Transcriptomic analysis to identify candidate genes conferring gossypol tolerance in *Fusarium oxysporum* f. sp. *vasinfectum*

By

Ambika Pokhrel

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
December 15, 2018

Keywords: *Fusarium oxysporum* f. sp. *vasinfectum,* gossypol, cotton, tolerance, RNA-seq, RT-qPCR

Copyright 2018 by Ambika Pokhrel

Approved by

Jeffrey J. Coleman, Assistant Professor of Plant Pathology
Kathy S. Lawrence, Professor of Plant Pathology
Mark R. Liles, Professor of Biology

Abstract

Fusarium oxysporium f.sp *vasinfectum* (*Fov*) is a soilborne filamentous fungus that causes vascular wilt on cotton. In response to pathogens such as *Fov*, cotton plants produce the phytoalexin gossypol as a defense mechanism. This research aims to explore the molecular mechanism(s) utilized by *Fov* to tolerate the antimicrobial action of gossypol during infection and colonization of cotton. Candidate genes that could be responsible for gossypol tolerance were identified through RNA sequencing. Four RNA samples were extracted from germlings of a highly virulent race 4 genotype *Fov* isolate that was grown in minimal medium in the presence of 80 µg/mL of gossypol for 1, 2, and 4 hours as well as untreated control. RNA-seq data showed upregulation of ABC and major facilitator transporters, fungal transcription factors, cytochrome P450s, and several classes of dehydrogenases when compared with transcripts isolated from the non-treated *Fov* RNA sample. KEGG pathway analysis identified genes involved in various metabolic pathways, including the biosynthesis of secondary metabolites and antibiotics. The RNA-seq data was validated through RT-qPCR where out of the selected 29 upregulated genes, 16 were significantly expressed through a paired t-test. Even though some genes were not significantly expressed, the expression was increased 70 to 240-fold for at least a single time point. Importantly, candidate genes and gene classes that could be involved in conferring gossypol tolerance were identified Including a beta lactamse, a tannase, several dehydrogenases, transcription factors, cytochrome P450s, and multiple ABC and major

facilitator superfamily transporters. Collectively, these findings provide a framework for further investigation into *Fov* virulence and the mechanisms underlying gossypol tolerance.

Keywords: cotton, Fusarium oxysporum f.sp. *vasinfectum, gossypol, tolerance*

Acknowledgements

I would like to thank my major advisor, Dr. Jeffrey Coleman for giving me the great opportunity to work on this interesting project. His patience, understanding, support and invaluable suggestions kept me going through the challenging phases of this study. I would like to thank my committee members Dr. Kathy Lawrence and Dr. Mark Liles for their comments and valuable suggestions. I would also like to acknowledge my lab members Seungyeon, Sahar and Qiang for suggestions, help and encouragement during my research work. I am grateful for my friends Laura and Qing for being there during my hard and happy times. In the times when I was lost and frustrated, Rishi and my whole auburn Nepali family was there to remind me my worth and expectations.

I owe all my success to my parents, brothers and sisters for their support and unconditional love. Finally, this thesis is dedicated to my dearest Mom and in memory of my beloved Father.

Table of Contents

Abstract ... ii

Acknowledgements ... iv

Table of Contents ... v

List of Tables ... vii

List of Figures ... ix

CHAPTER ONE: Introduction and Literature Review ... 1

1.1 *Fusarium* ... 1

1.2 *Fusaium oxysporum* ... 2

1.3 *Fusarium oxysporum* f. sp. *vasinfectum* ... 2

1.3.1 Environmental factors ... 4

1.3.2 Interaction with nematodes ... 5

1.3.3 Development and distribution of different races ... 6

1.4 Phytoalexins ... 8

1.5 Gossypol ... 10

1.5.1 Biological activity of gossypol ... 11

1.6 Phytoalexin tolerance by plant pathogens ... 12

1.7 References ... 17

CHAPTER TWO: Transcriptomic analysis to identify candidate genes conferring gossypol tolerance in *Fusarium oxysporum* f. sp. *vasinfectum* ... 26

Abstract ... 26

2.1 Introduction ... 27

2.2 Gossypol tolerance by *Fov* ... 29

2.3 Results ... 29

2.3.1 Gene expression profile in *Fusarium oxysporum* f. sp. *vasinfectum* when exposed to gossypol ... 29

2.3.2 Functional analysis of differentially expressed genes ... 32

2.3.3 Gene expression patterns ... 34

2.3.4 Validation of DEGs from RNA-seq through RT-qPCR 35
2.4 Discussion 44
2.5 Materials and methods 50
2.5.1 Fungal culture and RNA-seq sample preparation 50
2.5.2 RNA isolation and RNA sequencing 50
2.5.3 Bioinformatics and statistical data analysis 51
2.5.4 Experimental setup and sample preparation for RT-qPCR 51
2.5.5 RNA isolation and cDNA preparation 52
2.5.6 RT-qPCR assay 52
2.5.7 RT-qPCR data analysis 53
2.6 Acknowledgements 53
2.7 References 53
SUPPLEMENTARY MATERIAL 59

List of Tables

Table 2-1: Numbers of differentially expressed genes under various Pfam domain categories 33
Table 2-2: Fold change of selected candidate genes at timepoints T0, T1, T2, T4, T8, T12 and T24 timepoints.. 41
Table S-1: Number of reads survived after trimming and proportion of reads mapped to the genome 59
Table S-2: Common 68 upregulated genes with Pfam domain and expression values at timepoints T1, T2, and T4 from RNA-seq data 59
Table S-3: Common 6 downregulated genes with Pfam domain and expression values at timepoints T1, T2, and T4 from RNA-seq data 65
Table S-4: Upregulated ABC, sugar, and other transporters from RNA-seq data with Pfam domains..65
Table S-5: Downregulated ABC, sugar, and other transporters from RNA-seq data with Pfam domains..67
Table S-6: Upregulated Major facilitator superfamily transporters from RNA-seq data with Pfam domains 67
Table S-7: Downregulated Major facilitator superfamily transporters from RNA-seq data with Pfam domains 68
Table S-8: Upregulated transcription factors from RNA-seq data with Pfam domains 69
Table S-9: Downregulated transcription factor from RNA-seq data with Pfam domain 71
Table S-10: Upregulated cytochrome P450s form RNA-seq data with Pfam domains 71
Table S-11: Downregulated cytochrome P450s form RNA-seq data with Pfam domains 72
Table S-12: Upregulated dehydrogenases from RNA-seq data with Pfam domains 72
Table S-13: Downregulated dehydrogenases from RNA-seq data with Pfam domains..........77
Table S-14: Upregulated genes assigned with KEGG orthology ID through KEGG analysis 79
Table S-15: Downregulated genes assigned with KEGG orthology ID through KEGG analysis 83

Table S-16: Upregulated genes involved in metabolic pathways with KEGG ID, gene name and function identified through KEGG analysis 84
Table S-17: Upregulated genes involved in microbial metabolism in diverse environments with KEGG ID, gene name and function identified through KEGG analysis 86
Table S-18: Upregulated genes involved in biosynthesis of secondary metabolites with KEGG ID, gene name and function identified through KEGG analysis 87
Table S-19: Upregulated genes involved in biosynthesis of antibiotics with KEGG ID, gene name and function identified through KEGG analysis 88
Table S-20: Upregulated genes involved in carbon metabolism with KEGG ID, gene name and function identified through KEGG analysis 88
Table S-21: List of primers and amplicon length of candidate genes selected for RT-qPCR...89

List of Figures

Figure 1-1: Structure of gossypol..10
Figure 1-2: Enzymatic detoxification of phytoalexins to more polar compounds 14
Figure 1-3: Proposed three main mechanisms of phytoalexin tolerance by fungi 16
Figure 2-1: Structure of gossypol..28
Figure 2-2: Differentially expressed genes (DEGs) at 1 hour, 2 hours, and 4 hours after gossypol treatment when compared with the untreated control 31
Figure 2-3: Venn diagrams illustrating the number of upregulated (A) and downregulated (B) genes in Fov at 1 hour, 2 hours, and 4 hours after gossypol exposure. 31
Figure 2-4: Classification of up- and down-regulated genes by different pathways identified by KEGG analysis 34
Figure 2-5: RNA-seq data heatmap illustrating log2 fold change of 50 DEGs (*p*-value < 0.05) in all 16 samples...37
Figure 2-6: Comparison of DCT values between gossypol treated and DMSO treated samples at T1 from RT-qPCR data...38
Figure 2-7: Comparison of relative log2 fold changes between RNA-seq and RT-qPCR results at T2 after gossypol treatment in *Fov*...39
Figure 2-8: Heatmap of RT-qPCR analysis of candidate genes in *Fov* under gossypol treatment, with three biological and technical replicates 40

CHAPTER ONE

1. Introduction and Literature Review

1.1 Fusarium

Fusarium is a diverse group of filamentous ascomycete fungi that includes many plant pathogens of agricultural and clinical importance (Ma et al. 2013). *Fusarium* species have been regarded as economically important due to the variety of diseases they cause on various plants including crown rot, scab, and head blight on cereals; vascular wilts on horticultural and agriculturally important crops; root rots, and cankers (Nelson et al. 1994). *Fusarium* species have also been studied for their clinical importance in immunocompromised patients and are responsible for a wide range of infections including keratitis, onychomycosis, sinusitis, fusariosis, and mycotoxicosis (Kosmidis and Denning 2017).

As a genus *Fusarium* was first described in the early 19th century. In 1935, the genus was organized into 16 sections by Wollenweber and Reinking which were composed of 65 species, 55 varieties, and 22 forms based on morphological differences (Wollenweber and Recking 1935). Later it was reclassified into 70 species based on morphological and phylogenetic information (Leslie and Summerell 2006). With the emergence of modern sequencing technologies, 22 different species complexes are now currently recognized in the genus *Fusarium* with more than 200 species. All of the species complexes differ in morphology, molecular parameters, and host associations (Laurence et al. 2016).

1.2 Fusarium oxysporum

One of the species complex is the *Fusarium oxysporum* species complex (FOSC) which is a widely studied group of ascomycetous fungi that encompasses genetically and phenotypically diverse strains. Members of the FOSC are soil-borne and saprophytic, where they can survive on organic matter in the soil and rhizosphere for extended period of time (Chantal, et.al., 2018; Kang, Demers, del Mar Jimenez-Gasco, & Rep, 2014). The species complex comprises devastating plant pathogens causing wilt and rot diseases on more than 100 cultivated plant species and are considered one of the world's most economically important soil-borne plant pathogens in the world (Gordon and Martyn 1997; Leslie and Summerell 2006). Some isolates of *F. oxysporum* can penetrate roots and invade the vascular system of the host plant, while other isolates penetrate the roots but are unable to invade the vascular system and cause disease (Chantal et al. 2018). Pathogenic isolates of *F. oxysporum* show a high degree of host specificity and they are classified into more than 120 *formae speciales* and races depending on the plant species and cultivar they can infect (Armstrong and Armstrong 1981).

1.3 *Fusarium oxysporum* f.sp. *vasinfectum*

Fusarium oxysporum Schlechtend.:Fr. f. sp. *vasinfectum* (Atk.) W.C. Snyder & H.N. Hans., is a pathogen responsible for vascular wilt disease in cotton (*Gossypium*. spp.), an important economic crop, the major source of natural fiber and a cosmopolitan *forma specialis* that causes significant crop losses in most cotton growing areas of the world (Assigbetse et al. 1994). *F. oxysporum* f. sp. *vasinfectum* (*Fov*) is a soil borne pathogen, but can persist as a saprophyte that is able to survive for longer period of time as large thick cell walled resting spores, chlamydospores in the soil (Ragazzi and Vecchio 1992; Devay et al.

1997). The fungus is able to be disseminated through production of macroconidia and microconidia. Macroconidia are falcate shaped, moderately curved, sub-cylindrical, gradually tapering towards both ends with a distinct pedicellate basal cell and pointed apical cell. They are composed of three to five cells and are around 27-48 x 2.5-4.5 µm in size and borne in sporodociums or on conidiophores (Nelson et al. 1981). Microconidia are 5-20 x 2.2-3.5 µm in size and composed of one or two cells, where their shape can vary from cylindrical to ellipsoid, oval or straight to slightly curved. They are borne on phialides arising from short conidiophores (Nelson et al. 1981). Chlamydospores are single celled of about 7-11 µm in size, and can be smooth or rough walled, globose to sub-globose, and can be found in pairs, short chains, or in clusters (Nelson et al. 1981). They are readily formed in old cultures, but are better known as resting/over wintering spores that can remain dormant in soil for longer periods and germinate in various types of soils (Smith and Snyder 1972). The short micro conidiophores and chlamydospores are the main morphological features that distinguish *F. oxysporum* from other fusaria (Hillocks 1992).

The chlamydospores in the soil germinate when the nutrients and environmental conditions are favorable for pathogen growth (Smith and Snyder 1972). Once germinated, they can infect either intact or wounded cotton roots (Rodriguez-Galvez and Mendgen 1995). *Fov* forms a compact mycelium or a hyphal net on the cotton root surface that facilitates the infection of the root surface and promotes competition against other microbes within the rhizosphere (Rodriguez-Galvez and Mendgen 1995). The penetration process starts with the development of specialized penetration hyphae (Rodriguez-Galvez and Mendgen 1995). Both intercellular and intracellular penetration of the root occurs primarily in the meristematic zone and to a lesser extent the zones of elongation and maturation (Rodriguez-Galvez and Mendgen 1995). The hyphal growth occurs along the

depression in the cell wall junctions which results in the ingress of hyphae between adjoining epidermal cells. Alternatively, direct penetration of the epidermal cells can take place immediately after conidial germination. The fungus enters through the root cortex and follows both inter and intracellular routes (Hall et al. 2013). The hyphae colonize the xylem vessels and invades adjacent xylem vessels by growing through the pits or by directly penetrating the vessel walls (Hall et al. 2013). The fungus spreads systemically inside plants via transport of conidia and mycelial growth in the vessels and cortex region. Vascular occlusion occurs due to the release of conidia by the pathogen and the simultaneous release of secondary compounds by the plant causing disruption of the water transport system (Turco et al. 2007a). This leads to the development of the characteristic symptoms of *Fusarium* wilt of cotton; chlorosis and necrosis of leaves and vascular browning of xylem, and ultimately results in either a physiologically compromised plant yielding a suboptimal quantity and/or quality lint production or a completely collapsed plant. The incidence and severity of *Fusarium* wilt outbreaks in cotton are a result of the pathogen density, the genetic resistance of the plant, and the prevailing environmental conditions (Devay et al. 1997; Mcfadden et al. 2004).

1.3.1 Environmental Factors

Fov is a warm temperature favoring pathogen. The optimum temperature required for *Fusarium* spore germination in soil is ~25°C (Nelson et al. 1990) and the optimum temperature for hyphal penetration ranges from 28 to 30°C (Rodriguez-Galvez and Mendgen 1995). *F. oxysporum* can grow in temperatures ranging from 10 to 32°C, while growth inhibition occurs at temperatures below or above this range (Larkin and Fravel 2002). The optimum moisture holding capacity of the soil for spore germination and fungal

growth is 40% and the fungal activity is favored by high humidity (El-Abyad and Saleh 1971). The disease is most damaging in acidic and sandy soils with a pH of 5.0 to 6.5 (Bell et al. 2003) while the disease is less of a problem in heavier clay soils (Larkin 1993).

Disease suppression of different types of soil on *Fusarium* wilt of cotton has been known to occur (Larkin 1993), as red lateritic clay soils are suppressive to *Fusarium* wilt (Smith and Snyder 1972). Disease suppression activity is commonly associated with physical characteristics like pH, soil type, and organic matter content in the soil (Larkin and Fravel 2002).

1.3.2 Interaction with nematodes

Infection of some isolates of *Fov* is associated with the root-knot nematode, *Meloidogyne incognita* (Kofoid and White) Chitwood, for development of *Fusarium* wilt in some cotton cultivars. Initial cotton root infection by *M. incognita* increases the incidence of secondary infection and colonization by *Fov* (Garber et al. 1978). Cotton plants infected with both *Fov* and root-knot nematodes are more stunted, resulting in greater yield losses than plants infected with *Fov* alone (Garber et al. 1978). The control of root knot nematodes by nematode resistant cultivars, nematicides, and crop rotation results in marked reduction in the incidence and the severity of *Fusarium* wilt of cotton (Kim 2005). In soil infested with both pathogens, the extent of fungal invasion and colonization is correlated with the degree of root galling by nematodes; however, the precise mechanism(s) involved in the interaction between *Fov* and *M. incognita* that favor disease progression is not fully understood (Garber et al. 1978).

1.3.3 Development and distribution of different races

Races of fungal phytopathogens are defined as a population or biotypes that carry the same combination of virulence genes and are capable of attacking certain varieties of susceptible host species (Parlevliet 1985; Dutta and Garber 1961). Pathogenic races can be of two different types; those that interact differentially with varieties of the host plant, and those that do not (Plank 1969). In *F. oxysporum* races had been identified inconsistently (Kistler 1997). In some instances, it has been defined as strains compatible to specific cultivars of the host genotype (Kistler 1997). This definition of race is common in plant pathology; however, in some cases race has also been defined as selectivity of isolates to distinct plant species (Armstrong and Armstrong 1981). As a result, race designation was given for isolates that were specific to genotypes within a plant species.

Eight different races within *Fov* have been described based on the geographic location of their initial isolation and pathogenicity tests on cotton and non-cotton hosts (Armstrong and Armstrong 1958, 1960; Ibrahim 1966; Armstrong and Armstrong 1978; Qiying et al. 1985). Races 1 and 2 were identified from the United States (Armstrong and Armstrong 1958), race 3 in Egypt (Armstrong and Armstrong 1960), race 4 in India (Armstrong and Armstrong 1960), race 5 in Sudan (Ibrahim 1966), race 6 in Brazil (Armstrong and Armstrong 1978), and races 7 and 8 in China (Qiying et al. 1985). Originally, all of these races had distinct geographic locations but with the global trade of cotton seeds their distribution has changed. In the United States, only races 1 and 2 were known to occur until early 2000s (Smith et al. 1981; Devay 1986) where eventually races 3, 4, and 8 were identified in California in 2003 (Kim 2005). Among all the races, race 4 is the most virulent strain of *Fov* (Ulloa et al. 2013). As reported by (Cianchetta et al. 2015) race 4 was not

documented outside of California in the United States until 2017 when it was reported in western Texas (Halpern et al. 2018). Unlike the other races of *Fov*, race 4 can cause extensive disease symptoms in cotton plants even in the absence of root knot nematodes (Kim 2005).

In addition to the eight races of *Fov*, in 1993 two closely related biotypes of *Fov* populations were discovered on cotton crops in Australia (Kochman 1995). Since the term "race" in regards to *Fov* was deemed invalid, no race designation was made for the Australian biotypes (Davis et al. 2006, 1996). Some studies have indicated that the Australian isolates behave similar to race 6 on differential hosts (Davis et al. 1996) while other studies have suggested that the Australian *Fov* isolates evolved indigenously (Wang et al. 2006). Overall, knowledge on the Australian *Fov* genotypes is limited.

Previously the race designation of *Fov* isolates was based loosely on pathogenicity tests using different cotton cultivars and non-cotton hosts. However, these tests are time consuming and require a standardized procedure. The classification of *Fov* races might be ambiguous if only based on a pathogenicity test on a few cultivars (Davis et al. 2006). Even the simple difference in inoculation densities (Devay et al. 1997) and inoculation methods (Ibrahim and Nirenberg 1993) may alter the aggressiveness of *Fov* towards some cultivars. Since the 1990s, pathogenicity tests in conjunction with different DNA-based techniques have been employed to distinguish among different races of *Fov* (Bridge et al. 1993; Assigbetse et al. 1994; Egamberdiev et al. 2013; Skovgaard et al. 2001). In 1993, vegetative compatibility groups (VCGs) among races 1, 2, 3, and 4 of *Fov* were investigated and concluded that race 1 and 2 belong to one VCG and race 3 and 4 belong to a separate VCG (Bridge et al. 1993). Random amplified polymorphic DNA (RAPD) markers along with

pathogenicity tests were also used to distinguish *Fov* races; however no strict correlation was found between races and RFLPs (Assigbetse et al. 1994). Amplified fragment length polymorphism (AFLP) markers have also been used to find intraspecific genetic variation in fungi (Mueller and Wolfenbarger 1999). Recently AFLP technology has been used to distinguish between different races of *Fov* (Egamberdiev et al. 2013; Wang et al. 2006).

Multigene genealogy analysis has been conducted on DNA sequences encoding elongation factor (*EF-1*), beta tubulin (*BT*), and phosphate permease (*PHO*) (Skovgaard et al. 2001). Phylogenetic analysis based on regions within the coding regions of *EF-1*, *BT*, and *PHO* genes sorted *Fov* races into four lineages. Lineage I with race 3 and 5; lineage II with race 1,2, and 6; lineage III with race 8, and lineage IV with race 4 and 7 (Skovgaard et al. 2001).

1.4 Phytoalexins

Plants are continuously challenged with many pathogenic microorganisms in their natural environment. They have developed a variety of resistance mechanisms, innate and induced, to cope with these stresses. One of these mechanisms is the production of low-molecular-weight antimicrobial or anti-oxidative compounds termed phytoalexins, as a response to pathogenic microorganisms and abiotic stresses (Ahuja et al. 2012; Jeandet et al. 2013). As such they take part in an intricate defense system enabling plants to impede the invasion of microorganisms (Jeandet and Philippe 2015). The concept of phytoalexins as a defense response was first introduced in 1940 by Müller and Börger when they observed that potato tubers infected with a strain of *Phytophthora infestans* is capable of initiating a hypersensitive reaction that significantly inhibited the effect of a subsequent infection with another strain of *P. infestans* (Muller 1940). This inhibition was called a “principle” produced

by the plant cells reacting in a hypersensitive manner that they named phytoalexin (Deverall 1972). After Muller introduced the concept of phytoalexin, these biologically active compounds have been shown to have a number of applications including health promotion in humans (Pedras et al. 2011; Yang et al. 2009; Boue et al. 2009; Smoliga et al. 2011). For example, resveratrol from grapevines has anticarcinogenic, anti-aging, antioxidant, and anti-inflammatory properties (Smoliga et al. 2011), glyceollin from soybeans has antitumor and antiproliferative actions (Ng et al. 2011), and 3-deoxyanthocyanins from sorghum are known to reduce gastrointestinal cancer (Yang et al. 2009).

A range of phytoalexins have been described that are produced by crop plants in the families of Leguminosae, Solanaceae, Brassicaceae, Malvaceae, and Poaceae in response to pathogen infection or elicitor treatment (Ahuja et al. 2012; Jeandet et al. 2014). Several parts of a plant including the leaves, stems, roots, flowers, and seeds, are capable of producing phytoalexins, and their production can be influenced by several factors such as humidity, temperature, and water availability (Mikkelsen et al. 2003). Phytoalexins have been categorized into several different classes based on the structure and the active side chains in the compound. Flavonoids, alkaloids, terpenoids, coumarins, apigeniden, phenolic compounds, apigeninidin, and luteolinidin and more than 300 phytoalexins have been characterized within these classes (Harborne 1999; Araujo and Menezes 2009). Among these classes, terpenoids constitute the largest group and play varied roles from plant growth and development, to plant protection from herbivores and pathogens (Tholl 2015). The terpenoid phytoalexins have higher commercial value due to their industrial and pharmacological uses; for instance paclitaxel, a diterpene alkaloid, is used clinically for the treatment of different types of cancers (Guchelaar et al. 1994), and artemisinin, a sesquiterpene lactone, is used clinically for the treatment of malaria caused by *Plasmodium*

falciparum (Paddon and Keasling 2014). Cotton plants, produce gossypol, a sesquiterpenoid phytoalexin (Tian et al. 2016).

1.5 Gossypol

Gossypol is a yellow colored phenolic compound occurring naturally in small intercellular pigment glands of cotton plants (Liu et al. 1999). It was first isolated by Longmore (1886) as a crude pigment from cottonseed oil. The name is derived from the scientific name of cotton plants, *Gossypium,* combined with 'ol' from phenol (Spinosa et al. 2008). It is crystalline in nature, insoluble in water and hexane, but soluble in ether, chloroform, acetone, methyl ethyl ketone, and partially soluble in crude vegetable oils. It has a molecular weight of 518.55 Daltons with a chemical formula $C_{30}H_{30}O_8$ and structural formula of 2,2′-bis(8-formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphthalene); Figure 1-1 (Gadelha et al. 2014; Abou-Donia 1976; Rogers et al. 2002).

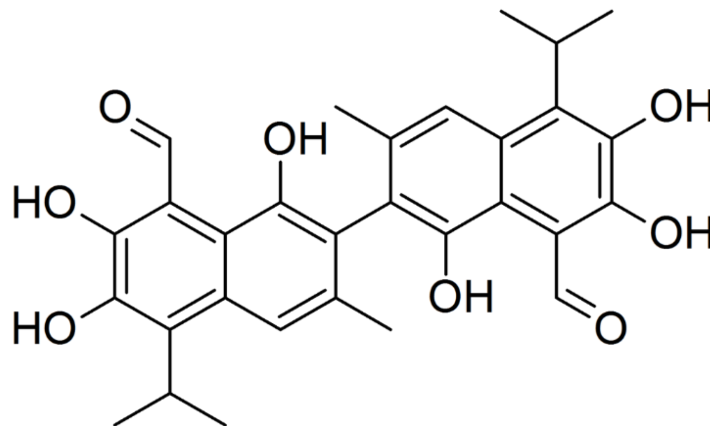

Figure 1-1: Structure of gossypol

Gossypol is the combination of two enantiomers, (−) and (+) gossypol, where the (-) isomer is the more physiologically active form (Hron et al. 1999) and more toxic than the (+) isomer (Lordelo et al. 2005). Both isomers are produced by cotton species in varied proportions and is genetically determined by the alleles Gl_2 and Gl_3 (Spinosa et al. 2008; Lee 1973). In upland cotton (*Gossypium hirsutum*), Gl_2 is expressed higher than Gl_3 and usually

produces more of the (+) enantiomer; while in the Pima cultivar (*G. barbadense*) both the Gl_2 and Gl_3 alleles are equally expressed and synthesize more of the (-) enantiomer (Hron et al. 1999; Lee 1973).

Gossypol is produced by several plant parts, stems, leaves, flower buds, roots, and seeds, where the highest concentration is found in the seeds (Abou-Donia 1976; Kenar 2006; Alexander et al. 2008; Hunter et al. 1978). Gossypol and derivative compounds like desoxyhemigossypol, desoxy-6-methoxyhemigossypol, hemigossypol, 6-methoxyhemigossypol, etc. are produced and released by cotton roots in the form of root exudates (Hunter et al. 1978). These compounds tend to occur in greater concentration in the roots of *G. barbadense* varieties (Stipanovic et al. 1977). The gossypol content of the root bark increases as the plant matures and can constitute about 1.8% of total amount of gossypol in cotton (Royce et al. 1941). The content of gossypol found in seeds varies among different cotton varieties (Alexander et al. 2008; Randel et al. 1992) and can range from 0.02 to 6.64% (Price et al. 1993). The seeds of *G. barbadense* alone may synthesize up to 34g of gossypol per kg of seeds (Percy et al. 1996). In most cotton cultivars, seeds might contain more than 7 g/kg of free gossypol and more than 14 g/kg of total gossypol (Alexander et al. 2008).

1.5.1 Biological activity of gossypol

Gossypol is hypothesized to be a part of the defense system of the cotton plant through anti-insect activity (Bottger et al. 1964) as well as antimicrobial activity (Mellon et al. 2011). Gossypol is toxic to humans as well as animals (Gadelha et al. 2014; Alexander et al. 2008), as gossypol poisoning has been reported in goats, pigs (Haschek et al. 1989), broiler chickens (Henry et al. 2001), sheep (Morgan et al. 1988), and dogs (West 1940). In

mammals, gossypol is reported to have an inhibitory effect in spermatogenesis in males and interferes with the estrous cycle, pregnancy, and early embryonic development in females (Randel et al. 1992). In addition to its antimicrobial activity, gossypol has anticancer and antiviral activity (Wang et al. 2008; Lee et al. 2017; Polsky et al. 1989; Gilbert et al. 1995). Gossypol has a cytotoxic effect on tumor cells and therefore may have a therapeutic benefit for the treatment of colon cancer (Wang et al. 2000). Literature shows that gossypol enantiomers contribute unevenly to these biological activities. In *Aspergillus flavus*, (-) gossypol was four times more active than (+) gossypol in inhibitory activity on conidial germination, mycelial growth, and conidiophore development (Mellon et al. 2003). Whereas in the fungal pathogen *Rhizoctonia solani,* (+) gossypol, (-) gossypol, as well as a racemic mixture were found to be equally effective in inhibiting growth of this pathogen (Puckhaber et al. 2002). Gossypol is a potent growth inhibitor of the pathogens *Pythium irregulare, P. ultimum,* and *F. oxysporum* when tested at a concentration of 100 μg ml^{-1} ,whereas the pathogens *R. solani* and *Mortierella* sp. growth inhibition was only around 60 to75% (Mellon et al. 2014). *Fov* is able to overcome gossypol inhibition over time suggesting *Fov* resilience towards gossypol might be due to detoxification of gossypol by *Fov* or due to the breakdown of gossypol naturally (Turco et al. 2007).

1.6 Phytoalexin tolerance by plant pathogens

The interaction between plants and pathogens has been referred to as a molecular arms race and has evolved for centuries and continues to escalate (Maor and Shirasu 2005). Although phytoalexins have been regarded as a means to defend plants against pathogens, their actual role in disease resistance is controversial (Vanetten et al. 1989). The ability of pathogens to tolerate the effect of phytoalexins is one of the determinants of host-

pathogen interactions (Wu and Vanetten 2004; Cho et al. 2014). With his work on *Monilinia fructicola* and pisatin, the phytoalexin of pea, I.A.M. Cruickshank established the concept of phytoalexin tolerance in pathogens (Cruickshank 1962). Muller had also advised that pathogens can “adapt to” or degrade phytoalexins (Muller 1958). However, many detoxification mechanisms of phytoalexins by fungi are not clearly understood (Cho et al. 2014).

The first enzymatic detoxification of a phytoalexin was reported by Uehera in 1964 concerning the detoxification of pisatin to non-toxic compounds by *F. oxysporum* and *Ascochyta pisi* (Uehara 1964). Since then, the most studied mechanism of phytoalexin tolerance is enzymatic degradation of phytoalexins to less or non-toxic compounds (VanEtten et al. 2001). Phytoalexins are lipophilic in nature and can efficiently penetrate cell membranes. The metabolism of phytoalexins by fungi generally involves their conversion to more polar products, and chemical reactions like reduction (Pedras and Suchy 2005), hydration (Li et al. 1995), glycosylation (Pezet et al. 2004), oxidation (Pedras et al. 2008), demethylation (Delserone et al. 1999), and oxidative dimerization (Breuil et al. 1998) are involved in catabolism by fungi. The formation of new hydroxyl groups by demethylation and oxygenation, glycosylation, hydration of double bonds, or reduction of aldehydes and ketones increases the degree of polarity of phytoalexins (Jeandet et al. 2014). An example is the oxidation of brassinin, a phytoalexin in crucifers, by the enzyme brassinin oxidase in *Leptosphaeria maculans* into the less fungitoxic compound indol-3-carboxaldehyde (Figure 1-2) (Pedras et al. 2008). Similarly, in *Fusarium pseudograminearum* the enzyme *N*-malonyltransferase is involved in the detoxification of benzoxazolinones, the phytoalexins in wheat (Kettle et al. 2015). Demethylation of pisatin by the cytochrome P450 pisatin demethylase into 6a-hydroxymaackiain and 3-hydroxymaackiain-isoflavan has been

characterized in *Nectria haematococca* and *F. oxysporum* f. sp. *pisi* (Delserone et al. 1999). Non-methylated phytoalexins are known to be less fungitoxic than methylated since methylation augments their lipophilic character (Jeandet et al. 2014). Hydration of the bean phytoalexin kievitone by the enzyme kievitone hydratase of *F. solani* produces a less toxic compound (Li et al. 1995). Similarly, oxidative dimerization of stilbene phytoalexins by a laccase-like stilbene oxidase from *Botrytis cinera* has also been reported (Breuil et al. 1998; Kettle et al. 2016).

In addition to enzymatic detoxification, a second mode of phytoalexin tolerance is a non-degradative mechanism, and is common in both prokaryotes and eukaryotes through the activation of transporters (Bolhuis et al. 1997). Transporters can nullify the effect of phytoalexins by isolating toxic compounds into a vacuole or extruding them outside the cell (Jeandet et al. 2014). The existence of non-degradative tolerance was first described in *N. haematococca* in an isolate that was unable to metabolize the phytoalexin pisatin (Denny

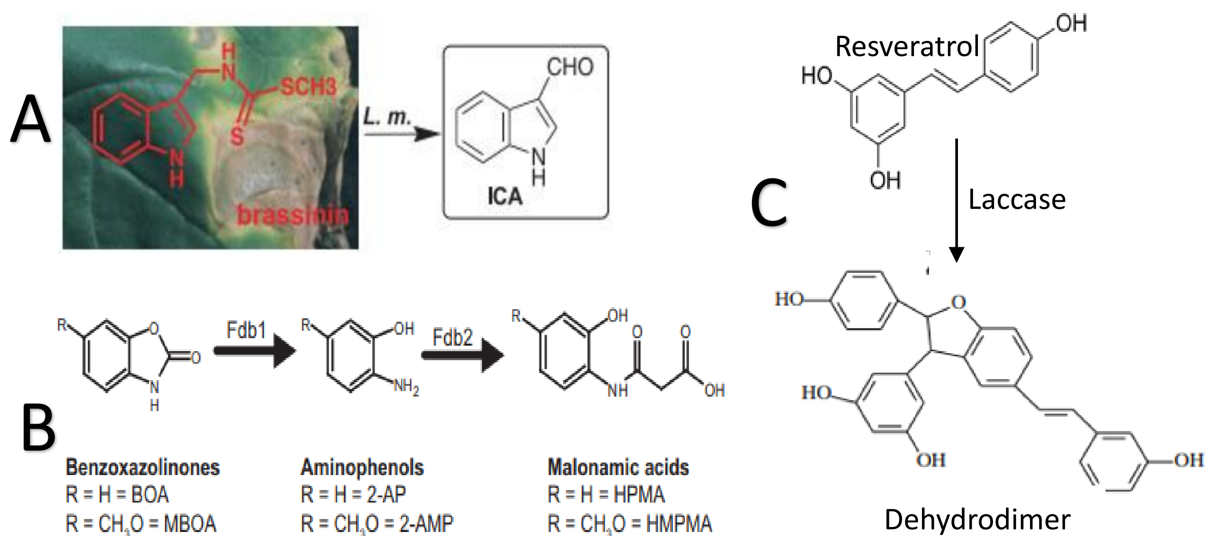

Figure 1-2: Enzymatic detoxification of phytoalexins to more polar compounds. (A) oxidation of brassinin to indol-3-carboxaldehyde by a brassinin oxidase. (B)degradation of benzoxazolinones to malonamic acid by a *N*-malonyltransferase. (C) oxidative dimerization of resveratrol to dehydrodimer by a laccase-like stilbene oxidase.

and Vanetten 1983). The transporters are expressed in fungi after elicitation by phytoalexins and other toxic compounds (Urban et al. 1999; Schoonbeek et al. 2001; Coleman et al. 2011a). The ABC transporter, NhABC1 of *N. haematococca*, GpABC1 of *Gibberella pulicaris*, and BcatrB of *Botrytis cinerea* confer tolerance to the phytoalexins pisatin, rishitin, and resveratrol, respectively (Schoonbeek et al. 2001; Coleman et al. 2011a; Fleisner et al. 2002). In addition, *NhABC1*, *BcatrB*, and *GpABC1* mutants had reduced virulence on pea, grape leaves, and potato respectively (Schoonbeek et al. 2001; Coleman et al. 2011a; Fleisner et al. 2002). Besides ABC transporters, members of the major facilitator superfamily (MFS) of transporters are also known to be involved in multidrug, toxin, and fungicide resistance through efflux of small molecules (Callahan et al. 1999; Roohparvar et al. 2008; dos Santos et al. 2014). *Bcmfs1*, a MFS transporter from *B. cinerea* was found to provide tolerance to camptothecin, a natural toxin produced by the plant *Camptotheca acuminata* (Hayashi et al. 2002). When the MFS transporter Pep5 from *N. haematococca* was added to a nonpathogenic pea isolate it was able to confer an increase in pathogenicity on pea (Han et al. 2001). *PEP5*, the MFS encoding gene was induced after exposure to the phytoalexin pisatin of pea (Liu et al. 2003); however, the molecular mechanism responsible for the increase in virulence of *PEP5* on pea is unknown.

Another mechanism of drug tolerance in microbes is through the alteration of the targeted cellular components (Bolhuis et al. 1997). However, no examples of phytoalexin tolerance mechanisms in fungi have been reported due to natural modification of the targeted cellular component. Studies on mutants of *N. haematococca* and tomatine, a phytoanticipin in tomato, demonstrated how changes in the target site could influence virulence (Défago and Kern 1983). It was found that tomatine tolerant mutants of this fungus were reduced in sterol content, and were more virulent on green tomato fruit, which

contain high concentrations of tomatine. This was because tomatine interacts directly with sterols in the cell membrane and a slight difference in sterol content affects fungal sensitivity to tomatine (Défago and Kern 1983; Défago et al. 1983). These three different mechanisms of phytoalexin tolerance by pathogens is represented in Figure 1-3 (Vanetten et al. 2001).

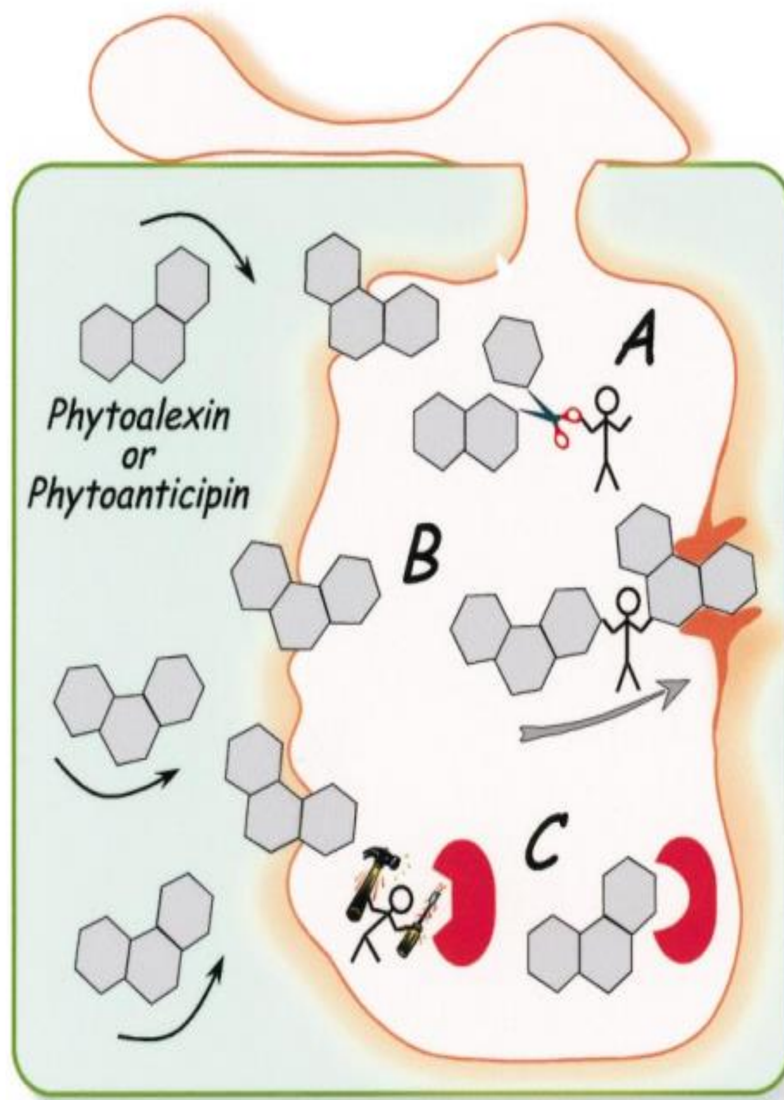

Figure 1-3: Proposed three main mechanisms of phytoalexin tolerance by fungi; (A) detoxification mediated by enzymatic modification; (B) non-degradative tolerance or exclusion of phytoalexins mediated by an efflux pump; and (C) alteration of the cellular component (red structure) targeted by the toxin.

1.7 References

Abou-Donia, M. B. 1976. Physiological effects and metabolism of gossypol. Pages 125-160. In:*Residue Reviews*, Springer New York, New York, NY

Ahuja, I., Kissen, R., and Bones, A. M. 2012. Phytoalexins in defense against pathogens. Trends Plant Sci. 17:73-90

Alexander, J., Benford, D., Cockburn, A., Cravedi, J.-P., Dogliotti, E., Domenico, A. Di, Luisa Férnandez-Cruz, M., Fürst, P., Fink-Gremmels, J., Corrado, L., Galli, P., Grandjean, J., Gzyl, G., Heinemeyer, N., Johansson, A., Mutti, J., Schlatter, R., Van Leeuwen, C. Van, and Peteghem, P. V. 2008. Scientific opinion of the panel on contaminants in the food chain. The EFSA Journal. 908:1-55

Araujo, F. F. de, and Menezes, D. 2009. Indução de resistência a doenças foliares em tomateiro por indutores biótico (*Bacillus subtilis*) e abiótico (Acibenzolar-S-Metil). Summa Phytopathol. 35:169-172

Armstrong, G., and Armstrong, J. 1981. Formae speciales and races of *Fusarium oxysporum* causing wilt diseases. Nelson PE, Toussoun TA, Cook RJ, eds. *Fusarium* Dis. Biol. Taxon. Univ. Park. PA, USA State Univ. Press. :391-399

Armstrong, G., and Armstrong, J. 1960. American, Egyptian, and Indian cotton-wilt fusaria: their pathogenicity and relationship to other wilt fusaria. U.S. Department of Agriculture Technology Bulletin 1219

Armstrong, G. M., and Armstrong, J. K. 1978. A new race (race 6) of the cotton-wilt *Fusarium* from Brazil. Plant Dis. Report. 62:421-423

Armstrong, J. K., and Armstrong, G. M. 1958. A race of the cotton-wilt *Fusarium* causing wilt of Yelredo soybean and flue-cured tobacco. Plant Dis Report. 42:147-151

Assigbetse, K. B., Fernandez, D., Dubois, M. P., Geiger, J., Medani, W., Katan, J., and Nicole, M. 1994. Differentiation of *Fusarium oxysporum* f. sp. *vasinfectum* races on cotton by random amplified polymorphic DNA (RAPD) analysis. Mol. Plant Pathol. 84:622-626

Bell, A. A., Wheeler, M. H., Liu, J., Stipanovic, R. D., Puckhaber, L. S., and Orta, H. 2003. United States Department of Agriculture-Agricultural Research Service studies on polyketide toxins of *Fusarium oxysporum* f.sp. *vasinfectum*: potential targets for disease control. Pest Manag. Sci. 59:736-47

Bolhuis, H., van Veen, H. W., Poolman, B., Driessen, A. J. M., and Konings, W. N. 1997. Mechanisms of multidrug transporters. FEMS Microbiol. Rev. 21:55-84

Bottger, G. T., Sheehan, E. T., and Lukefahr, M. J. 1964. Relation of gossypol content of cotton plants to insect resistance. Econ. Entomol. 57:283-285

Boue, S. M., Cleveland, T. E., Carter-Wientjes, C., Shih, B. Y., Bhatnagar, D., McLachlan, J. M., and Burow, M. E. 2009. Phytoalexin-enriched functional foods. J. Agric. Food Chem. 57:2614-2622

Breuil, A.-C., Adrian, M., Pirio, N., Meunier, P., Bessis, R., and Jeandet, P. 1998. Metabolism of stilbene phytoalexins by *Botrytis cinerea*: 1. Characterization of a resveratrol dehydrodimer. Tetrahedron Lett. 39:537-540

Bridge, P. D., Ismail, M. A., and Rutherford, M. A. 1993. An assessment of aesculin hydrolysis, vegetative compatibility and DNA polymorphism as criteria for characterizing pathogenic races within *Fusarium oxysporum* f.sp. *vasinfectum*. Plant Pathol. 42:264-269

Callahan, T. M., Rose, M. S., Meade, M. J., Ehrenshaft, M., and Upchurch, R. G. 1999. CFP, the putative cercosporin transporter of *Cercospora kikuchii*, is required for wild type cercosporin production, resistance, and virulence on soybean. Mol. Plant-Microbe Interact. 12:901-910

Chantal, and Alabouvette, C. 1997. Colonization of tomato root by a non-pathogenic strain of *Fusarium oxysporum*. New Phytologist. 137:481-494

Cho, Y., Ohm, R., Devappa, R., Lee, H., Grigoriev, I., Kim, B., and Ahn, J. 2014. Transcriptional responses of the *Bdtf1*-deletion mutant to the phytoalexin brassinin in the necrotrophic fungus *Alternaria brassicicola*. Molecules. 19:10717-10732

Cianchetta, A. N., Allen, T. W., Hutmacher, R. B., Kemerait, R. C., Kirkpatrick, T. L., Lawrence, G. W., Lawrence, K. S., Mueller, J. D., Nichols, R. L., Olsen, M. W., Overstreet, C., Woodward, J. E., Davis, R. M., Cianchetta, A. N., and Mueller, 2015*. Survey of Fusarium oxysporum* f. sp. *vasinfectum in the United States*. The journal of Cotton Science. 19:328-336

Coleman, J. J., White, G. J., Rodriguez-Carres, M., and Vanetten, H. D. 2011. An ABC transporter and a cytochrome P450 of *Nectria haematococca* MPVI are virulence factors on pea and are the major tolerance mechanisms to the phytoalexin pisatin. Mol. Plant-Microbe Interact. 24:368-376

Cruickshank, I. 1962. Studies on phytoalexins IV. the antimicrobial spectrum of pisatin. Aust. J. Biol. Sci. 15:147-159

Davis, R. M., Colyer, P. D., Rothrock, C. S., and Kochman, J. K. 2006. *Fusarium* wilt of cotton: population diversity and implications for management. Plant Dis. 90:692-703

Davis, R., Moore, N., and Kochman, J. 1996. Characterisation of a population of *Fusarium oxysporum* f.sp. *vasinfectum* causing wilt of cotton in Australia. Aust. J. Agric. Res. 47:1143

Défago, G., and Kern, H. 1983. Induction of *Fusarium solani* mutants insensitive to tomatine, their pathogenicity and aggressiveness to tomato fruits and pea plants. Physiol. Plant Pathol. 22:29-37

Défago, G., Kern, H., and Sedlar, L. 1983. Genetic analysis of tomatine insensitivity, sterol content and pathogenicity for green tomato fruits in mutants of *Fusarium solani*. Physiol. Plant Pathol. 22:39-43

Delserone, L., McCluskey, K., Matthews, D., and Vanetten, H. 1999. Pisatin demethylation by fungal pathogens and nonpathogens of pea: association with pisatin tolerance and virulence. Physiol. Mol. Plant Pathol. 55:317-326

Denny, T. P., and Vanetten, H. D. 1983. Characterization of an inducible, nondegradative tolerance of *Nectria haematococca* MPVI to phytoalexins. J. Gen. Micro. 129:2903-2913

Devay, J. E. 1986. Half a century dynamics and control of cotton diseases: *Fusarium* and *Verticillium* wilt. Proc. Beltwide Cott. Prod. Res. Conf, Natl Cotton Council, America, Memphis, TN, p 35

Devay, J. E., Gutierrez, A. P., Pullman, G. S., Wakeman, R. J., Garber, R. H., Jeffers, D. P., Smith, S. N., Goodell, P. B., Roberts, P. A., and N, S. S. 1997. Inoculum densities of *Fusarium oxysporum* f. sp. *vasinfectum* and *Meloidogyne incognita* in relation to the development of *Fusarium* wilt and the phenology of cotton plants (*Gossypium hirsutum*). Phytopathology. 87:341-346

Deverall, B. J. 1972. Phytoalexins and disease resistance. Proc. R. Soc. B Biol. Sci. 181:233-246

Dutta, S. K., and Garber, E. D. 1961. The identification of physiological races of a fungal phytopathogen using strains of the slime mold *Acrasis rosea*. Proc. Natl. Acad. Sci. 47:990-993

Egamberdiev, S. S., Ulloa, M., Saha, S., Salakhutdinov, I. B., Abdullaev, A., Glukhova, L. A., Adylova, A. T., Scheffler, B. E., Jenkins, J. N., and Abdurakhmonov, I. Y. 2013. Molecular characterization of uzbekistan isolates of *Fusarium oxysporum* f. sp. *vasinfectum*. J. Plant Sci. Mol. Breed. 2:3

El-Abyad, M. S., and Saleh, Y. E. 1971. Studies with *Fusarium oxysporum* f.sp. *vasinfectum*, the cause of cotton wilt in Egypt. Germination, sporulation and growth. Trans. Br. Mycol. Soc. 57:427-437

Fleisner, A., Sopalla, C., and Weltring, K.-M. 2002. An ATP-binding cassette multidrug-resistance transporter is necessary for tolerance of *Gibberella pulicaris* to phytoalexins and virulence on potato tubers. Mol. Plant-Microbe Interact. 15:102-108

Gadelha, I. C. N., Fonseca, N. B. S., Oloris, S. C. S., Melo, M. M., and Soto-Blanco, B. 2014. Gossypol toxicity from cottonseed products. Scientific World Journal. 2014:1-11

Garber, R. H., Jorgenson, E. C., Smith, S., and Hyer, A. H. 1978. Interaction of population levels of *Fusarium oxysporum* f. sp. *vasinfectum* and *Meloidogyne incognita* on cotton. J. Nematol. 11:133-137

Gilbert, N. E., O'Reilly, J. E., Chang, C. J. G., Lin, Y. C., and Brueggemeier, R. W. 1995. Antiproliferative activity of gossypol and gossypolone on human breast cancer cells. Life Sci. 57:61-67

Gordon, T. R., and Martyn, R. D. 1997. The evolutionary biology of *Fusarium oxysporum*. Annu. Rev. Phytopathol. 35:111-128

Guchelaar, H. J., ten Napel, C. H., de Vries, E. G., and Mulder, N. H. 1994. Clinical, toxicological and pharmaceutical aspects of the antineoplastic drug taxol: a review. Clin. Oncol. (R. Coll. Radiol). 6:40-8

Hall, C., Heath, R., and Guest, D. 2013. The infection process of *Fusarium oxysporum* f.sp. *vasinfectum* in Australian cotton. Australas. Plant Pathol. 42:1-8

Halpern, H. C., Bell, A. A., Wagner, T. A., Liu, J., Nichols, R. L., Olvey, J., Woodward, J. E., Sanogo, S., Jones, C. A., Chan, C. T., and Brewer, M. T. 2018. First report of *Fusarium* wilt of cotton caused by *Fusarium oxysporum* f. sp. *vasinfectum* race 4 in Texas, U.S.A. Plant Dis. 102:446-446

Han, Y., Liu, X., Benny, U., Kistler, H. C., and Vanetten, H. D. 2001. Genes determining pathogenicity to pea are clustered on a supernumerary chromosome in the fungal plant pathogen *Nectria haematococca*. Plant J. 25:305-314

Harborne, J. B. 1999. The comparative biochemistry of phytoalexin induction in plants. Biochem. Syst. Ecol. 27:335-367

Haschek, W. M., Beasley, V. R., Buck, W. B., and Finnell, J. H. 1989. Cottonseed meal (gossypol) toxicosis in a swine herd. J. Am. Vet. Med. Assoc. 195:613-5

Hayashi, K., Schoonbeek, H.-J., and De Waard, M. A. 2002. *Bcmfs1*, a novel major facilitator superfamily transporter from *Botrytis cinerea*, provides tolerance towards the natural toxic compounds camptothecin and cercosporin and towards fungicides. Appl. Environ. Microbiol. 68:4996-5004

Henry, M. H., Pesti, G. M., and Brown, T. P. 2001. Pathology and histopathology of gossypol toxicity in broiler chicks. Avian Dis. 45:598-604

Hillocks, R. J. 1992. *Fusarium* wilt. In: Hillocks RJ, ed. Cotton Diseases. Wallingford, UK: CAB International, 127–60.

Hron, R. J., Kim, H. L., Calhoun, M. C., and Fisher, G. S. 1999. Determination of (+)-, (−)-, and total gossypol in cottonseed by high-performance liquid chromatography. J. Am. Oil Chem. Soc. 76:1351-1355

Hunter, R. E., Halloin, J. M., Veech, J. A., and Carter, W. W. 1978. Exudation of terpenoids by cotton roots. Plant Soil. 50:237-240

Ibrahim, F. M. 1966. A new race of cotton-wilt *Fusarium* in the Sudan Gezira. Emp. Cott. Grow. Rev. 43:296-299

Ibrahim, G., and Nirenberg, H. I. 1993. Response of some Sudanese cotton cultivars to race 1 and 5 of *Fusarium oxysporum* f. sp. *vasinfectum*. J. Plant Dis. Prot. 100:645-651

Jeandet, P., Clément, C., Courot, E., and Cordelier, S. 2013. Modulation of phytoalexin biosynthesis in engineered plants for disease resistance. Int. J. Mol. Sci. 14:14136-14170

Jeandet, P., Hébrard, C., Deville, M.-A., Cordelier, S., Dorey, S., Aziz, A., and Crouzet, J. 2014. Deciphering the role of phytoalexins in plant-microorganism interactions and human health. Molecules. 19:18033-18056

Jeandet, P., and Philippe. 2015. Phytoalexins: current progress and future prospects. Molecules. 20:2770-2774

West, J. 1940. Lesions of gossypol poisoning in the dog. J. Am. Vet. Med. Assoc. 96:74-76

Kang, S., Demers, J., del Mar Jimenez-Gasco, M., and Rep, M. 2014. *Fusarium oxysporum*. In:*Genomics of plant-associated fungi and oomycetes: dicot pathogens*, Springer Berlin Heidelberg, Berlin, Heidelberg. 99-119

Kenar, J. A. 2006. Reaction chemistry of gossypol and its derivatives. J. Am. Oil Chem. Soc. 83:269-302

Kettle, A. J., Batley, J., Benfield, A. H., Manners, J. M., Kazan, K., and Gardiner, D. M. 2015. Degradation of the benzoxazolinone class of phytoalexins is important for virulence of *Fusarium pseudograminearum* towards wheat. Mol. Plant Pathol. 16:946-962

Kettle, A. J., Carere, J., Batley, J., Manners, J. M., Kazan, K., and Gardiner, D. M. 2016. The *Fdb3* transcription factor of the *Fusarium* detoxification of benzoxazolinone gene cluster is required for MBOA but not BOA degradation in *Fusarium pseudograminearum*. Fungal Genet. Biol. 88:44-53

Kim, Y., Hutmacher, R.B., and Davis, R.M. 2005. Characterization of California isolates of *Fusarium oxysporum* f. sp. *vasinfectum*. Plant Dis. 89:366-372

Kistler, H. C. 1997. Genetic diversity in the plant-pathogenic fungus *Fusarium oxysporum*. Phytopathology. 87:474-479

Kochman, J. 1995. *Fusarium* wilt in cotton - a new record in Australia. Australas Plant Pathol. 24:74

Kosmidis, C., and Denning, D. W. 2017. Opportunistic and Systemic Fungi. In: *Infectious Diseases*, Pages 1681-1709

Larkin, R. P. 1993. Ecology of *Fusarium oxysporum* f. sp. *niveum* in soils suppressive and conducive to *Fusarium* wilt of watermelon. Phytopathology. 83:1097-1105

Larkin, R. P., and Fravel, D. R. 2002. Effects of varying environmental conditions on biological control of *Fusarium* wilt of tomato by nonpathogenic *Fusarium* spp. Phytopathology. 92:1160-1166

Laurence, M. H., Walsh, J. L., Shuttleworth, L. A., Robinson, D. M., Johansen, R. M., Petrovic, T., Vu, T. T. H., Burgess, L. W., Summerell, B. A., and Liew, E. C. Y. 2016. Six novel species of *Fusarium* from natural ecosystems in Australia. Fungal Divers. 77:349-366

Lee, J. 1973. The inheritance of gossypol level in *Gossypium*. II. Inheritance of seed gosypol in two trains of cultivated *Gossypoium barbadense* L. Genetics. 75:259-64

Lee, W., Basri, D., and Ghazali, A. 2017. Bactericidal effect of pterostilbene alone and in combination with gentamicin against human pathogenic bacteria. Molecules. 22:463

Leslie, J. F., and Summerell, B. A. 2006. The *Fusarium* laboratory manual. Blackwell Publishing, Ames, Iowa, USA.

Li, D., Chung, K. R., Smith, D. A., and Schardl, C. L. 1995. The *Fusarium solani* gene encoding kievitone hydratase, a secreted enzyme that catalyzes detoxification of a bean phytoalexin. Mol. Plant. Microbe. Interact. 8:388-97

Liu, X., Inlow, M., and VanEtten, H. D. 2003. Expression profiles of pea pathogenicity (*PEP*) genes in vivo and in vitro, characterization of the flanking regions of the *PEP* cluster and evidence that the PEP cluster region resulted from horizontal gene transfer in the fungal pathogen *Nectria haematococca*. Curr. Genet. 44:95-103

Liu, Benedict, Stipanovic, and Bell. 1999. Purification and characterization of s-adenosyl-l-methionine: desoxyhemigossypol-6-o-methyltransferase from cotton plants. An enzyme capable of methylating the defense terpenoids of cotton. Plant Physiol. 121:1017-1024

Lordelo, M. M., Davis, A. J., Calhoun, M. C., Dowd, M. K., and Dale, N. M. 2005. Relative toxicity of gossypol enantiomers in broilers. Poult. Sci. 84:1376-1382

Ma, L., Geiser, D. M., Proctor, R. H., Rooney, A. P., Donnell, K. O., Trail, F., Gardiner, D. M., Manners, J. M., and Kazan, K. 2013. *Fusarium* Pathogenomics. Annu Rev Microbiol. 67:399-416

Maor, R., and Shirasu, K. 2005. The arms race continues: battle strategies between plants and fungal pathogens. Curr. Opin. Microbiol. 8:399-404

Mcfadden, H., Beasley, D., and Brubaker, C. L. 2004. Assessment of *Gossypium sturtianum* and *G. australe* as potential sources of *Fusarium* wilt resistance to cotton. Euphytica. 138:61-72

Mellon, J. E., Cotty, P. J., and Dowd, M. K. 2003. Effects of optically active gossypol on conidia germination and growth of *Aspergillus flavus*. In: Proc. Beltwide Cott. Conf, Nashville, TN.

Mellon, J. E., Dowd, M. K., Beltz, S. B., and Moore, G. G. 2014. Growth inhibitory effects of gossypol and related compounds on fungal cotton root pathogens. Lett. Appl. Microbiol. 59:161-168

Mellon, J. E., Zelaya, C. A., and Dowd, M. K. 2011. Inhibitory effects of gossypol-related compounds on growth of *Aspergillus flavus*. Lett. Appl. Microbiol. 52:406-412

Mikkelsen, M. D., Petersen, B. L., Glawischnig, E., Jensen, A. B., Andreasson, E., and Halkier, B. A. 2003. Modulation of CYP79 genes and glucosinolate profiles in Arabidopsis by defense signaling pathways. Plant Physiol. 131:298-308

Morgan, S., Stair, E., Martin, T., Edwards, W., and Morgan, G. 1988. Clinical, clinicopathologic, pathologic, and toxicologic alterations associated with gossypol toxicosis in feeder lambs. Am J Vet Res. 49:493-499

Mueller, and Wolfenbarger. 1999. AFLP genotyping and fingerprinting. Trends Ecol. Evol. 14:389-394

Muller, K. 1940. Experimentelle untcrsuchungcn uber die *Phytophthora*-resistem der kartoffel. Reichsasnstalt Landw Forstw Berlin Arb Biol. 23:189-231

Muller, K. 1958. Studies on Phytoalexins I. The Formation and the immunological significance of phytoalexin produced by *Phaseolus vulgaris* in response to infections with *Sclerotinia fructicola* and *Phytophthora infestans*. Aust. J. Biol. Sci. 11:275-300

Nelson, P. E., Burgess, L. W., and Summerell, B. A. 1990. Some morphological and physiological characters of *Fusarium* species in sections *Liseola* and *Elegans* and similar species. Mycologia. 82:99-106

Nelson, P. E., Dignani, M. C., and Anaissie2, E. J. 1994. Taxonomy, biology, and clinical aspects of *Fusarium* species. 7:479-504

Nelson, P. E., Toussoun, T. A., and Cook, R. J. 1981. *Fusarium*: diseases, biology, and taxonomy. The Pennsylvania State University Press, University Park.

Ng, T. B., Ye, X. J., Wong, J. H., Fang, E. F., Chan, Y. S., Pan, W., Ye, X. Y., Sze, S. C. W., Zhang, K. Y., Liu, F., and Wang, H. X. 2011. Glyceollin, a soybean phytoalexin with medicinal properties. Appl. Microbiol. Biotechnol. 90:59-68

Paddon, C. J., and Keasling, J. D. 2014. Semi-synthetic artemisinin: a model for the use of synthetic biology in pharmaceutical development. Nat. Rev. Microbiol. 12:355-367

Parlevliet, J. E. 1985. Race and pathotype concepts in parasitic fungi. Eppo Bull. 15:145-150

Pedras, M. S. C., Minic, Z., and Jha, M. 2008. Brassinin oxidase, a fungal detoxifying enzyme to overcome a plant defense - purification, characterization and inhibition. FEBS J. 275:3691-3705

Pedras, M. S. C., and Suchy, M. 2005. Detoxification pathways of the phytoalexins brassilexin and sinalexin in *Leptosphaeria maculans*: isolation and synthesis of the elusive intermediate 3-formylindolyl-2-sulfonic acid. Org. Biomol. Chem. 3:2002-2007

Pedras, M. S. C., Yaya, E. E., and Glawischnig, E. 2011. The phytoalexins from cultivated and wild crucifers: chemistry and biology. Nat. Prod. Rep. 28:381-405

Percy, R., Calhoun, M., Science, H. K.-C., and 1996, U. 1996. Seed gossypol variation within *Gossypium barbadense* L. cotton. Crop Science. 36:193-197

Pezet, R., Gindro, K., Viret, O., and Spring, J.-L. 2004. Glycosylation and oxidative dimerization of resveratrol are respectively associated to sensitivity and resistance of grapevine cultivars to downy mildew. Physiol. Mol. Plant Pathol. 65:297-303

Plank, J. E. 1969. Pathogenic races, host resistance, and an analysis of pathogenicity. Netherlands J. Plant Pathol. 75:45-52

Polsky, B., Segal, S. J., Baron, P. A., Gold, J. W. M., Ueno, H., and Armstrong, D. 1989. Inactivation of human immunodeficiency virus in vitro by gossypol. Contraception. 39:579-587

Price, W. D., Lovell, R. A., and McChesney, D. G. 1993. Naturally occurring toxins in feedstuffs: Center for Veterinary Medicine Perspective. J. Anim. Sci. 71:2556-2562

Puckhaber, L. S., Dowd, M. K., Stipanovic, R. D., and Howell, C. R. 2002. Toxicity of (+)- and (−)-Gossypol to the Plant Pathogen, *Rhizoctonia solani*. Agric. Food Chem. 50:7017-7021

Qiying, C., Xiuqen, J., and Sun Wenji. 1985. Identification of races of cotton-wilt *Fusarium* in China. Sci. Agric. Sin.6:1-6

Ragazzi, A., and Vecchio, V. 1992. Behaviour of chlamydospore of *Fusarium oxysporum* f. sp. *vasinfectum* in substrates containing sodium chloride. Phytopathol. Mediterr. 31:85-87

Randel, R. D., Chase, C. C., and Wyse, S. J. 1992. Effects of gossypol and cottonseed products on reproduction of mammals. J. Anim. Sci. 70:1628-1638

Rodriguez-Galvez, E., and Mendgen, K. 1995. The infection process of *Fusarium oxysporum* in cotton root tips. Protoplasma. 189:61-72

Rogers, G. M., Poore, M. H., and Paschal, J. C. 2002. Feeding cotton products to cattle. Vet Clin North Am Food Anim Pract. 18:267-294

Roohparvar, R., Mehrabi, R., Van Nistelrooy, J. G., Zwiers, L.-H., and De Waard, M. A. 2008. The drug transporter MgMfs1 can modulate sensitivity of field strains of the fungal wheat pathogen *Mycosphaerella graminicola* to the strobilurin fungicide trifloxystrobin. Pest Manag. Sci. 64:685-693

Royce, H. D., Harrison, J. R., and Hahn, E. R. 1941. Cotton-root bark as a source of gossypol. Oil Soap. 18:27-29

Dos Santos, S. C., Teixeira, M. C., Dias, P. J., and SÃ¡-Correia, I. 2014. MFS transporters required for multidrug/multixenobiotic (MD/MX) resistance in the model yeast: understanding their physiological function through post-genomic approaches. Front. Physiol. 5:180

Schoonbeek, H., Del Sorbo, G., and De Waard, M. A. 2001. The ABC transporter *BcatrB* affects the sensitivity of *Botrytis cinerea* to the phytoalexin resveratrol and the fungicide fenpiclonil. Mol. Plant-Microbe Interact. 14:562-571

Skovgaard, K., Nirenberg, H. I., O'donnell, K., and Rosendahl, S. 2001. Evolution of *Fusarium oxysporum* f. sp. *vasinfectum* races inferred from multigene genealogies. 91:1231-1237

Smith, S., Ebbels, D., Garger, R., and Kappelman, A. 1981. *Fusarium* wilt of cotton. The Pennsylvania State University Press, University Park, London

Smith, S. N., and Snyder, W. C. 1972. Germination of *Fusarium oxysporum* chlamydospores in soils favorable and unfavorable to wilt establishment. Phytopathology. 62:273-277

Smoliga, J. M., Baur, J. A., and Hausenblas, H. A. 2011. Resveratrol and health - A comprehensive review of human clinical trials. Mol. Nutr. Food Res. 55:1129-1141

Spinosa, H., Górniak, S., and Benito Soto-Blanco. 2008. Gossypol and antinutritional factors of soybean. Pages 531-545. In: *Toxicologia Aplicada à Medicina Veterinária*. B.S.-B. HS Spinosa, SL Górniak, ed. Manole

Stipanovic, R. D., Bell, A. A., and Lukefahr, M. J. 1977. Natural insecticides from cotton (*Gossypium*). ACS Symp. Ser. 62:197-214

Tholl, D. 2015. Biosynthesis and biological functions of terpenoids in plants. Adv Biochem Eng Biotechnol. 148:63-106

Tian, Ruan, X., Huang, J., Fang, J., Mao, X., Wang, Y., Chen, L., and Yang, X. 2016. Gossypol: phytoalexin of cotton. Sci China Life Sci. 59:122-129

Turco, E., Vizzuso, C., Franceschini, S., Ragazzi, A., and Stefanini, F. M. 2007. The in vitro effect of gossypol and its interaction with salts on conidial germination and viability of *Fusarium oxysporum* sp. *vasinfectum* isolates. J. Appl. Microbiol. 103:2370-2381

Uehara, K. 1964. Relationship between the host specificity of pathogen and phytoalexin. Japanese J. Phytopathol. 29:103-110

Ulloa, M., Hutmacher, R. B., Roberts, P. A., Wright, S. D., Nichols, R. L., and Michael Davis, R. 2013. Inheritance and QTL mapping of *Fusarium* wilt race 4 resistance in cotton. Theor. Appl. Genet. 126:1405-1418

Urban, M., Bhargava, T., and Hamer, J. E. 1999. An ATP-driven efflux pump is a novel pathogenicity factor in rice blast disease. EMBO J. 18:512-21

Vanetten, H. D., Matthews, D. E., and Matthews, P. S. 1989. Phytoalexin detoxification: importance for pathogenicity and practical implications. Annu Rev Phytopathol. 27:143-164

VanEtten, H., Temporini, E., and Wasmann, C. 2001. Phytoalexin (and phytoanticipin) tolerance as a virulence trait: why is it not required by all pathogens? Physiol. Mol. Plant Pathol. 59:83-93

Wang, B., Brubaker, C. L., Tate, W., Woods, M. J., Matheson, B. A., and Burdon, J. J. 2006. Genetic variation and population structure of *Fusarium oxysporum* f.sp. *vasinfectum* in Australia. Plant Pathol. 55:746-755

Wang, X., Beckham, T. H., Morris, J. C., Chen, F., and Gangemi, J. D. 2008. Bioactivities of Gossypol, 6-Methoxygossypol, and 6,6′-Dimethoxygossypol. J. Agric. Food Chem. 56:4393-4398

Wang, X., Wang, J., Wong, S. C. H., Chow, L. S. N., Nicholls, J. M., Wong, Y. C., Liu, Y., Kwong, D. L. W., Sham, J. S. T., and Tsao, S. W. 2000. Cytotoxic effect of gossypol on colon carcinoma cells. Life Sci. 67:2663-2671

Wollenweber, H. W., and Recking, O. A. 1935. The fusaria; their description, injurious effects, and control. Paul Parey, Berlin

Wu, Q., and VanEtten, H. D. 2004. Introduction of plant and fungal genes into pea (*Pisum sativum* L.) hairy roots reduces their ability to produce pisatin and affects their response to a fungal pathogen. Mol. Plant-Microbe Interact. 17:798-804

Yang, L., Browning, J. D., and Awika, J. M. 2009. Sorghum 3-deoxyanthocyanins possess strong phase II enzyme inducer activity and cancer cell growth inhibition properties. J. Agric. Food Chem. 57:1797-1804

CHAPTER TWO

2. Transcriptomic analysis to identify candidate genes conferring gossypol tolerance in *Fusarium oxysporum* f. sp. *vasinfectum*

Abstract

Fusarium oxysporium f.sp *vasinfectum* (*Fov*) is a soilborne filamentous fungus that causes vascular wilt on cotton. In response to pathogens such as *Fov*, cotton plants produce the phytoalexin gossypol as a defense mechanism. This research aims to explore the molecular mechanism(s) utilized by *Fov* to tolerate the antimicrobial action of gossypol during infection and colonization of cotton. Candidate genes that could be responsible for gossypol tolerance were identified through RNA sequencing. Four RNA samples were extracted from germlings of a highly virulent race 4 genotype *Fov* isolate that was grown in minimal medium in the presence of 80 µg/mL of gossypol for 1, 2, and 4 hours as well as untreated control. RNA-seq data showed upregulation of ABC and major facilitator transporters, fungal transcription factors, cytochrome P450s, and several classes of dehydrogenases when compared with transcripts isolated from the non-treated *Fov* RNA sample. KEGG pathway analysis identified genes involved in various metabolic pathways including the biosynthesis of secondary metabolites and antibiotics. The RNA-seq data was validated through RT-qPCR where out of the selected 29 upregulated genes, 16 were significantly expressed through a paired t-test. Even though some genes were not significantly expressed, the expression was increased 70 to 240-fold for at least a single time point. Importantly, candidate genes and gene classes that could be involved in conferring

gossypol tolerance were identified Including a beta lactamse, a tannase, several dehydrogenases, transcription factors, cytochrome P450s, and multiple ABC and major facilitator superfamily transporters. Collectively, these findings provide a framework for further investigation into *Fov* virulence and the mechanisms underlying gossypol tolerance.

Keywords: Fusarium oxysporum f.sp. *vasinfectum, gossypol, cotton, tolerance*

2.1 Introduction

Fusarium oxysporum Schlechtend.:Fr. f. sp. *vasinfectum* (Atk.) W.C. Snyder & H.N. Hans., is a pathogen causing vascular wilting disease in cotton (*Gossypium*. spp.), a major source of natural fiber and an important economic crop. It is cosmopolitan in nature and causes significant crop losses in most cotton growing areas of the world (Assigbetse et al. 1994). Being a soil borne pathogen, it is able to survive in the soil as chlamydospores for a long period of time (Ragazzi and Vecchio 1992). Once germinated it infects either intact or wounded cotton roots (Rodriguez-Galvez and Mendgen 1995). After infecting the root tissue, hyphae invades the xylem vessels causing vascular occlusion due to the release of conidia by the pathogen and the simultaneous release of secondary compounds by the plant causing disruption in the water transport system (Turco et al. 2007). This leads to the development of the characteristic symptoms of Fusarium wilt of cotton, chlorosis and necrosis of leaves and vascular browning of xylem, ultimately resulting in a physiologically compromised plant with poor-quality/quantity of lint production or a completely collapsed plant. The incidence and severity of *Fusarium* wilt outbreaks in cotton are a result of pathogen density, genetic resistance of the plant, and prevailing environmental conditions (Devay et al. 1997; Mcfadden et al. 2004).

Plants have developed a variety of resistance mechanisms, innate and induced, to cope with pathogens and stresses. One of these mechanisms is the production of low-molecular-weight antimicrobial compounds termed phytoalexins, as a response to pathogenic microorganisms as well as abiotic stresses (Ahuja et al. 2012; Jeandet et al. 2013). The compounds are part in an intricate defense system enabling plants to impede the invasion of microorganisms (Jeandet and Philippe 2015). Production of phytoalexins in the vascular tissues is one of the defense strategy adopted by cotton plants against *Fusarium* wilt pathogens which leads to the vascular browning and occlusion symptoms (Kaufman et al. 1981; Bugbee 1970). Among different classes of phytoalexins, terpenoids constitute the largest group and play varied roles from plant growth and development to plant protection from herbivores and pathogens (Tholl 2015). Cotton plants, an important source of natural fiber, produce gossypol, a sesquiterpenoid phytoalexin (Tian et al. 2016). Gossypol is a yellow colored phenolic compound occurring naturally in small intercellular

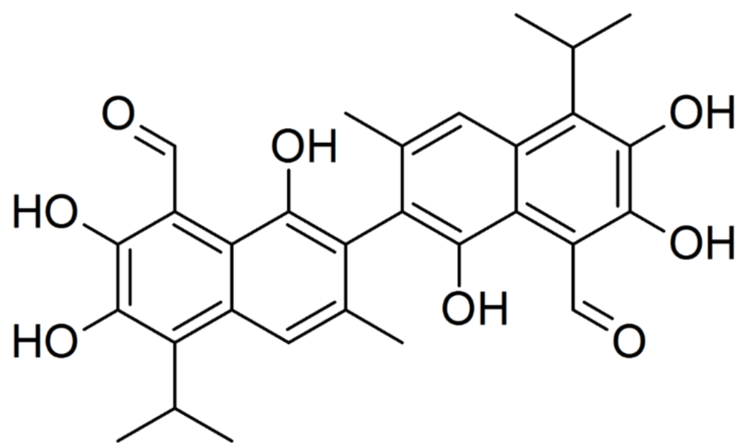

Figure 2-1: Structure of gossypol

pigment glands of cotton plants (Liu et al. 1999). It has a molecular weight of 518.55 Daltons with a chemical formula $C_{30}H_{30}O_8$ and a structural formula of 2,2'-bis(8-formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphthalene)[Figure 2-1; (Gadelha et al. 2014; Abou-Donia 1976; Rogers et al. 2002)]. Gossypol is hypothesized to be a part of the defense system of

the cotton plant as it has anti-insecticidal (Bottger et al. 1964) and antifungal activity (Mellon et al. 2011). It also displays a wide range of biological activity including anticancer, spermicidal, and antiviral effects (Wang et al. 2008; Kim et al. 1984; Lee et al. 2017; Polsky et al. 1989; Gilbert et al. 1995).

2.2 Gossypol tolerance by *Fov*

A previous study has indicated that gossypol is able to initially inhibit the growth of *Fov* but the ability of *Fov* to tolerate gossypol increases over time suggesting that resilience towards gossypol might be due to detoxification of gossypol or due to the breakdown of gossypol naturally (Turco et al. 2007). This study presents the differential gene expression analysis of *Fov* when the pathogen is exposed to gossypol.

2.3 Results

2.3.1 Gene expression profile in *Fusarium oxysporum* f.sp. *vasinfectum* when exposed to gossypol

Previous research has indicated that, *Fov* tolerance to gossypol increases with time (Turco et al. 2007). In order to better understand the molecular mechanisms underlying gossypol tolerance in *Fov,* a transcriptomic approach was undertaken using mycelia collected 1 hour (T1), 2 hours (T2) and 4 hours (T4) after the exposure to 80 μg/mL of gossypol.

The number of genes upregulated at 1 hour, 2 hours, and 4 hours after gossypol treatment when compared with control were 501, 226, and 261 genes, respectively (Figure 2-2). There was a total of 707 genes that were upregulated for at least one gossypol treatment time point and 68 upregulated genes that were common in all three timepoints

(Figure 2-3). Similarly, the number of downregulated genes at 1 hour, 2 hours, and 4 hours when compared to the untreated sample were 102, 69, and 51, respectively. Among these downregulated genes, a total of 169 different genes were downregulated in at least one time point after gossypol treatment, and only 6 genes were common at all three timepoints (Figure 2-3). Of the total differentially expressed genes (707 upregulated genes and 169 downregulated genes), approximately 70.86% of the upregulated genes and 60.35% of downregulated genes were differentially expressed the first hour (T1) after gossypol treatment. Common genes upregulated and downregulated in all three timepoints with putative Pfam functions and expression are shown in supplementary material Table S-2 and Table S-3.

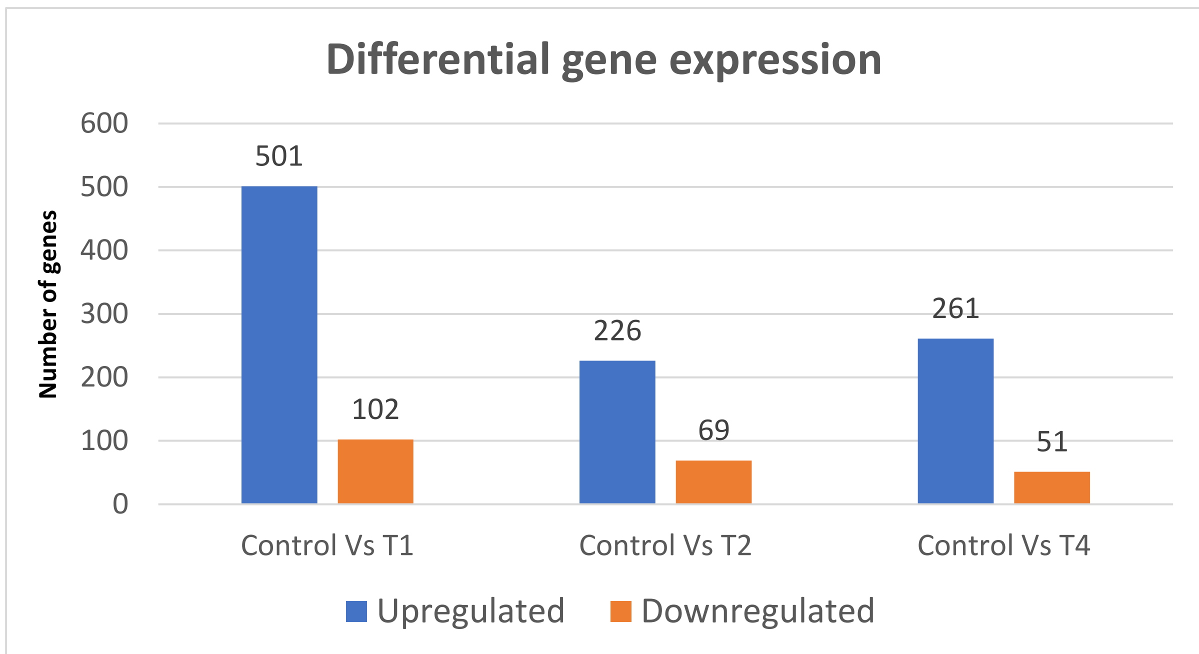

Figure 2-2: Differentially expressed genes (DEGs) at 1 hour, 2 hours, and 4 hours after gossypol treatment when compared with the untreated control. Numbers above each bar is the number of genes. DEGs were identified using DESeq2 at a *p*-value < 0.05 and Log fold change threshold equals 2. Blue color represents upregulated genes and orange color represents downregulated genes.

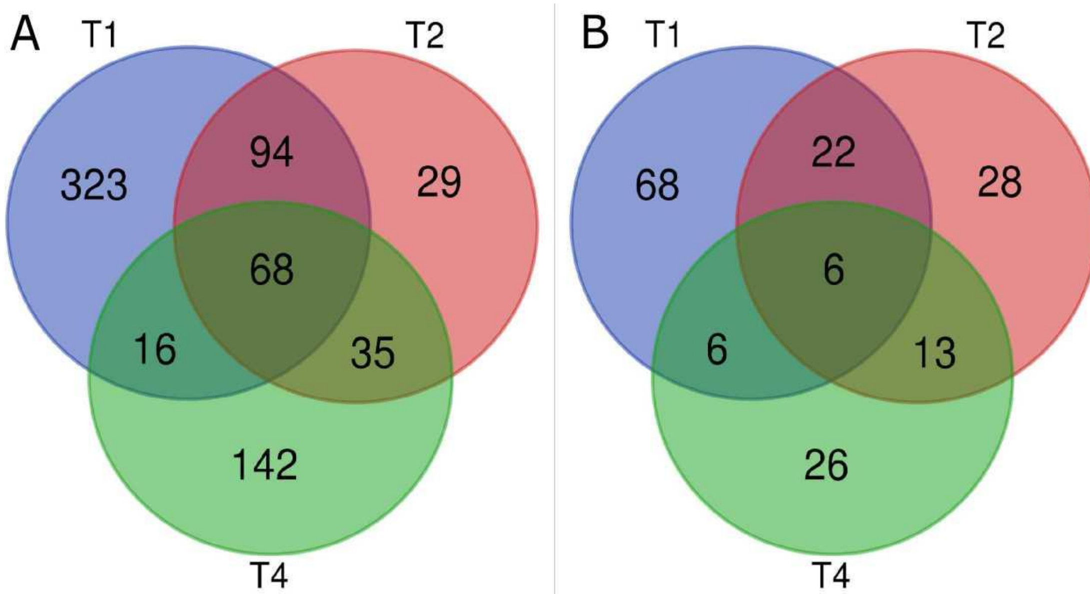

Figure 2-3: Venn diagrams illustrating the number of upregulated (A) and downregulated (B) genes in *Fov* at 1 hour, 2 hours, and 4 hours after gossypol exposure.

2.3.2 Functional analysis of differentially expressed genes

Functional evaluation of the DEGs was conducted by Pfam domain analysis using InterProScan. A total of 127 dehydrogenases were upregulated while 17 were downregulated and included zinc binding dehydrogenases (PF00107), short chain dehydrogenases (PF00106) and those containing FAD binding domains (PF01494) and NADP(H) binding domains (PF13460), aldehyde dehydrogenases (PF00171), acyl dehydrogenases (PF02771, PF02770, PF00441) and alcohol dehydrogenases (PF08240, PF00465) (Table S-12, S-13). There were 52 transcription factors that were upregulated while only a single transcription factor was downregulated. Those upregulated included fungal specific transcription factors (PF11951), and transcription factors containing a zinc binuclear cluster domain (PF00172) or C2H2 type zinc finger domain (PF00096) (Table S-8, S-9).

A total of 37 major facilitator superfamily (MFS) transporters (PF07960) were differentially expressed (30 upregulated and 7 downregulated) (Table S-6, S-7). Similarly, 28 transporters from other families were upregulated and 8 were downregulated (Table S-4, S-5). These transporters mostly include ABC transporters, sugar transporters, cation transporters, and potassium transporters. The numbers of cytochrome P450s that were differentially expressed were relatively low compared to dehydrogenases, transporters, and transcription factors, 10 cytochrome P450s were upregulated and 3 were downregulated (Table S-10, S-11).

Table 2-1: Numbers of differentially expressed genes under various Pfam domain categories

Classes	Upregulated	Downregulated	Total DEGs
ABC and sugar transporters	28	8	36
Major Facilitator Superfamily	30	7	37
Fungal Transcription Factors	52	1	53
Cytochrome P450	10	3	13
Dehydrogenase	127	17	144

Based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) protein interaction pathways, the top 15 functional categories of DEGs with best hits were determined (Figure 2-4). KEGG pathway analysis of DEGS demonstrated that enzymes involved in metabolism were highly expressed, followed by genes involved in the biosynthesis of secondary metabolites and carbon metabolism (Table S-16 to S-20). Automatic annotation of upregulated and downregulated genes was conducted using the KEGG Automatic Annotation Server (KAAS)and revealed that out of 707 upregulated genes only 123 genes were annotated. Similarly, out of 169 downregulated genes only 36 were annotated. The KAAS annotated genes with Gene ID and KEGG orthology ID are given in supplementary material Table S-14 and Table S-15.

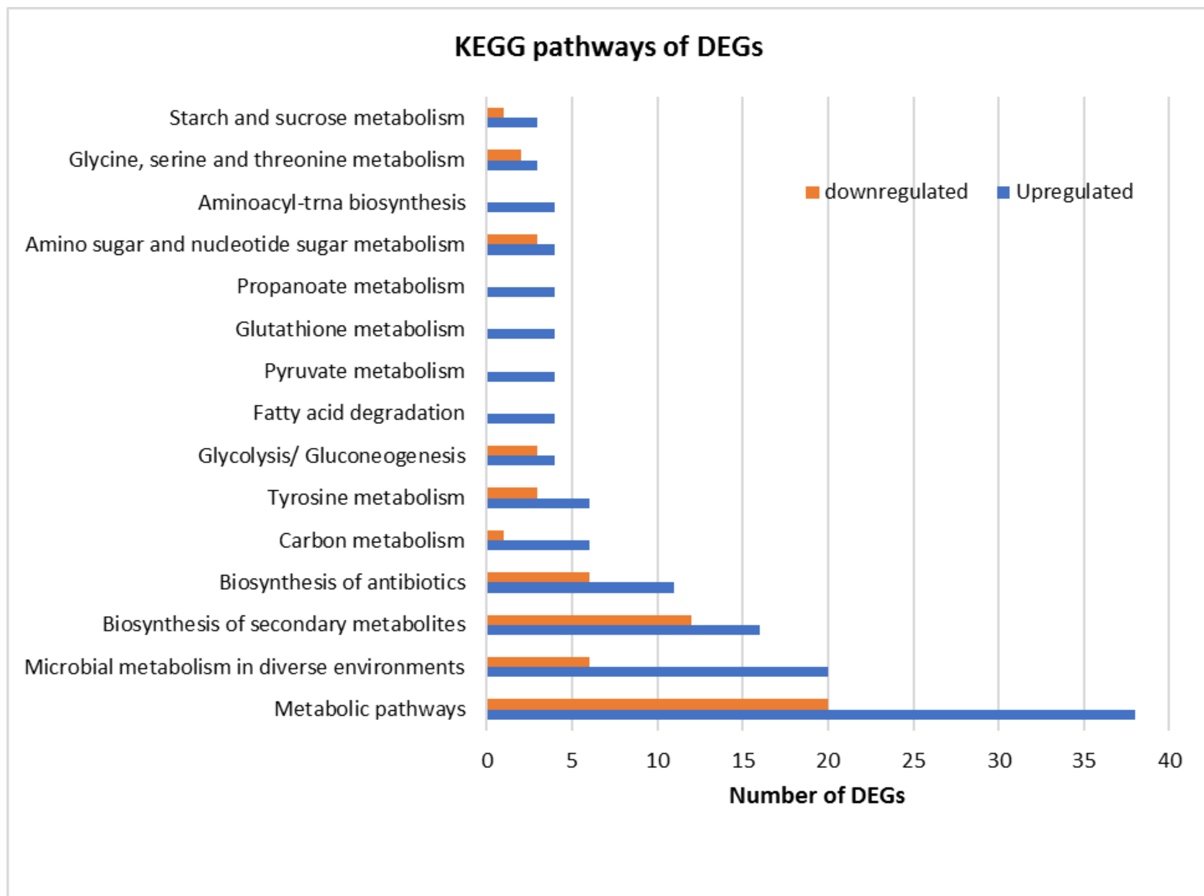

Figure 2-4: Classification of up- and down-regulated genes of different pathways by KEGG analysis.

2.3.3 Gene expression patterns

Hierarchical distribution was done for the top 50 genes that had significant expression (p-value < 0.05) with a high variance across all timepoints (Figure 2-5). Three different patterns of gene expression can be seen based on gene expression across all conditions (T0, T1, T2, and T4). One group encompassing 40 genes are genes where their expression gradually increases over the first two timepoints and peaks at T4. The second group of three genes are downregulated where the expression gradually decreases over the timepoints. In the final group of seven genes, the expression is high one hour after gossypol treatment but then gradually declines over the remaining time course. Vertical clustering of columns or timepoints suggest that timepoints T1 and T2 cluster together while T4 and the

control group cluster separately (Figure 2-5). The clustering is based on Euclidean distance between each log transformed points, and the three clusters can be described as moderately upregulated, highly upregulated, and downregulated gene clusters.

2.3.4 Validation of DEGs from RNA-seq through RT-qPCR

Reverse Transcription Quantitative PCR (RT-qPCR) was used to validate a total of 31 DEGs (29 upregulated and 2 downregulated genes). The genes were selected based on their expression values in all three timepoints and their predicted Pfam domains. Two housekeeping genes *EF-1* and *GAPDH* were used for normalization. Specific primers were designed for each gene (Table S-21) and primer efficiency and specificity were evaluated and verified for all primer pairs.

The inclusion of a negative control that received solvent treatment for each timepoint, 0 hour (T0), 1 hour (T1), 2 hours (T2), 4 hours (T4), 8 hours (T8), 12 hours (T12) and 24 hours (T24), allowed to determine if the change in gene expression was due to the solvent stress response to DMSO or part of the transcriptional response to gossypol. Paired two tailed t-tests were performed on delta Ct (DCT) values of all the selected genes to evaluate the significance of expression between negative control and gossypol treated conditions. From t-test at timepoint T1 it was found that out of 29 upregulated genes, 3 genes were significantly expressed at p value < 0.001, 7 genes were significantly expressed at p value < 0.0,1 and 11 genes were significantly expressed at p value < 0.05 (Figure 2-6). However, 8 genes were not significantly expressed at timepoint T1, suggesting that expression of these genes in the RNA-seq data at T1 was most likely due to induction from the DMSO solvent. The bar chart shows the DCT values of all genes at gossypol and DMSO

treated conditions. It should be noted that the lesser the DCT value, the more highly the gene is expressed.

At timepoint T2 (two hours after gossypol treatment), the log2fold change expression values of selected candidate genes for the RNA-seq and the RT-qPCR data was compared (Figure 2-7). The relative level of gene expression in both the RNA-seq and RT-qPCR was approximately the same for 20 of the assessed genes (G7914, G14616, G16397, G17308, G14232, G13126, G13127, G5244, G5243, G9742, G9749, G16843, G10034, G1781, G1443, G17337, G2070, G13853, G12645, and G11668); however for 11 of the assayed genes (G17064, G3905, G10333, G17063, G18019, G17062, G14749, G4637, G4633, G14661, and G16581) the level of expression determined by RT-qPCR was comparatively lower than RNA-seq. One possible explanation for this variation is the inclusion of the negative solvent only DMSO controls for each timepoint in the RT-qPCR data analysis which was absent in the RNA-seq data set.

The trend of gene expression from the RT-qPCR data indicates that immediately after gossypol treatment, there is increase in expression of genes (Figure 2-8). Gene expression is at its highest level soon after addition of gossypol (T1, T2 and T4) for most genes whereas at T0, T8, and T12, gene expression is comparatively lower. As determined using the Least Significance Difference test, gene expression at timepoints T1, T2, T4, and T24 was significantly higher than timepoints T0, T8 and T12 (p-value < 0.05). The heatmap shows the hierarchical distribution of genes where rows are clustered based on the distance between points (Figure 2-8).

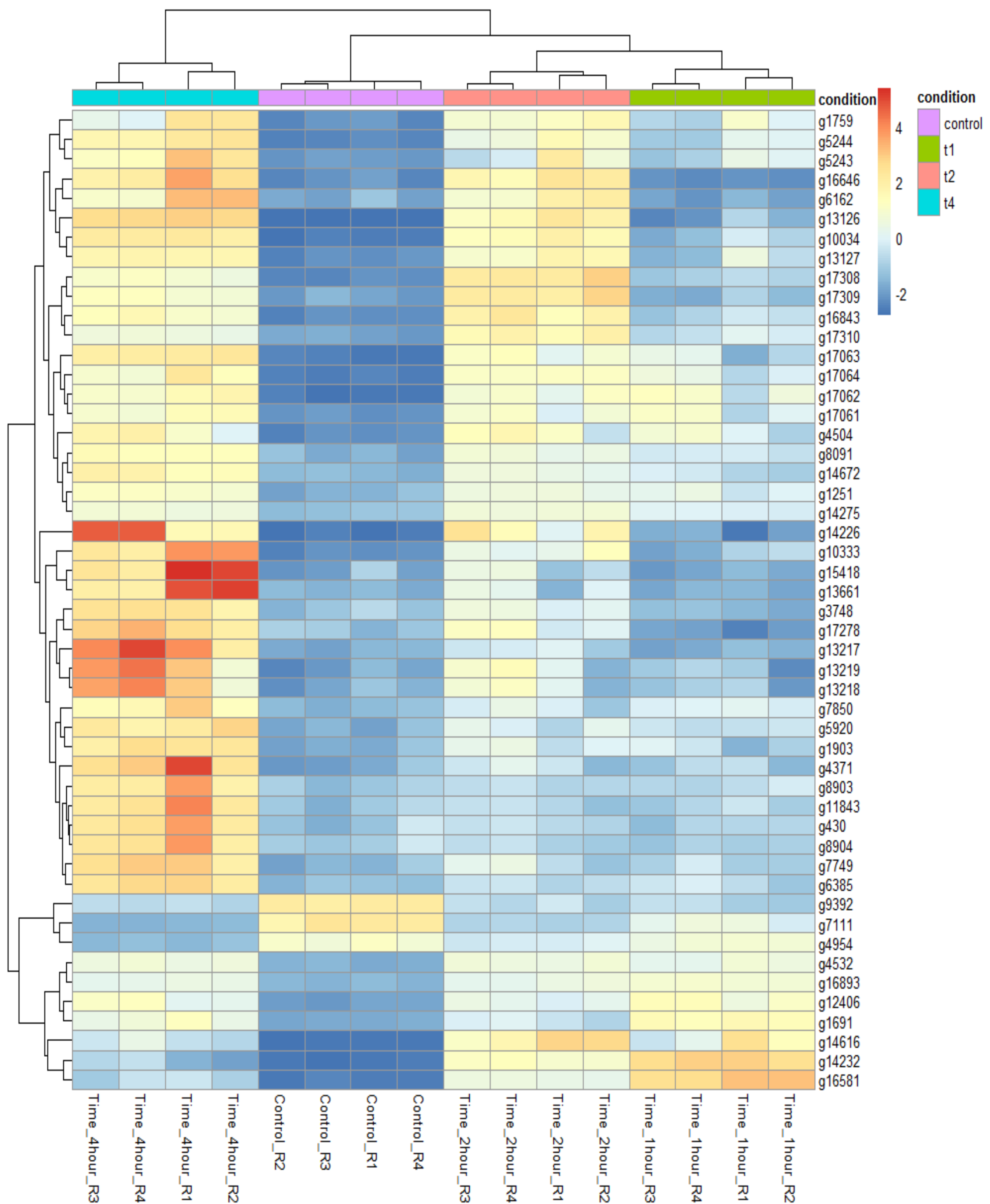

Figure 2-5: RNA-seq data heatmap illustrating log2 fold change of 50 DEGs (p-value < 0.05) in all 16 samples. The top 50 genes were selected based on the highest variance. Each row represents a gene and each column represents the sample condition. The genes cluster together based on similarity of fold change among the 16 samples. Blue color represents lower log2 fold change while red indicated a higher log2 fold change. The scale on the right indicate the colors to the log2 fold change values.

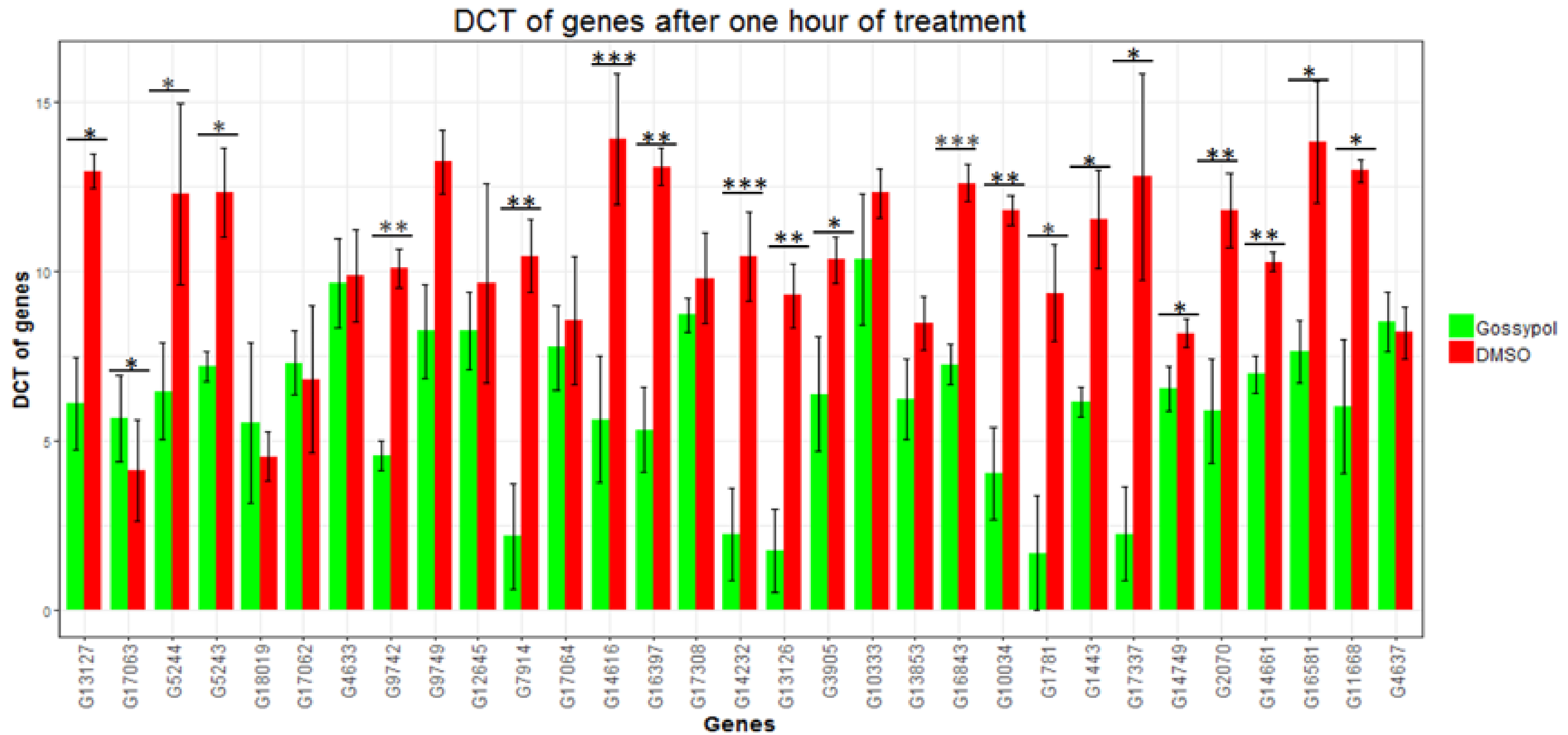

Figure 2-6: Comparison of DCT values between gossypol treated and DMSO treated samples at T1 from RT-qPCR data. Data represent means and standard deviation from three biological replicates. Each biological sample was repeated twice to have three technical replicates. The asterisks denote the level of significance for Student's t-test (**p*-value < 0.05, ***p*-value < 0.01, and ****p*-value < 0.001).

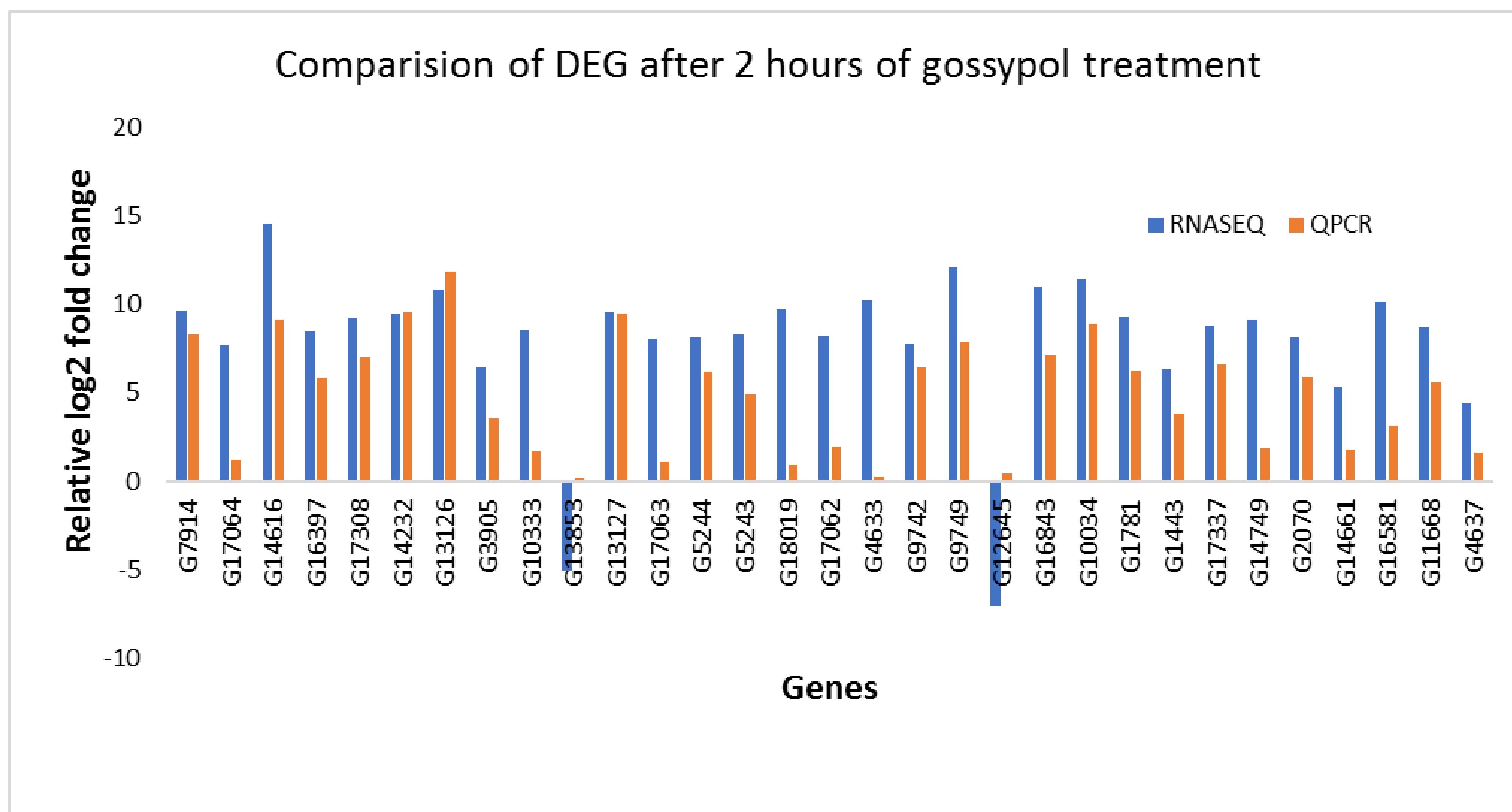

Figure 2-7: Comparison of relative log2 fold changes between RNA-seq and RT-qPCR results at 2 hours after gossypol treatment in *Fov*. Fold changes in RT-qPCR are calculated using double delta Ct (ΔΔCT) method where each gossypol treated sample was compared to the negative control group (DMSO treatment alone) as normalized with the reference genes *EF-1* and *GAPDH*. Fold change in RNA-seq was calculated using the DESeq2 package from R after performing DESeq2 normalization.

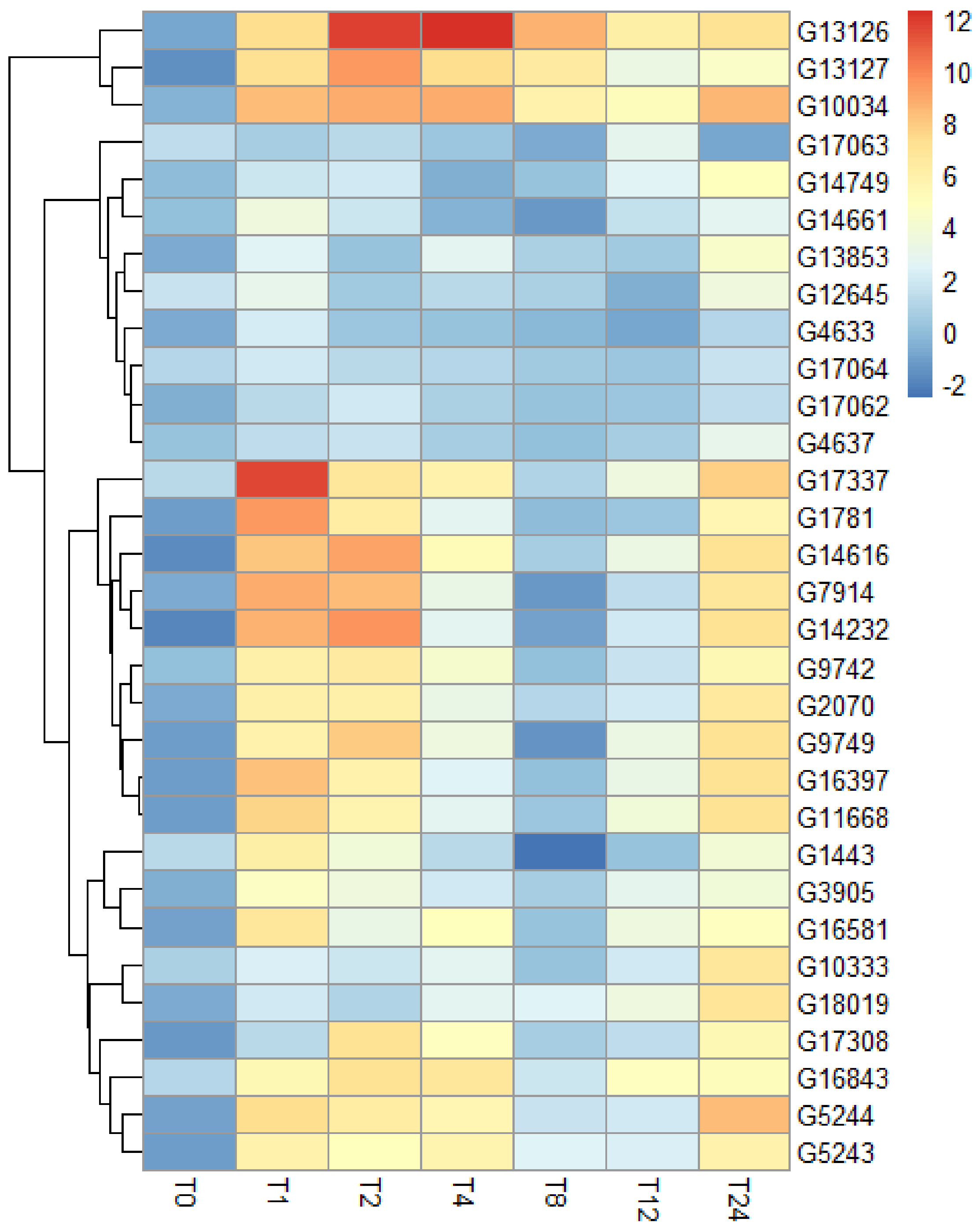

Figure 2-8: Heatmap of RT-qPCR analysis of candidate genes in *Fov* under gossypol treatment, with three biological and technical replicates. Genes are hierarchically distributed and clustered based on the distance between each timepoint. Each row represents a single gene and each column represents a timepoint. Squares indicated in red color represents genes with higher log2 fold change and blue color represents genes with lower log2 fold change values.

Table 2-2: Fold change of selected candidate genes at timepoints T0, T1, T2, T4, T8, T12, and T24

Putative Pfam function	Gene ID	Timepoints						
		T0	T1	T2	T4	T8	T12	T24
Aldo/keto reductase family	G7914*	0.57	440.61	318.59	8.55	0.4	2.38	101.98
AMP-binding enzyme	G17064	2	3.95	2.27	1.99	1.35	1.26	2.96
NAD(P)H-binding	G14616*	0.29	259.29	568.12	37.31	1.51	9.48	123.77
Major Facilitator Superfamily	G16397	0.44	279.44	56.41	5	0.94	9.13	123.59
Sugar (and other) transporter	G17308*	0.396	2.32	126.76	26.56	1.54	2.6	40.31
Major Facilitator Superfamily	G14232*	0.26	405.47	759.47	6.6	0.46	3.84	123.59
Zinc-binding dehydrogenase	G13126***	0.52	165.41	3547.43	5068.23	386.78	67.4	127.65
Calcineurin-like phosphoesterase	G3905*	0.63	23.97	11.5	3.93	1.54	7.09	13.79
GMC oxidoreductase	G10333	1.69	4.51	3.34	6.27	1.06	3.79	107.25
Glycosylhydrolasesfamily18	G13853	0.56	5.86	1.15	6.35	1.60	1.29	19.11
	G13127***	0.30	137.43	695.59	159.21	87.69	9.48	21.52
Acetyl transferase	G17063	2.63	1.53	2.22	1.24	0.61	7.08	0.53
NAD dependent epimerase/dehydratase family	G5244**	0.47	156.67	73.89	45.43	3.10	3.97	331.56
Zinc binding dehydrogenase	G5243**	0.44	55.32	30.48	50.26	5.07	4.58	55.73

Putative Pfam function	Gene ID	Timepoints						
		T0	T1	T2	T4	T8	T12	T24
Glutathione-dependent formaldehyde activating enzyme	G18019	0.59	3.87	1.90	6.20	5.23	10.78	118.06
ABC transporter	G17062	0.67	2.19	3.82	1.73	1.05	1.18	2.42
Aldehyde dehydrogenase	G4633	0.58	4.27	1.18	1.09	0.83	0.53	1.95
Aldehyde dehydrogenase family	G9742**	0.95	64.81	87.04	18.71	1.02	3.13	39.56
FAD binding domain	G9749	0.42	58.53	238.77	10.20	0.35	9.46	130.68
NADH:flavinoxidoreductase/NADH oxidase family	G12645	3.10	7.87	1.37	2.18	1.70	0.65	12.00
Tannase and feruloyl esterase	G16843**	1.96	39.40	134.85	99.77	3.32	28.35	33.86
Beta lactamase	G10034***	0.70	317.31	459.53	466.87	58.85	33.42	355.10
SRR1, circadian clock	G1781**	0.44	675.76	78.19	6.24	0.90	1.27	45.05
Cytochrome P450	G1443	2.33	71.25	13.74	2.25	0.16	1.10	13.99
Short chain dehydrogenase	G17337*	2.34	3308.49	99.25	54.69	1.79	10.57	218.82
Short chain dehydrogenase	G14749	0.90	3.30	3.75	0.63	1.13	5.55	30.99
Short chain dehydrogenase	G2070*	0.60	65.10	61.02	8.70	2.11	3.73	97.52
Cytochrome P450	G14661	1.02	11.43	3.38	0.74	0.40	2.89	6.13
FAD binding	G16581	0.50	102.60	9.03	29.56	1.13	10.57	28.40
Zinc binding dehydrogenase	G11668*	0.43	203.60	48.20	6.31	1.21	12.88	128.93

Putative Pfam function	Gene ID	Timepoints						
		T0	T1	T2	T4	T8	T12	T24
Fungal specific transcription factor	G4637	1.09	2.41	3.13	1.44	0.95	1.57	7.85

*statistically significant at the level 0.05 (paired two tailed t-test)

**statistically significant at the level 0.01 (paired two tailed t-test)

***statistically significant at the level 0.001 (paired two tailed t-test)

Several genes were found to be greatly induced after treatment with gossypol (Table 2-2). The genes G13126 (a putative zinc binding dehydrogenase) and G17337 (a putative short chain dehydrogenase) are expressed over a thousand fold in the presence of gossypol while several other genes have over a 500 fold increase in expression (G14616, G14232, G1781, and G13127). While some genes (G16397, G10333, G18019, G9749, G1443, and G16581) were not significantly upregulated after gossypol treatment, at some timepoints the fold change was high ranging from 71 to 238 fold.

2.4 Discussion

Cotton plants have developed a variety of resistance mechanisms, innate and induced, against fungal pathogens. One of these mechanisms is the production of phytoalexins, e.g. gossypol which has antimicrobial properties. It has been documented that the most successful plant pathogens have developed mechanisms to detoxify phytoalexins produced by their host plants (Morrissey and Osbourn 1999; Vanetten et al. 2001). The ability of *Fov* to overcome gossypol inhibition over time has been previously described (Turco et al. 2007); however the molecular mechanism(s) reposnsible for conferring this tolerance remain unknown.

In order to further investigate the molecular aspects responsible for gossypol tolerance in *Fov*, the gene expression profile changes immediately after treatment of gossypol were assessed by RNA-seq. Analysis of the *Fov* transcriptome identified 501, 226, and 261 genes upregulated 1 hour, 2 hours, and 4 hours after gossypol treatment. Conversely, 102, 69, and 51 genes were down regulated 1 hour, 2 hours, and 4 hours after gossypol treatment. The DEGs identified were different classes of dehydrogenases, transporters, transcription factors, cytochrome P450s and other various enzyme families.

RT-qPCR analysis of a select subset of 31 genes identified in the RNA-seq data set was used to verify the results from the transcriptomic study. Of the 29 selected upregulated genes, 16 were significantly upregulated in the time course RT-qPCR experiment. The use of a gossypol stock solution that was prepared in DMSO to treat the fungal culture made it essential to include negative DMSO control samples in the RT-qPCR analysis for confirmation of the RNA-seq data.

Classification of DEGs based on their Pfam function identified several differentially expressed dehydrogenases at all timepoints. The fold change expression of a zinc binding dehydrogenase (G13126) and a short chain dehydrogenase (G17337) was calculated to be more than thousand-fold in *Fov* after gossypol treatment. Short chain dehydrogenases, also known as reductases, are known for their critical roles in lipid, carbohydrate, amino acid, cofactor, hormones, and xenobiotic metabolism as well as enzymatic reduction (Kavanagh et al. 2008). Zinc dehydrogenases or alcohol dehydrogenases are known for the oxidation of alcohols (primary or secondary) to corresponding ketones and aldehydes (Crichton 2012). The conversion of toxic compounds to less lipophilic and more soluble compounds is mediated by enzymatic action of various dehydrogenases (Maser and Bannenberg 1994). Thus, oxidation, reduction, and further metabolism of gossypol to less toxic compound(s) might be catalyzed by these dehydrogenases in *Fov* and may promote pathogen growth in planta increasing virulence.

Transcription factors are another of the classes of highly upregulated genes in the gossypol treated *Fov* samples. Fungal specific transcription factors including those containing either a Zn(2)-Cys(6)binuclear cluster or a C2H2 zinc finger domain were differentially expressed in the presence of gossypol. Only one transcription factor was

significantly downregulated after treatment with gossypol suggesting the importance of transcription factors in regulating gene expression during fungal infection. In fungi, transcription factors are known for their role in coordinating the responses to external stimuli (Yin and Keller 2011). Transcription factors in fungi are also found to be involved in detoxification of phytoalexins in various plants (Kettle et al. 2016; Srivastava et al. 2013). For example, the zinc finger transcription factor Fdb3 in *F. pseudograminearum* has been reported for its ability to control the detoxification of 6-methoxy-benzoxazolin-2-one (MBOA), a phytoalexin in wheat (Kettle et al. 2016). Similarly, in the necrotrophic fungus *Alternaria brassicicola,* it was found that brassinin detoxification factor 1 (Bdtf1) was involved in the detoxification of the phytoalexin brassinin in *Brassica* plants (Srivastava et al. 2013). Upregulation of transcription factors is also important to regulate gene expression in fungi. Therefore, transcription factors could also be involved in similar roles in *Fov* and be important mediators for gossypol tolerance.

Several major facilitator superfamily (MFS) transporters were also significantly expressed in *Fov* when exposed to gossypol through RNA-seq analysis, and RT-qPCR analysis confirmed that MFS transporters were increased in expression from 200 to 700 fold. MFS transporters are known to be involved in multidrug, toxin, and fungicide resistance through transportation of small molecules (Callahan et al. 1999; Roohparvar et al. 2008; dos Santos et al. 2014). For example, BcMfs1, a MFS transporter from *Botrytis cinerea*, was found to provide tolerance to camptothecin, a natural toxin produced by the plant *Camptotheca acuminata* (Hayashi et al. 2002). The expression of the putative MFS encoding gene *PEP5* in *N. haematococca* has been reported when this pathogen was exposed to phytoalexin pisatin (Liu et al. 2003). Direct involvement of MFS transporters in phytoalexin tolerance has not been reported yet; however, MFS transporters could be

potential candidates involved in efflux of gossypol outside of the fungal cell and confer gossypol tolerance during colonization of *Fov*.

There were 28 transporters upregulated in the RNA-seq data including ABC, sugar, and cation transporters (Table S-4). One ABC transporter (G17062) and one Sugar transporter (G17308) was selected for validation by qPCR. G17308 was increased in expression by more than 100 fold, while G17062 was found only to have been increased in expression less than 5 fold. The less than expected increase in expression of G17062 is likely due to the observed increase in the RNA-seq data was the result of the response of the solvent, DMSO. Many ABC transporters are induced in *Fov* in response to gossypol treatment (Table S-4). ABC transporters of the pleiotropic drug resistance and multidrug resistance families are important in conferring resistance to xenobiotics (Kretschmer et al. 2009); and ABC transporters from multiple fungal pathogens are known to be involved in phytoalexin tolerance (Fleisner et al. 2002; Coleman et al. 2011). Sugar transporters are involved in efflux of glucose and in pathogen nutrition (Chen et al. 2010). The involvement of transporters containing a sugar transporter domain in tolerance of phytoalexins has not been reported although these transporters could play an important role in gossypol tolerance by *Fov*.

Similarly, after gossypol treatment there were 10 *Fov* cytochrome P450 upregulated in expression in the RNA-seq data (Table S-10), of which two CYPs (G1443 and G14661) were selected for validation by RT-qPCR. Both of these CYPS were confirmed to have higher levels of expression after treatment with gossypol. CYPs are a large group of diverse enzymes involved in primary and secondary metabolism and xenobiotic detoxification in fungi (Lah et al. 2011) and have been shown to be involved in phytoalexin detoxification in *F. oxysporum*

and *N. haematococca* (Coleman et al. 2011). Therefore, CYPs could be involved in a similar role in *Fov* and be important mediators involved in gossypol tolerance.

Among the upregulated genes from KEGG pathway analysis, 38 of them were involved in various metabolic pathways and 20 in microbial metabolism in adverse environments (Table S-16, Table S-17). Various dehydrogenases, oxidases, CYPs, etc. are involved in these pathways and metabolism of phytoalexins can be mediated by various enzymes like oxidases and CYPs (Schafer et al. 1989; Pezet et al. 1991). Many of the dehydrogenases, hydroxylases, oxygenases, CYPs, and reductases that are upregulated in *Fov* after gossypol exposure could be involved in various metabolic pathways that leads to the detoxification of gossypol to less toxic compounds.

Although not the primary focus of the study, the upregulated 16 genes involved in the biosynthesis of secondary metabolites and 11 genes were involved in the biosynthesis of antibiotics through the KEGG analysis are intriguing. Secondary metabolites, including antibiotics, are produced by fungi as competitive weapons against other microorganisms or plants during their growth (Demain and Fang 2000); perhaps the expression of these gene for the synthesis of secondary metabolites and antibiotics might aid *Fov* to establish successfully inside cotton plants.

Hierarchical clustering of genes by heatmaps from RNA-seq and qPCR data indicates that the zinc binding dehydrogenase (G13126) and a beta lactamase (G10034) cluster together. G10034 is also significantly expressed at all timepoints after gossypol treatment in both RNA-seq and RT-qPCR. In bacteria, β-lactamases have been studied for their role in β-lactam antibiotic resistance (Kotra and Mobashery 1998). Similarly, in fungi two related lactamases were found to function in xenobiotic hydrolysis. *F. verticillioides* encoded

a lactamase that was responsible for the degradation of 2-benzoxazolinone (BOA) in maize (Glenn et al. 2016). Likewise, *F. pseudograminearum* encoded a lactamase that was involved in the degradation of benzoxazolinones, a class of phytoalexins in wheat (Kettle et al. 2015). While the role that β-lactamase fulfills in *Fov* is not known, the high expression of the gene after treatment with gossypol is intriguing; however, a lactam ring is not present in gossypol. This does not rule out the presence of a lactam ring being formed further in an unknown detoxification pathway of gossypol.

Similarly, another gene encoding an enzyme with a tannase and feruloyl esterase domain (G16843) is also expressed at a higher fold in both the RNA-seq and RT-qPCR data. Tannases are known to catalyze the hydrolysis of bonds present in tannins and gallic acid ester (Lekha and Lonsane 1997). It is known that gossypol is sensitive to oxidation and has a tendency to react and degrade in the presence of several reagents; therefore hydroxyl groups of gossypol are first protected by converting them to ester derivatives (Kenar 2006). No tannase enzymes have been reported to be involved in phytoalexin tolerance but based on the structure of gossypol and the transcriptomic data, the tannase might be involved in hydrolysis of ester bonds in gossypol derivatives.

This transcriptomic data with *Fov* and gossypol interaction supports the hypothesis that most successful fungal pathogens have developed resistance mechanisms against phytoalexins. The phytoalexin tolerance of *Fov* could be the result of several genes collectively acting synergistically, including different classes of genes like transcription factors, dehydrogenases, major facilitator transporters, ABC transporters, cytochrome P450s, and various enzymes. Further studies need to be conducted to clarify if these *Fov* genes are important virulence traits facilitating the fungus to colonize cotton plants.

2.5 Materials and methods

2.5.1 Fungal culture and RNA-seq sample preparation

Fungal cultures of the race 4 *Fov* isolate 89-1A were maintained at room temperature on V8 medium. Conidia were collected from two weeks old V8 plates and inoculated into 100 ml of liquid M100 (minimal) medium. The conidia were allowed to germinate on a rotary shaker at 150 rpm for 24 hours at room temperature. After 24 hours, a 25 ml aliquot of the culture was collected and frozen at -80°C for RNA extraction. The remaining 75 ml of the 89-1A culture was treated with gossypol (dissolved in DMSO) to obtain a final concentration of 80 μg/ml. Gossypol treated mycelium was collected in 25 ml aliquots from the remaining culture at 1 hour, 2 hours, and 4 hours after treatment. All of these collected samples were snap frozen at -80°Cfor RNA extraction.

2.5.2 RNA isolation and RNA sequencing

RNA was extracted from frozen mycelial samples using 0.5 mm glass beads (Sigma-Aldrich) and agitated for 50 seconds in two different cycles with 5 seconds interval in between (Leite et al. 2012). This was followed by phenol-chloroform purification method and RNA was eluted in DEPC (MP Biomedicals) treated water. DNA contamination was removed using DNase I (New England Biolabs). RNA quality and integrity was verified by gel electrophoresis and confirmed by Bioanalyzer before sending for sequencing. cDNA library preparation and RNA sequencing were performed at the Genomic Services Lab at Hudson Alpha Institute of Biotechnology. Illumina 1.9, paired end, directional RNA-seq was done with 50 bp read length and 25 million reads per sample. The RNA-seq experiment had 4 different biological replicates and each replicate had 4 different sample conditions.

2.5.3 Bioinformatics and statistical data analysis

The quality of 50 base pairs reads from the 16 separate libraries were checked by FastQC and all the low quality bases, adapter sequences, and sequencing artifacts were removed using Trimmomatic, version 0.35 (Bolger et al. 2014). The number of reads that survived after trimming across all the samples in each biological replicate is shown in supplementary material Table S-1. Of the remaining reads, the proportion of reads mapped to the genome ranged from 70.13% to 89.07% (Table S-1). After filtering, the reads were aligned to the reference genome sequence of 89-1A (Seungyeon Seo, unpublished data) using STAR (Dobin et al. 2013). The number of reads mapped to each gene were counted using the python package HTSeq (Anders et al. 2015). Data were normalized and differential gene expression was calculated using Bioconductor package DESeq2 (Love et al. 2014). The genes with Log2Fold change > 2 and p-value < 0.05 were considered as differentially expressed. The Pfam domain scan of differentially expressed genes was conducted using the program InterProScan (Zdobnov and Apweiler 2001).

2.5.4 Experimental setup and sample preparation for RT-qPCR

The experimental setup for sample preparation for the RT-qPCR was done in a similar way as for the RNA-seq experimental setup. Conidia were collected from two week old V8 plates and inoculated in two flasks Erlenmeyer flasks each containing 175 ml of liquid M100 (minimal) medium. The cultures were left to grow on the shaker at 150 rpm for 24 hours at room temperature. After 24 hours, the growing mycelium was collected from 25ml of the culture from both flasks separately. At this time to the remaining growing cultures: one flask was treated with gossypol stock solution (gossypol in DMSO) to final concentration of 80 μg/ml of gossypol and 4% DMSO and another flask was only treated with final

concentration of 4% DMSO. The samples collected from the DMSO treated flasks were denoted as negative controls. Aliquots of 25 mL of the mycelium from both flasks were collected from the remaining cultures at 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, and 24 hours after treatment. All of these collected samples were snap frozen at -80°C for RNA extraction.

2.5.5 RNA isolation and cDNA preparation

RNA was extracted from the frozen mycelial samples using 0.5 mm glass beads (Sigma-Aldrich) and agitated for 50 seconds in two different cycles with 5 seconds interval in between (Leite et al. 2012). This was followed by phenol-chloroform purification method and RNA was eluted in DEPC treated water. DNA contamination was removed using DNase I (New England Biolabs). RNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and RNA quality was evaluated by gel electrophoresis. cDNA was synthesized from 1µg of total RNA by reverse transcriptase using the QuantiNova Reverse Transcription Kit (Qiagen).

2.5.6 RT-qPCR assay

RT-qPCR was performed in skirted white 96-well plates (VWR) on the CFX96 Real-Time System (Bio-Rad). The reaction mix was made using: 10µl of PerfeCTa SYBR Green FastMix (QuantaBio), 1.5 µl of 10 µM primer mix, 1 µl of a diluted 1:10 cDNA and water to complete a final volume of 20 µl. Cycling conditions were 95 °C for 5 minutes, and 40 cycles of 95 °C for 5 s, 60 °C for 30 s. A total of 29 upregulated genes and 2 downregulated genes determined by DESeq2 were selected for validation by RT-qPCR. The primers for all selected genes were designed using Primer quest tool (Integrated DNA Technology) [Table S-21]. Efficiency of each primer were calculated by performing five

series of 10 fold serial dilution RT-qPCR assay and analyzing the melt curve peaks. The expression of each gene under DMSO and gossypol treatment was assayed using three biological and three technical replicates.

2.5.7 RT-qPCR data analysis

Data obtained from RT-qPCR was analyzed using double delta Ct method. Two housekeeping genes: *EF-1* and *GAPDH* were used for data normalization. Relative gene fold change was calculated using formula $2^{(-\Delta\Delta ct)}$ (Livak and Schmittgen 2001). The R graphics program ggplot2 (Wickham 2009) was used to generate bar plots.

2.6 Acknowledgements

This study was supported by Alabama Agriculture Experiment Station and National Institute of Food and Agriculture (NIFA), USDA.

2.7 References

Abou-Donia, M. B. 1976. Physiological effects and metabolism of gossypol. Pages 125-160. In:*Residue Reviews*, Springer New York, New York, NY.

Ahuja, I., Kissen, R., and Bones, A. M. 2012. Phytoalexins in defense against pathogens. Trends Plant Sci. 17:73-90

Anders, S., Pyl, P. T., and Huber, W. 2015. HTSeq--a python framework to work with high-throughput sequencing data. Bioinformatics. 31:166-9

Assigbetse, K., Fernandez, D., Dubois, M., and Geiger, J. P. 1994. Differentiation of *Fusarium oxysporum* f. sp. *vasinfectum* races on cotton by random amplified polymorphic DNA (RAPD) analysis. Mol Plant Path. 84:622-626

Bolger, A. M., Lohse, M., and Usadel, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 30:2114-20

Bolhuis, H., van Veen, H. W., Poolman, B., Driessen, A. J. M., and Konings, W. N. 1997. Mechanisms of multidrug transporters. FEMS Microbiol. Rev. 21:55-84

Bottger, G. T., Sheehan, E. T., and Lukefahr, M. J. 1964. Relation of gossypol content of cotton plants to insect resistance. Econ. Entomol. 57:283-285

Breuil, A.-C., Adrian, M., Pirio, N., Meunier, P., Bessis, R., and Jeandet, P. 1998. Metabolism of stilbene phytoalexins by *Botrytis cinerea*: 1. Characterization of a resveratrol dehydrodimer. Tetrahedron Lett. 39:537-540

Bugbee, W. M. 1970. Vascular response of cotton to infection by *Fusarium oxysporum* f.sp. *vasinfectum*. Phytopathology. 60:121-123

Callahan, T. M., Rose, M. S., Meade, M. J., Ehrenshaft, M., and Upchurch, R. G. 1999. *CFP*, the putative cercosporin transporter of *Cercospora kikuchii*, is required for wild type cercosporin production, resistance, and virulence on soybean. Mol. Plant-Microbe Interact. 12:901-910

Chen, L.-Q., Hou, B.-H., Lalonde, S., Takanaga, H., Hartung, M. L., Qu, X.-Q., Guo, W.-J., Kim, J.-G., Underwood, W., Chaudhuri, B., Chermak, D., Antony, G., White, F. F., Somerville, S. C., Mudgett, M. B., and Frommer, W. B. 2010. Sugar transporters for intercellular exchange and nutrition of pathogens. Nature. 468:527-32

Cho, Y., Ohm, R., Devappa, R., Lee, H., Grigoriev, I., Kim, B., and Ahn, J. 2014. Transcriptional responses of the *Bdtf1*-deletion mutant to the phytoalexin brassinin in the necrotrophic fungus *Alternaria brassicicola*. Molecules. 19:10717-10732

Coleman, J. J., White, G. J., Rodriguez-Carres, M., and Vanetten, H. D. 2011. An ABC transporter and a cytochrome P450 of *Nectria haematococca* MPVI are virulence factors on pea and are the major tolerance mechanisms to the phytoalexin pisatin. Mol. Plant-Microbe Interact. 24:368-376

Crichton, R. R. 2012. Zinc - lewis acid and gene regulator. Biol. Inorg. Chem. 229-246

Cruickshank, I. 1962. Studies on phytoalexins IV. the antimicrobial spectrum of pisatin. Aust. J. Biol. Sci. 15:147-159

Demain, A. L., and Fang, A. 2000. The natural functions of secondary metabolites. Adv. Biochem. Eng. Biotechnol. 69:1-39

Devay, J. E., Gutierrez, A. P., Pullman, G. S., Wakeman, R. J., Garber, R. H., Jeffers, D. P., Smith, S. N., Goodell, P. B., Roberts, P. A., and N, S. S. 1997. Inoculum densities of *Fusarium oxysporum* f. sp. *vasinfectum* and *Meloidogyne incognita* in relation to the development of *Fusarium* wilt and the phenology of cotton plants (*Gossypium hirsutum*). Phytopathology. 87:341-6

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T. R. 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 29:15-21

Vanetten, H. D., Pueppke, S. G., and Kelsey, T. C. 1975. 3,6a-dihydroxy-8,9-methylenedioxypterocarpan as a metabolite of pisatin produced by *Fusarium solani* f. sp. *pisi*. Phytochemistry. 14:1103-1105

Fleisner, A., Sopalla, C., and Weltring, K.-M. 2002. An ATP-binding cassette multidrug-resistance transporter is necessary for tolerance of *Gibberella pulicaris* to phytoalexins and virulence on potato tubers. Mol. Plant-Microbe Interact. 15:102-108

Gadelha, I. C. N., Fonseca, N. B. S., Oloris, S. C. S., Melo, M. M., and Soto-Blanco, B. 2014. Gossypol toxicity from cottonseed products. Scientific World Journal. vol. 2014, Article ID 231635, 11 pages

Gardner, H. W., Desjardins, A. E., McCormick, S. P., and Weisleder, D. 1994. Detoxification of the potato phytoalexin rishitin by *Gibberella pulicaris*. Phytochemistry. 37:1001-1005

Gilbert, N. E., O'Reilly, J. E., Chang, C. J. G., Lin, Y. C., and Brueggemeier, R. W. 1995. Antiproliferative activity of gossypol and gossypolone on human breast cancer cells. Life Sci. 57:61-67

Glenn, A. E., Davis, C. B., Gao, M., Gold, S. E., Mitchell, T. R., Proctor, R. H., Stewart, J. E., and Snook, M. E. 2016. Two horizontally transferred xenobiotic resistance gene clusters associated with detoxification of benzoxazolinones by *Fusarium* species. PLoS One. 11:e0147486

Hayashi, K., Schoonbeek, H.-J., and De Waard, M. A. 2002. *Bcmfs1*, a novel major facilitator superfamily transporter from *Botrytis cinerea*, provides tolerance towards the natural toxic compounds camptothecin and cercosporin and towards fungicides. Appl. Environ. Microbiol. 68:4996-5004

Jeandet, P., Clément, C., Courot, E., and Cordelier, S. 2013. Modulation of phytoalexin biosynthesis in engineered plants for disease resistance. Int. J. Mol. Sci. 14:14136-14170

Jeandet, P., and Philippe. 2015. Phytoalexins: current progress and future prospects. Molecules. 20:2770-2774

Kaufman, Z., Netzer, D., and Barash, I. 1981. The apparent involvement of phytoalexins in the resistance response of cotton plants to *Fusarium oxysporum* f. sp. *vasinfectum*. J. Phytopathol. 102:178-182

Kavanagh, K. L., Jörnvall, H., Persson, B., and Oppermann, U. 2008. Medium- and short-chain dehydrogenase/reductase gene and protein families: the SDR superfamily: functional and structural diversity within a family of metabolic and regulatory enzymes. Cell. Mol. Life Sci. 65:3895-906

Kenar, J. A. 2006. Reaction chemistry of gossypol and its derivatives. J. Am. Oil Chem. Soc. 83:269-302

Kettle, A. J., Batley, J., Benfield, A. H., Manners, J. M., Kazan, K., and Gardiner, D. M. 2015. Degradation of the benzoxazolinone class of phytoalexins is important for virulence of *Fusarium pseudograminearum* towards wheat. Mol. Plant Pathol. 16:946-962

Kettle, A. J., Carere, J., Batley, J., Manners, J. M., Kazan, K., and Gardiner, D. M. 2016. The *Fdb3* transcription factor of the *Fusarium* detoxification of benzoxazolinone gene cluster is required for MBOA but not BOA degradation in *Fusarium pseudograminearum*. Fungal Genet. Biol. 88:44-53

Kim, I. C., Waller, D. P., Marcelle, G. B., Cordell, G. A., Fong, H. H. S., Pirkle, W. H., Pilla, L., and Matlin, S. A. 1984. Comparative spermicidal effects of (+)-gossypol, (+)-gossypol, (−)-gossypol and gossypolone. Contraception. 30:253-259

Kotra, L. P., and Mobashery, S. 1998. β-Lactam antibiotics, β-lactamases and bacterial resistance. Bull. Inst. Pasteur. 96:139-150

Kretschmer, M., Leroch, M., Mosbach, A., Walker, A.-S., Fillinger, S., Mernke, D., Schoonbeek, H.-J., Pradier, J.-M., Leroux, P., De Waard, M. A., and Hahn, M. 2009.

Fungicide-driven evolution and molecular basis of multidrug resistance in field populations of the grey mould fungus *Botrytis cinerea* A. PLoS Pathog. 5:e1000696

Lah, L., Podobnik, B., Novak, M., Korošec, B., Berne, S., Vogelsang, M., Kraševec, N., Zupanec, N., Stojan, J., Bohlmann, J., and Komel, R. 2011. The versatility of the fungal cytochrome P450 monooxygenase system is instrumental in xenobiotic detoxification. Mol. Microbiol. 81:1374-1389

Lee, W., Basri, D., and Ghazali, A. 2017. Bactericidal effect of pterostilbene alone and in combination with gentamicin against human pathogenic bacteria. Molecules. 22:463

Leite, G. M., Magan, N., and Medina, Á. 2012. Comparison of different bead-beating RNA extraction strategies: An optimized method for filamentous fungi. J. Microbiol. Methods. 88:413-418

Lekha, P. K., and Lonsane, B. K. 1997. Production and application of tannin acyl hydrolase: state of the art. Adv. Appl. Microbiol. 44:215-60

Liu, X., Inlow, M., and Vanetten, H. D. 2003. Expression profiles of pea pathogenicity (PEP) genes in vivo and in vitro, characterization of the flanking regions of the PEP cluster and evidence that the PEP cluster region resulted from horizontal gene transfer in the fungal pathogen *Nectria haematococca*. Curr. Genet. 44:95-103

Liu, Benedict, Stipanovic, and Bell. 1999. Purification and characterization of s-adenosyl-l-methionine: desoxyhemigossypol-6-o-methyltransferase from cotton plants. An enzyme capable of methylating the defense terpenoids of cotton. Plant Physiol. 121:1017-1024

Livak, K. J., and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative pcr and the 2−ΔΔCT method. Methods. 25:402-408

Love, M. I., Huber, W., and Anders, S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15:550

Maser, E., and Bannenberg, G. 1994. 11β-hydroxysteroid dehydrogenase mediates reductive metabolism of xenobiotic carbonyl compounds. Biochem. Pharmacol. 47:1805-1812

Mcfadden, H., Beasley, D., and Brubaker, C. L. 2004. Assessment of *Gossypium sturtianum* and *G. australe* as potential sources of *Fusarium* wilt resistance to cotton. Euphytica. 138:61-72

Mellon, J. E., Zelaya, C. A., and Dowd, M. K. 2011. Inhibitory effects of gossypol-related compounds on growth of *Aspergillus flavus*. Lett. Appl. Microbiol. 52:406-412

Morrissey, J. P., and Osbourn, A. E. 1999. Fungal resistance to plant antibiotics as a mechanism of pathogenesis. Microbiol. Mol. Biol. Rev. 63:708-24

Muller, K. 1958. Studies on Phytoalexins I. The formation and the immunological significance of phytoalexin produced by *Phaseolus vulgaris* in response to infections with *Sclerotinia fructicola* and *Phytophthora infestans*. Aust. J. Biol. Sci. 11:275

Pezet, R., Pont, V., and Hoang-Van, K. 1991. Evidence for oxidative detoxication of pterostilbene and resveratrol by a laccase-like stilbene oxidase produced by *Botrytis cinerea*. Physiol. Mol. Plant Pathol. 39:441-450

Polsky, B., Segal, S. J., Baron, P. A., Gold, J. W. M., Ueno, H., and Armstrong, D. 1989. Inactivation of human immunodeficiency virus in vitro by gossypol. Contraception. 39:579-587

Ragazzi, A., and Vecchio, V. 1992. Behaviour of chlamydospore of *Fusarium oxysporum* f. sp. *vasinfectum* in substrates containing sodium chloride. Phytopathol. Mediterr. 31:85-87

Rodriguez-Galvez, E., and Mendgen, K. 1995. The infection process of *Fusarium oxysporum* in cotton root tips. Protoplasma. 189:61-72

Rogers, G. M., Poore, M. H., and Paschal, J. C. 2002. Feeding cotton products to cattle. The veterinary clinics of north america food animal practice. 18:267-94

Roohparvar, R., Mehrabi, R., Van Nistelrooy, J. G., Zwiers, L.-H., and De Waard, M. A. 2008. The drug transporter *MgMfs1* can modulate sensitivity of field strains of the fungal wheat pathogen *Mycosphaerella graminicola* to the strobilurin fungicide trifloxystrobin. Pest Manag. Sci. 64:685- 693

Dos Santos, S. C., Teixeira, M. C., Dias, P. J., and Sa Correia, I. 2014. MFS transporters required for multidrug/multixenobiotic (MD/MX) resistance in the model yeast: understanding their physiological function through post-genomic approaches. Front. Physiol. 5:180

Schafer, W., Straney, D., Ciuffetti, L., Van Etten, H. D., and Yoder, O. C. 1989. One enzyme makes a fungal pathogen, but not a saprophyte, virulent on a new host plant. Science. 246:247-249

Srivastava, A., Cho, I. K., and Cho, Y. 2013. The *Bdtf1* gene in *Alternaria brassicicola* is important in detoxifying brassinin and maintaining virulence on *Brassica* species. Mol. Plant-Microbe Interact. 26:1429-1440

Tholl, D. 2015. Biosynthesis and biological functions of terpenoids in plants. Adv Biochem Eng Biotechnol. 148:63-106.

Tian, Ruan, X., Huang, J., Fang, J., Mao, X., Wang, Y., Chen, L., and Yang, X. 2016. Gossypol: phytoalexin of cotton. Sci China Life Sci. 59:122-129

Turco, E., Vizzuso, C., Franceschini, S., Ragazzi, A., and Stefanini, F. M. 2007. The in vitro effect of gossypol and its interaction with salts on conidial germination and viability of *Fusarium oxysporum* sp. *vasinfectum* isolates. J. Appl. Microbiol. 103:2370-2381

Uehara, K. 1964. Relationship between the host specificity of pathogen and phytoalexin. Japanese J. Phytopathol. 29:103-110

Vanetten, H. D., Matthews, D. E., and Matthews, P. S. 1989. Phytoalexin detoxification: importance for pathogenicity and practical implications. Annu Rev Phytopathol. 27:143-164

Vanetten, H., Temporini, E., and Wasmann, C. 2001. Phytoalexin (and phytoanticipin) tolerance as a virulence trait: why is it not required by all pathogens? Physiol. Mol. Plant Pathol. 59:83-93

Wang, X., Beckham, T. H., Morris, J. C., Chen, F., and Gangemi, J. D. 2008. Bioactivities of gossypol, 6- methoxygossypol, and 6,6′-dimethoxygossypol. J. Agric. Food Chem. 56:4393-4398

Wang, X., Wang, J., Wong, S. C. H., Chow, L. S. N., Nicholls, J. M., Wong, Y. C., Liu, Y., Kwong, D. L. W., Sham, J. S. T., and Tsao, S. W. 2000. Cytotoxic effect of gossypol on colon carcinoma cells. Life Sci. 67:2663-2671

Wickham, H. 2009. ggplot2: Elegant graphics for data analysis. Springer Publishing Company, Incorporated.

Yin, W., and Keller, N. P. 2011. Transcriptional regulatory elements in fungal secondary metabolism. J. Microbiol. 49:329-339

Zdobnov, E. M., and Apweiler, R. 2001. InterProScan - an integration platform for the signature-recognition methods in InterPro. Bioinformatics. 17:847-848

SUPPLEMENTARY MATERIAL

Table S-1: Number of reads survived after trimming and proportion of reads mapped to the genome

Samples	Reads survived after trimming	Proportion of uniquely mapped reads
T0 R1	23523510	87.07%
T0 R2	43756775	89.07%
T0 R3	26264144	70.13%
T0 R4	31940447	72.97%
T1 R1	43629450	84.35%
T1 R2	42388166	87.13%
T1 R3	49930009	78.41%
T1 R4	33762920	76.17%
T2 R1	44958722	81.50%
T2 R2	41340700	85.38%
T2 R3	34312966	79.94%
T2 R4	39542066	79.02%
T4 R1	62679395	82.28%
T4 R2	49942782	85.76%
T4 R3	29276669	77.56%
T4 R4	39874612	79.01%

Table S-2: Common 68 upregulated genes with Pfam domain and expression values at timepoints T1, T2, and T4 from RNA-seq data

Gene ID	Pfam ID	Function	T1 expression	T2 expression	T4 expression
G16893			3.97	3.31	3.24
G14810	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain	3.29	3.28	3.25
G8072			3.82	4.10	3.84

Gene ID	Pfam ID	Function	T1 expression	T2 expression	T4 expression
G15647	PF04082, PF00096	Fungal specific transcription factor domain, Zinc finger, C2H type	4.15	4.51	3.91
G4532			3.72	3.94	3.93
G11615	PF00689, PF00122, PF00702, PF00690	Cation transporting ATPase,C-terminus,N-terminus, halo acid dehalogenase-like hydrolase, E1-E2 ATPase	5.07	4.44	3.97
G11765	PF02538, PF05378, PF01968	HydantoinaseB/oxoprolinase	3.53	5.51	4.04
G16103	PF11917	Protein of unknown function (DUF3435)	5.09	5.89	4.19
G5799	PF07690	Major Facilitator Superfamily	5.90	5.13	4.34
G4637	PF04082, PF00172	Fungal specific transcription factor domain, Fungal Zn(2)-Cys(6)binuclear cluster domain	5.64	4.43	4.34
G3976	PF00106	short chain dehydrogenase	7.79	7.95	4.34
G6536	PF08325, PF13649	WLM domain, Methyl transferase domain	4.27	4.60	4.66
G17310	PF04082	Fungal specific transcription factor domain	3.41	6.26	4.71

Gene ID	Pfam ID	Function	T1 expression	T2 expression	T4 expression
G12406	PF00173, PF01070	Cytochrome b5-like Heme/Steroid binding domain, FMN-dependent dehydrogenase	5.38	3.97	4.82
G14228	PF00501, PF13193	AMP-binding enzyme	6.33	4.78	4.89
G13181	PF00083	Sugar (and other) transporter	6.02	4.82	5.01
G13718			8.55	6.48	5.05
G11616	PF02705, PF02386	K+ potassium transporter, Cation transporter protein	5.32	6.05	5.05
G9742	PF00171	Aldehyde dehydrogenase family	7.36	7.80	5.20
G3132			4.75	5.85	5.25
G4103			9.96	7.38	5.37
G14856	PF01161, PF09056	Phosphatidyl ethanolamine-binding protein, Prokaryotic phospholipase A2	5.02	5.34	5.41
G2387	PF03062	MBOAT,membrane-bound O-acyl transferase family	8.52	6.96	5.50
G11320	PF13577	SnoaL-like domain	9.79	7.02	5.72
G1272	PF00732	GMC oxidoreductase	6.46	7.83	5.82
G9263			6.38	6.98	5.96
G17061	PF07690	Major Facilitator Superfamily	5.11	5.34	5.97
G8733	PF01979	Amido hydrolase family	9.63	7.65	6.15

Gene ID	Pfam ID	Function	T1 expression	T2 expression	T4 expression
G9846	PF00107	Zinc-binding dehydrogenase	8.60	7.50	6.17
G14232	PF07690	Major Facilitator Superfamily	11.67	9.52	6.20
G16420	PF13489	Methyl transferase domain	9.27	7.88	6.40
G11668	PF00107	Zinc-binding dehydrogenase	10.38	8.69	6.55
G2070	PF00106	Short chain dehydrogenase	9.42	8.10	6.57
G4638	PF02629, PF00549	CoA binding domain, CoA-ligase	7.76	7.90	6.57
G6112	PF07859	alpha/beta hydrolase fold	7.62	7.74	6.66
G14618	PF05368	NmrA-like family	7.50	7.12	6.66
G18019	PF04828	Glutathione-dependent formaldehyde-activating enzyme	8.69	9.72	6.70
G14619	PF00561	alpha/beta hydrolase fold	6.39	6.95	6.75
G4635	PF03328	HpcH/HpaIaldolase/citra telyasefamily	6.40	7.37	6.79
G16397	PF07690	Major Facilitator Superfamily	10.40	8.45	6.91
G14759	PF00848, PF00355	Ring hydroxylating alpha subunit (catalytic domain), Rieske[2Fe-2S] domain	6.31	6.34	7.03

Gene ID	Pfam ID	Function	T1 expression	T2 expression	T4 expression
G17335	PF13688, PF00200	Metallo-peptidasefamilyM12, Disintegrin	10.04	8.51	7.09
G17308	PF00083	Sugar (and other) transporter	4.42	9.19	7.22
G9997			5.80	7.98	7.54
G17337	PF00106, PF04082	Short chain dehydrogenase, Fungal specific transcription factor domain	10.97	8.79	7.69
G14750	PF05368	NmrA-like family	7.85	7.43	7.73
G16926	PF12697	Alpha/beta hydrolase family	9.25	8.09	7.80
G1759	PF03595	Voltage-dependent anion channel	5.57	7.10	7.81
G2286	PF13561	Enoyl- (Acyl carrier protein) reductase	6.58	10.09	7.87
G5740	PF13460	NAD(P)H-binding	11.23	9.90	8.12
G17064	PF00668, PF00501, PF00550	Condensation domain, AMP-binding enzyme, Phosphopantetheine attachment site	6.30	7.69	8.23
G4503	PF02771	Acyl-CoA dehydrogenase, N-terminal domain	7.45	8.26	8.26
G9749	PF01494	FAD binding domain	10.79	12.06	8.27
G16581	PF01494	FAD binding domain	13.40	10.13	8.42
G1781	PF07985	SRR1	10.42	9.29	8.43

Gene ID	Pfam ID	Function	T1 expression	T2 expression	T4 expression
G14749	PF00106	Short chain dehydrogenase	8.70	9.10	8.47
G4506			6.69	8.41	8.57
G17062	PF00005, PF00664	ABC transporter, ABC transporter transmembrane region	8.01	8.17	8.88
G4633	PF00171	Aldehyde dehydrogenase family	9.23	10.22	9.15
G5244	PF01370	NAD dependent epimerase/dehydratase family	6.24	8.11	9.56
G17063	PF13523	Acetyltransferase(GNAT) domain	6.39	8.06	9.69
G4504	PF02515	CoA-transferase family III	8.33	9.39	9.73
G5243	PF00107	Zinc-binding dehydrogenase	6.34	8.25	9.91
G13127			6.89	9.52	9.94
G16843	PF07519	Tannase and feruloyl esterase	7.24	11.00	10.10
G14616	PF13460	NAD(P)H-binding	13.43	14.51	10.99
G13126	PF00107, PF08240	Zinc-binding dehydrogenase, Alcohol dehydrogenase GroES-like domain	5.67	10.86	12.08
G10034	PF12706	Beta-lactamase superfamily domain	7.77	11.37	12.09

Table S-3: Common 6 downregulated genes with Pfam domain and expression values at timepoints T1, T2, and T4 from RNA-seq data

Gene ID	Pfam ID	Function	T1 expression	T2 expression	T4 expression
G12645	PF00724	NADH: flavin oxidoreductase/ NADH oxidase family	-4.50	-7.07	-5.61
G3067	PF00083	Sugar (and other) transporter	-3.36	-4.80	-4.09
G9392			-4.24	-3.97	-4.06
G16857	PF07716	Basic region leucine zipper	-3.85	-4.74	-3.97
G13853	PF00704	Glycosyl hydrolases family18	-2.99	-5.10	-3.33
G8874			-3.09	-2.93	-4.51

Table S-4: Upregulated ABC, sugar, and other transporters from RNA-seq data with Pfam domains

Gene ID	Pfam ID	Function
G15525	PF01061	ABC-2 type transporter
G15525	PF14510	ABC-transporter extracellular N-terminal
G15525	PF00005	ABC transporter
G15525	PF06422	CDR ABC transporter
G14872	PF00083	Sugar (and other) transporter
G14773	PF00664	ABC transporter transmembrane region
G14773	PF00005	ABC transporter
G11513	PF00690	Cation transporter / ATPase, N-terminus
G1844	PF00083	Sugar (and other) transporter
G17308	PF00083	Sugar (and other) transporter
G16947	PF00083	Sugar (and other) transporter
G3073	PF00664	ABC transporter transmembrane region
G3073	PF00005	ABC transporter
G11615	PF00690	Cation transporter / ATPase, N-terminus
G14231	PF01061	ABC-2 type transporter
G14231	PF00005	ABC transporter

Gene ID	Pfam ID	Function
G14231	PF06422	CDR ABC transporter
G14231	PF14510	ABC-transporter extracellular N-terminal
G11616	PF02705	K+ potassium transporter
G8088	PF10337	Putative ER transporter, 6TM, N-terminal
G5761	PF00083	Sugar (and other) transporter
G3879	PF00083	Sugar (and other) transporter
G15640	PF00083	Sugar (and other) transporter
G6073	PF10337	Putative ER transporter, 6TM, N-terminal
G13181	PF00083	Sugar (and other) transporter
G16571	PF00083	Sugar (and other) transporter
G9755	PF00083	Sugar (and other) transporter
G4124	PF01061	ABC-2 type transporter
G4124	PF06422	CDR ABC transporter
G4124	PF00005	ABC transporter
G4124	PF14510	ABC-transporter extracellular N-terminal
G4732	PF00005	ABC transporter
G4732	PF01061	ABC-2 type transporter
G4732	PF06422	CDR ABC transporter
G4732	PF14510	ABC-transporter extracellular N-terminal
G4585	PF00083	Sugar (and other) transporter
G17062	PF00005	ABC transporter
G17062	PF00664	ABC transporter transmembrane region
G13990	PF06422	CDR ABC transporter
G13990	PF00005	ABC transporter
G13990	PF01061	ABC-2 type transporter
G11512	PF02705	K+ potassium transporter
G6162	PF01226	Formate / nitrite transporter
G1068	PF14510	ABC-transporter extracellular N-terminal
G1068	PF00005	ABC transporter
G1068	PF06422	CDR ABC transporter

Gene ID	Pfam ID	Function
G1068	PF01061	ABC-2 type transporter
G5833	PF00083	Sugar (and other) transporter

Table S-5: Downregulated ABC, sugar, and other transporters from RNA-seq data with Pfam domains

Gene ID	Pfam ID	Function
G225	PF01490	Transmembrane amino acid transporter protein
G15196	PF00690	Cation transporter / ATPase, N-terminus
G13568	PF04145	Ctr copper transporter family
G3067	PF00083	Sugar (and other) transporter
G12622	PF12848	ABC transporter
G12622	PF00005	ABC transporter
G10880	PF02535	ZIP Zinc transporter
G1912	PF03169	OPT oligopeptide transporter protein
G2915	PF01384	Phosphate transporter family

Table S-6: Upregulated Major facilitator superfamily transporters from RNA-seq data with Pfam domains

Gene ID	Pfam ID	Function
G8405	PF07690	Major Facilitator Superfamily
G15166	PF07690	Major Facilitator Superfamily
G2996	PF07690	Major Facilitator Superfamily
G1849	PF07690	Major Facilitator Superfamily
G12440	PF07690	Major Facilitator Superfamily
G5738	PF07690	Major Facilitator Superfamily
G13020	PF07690	Major Facilitator Superfamily
G17061	PF07690	Major Facilitator Superfamily
G12311	PF07690	Major Facilitator Superfamily
G15135	PF07690	Major Facilitator Superfamily
G5799	PF07690	Major Facilitator Superfamily

Gene ID	Pfam ID	Function
G14409	PF07690	Major Facilitator Superfamily
G12395	PF07690	Major Facilitator Superfamily
G3773	PF07690	Major Facilitator Superfamily
G3190	PF07690	Major Facilitator Superfamily
G1861	PF07690	Major Facilitator Superfamily
G15271	PF07690	Major Facilitator Superfamily
G14797	PF07690	Major Facilitator Superfamily
G16397	PF07690	Major Facilitator Superfamily
G14232	PF07690	Major Facilitator Superfamily
G8026	PF07690	Major Facilitator Superfamily
G11770	PF07690	Major Facilitator Superfamily
G5712	PF07690	Major Facilitator Superfamily
G8159	PF07690	Major Facilitator Superfamily
G17302	PF07690	Major Facilitator Superfamily
G9120	PF07690	Major Facilitator Superfamily
G16528	PF07690	Major Facilitator Superfamily
G1573	PF07690	Major Facilitator Superfamily
G13135	PF07690	Major Facilitator Superfamily
G15388	PF07690	Major Facilitator Superfamily

Table S-7: Downregulated Major facilitator superfamily transporters from RNA-seq data with Pfam domains

Gene ID	Pfam ID	Function
G11174	PF07690	Major Facilitator Superfamily
G16496	PF07690	Major Facilitator Superfamily
G12203	PF07690	Major Facilitator Superfamily
G11064	PF07690	Major Facilitator Superfamily
G16807	PF07690	Major Facilitator Superfamily
G11928	PF07690	Major Facilitator Superfamily
G12670	PF07690	Major Facilitator Superfamily

Table S-8: Upregulated transcription factors from RNA-seq data with Pfam domains

Gene ID	Pfam ID	Function
G10320	PF11951	Fungal specific transcription factor domain
G10320	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
G10851	PF11951	Fungal specific transcription factor domain
G10851	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
G11314	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
G11701	PF11951	Fungal specific transcription factor domain
G11701	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
G11830	PF04082	Fungal specific transcription factor domain
G1370	PF04082	Fungal specific transcription factor domain
G1398	PF04082	Fungal specific transcription factor domain
G1398	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
G13989	PF04082	Fungal specific transcription factor domain
G13989	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
G1407	PF04082	Fungal specific transcription factor domain
G14409	PF04082	Fungal specific transcription factor domain
G14409	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
G14751	PF11951	Fungal specific transcription factor domain
G14799	PF04082	Fungal specific transcription factor domain
G148	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
G14810	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
G15272	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
G1530	PF11951	Fungal specific transcription factor domain
G15386	PF11951	Fungal specific transcription factor domain
G15525	PF04082	Fungal specific transcription factor domain
G15525	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
G15647	PF04082	Fungal specific transcription factor domain
G15647	PF00096	Zinc finger, C2H2 type
G15666	PF04082	Fungal specific transcription factor domain

G15666	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
G16453	PF04082	Fungal specific transcription factor domain
G16453	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
G16716	PF11951	Fungal specific transcription factor domain
G17094	PF04082	Fungal specific transcription factor domain
G17310	PF04082	Fungal specific transcription factor domain
G17337	PF04082	Fungal specific transcription factor domain
G2028	PF04082	Fungal specific transcription factor domain
G2030	PF04082	Fungal specific transcription factor domain
G2299	PF04082	Fungal specific transcription factor domain
G2369	PF04082	Fungal specific transcription factor domain
G2369	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
G2995	PF04082	Fungal specific transcription factor domain
G3074	PF04082	Fungal specific transcription factor domain
G3213	PF04082	Fungal specific transcription factor domain
G3328	PF04082	Fungal specific transcription factor domain
G3416	PF04082	Fungal specific transcription factor domain
G3610	PF04082	Fungal specific transcription factor domain
G3610	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
G3735	PF04082	Fungal specific transcription factor domain
G3735	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
G3971	PF04082	Fungal specific transcription factor domain
G3971	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
G4107	PF04082	Fungal specific transcription factor domain
G4498	PF04082	Fungal specific transcription factor domain
G4498	PF00096	Zinc finger, C2H2 type
G4524	PF04082	Fungal specific transcription factor domain
G4524	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
G4637	PF04082	Fungal specific transcription factor domain
G4637	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
G5923	PF04082	Fungal specific transcription factor domain

G6278	PF04082	Fungal specific transcription factor domain
G6345	PF04082	Fungal specific transcription factor domain
G6345	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
G6853	PF00096	Zinc finger, C2H2 type
G7331	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
G8136	PF04082	Fungal specific transcription factor domain
G8567	PF04082	Fungal specific transcription factor domain
G9152	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
G9711	PF04082	Fungal specific transcription factor domain
G9711	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain
G9771	PF11951	Fungal specific transcription factor domain
G9821	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain

Table S-9: Downregulated transcription factor from RNA-seq data with Pfam domain

Gene ID	**Pfam ID**	**Function**
G2849	PF00172	Fungal Zn(2)-Cys(6) binuclear cluster domain

Table S-10: Upregulated cytochrome P450s form RNA-seq data with Pfam domains

Gene ID	**Pfam ID**	**Function**
G9954	PF00067	Cytochrome P450
G1880	PF00067	Cytochrome P450
G4997	PF00067	Cytochrome P450
G2069	PF00067	Cytochrome P450
G6368	PF00067	Cytochrome P450
G14661	PF00067	Cytochrome P450
G4515	PF00067	Cytochrome P450
G1443	PF00067	Cytochrome P450
G4392	PF00067	Cytochrome P450
G11227	PF00067	Cytochrome P450

Table S-11: Downregulated cytochrome P450s form RNA-seq data with Pfam domains

Gene ID	Pfam ID	Function
G7160	PF00067	Cytochrome P450
G13893	PF00067	Cytochrome P450
G13959	PF00067	Cytochrome P450

Table S-12: Upregulated dehydrogenases from RNA-seq data with Pfam domains

Gene ID	Pfam ID	Function
G10035	PF13602	Zinc-binding dehydrogenase
G10035	PF08240	Alcohol dehydrogenase GroES-like domain
G10036	PF00106	short chain dehydrogenase
G10333	PF05199	GMC oxidoreductase
G10333	PF00732	GMC oxidoreductase
G10383	PF01494	FAD binding domain
G10383	PF13450	NAD(P)-binding Rossmann-like domain
G10445	PF08240	Alcohol dehydrogenase GroES-like domain
G10445	PF00107	Zinc-binding dehydrogenase
G10524	PF02826	D-isomer specific 2-hydroxy acid dehydrogenase, NAD binding domain
G10524	PF00389	D-isomer specific 2-hydroxy acid dehydrogenase, catalytic domain
G10557	PF00106	short chain dehydrogenase
G11181	PF00106	short chain dehydrogenase
G11216	PF00106	short chain dehydrogenase
G11304	PF01565	FAD binding domain
G11342	PF01370	NAD dependent epimerase / dehydratase family
G11344	PF00465	Iron-containing alcohol dehydrogenase
G11376	PF01565	FAD binding domain
G11376	PF02913	FAD linked oxidases, C-terminal domain
G11667	PF00171	Aldehyde dehydrogenase family

G11668	PF00107	Zinc-binding dehydrogenase
G11764	PF00970	Oxidoreductase FAD-binding domain
G11764	PF00175	Oxidoreductase NAD-binding domain
G12406	PF01070	FMN-dependent dehydrogenase
G1272	PF00732	GMC oxidoreductase
G1272	PF05199	GMC oxidoreductase
G13047	PF13738	Pyridine nucleotide-disulphide oxidoreductase
G13065	PF00393	6-phospho gluconate dehydrogenase, C-terminal domain
G13065	PF03446	NAD binding domain of 6-phospho gluconate dehydrogenase
G13126	PF00107	Zinc-binding dehydrogenase
G13126	PF08240	Alcohol dehydrogenase GroES-like domain
G13301	PF13460	NAD(P)H-binding
G13661	PF01565	FAD binding domain
G1370	PF08240	Alcohol dehydrogenase GroES-like domain
G1370	PF00107	Zinc-binding dehydrogenase
G1378	PF08240	Alcohol dehydrogenase GroES-like domain
G1378	PF00107	Zinc-binding dehydrogenase
G1396	PF00106	short chain dehydrogenase
G1398	PF01370	NAD dependent epimerase / dehydratase family
G13984	PF01370	NAD dependent epimerase / dehydratase family
G1409	PF00725	3-hydroxyacyl-CoA dehydrogenase, C-terminal domain
G1409	PF02737	3-hydroxyacyl-CoA dehydrogenase, NAD binding domain
G14190	PF00107	Zinc-binding dehydrogenase
G14190	PF08240	Alcohol dehydrogenase GroES-like domain
G14190	PF01370	NAD dependent epimerase / dehydratase family
G14263	PF07992	Pyridine nucleotide-disulphide oxidoreductase
G14287	PF01370	NAD dependent epimerase / dehydratase family
G14616	PF13460	NAD(P)H-binding

G14661	PF00667	FAD binding domain
G14661	PF00175	Oxidoreductase NAD-binding domain
G14671	PF00107	Zinc-binding dehydrogenase
G14671	PF08240	Alcohol dehydrogenase GroES-like domain
G14672	PF00724	NADH: flavin oxidoreductase / NADH oxidase family
G14677	PF08240	Alcohol dehydrogenase GroES-like domain
G14677	PF00107	Zinc-binding dehydrogenase
G14749	PF00106	short chain dehydrogenase
G14854	PF13738	Pyridine nucleotide-disulphide oxidoreductase
G14914	PF01565	FAD binding domain
G15163	PF08240	Alcohol dehydrogenase GroES-like domain
G15163	PF00107	Zinc-binding dehydrogenase
G15525	PF00106	short chain dehydrogenase
G15648	PF01494	FAD binding domain
G15649	PF00724	NADH: flavin oxidoreductase / NADH oxidase family
G1620	PF13460	NAD(P)H-binding
G16454	PF01494	FAD binding domain
G16581	PF01494	FAD binding domain
G16611	PF00106	short chain dehydrogenase
G16693	PF00106	short chain dehydrogenase
G16837	PF01565	FAD binding domain
G17285	PF00106	short chain dehydrogenase
G17309	PF01266	FAD dependent oxidoreductase
G17318	PF01494	FAD binding domain
G17330	PF05199	GMC oxidoreductase
G17330	PF00732	GMC oxidoreductase
G17336	PF00107	Zinc-binding dehydrogenase
G17336	PF08240	Alcohol dehydrogenase GroES-like domain
G17337	PF00106	short chain dehydrogenase
G17434	PF00106	short chain dehydrogenase
G1754	PF00732	GMC oxidoreductase

G1754	PF05199	GMC oxidoreductase
G1768	PF01370	NAD dependent epimerase / dehydratase family
G1777	PF00107	Zinc-binding dehydrogenase
G17960	PF07992	Pyridine nucleotide-disulphide oxidoreductase
G1816	PF01494	FAD binding domain
G1851	PF01565	FAD binding domain
G1892	PF01565	FAD binding domain
G2006	PF00106	short chain dehydrogenase
G2029	PF01408	Oxidoreductase family, NAD-binding Rossmann fold
G2070	PF00106	short chain dehydrogenase
G2131	PF08022	FAD-binding domain
G2166	PF00106	short chain dehydrogenase
G2638	PF07991	Aceto hydroxy acid isomer reductase, NADPH-binding domain
G291	PF08240	Alcohol dehydrogenase GroES-like domain
G291	PF00107	Zinc-binding dehydrogenase
G3010	PF00106	short chain dehydrogenase
G3117	PF00107	Zinc-binding dehydrogenase
G3117	PF08240	Alcohol dehydrogenase GroES-like domain
G3129	PF08240	Alcohol dehydrogenase GroES-like domain
G3171	PF08240	Alcohol dehydrogenase GroES-like domain
G3171	PF00107	Zinc-binding dehydrogenase
G3328	PF07992	Pyridine nucleotide-disulphide oxidoreductase
G3623	PF03807	NADPoxidoreductasecoenzymeF420-dependent
G3831	PF01266	FAD dependent oxidoreductase
G3872	PF00106	short chain dehydrogenase
G3911	PF01494	FAD binding domain
G3968	PF01494	FAD binding domain
G3976	PF00106	short chain dehydrogenase
G3977	PF00106	short chain dehydrogenase
G4049	PF00107	Zinc-binding dehydrogenase

G4049	PF08240	Alcohol dehydrogenase GroES-like domain
G4069	PF00465	Iron-containing alcohol dehydrogenase
G4100	PF00107	Zinc-binding dehydrogenase
G4100	PF08240	Alcohol dehydrogenase GroES-like domain
G4121	PF13602	Zinc-binding dehydrogenase
G4162	PF03358	NADPH-dependent FMN reductase
G4166	PF08240	Alcohol dehydrogenase GroES-like domain
G4166	PF00107	Zinc-binding dehydrogenase
G4167	PF00107	Zinc-binding dehydrogenase
G4167	PF08240	Alcohol dehydrogenase GroES-like domain
G4172	PF00724	NADH: flavin oxidoreductase / NADH oxidase family
G441	PF00106	short chain dehydrogenase
G4503	PF02771	Acyl-CoA dehydrogenase, N-terminal domain
G4503	PF02770	Acyl-CoA dehydrogenase, middle domain
G4503	PF00441	Acyl-CoA dehydrogenase, C-terminal domain
G4550	PF00106	short chain dehydrogenase
G4581	PF01370	NAD dependent epimerase / dehydratase family
G4633	PF00171	Aldehyde dehydrogenase family
G5012	PF00890	FAD binding domain
G5027	PF07992	Pyridine nucleotide-disulphide oxidoreductase
G5243	PF00107	Zinc-binding dehydrogenase
G5244	PF01370	NAD dependent epimerase / dehydratase family
G5269	PF01565	FAD binding domain
G5692	PF00890	FAD binding domain
G5740	PF13460	NAD(P)H-binding
G5793	PF00107	Zinc-binding dehydrogenase
G5834	PF01593	Flavin containing amine oxidoreductase
G5917	PF00107	Zinc-binding dehydrogenase
G5917	PF08240	Alcohol dehydrogenase GroES-like domain
G5920	PF00171	Aldehyde dehydrogenase family
G5988	PF00724	NADH: flavin oxidoreductase / NADH oxidase family

G6106	PF01370	NAD dependent epimerase / dehydratase family
G6230	PF00465	Iron-containing alcohol dehydrogenase
G6271	PF00107	Zinc-binding dehydrogenase
G6271	PF16884	N-terminal domain of oxidoreductase
G6659	PF00171	Aldehyde dehydrogenase family
G71	PF01494	FAD binding domain
G7845	PF08240	Alcohol dehydrogenase GroES-like domain
G7845	PF00107	Zinc-binding dehydrogenase
G8008	PF00106	short chain dehydrogenase
G8185	PF01070	FMN-dependent dehydrogenase
G8403	PF01565	FAD binding domain
G8618	PF00107	Zinc-binding dehydrogenase
G8618	PF08240	Alcohol dehydrogenase GroES-like domain
G8683	PF00106	short chain dehydrogenase
G8885	PF00171	Aldehyde dehydrogenase family
G8885	PF01408	Oxidoreductase family, NAD-binding Rossmann fold
G9013	PF00106	short chain dehydrogenase
G9522	PF00732	GMC oxidoreductase
G9522	PF05199	GMC oxidoreductase
G9742	PF00171	Aldehyde dehydrogenase family
G9749	PF01494	FAD binding domain
G9787	PF00171	Aldehyde dehydrogenase family
G9793	PF08240	Alcohol dehydrogenase GroES-like domain
G9793	PF00107	Zinc-binding dehydrogenase
G9846	PF00107	Zinc-binding dehydrogenase

Table S-13: Downregulated dehydrogenases from RNA-seq data with Pfam domains

Gene ID	Pfam ID	Function
G10481	PF08240	Alcohol dehydrogenase GroES-like domain
G10481	PF00107	Zinc-binding dehydrogenase

Gene ID	Pfam ID	Function
G11062	PF08022	FAD-binding domain
G11062	PF08030	Ferric reductase NAD binding domain
G11858	PF01593	Flavin containing amine oxidoreductase
G12645	PF00724	NADH: flavin oxidoreductase / NADH oxidase family
G13210	PF08240	Alcohol dehydrogenase GroES-like domain
G13210	PF00107	Zinc-binding dehydrogenase
G13650	PF07992	Pyridine nucleotide-disulphide oxidoreductase
G13783	PF08022	FAD-binding domain
G13783	PF08030	Ferric reductase NAD binding domain
G13862	PF00171	Aldehyde dehydrogenase family
G15182	PF00984	UDP-glucose/GDP-mannose dehydrogenase family, central domain
G15182	PF03721	UDP-glucose/GDP-mannose dehydrogenase family, NAD binding domain
G15182	PF03720	UDP-glucose/GDP-mannose dehydrogenase family, UDP binding domain
G224	PF01266	FAD dependent oxidoreductase
G2350	PF01565	FAD binding domain
G2725	PF00441	Acyl-CoA dehydrogenase, C-terminal domain
G3034	PF01266	FAD dependent oxidoreductase
G4736	PF08028	Acyl-CoA dehydrogenase, C-terminal domain
G4736	PF02771	Acyl-CoA dehydrogenase, N-terminal domain
G5825	PF08240	Alcohol dehydrogenase GroES-like domain
G5825	PF00107	Zinc-binding dehydrogenase
G7618	PF00106	short chain dehydrogenase
G8862	PF00171	Aldehyde dehydrogenase family

Table S-14: Upregulated genes assigned with KEGG orthology ID through KEGG analysis

Gene ID	KEGG ID
G4650	K00222
G659	K22285
G7378	K02510
G11480	K09419
G9122	K20826
G16539	K01568
G2877	K20121
G2638	K00053
G6271	K07119
G11765	K01469
G11829	K00681
G8611	K01876
G9522	K19069
G4140	K13333
G17285	K01188
G8176	K07407
G17350	K06978
G8085	K01899
G11513	K01536
G11508	K04450
G1880	K10437
G11817	K15102
G6296	K00799
G6536	K22685
G15496	K01607
G9713	K01555
G17189	K18195
G13159	K04630

Gene ID	KEGG ID
G7009	K12666
G12072	K20832
G17318	K03380
G17494	K10798
G6659	K00128
G3073	K05658
G17997	K15255
G11615	K01536
G11753	K05349
G11764	K00326
G15648	K00480
G243	K01621
G11616	K03549
G12406	K00101
G3171	K13953
G5761	K08139
G3879	K08141
G3850	K01078
G1816	K00480
G15640	K08141
G9789	K14333
G15135	K22134
G12305	K01702
G12395	K08158
G1518	K09705
G17064	K22148
G5547	K00750
G17960	K03885
G10383	K00480
G17063	K22151

Gene ID	KEGG ID
G4635	K11390
G4649	K00451
G13357	K09704
G1272	K17066
G8185	K00101
G4116	K00459
G109	K04711
G6227	K11159
G4659	K08678
G14661	K14338
G9714	K00451
G4515	K21293
G3117	K13953
G10381	K14333
G9742	K00128
G16823	K00799
G4503	K00249
G10266	K00799
G4638	K01899
G4585	K08141
G1398	K01183
G2387	K00637
G1395	K17740
G4633	K00135
G11320	K17740
G11530	K01887
G16397	K22134
G11372	K01560
G1670	K04618
G9738	K01875

Gene ID	KEGG ID
G3623	K00286
G17359	K17740
G11512	K03549
G7326	K14001
G5200	K06035
G4627	K03457
G3613	K03320
G9787	K00128
G4632	K01900
G9741	K00276
G9998	K00799
G4505	K11390
G17309	K00306
G10333	K00108
G8920	K01480
G6840	K01895
G5782	K03787
G16454	K03380
G4040	K01279
G17340	K00456
G3784	K20059
G14677	K13953
G13065	K00033
G13656	K01443
G3230	K03457
G16340	K11493
G4321	K01408
G8084	K01900
G17278	K01312
G11376	K00102

Gene ID	KEGG ID
G5917	K13953
G5988	K00354
G5920	K00128
G4166	K13953
G3954	K06035

Table S-15: Downregulated genes assigned with KEGG orthology ID through KEGG analysis

Gene ID	KEGG orthology
G8215	K00505
G13650	K17877
G15196	K01536
G15182	K00012
G11062	K21421
G12357	K00275
G16496	K03448
G7160	K05917
G7106	K01424
G7582	K17069
G8338	K03320
G14990	K07297
G1677	K11244
G13892	K16231
G14157	K00559
G13853	K01183
G261	K08994
G1018	K09829
G12694	K11976
G2531	K01613
G10880	K14715

Gene ID	KEGG orthology
G9451	K01785
G12641	K07393
G14022	K13690
G10481	K00002
G10990	K05928
G206	K09983
G12639	K09568
G7442	K12373
G13203	K05349
G224	K00306
G8862	K00129
G2915	K14640
G8860	K00276
G4954	K03146
G7827	K18278

Table S-16:Upregulated genes involved in metabolic pathways with KEGG ID, gene name and function identified through KEGG analysis

Gene ID	KEGG ID	Gene	Function
G13065	K00033	PGD	6-phospho gluconate dehydrogenase
G2638	K00053	ilvC	ketol-acid reducto isomerase
G12406, G8185	K00101	lldD	L-lactate dehydrogenase (cytochrome)
G10333	K00108	betA	Choline dehydrogenase
G6659, G9742, G9787, G5920	K00128	ALDH	Aldehyde dehydrogenase (NAD+)
G4633	K00135	gabD	succinate-semialdehyde dehydrogenase/glutarate-semialdehyde dehydrogenase
G4650	K00222	TM7SF2	Delta14-sterolreductase
G4503	K00249	ACADM	acyl-CoA dehydrogenase

Gene ID	KEGG ID	Gene	Function
G9741	K00276	AOC3	primary-amine oxidase
G3623	K00286	proC	pyrroline-5-carboxylate reductase
G17309	K00306	PIPOX	Sarcosine oxidase/L-pipecolate oxidase
G4649, G7914	K00451	HGD	homogentisate1,2-dioxygenase
G17340	K00456	CDO1	Cysteine dioxygenase
G15648, G1816, G10383	K00480	E1.14.13.1	Salicylate hydroxylase
G11829	K00681	ggt	gamma-glutamyl transpeptidase/glutathione hydrolase
G5547	K00750	GYG1	Glycogenin
G3850	K01078	PHO	Acid phosphatase
G1398	K01183	E3.2.1.14	Chitinase
G17285	K01188	E3.2.1.21	beta-glucosidase
G11765	K01469	OPLAH	5-oxoprolinase(ATP-hydrolysing)
G8920	K01480	speB	Agmatinase
G9713	K01555	FAH	Fumaryl acetoacetase
G11372	K01560	E3.8.1.2	2-haloaciddehalogenase
G16539	K01568	PDC	Pyruvate decarboxylase
G15496	K01607	pcaC	4-carboxy muconolactone decarboxylase
G243	K01621	xfp	xylulose-5-phosphate/fructose-6-phosphate phosphor ketolase
G12305	K01702	LEU1	3-isopropyl malate dehydratase
G6840	K01895	ACSS	acetyl-CoA synthetase
G8085, G4638	K01899	LSC1	succinyl-CoA synthetase alpha subunit
G4632, G8084	K01900	LSC2	succinyl-CoA synthetase alpha subunit
G5782	K03787	surE	5'-nucleotidase
G11753	K05349	bglX	beta-glucosidase
G8176	K07407	E3.2.1.22B	alpha-galactosidase
G4659	K08678	UXS1	UDP-glucuronate decarboxylase

Gene ID	KEGG ID	Gene	Function
G1880	K10437	PHAA	Phenyl acetate2-hydroxylase
G7009	K12666	OST1	Oligo saccharyl transferase complex subunit alpha (ribophorin I)
G3171, G3117, G14677, G5917, G4166	K13953	adhP	Alcohol dehydrogenase, propanol-preferring
G1272	K17066	MOX	Alcohol oxidase

Table S-17:Upregulated genes involved in microbial metabolism in diverse environments with KEGG ID, gene name and function identified through KEGG analysis

Gene ID	KEGG ID	Gene	Function
G13065	K00033	PGD	6-phospho gluconate dehydrogenase
G6659, G9742, G9787, G5920	K00128	ALDH	Aldehyde dehydrogenase (NAD+)
G4633	K00135	gabD	succinate-semialdehyde dehydrogenase/glutarate-semialdehyde dehydrogenase
G4649, G9714	K00451	HGD	homogentisate1,2-dioxygenase
G15648, G1816, G10383	K00480	E1.14.13.1	Salicylate hydroxylase
G9713	K01555	FAH	Fumaryl acetoacetase
G11372	K01560	E3.8.1.2	2-haloacid dehalogenase
G15496	K01607	pcaC	4-carboxymucono lactone decarboxylase
G243	K01621	xfp	xylulose-5-phosphate/fructose-6-phosphate phosphor ketolase
G6840	K01895	ACSS	acetyl-CoA synthetase
G8085, G4638	K01899	LSC1	succinyl-CoA synthetase alpha subunit
G4632, G8084	K01900	LSC2	succinyl-CoA synthetase beta subunit
G7378	K02510	hpaI	4-hydroxy-2-oxoheptanedioatealdolase

Gene ID	KEGG ID	Gene	Function
G17318, G16454	K03380	E1.14.13.7	phenol2-monooxygenase
G5200, G3954	K06035	DDI2_3	Cyanamide hydratase
G1880	K10437	PHAA	phenylacetate2-hydroxylase
G3171, G3117, G14677, G5917, G4166	K13953	adhP	Alcohol dehydrogenase, propanol-preferring
G9789, G10381	K14333	DHBD	2,3-dihydroxybenzoatedecarboxylase
G14661	K14338	cypDE	Cytochrome P450/NADPH-cytochrome P450 reductase
G1272	K17066	MOX	Alcohol oxidase

Table S-18: Upregulated genes involved in biosynthesis of secondary metabolites with KEGG ID, gene name and function identified through KEGG analysis

Gene ID	KEGG ID	Gene	Function
G13065	K00033	PGD	6-phospho gluconate dehydrogenase
G2638	K00053	ilvC	ketol-acid reducto isomerase
G6659, G9742, G9787, G5920	K00128	ALDH	Aldehyde dehydrogenase (NAD+)
G4650	K00222	TM7SF2	Delta14-sterolreductase
G4503	K00249	ACADM	acyl-CoA dehydrogenase
G9741	K00276	AOC3	primary-amine oxidase
G3623	K00286	proC	pyrroline-5-carboxylate reductase
G17285	K01188	E3.2.1.21	beta-glucosidase
G16539	K01568	PDC	Pyruvate decarboxylase
G12305	K01702	LEU1	3-isopropyl malate dehydratase
G6840	K01895	ACSS	acetyl-CoA synthetase
G8085, G4638	K01899	LSC1	succinyl-CoA synthetase alpha subunit
G4632, G8084	K01900	LSC2	succinyl-CoA synthetase beta subunit
G5782	K03787	surE	5'-nucleotidase

Gene ID	KEGG ID	Gene	Function
G11753	K05349	bglX	beta-glucosidase
G3171, G3117, G14677, G5917, G4166	K13953	adhP	Alcohol dehydrogenase, propanol-preferring

Table S-19:Upregulated genes involved in biosynthesis of antibiotics with KEGG ID, gene name and function identified through KEGG analysis

Gene ID	KEGG ID	Gene	Function
G13065	K00033	PGD	6-phospho gluconate dehydrogenase
G2638	K00053	ilvC	ketol-acid reducto isomerase
G6659, G9742, G9787, G5920	K00128	ALDH	Aldehyde dehydrogenase (NAD+)
G4650	K00222	TM7SF2	Delta14-sterol reductase
G4503	K00249	ACADM	acyl-CoA dehydrogenase
G3623	K00286	proC	pyrroline-5-carboxylate reductase
G13656	K01443	nagA	N-acetyl glucosamine-6-phosphate deacetylase
G6840	K01895	ACSS	acetyl-CoA synthetase
G8085, G4638	K01899	LSC1	succinyl-CoA synthetase alpha subunit
G4632, G8084	K01900	LSC2	succinyl-CoA synthetase beta subunit
G3171, G3117, G14677, G5917, G4166	K13953	adhP	Alcohol dehydrogenase, propanol-preferring

Table S-20: Upregulated genes involved in carbon metabolism with KEGG ID, gene name and function identified through KEGG analysis

Gene ID	KEGG ID	Gene	Function
G13065	K00033	PGD	6-phospho gluconate dehydrogenase
G4503	K00249	ACADM	acyl-CoA dehydrogenase

Gene ID	KEGG ID	Gene	Function
G6840	K01895	ACSS	acetyl-CoA synthetase
G8085, G4638	K01899	LSC1	succinyl-CoA synthetase alpha subunit
G4632, G8084	K01900	LSC2	succinyl-CoA synthetase beta subunit
G1272	K17066	MOX	Alcohol oxidase

Table S-21: List of primers and amplicon length of candidate genes selected for RT-qPCR

Gene ID	Forward Primer 5' → 3'	Reverse Primer 5' → 3'	Amplicon length (bp)
G10034	GAGAACCGAAGTATGCGATAGG	CTGCTCTCCTTTCACCCTTC	133
G13127	CCTACAGGGTTATGGTGTTCAG	CCATGGTGCTCCCAGATTAT	96
G5243	GGAGCGTAATGTTGGCATAATC	CTAGCTTCATTGCCCTCTTCT	96
G17063	AACTCAGACCGAGTCAACAAG	GAGTTCTCCGTTCCACTCAAA	126
G4633	GTCACGCTGGTACTCGTATTT	CCGTGTCGGGATCAAAGTT	114
G14749	CGGCCAGTTGTTGGTCTAAT	GCAGGTGAGTTCAGTCTCTTC	88
G17337	TGAAGGCGCTGAGGATTAAG	TAGCTCAGTCGACATAGTAGGG	101
G18019	TATAGGTGAGACCCGAACCTAC	CCGCAGTTACCACAGAAGTT	78
G16581	CATCTTCGCGACCTTCAATCT	TCATGGCGTTGGTCTTCTTC	113
G2070	CGGTAGGTCCTTGTTCTTCTTC	CTCGTATCATCAACGTGTCCAG	92
G11668	CCGAGAATGTCCATCTCCTAAAC	CGATAACAGTCTCTCCAGCTTTC	144
G14661	CATACGCAGGTGCAGAAAGA	GATTGACCAGCTCTGGGAATAG	117
G1443	GCAGGATCATGCTTCTCATACT	ACAGTGCCTTAAGCCTCATATC	129
G4637	GTTCAACGTCACAGTCCTCATC	GCGGCTATGGTGGTCAATATAC	95
G1781	CCAGAGCCTCGATATCTCTGTA	CAAGTGTCTCCTCGTCCATTATC	86
G17308	CCTGAGATTTCTGGTCTGAGTG	CGAAGTCCTCCTTACCATTCTC	127
G13126	CGTTGCAGCCTCCAGATAA	GGCTCTGTTGATGGCACTAT	96
G5244	GCTTGGCTCAACGACTATTTC	CATGTTTGCTCACCCTTGAC	119
G17064	CGGATGATGACGAGGAGATTAC	CGAGCCTCATGCTGGATTAT	100

Gene ID	Forward Primer 5' → 3'	Reverse Primer 5' → 3'	Amplicon length (bp)
G16843	GTTCTCTACTCCTGTGGTTGTG	CTGCAGGAGGTGTTGAAACT	99
G9742	CATCAGCGACTTGGAAGACTAC	CCTGAACATCGCGCTCAATA	87
G3905	CGAGCTGATACTCTGGTTCTTC	CGCTCTTGTCAACCCAGTTA	83
G17062	GTCGTGGCTCTTCGGTAAAT	GTCTTCTCGGTCACCTCAAAC	104
G16397	CCGATGAAACCCGTGTATGT	GTGATTGTCGGGCGTTCTAT	119
G9749	GCAACACGGATCAAGGGAAT	GGAAGCCAGCTCCAAGAAATAG	98
G7914	GAGACGACATCATCCCGATTC	TGCTTCCTTTCCTCATCTGTC	100
G14232	GAGACGACATCATCCCGATTC	TGCTTCCTTTCCTCATCTGTC	100
G14616	GGAATGCAGATCGACCTTGT	CAGGTTCCTGTACTCTGGAATG	114
G10333	GCATCGATGAGCAGTGTCTTA	GACCTCACTGGCTACGAATATG	104
G12645	CGGTGGTTTAGATAGTCGTCAG	CACAGCAACTCCGGTATCTT	103
G13853	CTTCCAGCTTCGTTGTAGACTC	CCTCAGTGAATGCCACACTATC	102
EF-1	TGAGTTCGAGGCTGGTATCT	CACTTGGTGGTGTCCATCTT	110
GAPDH	CCACCGTCCACTCCTACACT	GAGCTCAGGAATGACCTTGC	146