters per species. AntiSMASH results also revealed that *C. austroafricana*, *C. cubensis* and *C. deuterocubensis* had 7, 6, and 8 *NRPS* gene clusters, respectively (Appendix 1-3). The additional species, *Valsa mali* and *Diaporthe ampelina*, respectively had 7 and 8 *NRPS* gene clusters. *Valsa mali* and *Diaporthe ampelina* species are closely related to *Chrysoporthe* species. Most of the NRPS genes are putatively involved in the siderophore biosynthesis.

When only the biosynthetic, regulatory and additional biosynthetic genes flanking core genes were considered, the BLAST results indicated that almost all the genes were reciprocally present between the species. However, all the minimal genes from cluster 88 of *C. austroafricana* were absent from *C. cubensis* and minimal genes from cluster 50 of *C. deuterocubensis* were absent from *C. cubensis*. Subsequently, entire gene clusters were used as BLAST queries, revealing that almost all gene clusters were present between the three *Chrysoporthe* species (Table 6).

The largest *NRPS* gene amongst the three species spanned 14736 bp, and it was shared between the three species (Table 7). This gene forms part of cluster 72 of *C. austroafricana*, cluster 28 of *C. cubensis* and cluster 20 of *C. deuterocubensis*. The smallest *NRPS*

biosynthetic gene was from cluster 50 of *C. deuterocubensis* and spanned 2926 bp, followed by a gene from cluster 32 of *C. austroafricana* which spanned 3027 bp and a gene from cluster 57 of *C. cubensis* which spanned 4716 bp, respectively. The largest cluster spanned 54736 bp and was common amongst the three species, while the smallest was cluster 57 from *C. cubensis* (24607 bp).

PCR assays confirmed the presence or absence for almost all of the genes identified by reciprocal BLAST (Table 8). Cluster 88 from *C. austroafricana* and cluster 50 from *C. deuterocubensis* was found to be absent from *C. cubensis*. Additional gene from *C. deuterocubensis* Cluster 32B was found to be absent from *C. cubensis*. Only one gene from Cluster 32 of *C. deuterocubensis* did not amplify from *C. cubensis* genomic DNA.

Differential expression of gene clusters from *C. austroafricana*

Most of the *NRPS* genes were upregulated in minimal conditions compared to the complete media (with respect to nutrients). Only one gene was highly upregulated, with 5-fold upregulation. A fraction of gene profiles was not determined by the experiment (Table 9, *in vitro* column). RT-qPCR results of RNA extracted from two *Eucalyptus* clones (TAG5 and ZAG14) inoculated with *C. austroafricana* CMW2113 showed that most of the *NRPS* genes were undetermined (N/A). However, from the fraction of the genes that were quantified, most of the genes were highly upregulated (5-fold upregulation; Figure 1).

UV induced gene expression profiling

Quantification of secondary metabolite gene expression under ultraviolet light conditions showed that a fraction of genes was silent in all three species, while others were differentially expressed between the species (Table 10). Genes 28, 46, 52, and 72 remained silent in all three species. From the genes that were expressed and quantified, most were upregulated under UV exposure compared to the controls (Figure 1). Gene 20A was highly upregulated in all three *Chrysoporthe* species. Between the three species, *C. austroafricana* showed the highest expression of this gene, with 4,5-fold change compared to the control, followed by *C. deuterocubensis* with a 2-fold upregulation.

Discussion

In this study, we identified all the secondary metabolites gene clusters present in the draft genomes of three *Chrysoporthe* species, namely *C. austroafricana*, *C. cubensis*, *and C. deuterocubensis*. We found that these species possess different numbers of *NRPS* gene

clusters. *Chrysoporthe austroafricana* has 7 *NRPS* gene clusters, *C. cubensis* has 6 and *C. deuterocubensis* has 8 gene clusters. *NRPS* genes from *Chrysoporthe* species were very large, as expected. These findings are in line with that of Wei *et al.* (2005), from which the largest fungal *NRPS* gene of 15.8 kb was identified, spanning 18 modules.

BLAST assays revealed that *NRPS* gene clusters were mostly identical between the three species. However, we found three clusters that were unique between the three species. *Chrysoporthe cubensis* lacked one cluster that was present in both *C. austroafricana* and *C. deuterocubensis*. *Chrysoporthe austroafricana* possessed all the clusters that were present between the species, but two of the clusters from *C. deuterocubensis* were incomplete. One of these clusters lacked a few bases and the other lacked two genes. Most of the *NRPS* gene clusters predicted from all three species were putatively involved in siderophore biosynthesis. However, this conclusion is only based on *in silico* data and thus functional characterization of these *NRPS* gene clusters will be necessary. The results represent the first characterization of NRPS gene clusters in *Chrysoporthe* species.

A fraction of NRPS genes in *C. austroafricana* were upregulated when the fungus was grown in minimal nutrient media. Most of the genes were down-regulated. It is unclear whether variation in nutrient composition had a down-regulation effect, or whether it was the type of media used to grow the isolate. Results obtained from *in planta* RNA-seq data generated from two *Eucalyptus* clones (TAG5 and ZG14) were inconclusive because most of the gene expression profiles were undetermined. However, those that were profiled were upregulated, with higher levels of upregulation in TAG5 (tolerant) than in ZG14 (susceptible). This could mean that inoculation of *C. austroafricana* on *Eucalyptus* clones encourages upregulation of *NRPS* genes of the isolate, presumably for nutrient sequestration. These results represent the first comparison of *C. austroafricana NRPS* gene expression profiles from different growth conditions.

When *Chrysoporthe* species were grown under near-UV and exposed to brief UV, we observed that most genes were upregulated in the exposed isolates compared to control isolates. This suggests that UV light has the ability to upregulate *NRPS* genes. The findings are in line with those previously reported by Sebastian *et al.* (1990), which showed upregulation of *S. cerevisiae NRPS* genes when exposed to UV light. However, a fraction of genes was down-regulated compared to those that were not exposed to UV light. There were also differences in expression levels between the three species.

Conclusions

This study compared secondary metabolite gene clusters between three *Chrysoporthe* species, namely *C. austroafricana*, *C. cubensis*, and *C. deuterocubensis*. *NRPS* gene clusters were common and syntenous between the three species, except for one that was absent in the draft genome of *C. austroafricana*. The study also indicated that most of the *NRPS* gene clusters were siderophore-producing gene clusters (according to AntiSMASH and NCBI BLAST searches). Within the gene clusters, both regulatory and transport genes were present. The study also showed that exposure of *Chrysoporthe* isolates to UV light had an influence on the expression of *NRPS* genes, because most of the genes were upregulated when exposed to UV. However, we propose that functional studies of these siderophore producing genes should be conducted to elucidate their role during exposure to UV light.

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Tables and figures

Table 1 Isolates used for the confirmation of genes within gene clusters of *Chrysoporthe* species.

Species	Culture collection nr	Country of origin	Host	Collector
C. austroafricana	CMW 2113	South Africa	*Eucalyptus grandis*	MJ Wingfield
C. cubensis	CMW 10028	Colombia	*Eucalyptus* sp.	C Rodas
C. deuterocubensis	CMW 8650	Indonesia	*Syzygium* sp.	MJ Wingfield

Table 2 Primers designed in this study to amplify *NRPS* genes, core biosynthesis, additional biosynthesis and transport genes predicted by AntiSMASH from three *Chrysoporthe* species.

Species	Cluster	Primer ID	Sequence	T_m (°C)
C. austroafricana	13	F13Core	TCGCTATGTGTTCGTCTG	57.62
		R13Core	TCTTTGCTCTCGTTCTTTCT	56.3
		F13Add	TCCTACGGTCCATCACCA	59.9
		R13Add	GGATCGCTAGGCTACTTT	57.62
		F13Trans	TATTCTCCTTCTGCCACT	55.34
		R13Trans	TTCCGCCACTTTATGAAC	55.34
	32A	F32ACore	CGATACCAGAACAACACC	57.62
		R32ACore	GCGAAGAACGAAGAGTAG	57.62
		F32AAdd	AACAGGACGGAGCACGAGA	62.32
		R32AAdd	CGTGGATGTTGTGTGCGA	59.9
		F32ATrans	TGGGTATTGTGGTGGTGG	59.9
		R32ATrans	AGGTAGTGCGCTCGTAAA	57.62
	72	F72Core	GCAGTGAAGAAAGTCGAA	55.34
		R72Core	GCTCTCTACCTCAACAAA	55.34
		F72Add	CCCATCCAGATACCCATCA	60.16
		R72Add	TCAGGAACTCCCCTTTCA	57.62
	46	F46Core	TTTGGTCTCCCTCTTCCT	57.62
		R46Core	CTTCTCGTCCATTTTCGT	55.34
		F46Add	GGTGTTAGGCACTGATTT	55.34
		R46Add	GGTTGTTGGGATTGGTGA	57.62
	88	F88Core	TGCATTTCAGCGCGATTT	55.34
		R88Core	GCGCCCCTCATTAAGTAT	57.62
		F88Add	ACCCCCATTTCACCCACA	59.9
		R88Add	GGAAAAAGCCGGACACGA	59.9
	94	F94Core	GCGTTTTTCTTCGCGGTT	57.62
		R94Core	TCTTCAGGTTCCCAGGGTT	60.16
		F94Add	AGGTTCTGGTAGGATGGT	57.62
		R94Add	GAGTGTAGATGTTATGGGTGT	58.66
	98	F98Core	GATGACCGGAACGAGAGGA	62.32
		R98Core	GGGTACGGGTTGAAGTGGA	62.32
		F98Add	CACCTATCGCAGAGAATAA	55.85
		R98Add	GCCCAGTAGAGCTCATAA	57.62
		F98Trans	TTCTCACAATCGCACATC	55.34
		R98Trans	GCTTCTCCTCAAATTTTCC	55.85
C. cubensis	28	F28Core	CCCGAACAGACCTCAAAA	57.62
		R28Core	CGGATAGGAGAAGGTCAA	57.62
		F28Add	GTCTTGCTAATCTGGCTG	57.62
		R28Add	AATGCCCTCAAAACACTC	55.34
	37	F37Core	TGGCTCTGGGGATATCGT	59.9
		R37Core	TGTGCGCAGGATTGGATT	57.62
		F37Add	TAGAAGAGCAGCCAGCAA	57.62
		R37Add	GAGCACATGAGGGTCGAA	59.9
		F37Trans	TCTCCTTCATCTATCCCTCC	60.4
		R37Trans	CTTCTCCTCTGTGCTCTT	57.62
	47	F47Core	AGAGATCGAGGGAGAGAG	59.9
		R47Core	AAGGAAGAGGGAGACCAA	57.62
		F47Add	TTGCAAAGGTGATGCTGT	55.34
		R47Add	GCAAAGTTCGTCCGGTTA	57.62
		F47Trans	GAAAAACGTCTACATCGCT	55.85
		R47Trans	AATGAAGAGACGGGTAGG	57.62
	52	F52Core	CACTCCATCCTACGCACA	59.9
		R52Core	ATAGTCGTCCTCGTCCTC	59.9
		F52Add	CATTGGCTTTACGGAACT	55.34
		R52Add	GATGATGTTCTGGTGGCT	57.62
		F52Trans	GGGTTGTTCAGGAGTTGT	57.62

Table 2 continued

Species	Cluster	Primer ID	Sequence	T_m (°C)
		R52Trans	GTGTGAAAAGGAGGAAGG	57.62
	56	F56Core	GCTGGTGGGGTCTATGTT	59.9
		R56Core	CCGTCTGGGTTGTATCGT	59.9
		F56Add	TCACACGACAGCTCATCA	57.62
		R56Add	TACCAACCAATACCACCC	57.62
		F56Trans	CTTACCTCTTCCTCCTCC	59.9
		R56Trans	ACAATCTCTCTCTCTTCATC	56.3
	57	F57Core	GCGTTTTTCTTCGCGGTT	57.62
		R57Core	TCTTCAGGTTCCCAGGGTT	60.16
		F57Add	CCACTCTCGTTCTAGTCA	57.62
		R57Add	TCTCTCCATCTAATCCCA	55.34
C. deuterocubensis	3	F3Core	ACAACTTCAAGTCACTCCA	55.85
		R3Core	ACTCACATATCAGGCTCA	55.34
		F3Trans	ACCGGTCTCTTTATATGC	55.34
		R3Trans	TTAGCTGTGTTGGTATGG	55.34
	15	F15Core	ATAGGAAATGGAAGGGAC	55.34
		R15Core	CAAAATAGAGATGCCCAG	55.34
		F15Add	CCCTCTCCAACTTCTTCA	57.62
		R15Add	AATCTTTGCTCGTACTCC	55.34
	20	F20Core	GACAATAGGCGAGATGCTGA	60.4
		R20Core	GGCTGAACGAAGGGAAGA	59.9
		F20Add	AACCATTCTATCCACCTCC	58.01
		R20Add	TCTGGCCTCCTCCAATCA	59.9
	30	F30Core	AAAACTACCACGGGCACA	57.62
		R30Core	GCAACAAAGCCCATCTCA	57.62
		F30Add	TCCTATAACCTCACTTCCCC	60.4
		R30Add	CACCTTCCCACAGATCTT	57.62
	31	F31Core	TTTGAGAAGTCGAGGTGG	57.62
		R31Core	GGTCGTGGATAAAAGCAG	57.62
		F31Add	GGAAGAGCAACCAGCAAG	59.9
		R31Add	CCGAAGAGGCAGAGAACA	59.9
		F31Trans	TCTCCTTCATCTATCCCTCC	60.4
		R31Trans	CTTCTCCTCTGTGCTCTT	57.62
	32B	F32BCore	CACTCCATCCTACGCACA	59.9
		R32BCore	TCATAGTCGTCCTCATCCTC	60.4
		F32BAdd	TTTGGGCTGGAGTCATTT	55.34
		R32BAdd	TGGATCGCTAGGCTACTT	57.62
		F32BTrans	GTGCTGTTGTCTCTGGGT	59.9
		R32BTrans	GTGTGAAAAGGAGGAAGG	57.62
	40	F40Core	GCATGGGAGGAGACTGTA	59.9
		R40Core	GAGGATATGGGTGGTGTT	57.62
		F40Add	CATCGTCCATATCCACTT	55.34
		R40AAdd	TACCAACCAATACCACCC	57.62
		F40Trans	TACCTCCCACAAAACTCT	55.34
		R40Trans	AGATGCAGACGACCATGA	57.62
	50	F50Core	GAGACAAGAGACCGAACA	57.62
		R50Core	GTAAACCGCGACAATCCA	57.62
		F50Add	ACCGGAAGCAGCAATACA	57.62
		R50Add	AAGGAGGACATTCGCACA	57.62

Table 3 Primers used for RT-qPCR assays. All primer sequences were optimized for annealing at 62.5 °C.

Species	Gene ID	Primer ID	Primer Sequence
C. *austroafricana*			
Cluster 72	Core	F72Core	GCAGTGAAGAAAGTCGAA
		R72Core	GCTCTCTACCTCAACAAA
Cluster 46	Core	F46Core	TTTGGTCTCCCTCTTCCT
		R46Core	CTTCTCGTCCATTTTCGT
Cluster 94	Core	F94Core	GCGTTTTTCTTCGCGGTT
		R94Core	TCTTCAGGTTCCCAGGGTT
Cluster 98	Core	F98Core	GATGACCGGAACGAGAGGA
		R98Core	GGGTACGGGTTGAAGTGGA
	Trans	F98Trans	TTCTCACAATCGCACATC
		R98Trans	GCTTCTCCTCAAATTTTCC
C. *cubensis*			
cluster 28	Core	F28Core	CCCGAACAGACCTCAAAA
		R28Core	CGGATAGGAGAAGGTCAA
cluster 52	Trans	F52Trans	GGGTTGTTCAGGAGTTGT
		R52Trans	GTGTGAAAAGGAGGAAGG
C. *deuterocubensis*			
Cluster 3	Core	F3Core	ACAACTTCAAGTCACTCCA
		R3Core	ACTCACATATCAGGCTCA
	Trans	F3Trans	ACCGGTCTCTTTATATGC
		R3Trans	TTAGCTGTGTTGGTATGG
Cluster 20	Add	F20Add	AACCATTCTATCCACCTCC
		R20Add	TCTGGCCTCCTCCAATCA
Cluster 30	Core	F30Core	AAAACTACCACGGGCACA
		R30Core	GCAACAAAGCCCATCTCA
Cluster 31	Core	F31Core	TTTGAGAAGTCGAGGTGG
		R31Core	GGTCGTGGATAAAAGCAG
Cluster 32B	Core	F32BCore	CACTCCATCCTACGCACA
		R32BCore	TCATAGTCGTCCTCATCCTC
Cluster 50	Core	F50Core	GAGACAAGAGACCGAACA
		R50Core	GTAAACCGCGACAATCCA

Table 4 Internal control gene primers used for RT-qPCR assays.

Gene	Primer ID	Primer sequence (5'-3')	Temp
Actin	ACTINF	GAAGATCACTGCCTTGCTCC	62.5
	ACTINR	CGATAACAGCTCCTCTTGGC	62.5
Beta-tubulin	BT1F	AACCATCTCTGGCGAGCA	62.5
	BT1R	GACATACTTGTTGCCGGA	62.5
Beta-tubulin	BT2F	AGTTGACCCAGCAGATGTT	62.5
	BT2R	TCTGGACGTTGTTGGGGA	62.5
Elongation factor	EF1F	CGAGAAGGAAGGTATGTA	62.5
	EF1R	CTGTGCAACAAGGAATGT	62.5
18S	18SF	TCTCGAATCGCATGGCCT	62.5
	18SR	GCGCTCTACACGGGTCTTCA	62.5
28S	28SF	GGCCCGAGTTGTAATTTG	62.5
	28SR	TTCCTGCCGTTCTTATCC	62.5

Table 5 AntiSMASH results showing different classes and numbers of secondary metabolite gene clusters from three *Chrysoporthe* genomes.

Cluster	*C. austroafricana*	*C. cubensis*	*C. deuterocubensis*
T1PKS	20	20	24
T3PKS	1	1	1
NRPS	7	6	8
Indole	2	1	2
Terpene	8	8	9
Terpene-NRPS	1	1	0
T1PKS-NRPS	8	7	7
Indole-T1PKS	1	2	1
Lantipeptide-T1PKS	1	1	0
Phosphonate	1	1	1
Others	11	12	10

Table 6 Unique clusters in three *Chrysoporthe* spp.

Cluster Number	C. *austroafricana*	C. *cubensis*	C. *deuterocubensis*
88	Present (query)	Absent	Present
15	Present (few nucleotides missing)	Present	Present (query)
40	Present (with two adjacent genes missing)	Present	Present (query)
50	Present	Absent	Present (query)
17 more clusters	Present	Present	Present

Table 7 Sizes and locations of NRPS gene clusters and core biosynthesis genes found within three *Chrysoporthe* genomes.

	Cluster location		Scaffold number	Cluster size	Core biosynthetic gene location		Core biosynthetic gene size
C. austroafricana							
Cluster 13	4852041	4903959	scaffold1	51918	4872041	4883959	11918
Cluster 32	3209912	3261711	scaffold4	51799	3236609	3239636	3027
Cluster 46	2442137	2488040	scaffold5	45903	2462137	2468040	5903
Cluster 72	5341085	5395821	scaffold6	54736	5361085	5375821	14736
Cluster 88	1934493	1979327	scaffold8	44834	1954493	1959327	4834
Cluster 94	61692	108118	scaffold9	46426	81692	88118	6426
Cluster 98	762449	808806	scaffold9	46357	782449	788806	6357
C. cubensis							
Cluster 28	46143	100879	scaffold87	54736	66143	80879	14736
Cluster 37	1	45691	scaffold123	45690	17223	25691	8468
Cluster 47	21720	63472	scaffold212	41752	41720	46492	4772
Cluster 52	1	32280	scaffold263	32279	359	12280	11921
Cluster 56	1	31845	scaffold313	31844	13637	18732	5095
Cluster 57	1	24608	scaffold353	24607	7644	12360	4716
C. deuterocubensis							
Cluster 3	401525	438541	scaffold2	37016	421525	426061	4536
Cluster 15	118545	161021	scaffold44	42476	138545	142059	3514
Cluster 20	65435	120171	scaffold70	54736	85435	100171	14736
Cluster 30	53628	87197	scaffold142	33569	73628	84076	10448
Cluster 31	41972	79216	scaffold159	37244	61972	67734	5762
Cluster 32	1	45964	scaffold163	45963	14046	25964	11918
Cluster 40	1	38182	scaffold24	38181	13312	18182	4870
Cluster 50	4253	41257	scaffold32	37004	26158	29084	2926

Table 8 Reciprocal BLAST search results confirmation using PCR assays of *C. austroafricana*, *C. cubensis* and *C. deuterocubensis*. The blue boxes, positive (+) and negative (-) represent query species genes, presence of the query gene and absence of the query gene, respectively.

		Species		
	Gene	*C. austroafricana*	*C. cubensis*	*C. deuterocubensis*
C. austroafricana				
Cluster 13	Core		+	+
	Add		+	+
	Trans		+	+
Cluster 32A	Core		+	+
	Add		+	+
	Trans		+	+
Cluster 72	Core		+	+
	Add		+	+
Cluster 46	Core		+	+
	Add		+	+
Cluster 88	Core		-	+
	Add		-	+
Cluster 94	Core		+	+
	Add		+	+
Cluster 98	Core		+	+
	Add		+	+
	Trans		+	+

Table 8 continued

		Species		
	Genes	*C. Austrosfricana*	*C, cubensis*	*C. deuterocubensis*
C. *cubensis*				
Cluster 28	Core Add Core	+ + +		+ + +
Cluster 37	Add Trans Core	+ + +		+ + +
Cluster 47	Add Trans	+ +		+ +
Cluster 52	Core Add Trans	+ + +		+ + +
Cluster 56	Core Add Trans	+ + +		+ + +
Cluster 57	Core Add	+ +		+ +
C. *deuterocubensis*				
Cluster 3	Core Add	+ +	+ +	
Cluster 15	Core Add	+ +	+ +	

Table 8 continued

		Species		
	Genes	*C. austrosfricana*	*C, cubensis*	*C. deuterocubensis*
Cluster 20	Core	+	+	
	Add	+	+	
Cluster 30	Core	+	+	
	Add	+	+	
Cluster 31	Core	+	+	
	Add	+	+	
	Trans	+	+	
Cluster 32B	Core	+	+	
	Add	+	-	
	Trans	+	+	
Cluster 40	Core	+	+	
	Add	+	+	
	Trans	+	+	
Cluster 50	Core	+	-	
	Add	+	-	

Table 9 *NRPS* gene expression profiles of *C. austroafricana* gene clusters, *in vitro* and *in planta*. The expression number represents the regulation of genes in fold change.

	Expression		
Cluster	*In vitro*	TAG5 *in planta*	ZG14 *in planta*
Caus-CMW211300003308-Cluster94	-2.088418884	4.545853577	1.776680562
Caus-CMW211300003309-Cluster94	0.726259351	-0.530875159	N/A
Caus-CMW211300003310-Cluster94	-0.401368855	N/A	N/A
Caus-CMW211300003311-Cluster94	NA	N/A	N/A
Caus-CMW211300003313-Cluster94	-0.603024532	N/A	N/A
Caus-CMW211300003315-Cluster94	-0.425948481	N/A	N/A
Caus-CMW211300003316-Cluster94	1.555494912	N/A	N/A
Caus-CMW211300003317-Cluster94	-0.32265203	N/A	N/A
Caus-CMW211300003122-Cluster98	-0.242410937	N/A	N/A
Caus-CMW211300003123-Cluster98	0.484878223	N/A	N/A
Caus-CMW211300003124-Cluster98	0.172560391	N/A	N/A
Caus-CMW211300003125-Cluster98	0.850122008	N/A	N/A
Caus-CMW211300003126-Cluster98	-0.962197086	N/A	N/A
Caus-CMW211300003127-Cluster98	NA	N/A	N/A
Caus-CMW211300003128-Cluster98	-0.261767935	N/A	N/A
Caus-CMW211300003129-Cluster98	-0.425948481	N/A	N/A
Caus-CMW211300003130-Cluster98	-0.401368855	N/A	N/A
Caus-CMW211300003131-Cluster98	NA	N/A	N/A
Caus-CMW211300003132-Cluster98	-0.804230457	2.574701796	1.834715869
Caus-CMW211300001295-Cluster72	-0.362930138	N/A	N/A
Caus-CMW211300001296-Cluster72	1.210546179	N/A	N/A
Caus-CMW211300001297-Cluster72	0.364032003	N/A	N/A
Caus-CMW211300001298-Cluster72	0.160477327	N/A	N/A
Caus-CMW211300001299-Cluster72	1.573782626	N/A	N/A
Caus-CMW211300001300-Cluster72	0.578487423	N/A	N/A
Caus-CMW211300001302-Cluster72	0.205807904	N/A	N/A
Caus-CMW211300001303-Cluster72	-0.717584809	N/A	N/A
Caus-CMW211300006768-Cluster13	NA	N/A	-0.950240167
Caus-CMW211300006769-Cluster13	-0.401368855	0.471125149	-0.950240167
Caus-CMW211300006770-Cluster13	-0.014975705	-0.530875159	-0.811219037
Caus-CMW211300006771-Cluster13	1.207288345	4.576037899	5.422379716
Caus-CMW211300008596-Cluster32	5.837654935	N/A	N/A
Caus-CMW211300008597-Cluster32	-0.335929555	3.365251531	2.141645246
Caus-CMW211300008598-Cluster32	NA	N/A	N/A
Caus-CMW211300008599-Cluster32	NA	N/A	N/A
Caus-CMW211300008600-Cluster32	-0.108652937	N/A	N/A

Table 9 continued

	Expression		
Cluster	*In vitro*	TAG5 *in planta*	ZG14 *in planta*
Caus-CMW211300009228-Cluster88	NA	N/A	N/A
Caus-CMW211300009229-Cluster88	NA	N/A	N/A
Caus-CMW211300009230-Cluster88	NA	0.087508919	N/A
Caus-CMW211300002142-Cluster46	-0.837079945	N/A	N/A

Table 10 *NRPS* gene expression profiles from cultures of the three species grown under near UV light. The expression number represents the regulation of genes in fold change.

	Gene ID	*C. austroafricana*	*C. cubensis*	*C. deuterocubensis*
C. austroafricana				
Cluster 72	Core	N/A	N/A	N/A
Cluster 46	Core	N/A	N/A	N/A
Cluster 94	Core	0.000522658	1.439613785	1.252357067
Cluster 98	Core	2.291494043	1.811738518	1.290332297
	Trans	0.318595557	0.208321715	0.284419432
C. cubensis				
cluster 28	Core	N/A	N/A	N/A
cluster 52	Trans	N/A	N/A	N/A
C. deuterocubensis				
Cluster 3	Core	0.657175304	0.561282431	0.607812518
	Add	1.082886948	0.659838212	0.839411773
Cluster 20	Add	4.559998477	1.037596819	2.320007202
Cluster 30	Core	0.32930075	2.078828937	0.613027611
Cluster 31	Core	2.0574196	2.164480905	2.057446976
Cluster 32B	Core	1.063247238	2.542116974	2.069377002
Cluster 50	Core	1.79054E-05	0.623020298	0.230890149

Figure 1

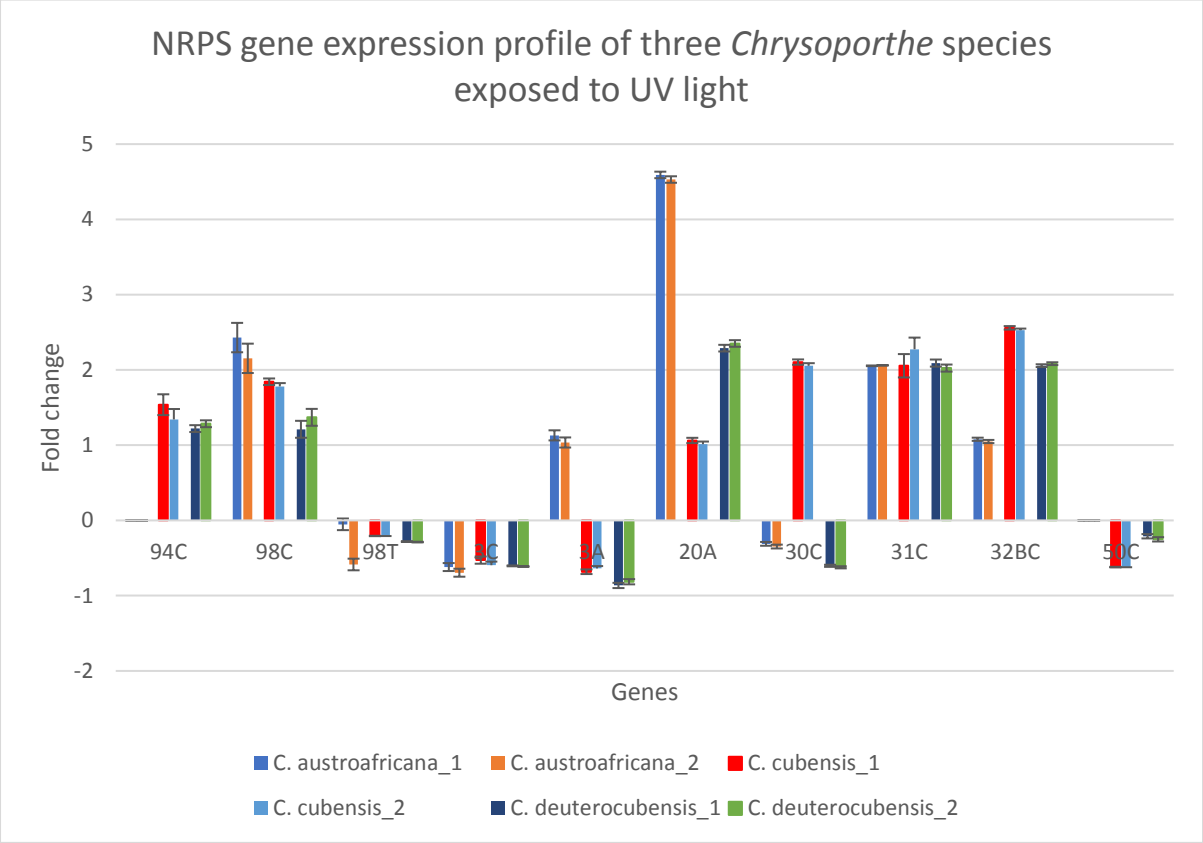
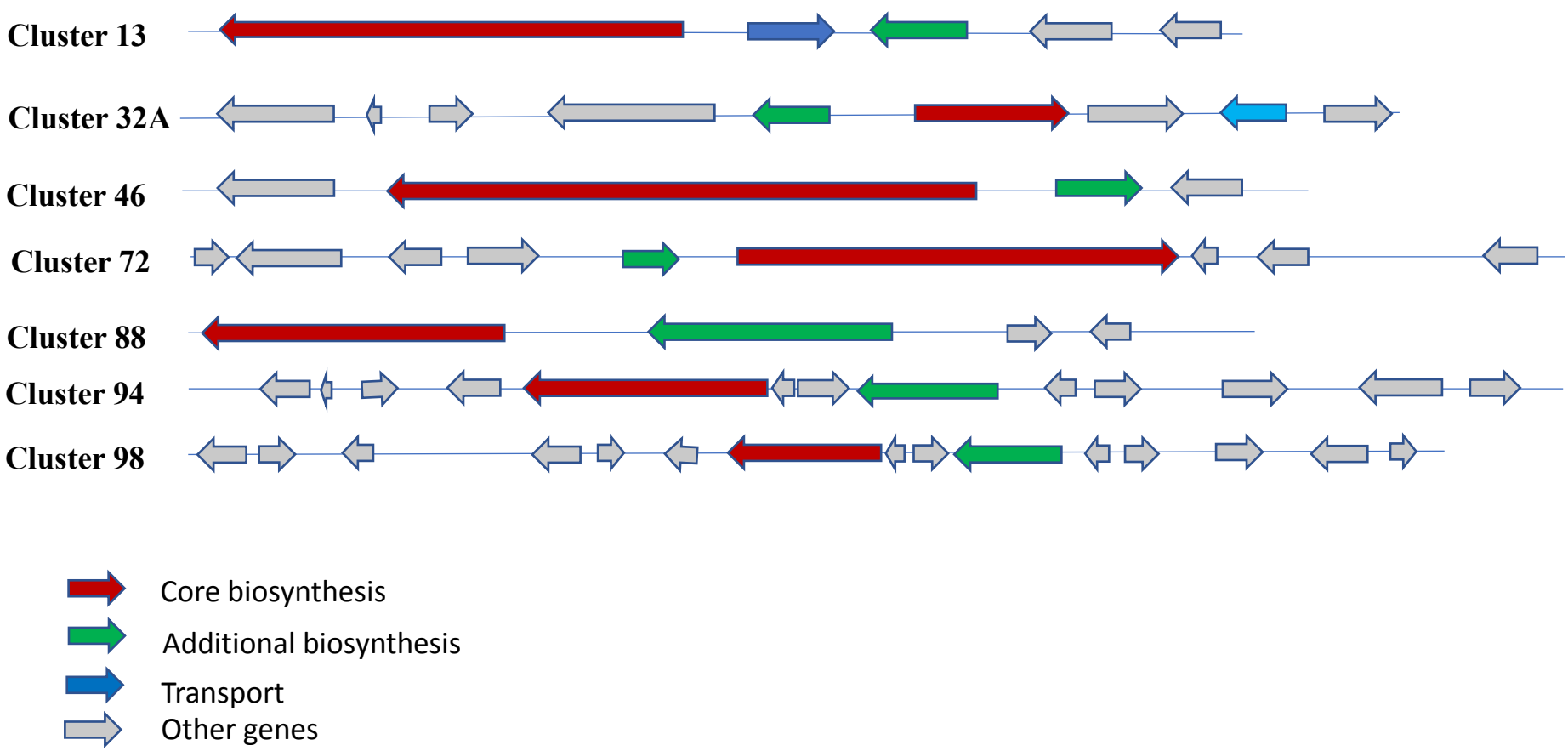


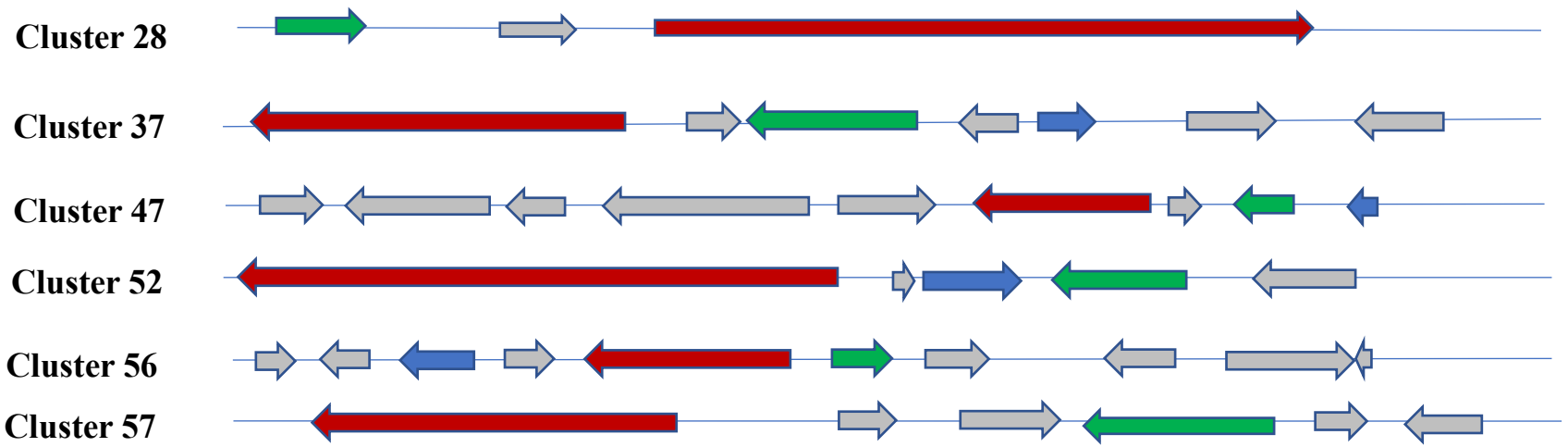
Figure 1: The graph represents the expression level of minimal genes (Table 9) from all three *Chrysoporthe* species (isolates), *C. austroafricana*, *C. cubensis* and *C. deuterocubensis*, when exposed to UV light compared to normal condition (in two replicates, 1 and 2).

Appendix

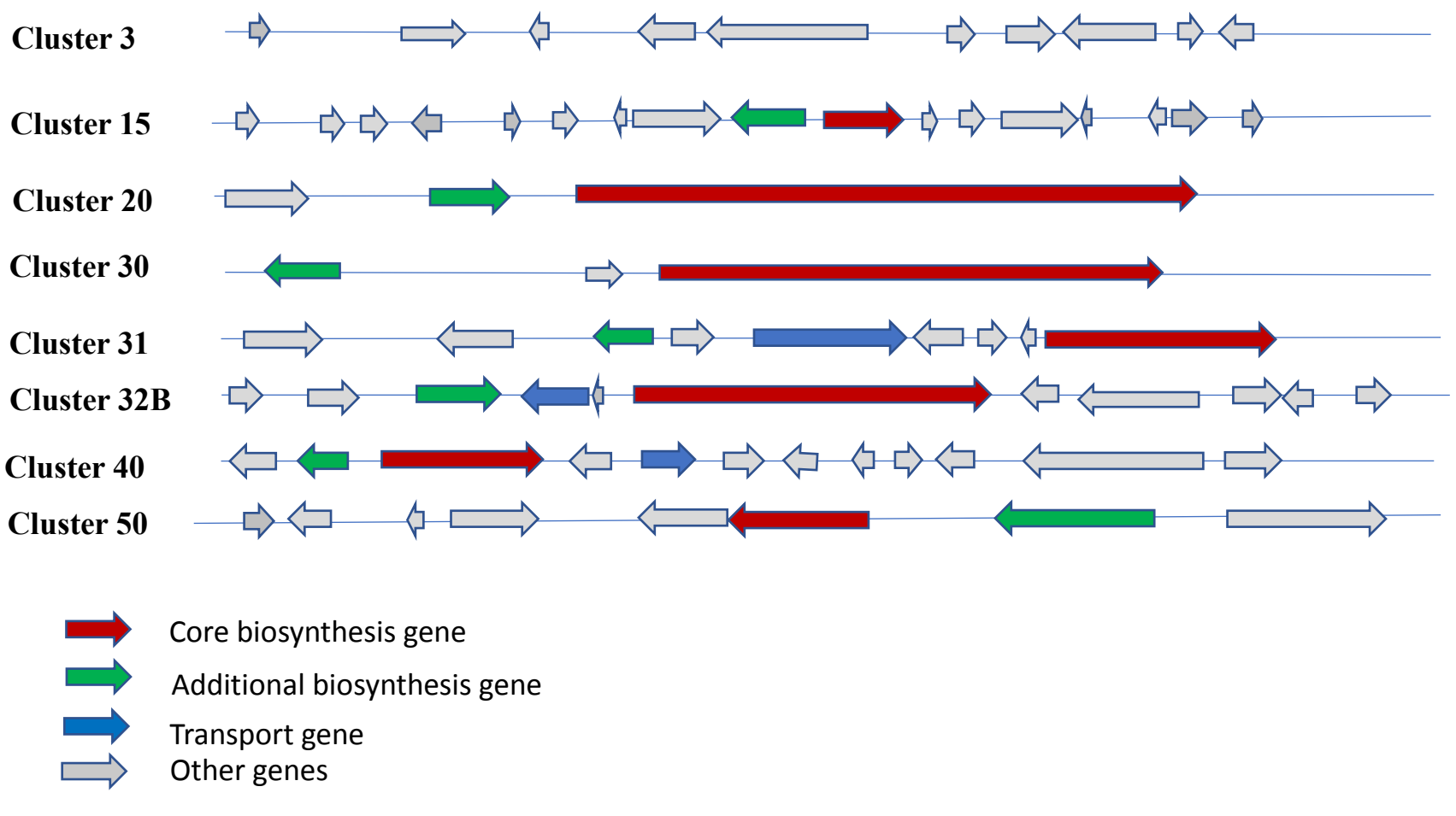
Appendix 1 *C. austroafricana*



Appendix 2 *C. cubensis*



Appendix 3 *C. deuterocubensis*



Summary

Chrysoporthe is a genus consisting of species that are pathogens to various plant hosts, including industrial *Eucalyptus* species. The pathogenicity of each species varies to different hosts and amongst themselves. The pathogenicity and virulence of most filamentous fungi is attributed to various factors, including siderophores (secondary metabolites). Secondary metabolites are bioactive compounds of small molecular weight. They are produced by genes that are located adjacent to each other and are co-regulated. Understanding the biology of fungi and their response to different environments is very crucial. This research was aimed at characterizing three *Chrysoporthe* species, *C. austroafricana*, *C. cubensis* and *C. deuterocubensis*, based on their secondary metabolite gene clusters with special focus on the Nonribosomal Peptide Synthetases (NRPS).

Results from the research chapter (chapter two) showed that the three *Chrysoporthe* species possess almost equal numbers of secondary metabolite gene clusters. With the special focus on NRPS, the study revealed that *NRPS* genes are very large as compared to other genes, as expected. The results also revealed that, within the *NRPS* gene clusters, putative siderophore producing gene clusters were also present. The second part of the research chapter showed that near UV light has the ability to induce *NRPS* genes, resulting in upregulation of their expression. This dissertation provides the first investigation of secondary metabolite gene clusters in *Chrysoporthe* species. Functional studies are proposed to further characterize upregulated *NRPS* genes.

Xikatsakanyo

Chrysoporthe I genus Leyi yi endliwaka hiti species leti ti tisaka mavabyi ka swimilana swo hlaya, ku katsa na ti *Eucalyptus* species, ximilana xo hlawuleka eka ti industry ta nhova. Vu pathogenicity bya ti species leti bya hambana eka swimilana na le xikharhi ka tona. Ti ndlela leti ti fungus ti tisaka mavabyi eka swimilana ti hlayile, ti katsa na ti siderophore (secondary metabolites). Secondary metabolites iti bioactive compounds ta ntikelo wale hansi. Ti endliwa hiti genes leti ti kumekaka kusuhi na kusuhi na swona ti lawuleka xikanwe. Ku twisisa biology yati fungi na leswi ti hlamurisaka xiswona kati ndhawu to hambana-hambana swina nkonka swinene. Nlavisiso lowu, wu kongomisiwile eka ku katsa ti species ti nharhu ta *Chrysoporthe*, *C. Austroafricana*, *C. Cubensis* and *C.deuterocubensis*, hiti secondary metabolites gene clusters ta tona. Na ku langutisa ngopfu-ngopfu eka ti nonribosomal peptide synthetases (NRPS).

Mbuyelo wa xilavisiso Lexi wu kombisile ku ti species leti tinharhu ta *Chrysoporthe* tina ti secondary metabolites gene clusters leti ati lavile ku ringana hiti nomboro. Ku langutisa ngopfu-ngopfu ka ti NRPS swi kombisile ku ti *NRPS* genes iti kulu ngopfu loko ti ringanisiwa na tinwani ta tona, na swona aswi languteriwile. Mbuyelo wu kombisile nakambe swaku, eka ti NRPS gene clusters, ti gene leti tingana vuswikoti byo endla ti siderophore ta kumeka. Xiphemu xavumbirhi xa nlavisiso xi kombisile swaku rivoni ra UV ra kota ku hlohlotela ti *NRPS* genes, swi endlaka leswaku ti tlakuseka kumbe ti hunguteka matirhelo ya tona. Matsalwa lawa ma tisa xilavisiso xo sungula xati secondary metabolites gene clusters eka ti species ta *Chrysoporthe*. Xilavisiso xama tirhelo yati genes leti tinga tlakusiwa xalaveka ku kota ku twisisa hivu enti ti *NRPS* genes leti tinga tlakusiwa eka mbuyelo lowu.