Investigating the genes and pathways involved in the biosynthesis of indole-3-acetic acid in *Fusarium graminearum.*

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Thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the degree of

Master of Biology

Under the auspices of the Ottawa-Carleton Institute for Biological Science

University of Ottawa
Ottawa, Ontario, Canada
April 2012

Abstract

The hormone indole-3-acetic acid (IAA) is utilized by some microorganisms as part of their infection strategy. This study investigated the enzymatic pathways and genes involved in the biosynthesis of IAA by *F. graminearum*. In culture, *F. graminearum* was able to produce IAA when supplied with tryptophan or intermediates found in the enzymatic pathways for the biosynthesis of IAA with the exclusion of indole-3-acetamide (IAM). Predicted gene function, gene expression profiling and RT-qPCR validation of specific gene expression patterns, allowed us to establish a list of candidate genes for the biosynthesis of IAA. A probable aldehyde dehydrogenase (fg02296) was inactivated by replacement in the *F. graminearum* genome. Results suggested that this gene is not required for IAA production or fitness level in *F. graminearum*. The role of IAA produced by this pathogen remains elusive however this study has provided some pertinent information on the biosynthesis of IAA in culture.

Acknowledgements

I would like to thank my supervisor Thérèse Ouellet for this opportunity, her help in designing this research project, her encouragement along the way and her financial contribution. As well, this project would not have been possible without the help and the expertise of the technicians in my lab. Margaret Balcerzak has been a great teacher and a mentor to me. She has helped me in the analysis of my results and has always made herself available. Hélène Rocheleau has also been a great teacher and I also have truly appreciated her support.

I would also like to thank those who have been very generous with their time and resources and have helped make this project possible. Gopal Subramaniam from Agriculture Canada and the members of his lab, thank you for letting me use your HPLC instrument, for introducing me to ATMT and helping me with transformations. Gopal Subramaniam has always made himself available to discuss ideas for my project and I have appreciated his suggestions and inputs along the way. Barbara Blackwell and Charles Séguin from Agriculture Canada, thank you for helping me setting up HPLC experiments and protocols, and for letting me use your incubator. Christopher Boddy from the University of Ottawa and the members of his lab, thank you for welcoming me in your lab, letting me use your LC-MS/MS instrument and teaching me the principles of LC-MS/MS.

I am very fortunate to have great friends who have always been positive and encouraging towards my studies, they are very important to me and I have appreciated their support over these past years. I am immensely appreciative for my boyfriend Jesse Wood who has stood by my side during the good and the difficult times. Thank you for never giving up on me and always being a motivator. Last but not least, I would like to thank my mother Francine Houle. I am tremendously grateful for her emotional and financial support. Thanks mom for believing in me and always being proud. I love you very much.

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ABA	abscisic acid
ARF	auxin response factor
ATMT	Agrobacterium tumefaciens
AuxRE	auxin response element
BAC	bacterial artificial chromosome
BLAST	basic local alignment search tool
BR	brassinosteroids
CK	cytokinins
CMC	carboxymethyl cellulose
Ct	quantification cycle
DAOM	Department of Agriculture, Ottawa, mycology
DFM	defined media
E	efficiency
ET	ethylene
FAD	flavin adenine dinucleotide
FHB	fusarium head blight
FGDB	*Fusarium graminearum* Genome Database
FRC	fusarium research center
GA	gibberellins
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCPSR	genealogical concordance phylogenetic species recognition
HPLC	high performance liquid chromatography
HPH	hygromycin phosphotransferase
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	knockout
LC-MS/MS	liquid chromatography-mass spectrophotometry/ mass spectrophotometry
IAA	indole-3-acetic acid
IaaM	tryptophan-2-monooxygenase
IaaH	indole-3-acetamide hydrolase
IAAld	indole-3-acetaldehyde
IAM	indole-3-acetamide
IAN	indole-3-acetonitrile
IAOx	indole-3-acetaldoxime
ILA	indole-3-lactic acid
IPA	indole-3-pyruvic acid
JA	jasmonic acid
L-TRP	L-tryptophan
MAMPs	microbe associated molecular patterns
MAT	mating-type gene

MIPS	Munich Information Center for Protein Sequences
N-TAM	N-hydroxyl tryptamine
NCBI	National Centre for Biotechnology Information
PCR	polymerase chain reaction
PDA	potato dextrose agar
PLP	pyridoxal phosphate
PR	pathogenesis-related protein
PRRs	pattern recognition receptors
R	resistance protein
RT-qPCR	reverse transcribed-quantitative PCR
SA	salicylic acid
SAR	systemic acquired resistance
T3SS	T3 secretion system
Ti	tumor-inducing
T-DNA	transfer-DNA
TAM	tryptamine
TOL	tryptophol
TPP	thiamine diphosphat
TRF	negative transformant
USER	uracil-specific excision reagent
WT	wild-type

Chapter 1 – Introduction

1.1 Fusarium head blight

Fusarium species are fungal pathogens for a large diversity of plant and thrive in a wide range of climatic conditions (Nelson et al., 1994). Among the various diseases caused by a *Fusarium* infection, fusarium head blight (FHB) is one that receives global attention as a devastating disease on cereal grains (Parry et al., 1995)

FHB is a disease affecting most cereal grains such as wheat, barley, oat, rye and maize, and its principal culprit is *F. graminearum* (Parry et al., 1995). Crops infected with FHB are demarked by premature head necrosis; infected grains are shriveled and white to pinkish in colour and can also contain a black discoloration from the fungus (Bushnell et al., 2003; Boddu et al., 2006). In all cases, infected crops typically result in lower grain quality and lower yield, and can have a tremendous economical impact (McMullen et al., 1997; Windels, 2000). In addition, toxins produced by the fungus and called mycotoxins accumulate during infection and can be a significant health concern. Contaminated crops are often too toxic and unsuitable for agricultural end product in human food or animal feed (Placinta et al., 1999). The consumption of contaminated crops by animals is correlated to various diseases associated with the type of mycotoxins ingested (Desjardins, 2006). In plants, some of these mycotoxins have been found to function as virulence factors during pathogenesis (reviewed in Desjardins and Hohn, 1997).

1.2 *F. graminearum*

As mentioned above, *F. graminearum* is responsible for FHB (Parry et al., 1995). Consequently, this species has been the focus of many studies concerned with the characteristics of its genome, its life cycle and its pathogenicity (O'Donnell et al., 2004, Cuomo et al., 2007, Desjardins and Proctor, 2007, Trail, 2009)

1.2.1 Brief overview of the *F. graminearum* genome

In 2003, the sequence of the *F. graminearum* genome was published by the Broad Institute of MIT and Harvard (http://www.broadinstitute.org/). The assembly generated a genome size of 36.1Mb distributed over a total of 4 chromosomes (Cuomo et al., 2007). Few high-identity duplicated sequences were found in the genome and only a few gene pairs originated from recent duplication (Cuomo et al., 2007). The total initial number of predicted genes was of 11640 (Cuomo et al., 2007). Gene functionality was inferred by conserved domains and these were compared to the closely related fungi *Neurospora crassa*, *Magnaporthe grisea* and *Aspergillus nidulans* (Cuomo et al., 2007). A second database for the annotation of the *F.graminearum* genome was developed as the Fusarium graminearum Genome Database (FGDB) at MIPS using a different system to annotate genes (mips.gsf.de/genre/proj/FGDB). The most recently updated version of the *F.graminearum* annotated genome is provided at MIPS and named FGDB v3.1. This version provides information on the full manually revised gene set based on the Broad Institute assembly FG3 genome sequence (Wong et al., 2011). The results of gene prediction tools were integrated with the help of comparative data on related species to result in a set of 13.718 annotated protein coding genes (Wong et al., 2011). An Affymetix gene chip was made available

following the release of the genome sequence combining both initial sets of gene calls from Broad Institute and MIPS, and is useful for expression analyses (Gueldener et al., 2006). Infection-related genes were overrepresented in distinct regions of high diversity and the opposite was true for highly conserved genes (Cuomo et al., 2007). This may explain the fungus' ability to quickly adapt to changing environments or hosts.

1.2.2 The *F. graminearum* life cycle and infection process

During the majority of its life cycle, *F. graminearum* remains haploid where it will produce copious asexual spores named macroconidia (Trail, 2009). These spores are produced in slimy masses on a cushion-shaped hyphal structure named the sporodochia (Trail, 2009). These may be produced on the surface of infected plants by colonized mycelia or on crop residue; they are associated with rain-splash dispersal and may serve in short-distance dispersal (Shaner, 2003; Deacon, 2006; Trail, 2009). The sexual development is initiated by an extended binucleate phase where binucleate hyphae are formed and eventually develop into flask-shaped sacs named perithecia (Guenther and Trail, 2005; Trail, 2009). This structure is filled with tubular sacs named asci, which are filled with sexual spores produced through meiosis (Trail, 2009). These sexual spores are named ascospores and they reach the host by being forcibly discharged into the air by the perithecia (Trail, 2009). This is thought to be the primary inoculum for the disease, although the relative contribution of the asexual spore conidia mentioned earlier and the sexual ascospore to the disease remains elusive (Trail, 2009).

In the field, *F. graminearum* can survive as a saprophyte on crop debris and spores can overwinter in soil (Guenther & Trail, 2005; Trail, 2009). Wheat is susceptible to disease during warm and moist weather conditions, when anthesis and spore dispersal coincides (Bushnell et al., 2003). Ascospores or conidia may at that point enter the openings of the flowering heads and infect. Symptoms of FHB, demarked by premature necrosis, may develop in spikelet tissue and spread through the entire spike (or head) (Boddu et al., 2006). The infected crop will be contaminated by mycotoxins and will often be too toxic and unsuitable in agricultural end products for human food or animal feed (Placinta et al., 1999). The infection strategy and mode of nutrition of *F. graminearum* during infection is still under debate, some have recognized this fungus to be hemi-biotrophic (reviewed in Bushnell et al., 2003).

Recently, Brown et al. (2010) have established the hyphal colonisation events occurring in all tissues of an *F. graminearum* infected wheat head. By doing so, they have also addressed the mode of fungal nutrition during infection (Brown et al., 2010). Their results show that, at the advancing front, intercellular colonising hyphae are surrounded by living host tissue and no host cell death is found ahead of the infection. Two-thirds of the colonized tissue early in the infection (<5dpi) is even asymptomatic. However behind the infection front, the fungus has colonized the vasculature and then the cortex intracellularly, at which point host cells have lost their entire cellular content and disease was apparent. As for the mode of nutrition, the authors state that their description does not resemble any defined mode of nutrient such as biotrophic, hemi-biotrophic or necrotophic according to classical definitions (Agrios 1997; Brown et al., 2010). However, their description fits some

definitions of a hemi-biotroph (Brown et al., 2010). It was shown that during early infection the asymptomatic state of intercellular hyphae always advanced through living host tissue, but once host cell death was initiated behind the infection front, *F. graminearum* colonized intracellularly and survived off the dead host cells (Brown et al., 2010). Thus during the initial stage of infection and at the advancing front, the fungus exists biotrophically but once symptomatic infection sets in, the fungus is necrotrophic. Nevertheless it is clear that when this pathogen invades the wheat head it is obviously very successful at defeating the wheat's defense system.

1.3 Plant defense system and biotic stress responses

In their natural environment plants are constantly exposed to various biotic stresses. Since plants do not have specialized cells to carry out immune functions, they have evolved defense mechanisms which prioritize defense over normal cellular functions (Spoel and Dong, 2008). A local infection can induce a systemic acquired resistance (SAR) which is effective against a broad spectrum of plant pathogens (Ryals et al., 1997; S. Spoel et al., 2003). This resistance involves the up-regulation of a large set of genes encoding pathogenesis-related proteins (PR) including some with antimicrobial activity, resistance (R) proteins which recognize specific effector molecules brought into the plant by the pathogen and others which may be involved in states of resistance (Van Loon and Van Strien, 1999; Spoel et al., 2003; Lopez et al., 2008). The onset of SAR is also accompanied by the local and systemic increase of hormone levels.

1.3.1 Hormones and plant defense

Induced resistance is regulated by a network of interconnected signal transduction pathways where hormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are the primary signals (Lopez et al., 2008). The plant will modify the relative abundance of these hormones and the expression of the genes regulated by them in order to activate distinct sets of defense-related genes (Spoel et al., 2003). In addition to these three key hormones, there are other hormones such as abscisic acid (ABA), gibberellin (GA), cytokinin (CK), brassinosteroid (BR) and of particular interest to this study, auxin; however their role in the defense response is less well understood (Lopez et al., 2008). Nevertheless, it has been shown that all of these hormones promote defense responses that are differentially effective against specific types of attackers (Robert-Seilaniantz et al., 2007; Lopez et al, 2008).

As mentioned in the previous section, nutritional lifestyles that have been identified for pathogenic fungi include biotrophic, hemi-biotrophic and necrotrophic (reviewed in Spoel et al. 2007). It is essential for the plant to activate the appropriate defense strategy according to the pathogen type or lifestyle. Biotrophic pathogens will be targeted by the SA hormone-mediated signalling pathway while necrotrophs will be sensitive to JA or ethylene-mediated responses (reviewed in Spoel and Dong, 2008). Thus the activation of one pathway as a defense response often suppresses the activation of the other. The other mentioned hormones, ABA, GA, CK, BR and auxin, are also implicated in either of the two types of responses (reviewed in Spoel and Dong 2008). Altogether these hormones form a network of interactions that target resistance from specific types of pathogens. However as discussed

earlier, not all pathogens fall in a distinct category, some will alternate depending on the stage of the infection and are recognized as hemi-biotrophic.

Some pathogens have developed the ability to manipulate this network to promote disease (Koornneef and Pieterse, 2008). The production of more than one hormone by a pathogen during infection is common. And the induction of auxin or auxin-related genes in particular is seen across a broad spectrum of pathogen as part of their infection strategy and promotes susceptibility.

1.4 Auxin

Auxin is a key plant hormone, regulating multiple facets of development in various cell types, including pattern formation during early development, elongation and branching of roots and shoots, development of vascular tissue and responses to light and gravity (Davies, 2004). It is produced in various regions of plants such as in the coleoptile tips of the grasses, in the apical meristem of shoots and roots, in leaf primordia, cotyledons of developing seeds and in fruits (Davies, 2004). Indole-3-acetic acid (IAA) is the major naturally occurring auxin in plants (Hobbie, 2007). It is generally found at concentrations of 10 to 100 ng/g fresh weight tissues in most monocots and dicots (Hobbie, 2007). The active form of IAA in plants is believed to be free IAA however IAA can be conjugated to amino acids and carbohydrates for several processes including IAA storage, transport, protection from enzymatic destruction, and targeting of the IAA for catabolism (reviewed in Woodward and Bartel, 2005).

1.4.1 The biochemistry of IAA biosynthesis

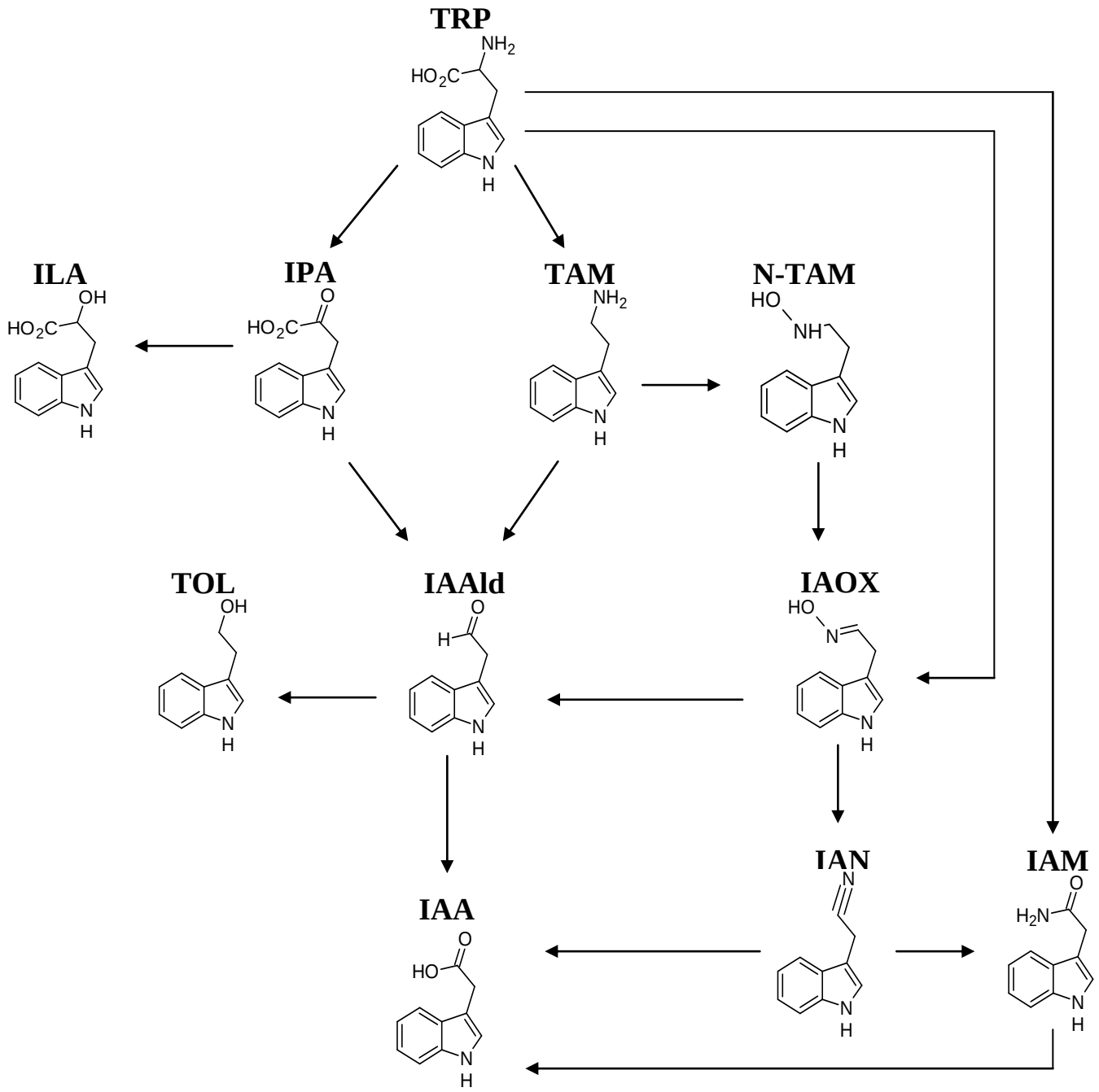
In plants, the synthesis of secondary metabolites stems from processes in the primary metabolism such as glycolysis and photosynthesis (Dewick, 2001). These processes contribute to the pentose phosphate cycle which produces D-erythrose-4-phosphate required by the shikimate pathway (Dewick, 2001). It is in the shikimate pathway that the secondary metabolite, shikimic acid, is formed and needed for the synthesis of various phenols, cinnamic acid derivatives, lignan and alkaloid compounds; including L-tryptophan (Dewick, 2001). L-tryptophan is one of the building blocks that form the basis of many natural product structures. When this aromatic amino acid undergoes decarboxylation, it provides the skeleton of an indole (Dewick, 2001). The synthesis of the auxin, indole-3-acetic acid involves a series of enzymatic reactions using multiple indolic intermediates.

IAA can be derived from either L-tryptophan (L-TRP)-dependent and/or L-TRP-independent pathways. The tryptophan-independent pathway, which may be utilized mostly by plants for biosynthesis of IAA, branches from indole-3-glycerolphosphate or other indoles (reviewed in Spaepen et al., 2007) but the significance of this pathway remains unclear especially in microorganisms. There are currently three main tryptophan-dependent pathways for the IAA biosynthesis in microorganisms (Fig. 1). These are named according to their first committed intermediate and they include: the indole-3-acetamide (IAM) pathway, the indole-3-pyruvic acid (IPA) pathway, and the trypamine (TAM) pathway. Indole-3-acetaldoxime (IAOx) is another intermediate; however its contribution is still uncertain (reviewed in Woodward and Bartel, 2005; Spaepen et al., 2007).

Among the IAA biosynthesis pathways and the steps needed to synthesize IAA, there are redundant and shared enzymatic reactions. The first pathway to be described is the IAM pathway and it involves only 2 enzymatic reactions using a mono-oxygenase and a hydrolase enzyme (Fig. 1). In this case the mono-oxygenase will catalyse the addition of oxygen from molecular oxygen to form IAM from L-TRP (Dewick, 2001). The hydrolase enzymes will then catalyse the hydrolysis at the carbonyl center of IAM and remove ammonia to form IAA (Dewick, 2001).

In the IPA pathway (Fig. 1), L-TRP is transformed into IPA with aminotransferase enzymes that exchange the amino group for an α-keto acid group. From this reaction, IPA can be transformed into two products: either a byproduct named indole-3-lactic acid (ILA), or indole-3-acetaldehyde (IAAld). ILA is formed with ILA reductase activity, where the α-ketone is reduced; however this enzymatic reaction is reversible (Dewick, 2001). IPA can also undergo a decarboxylation process that depends on thiamine diphosphate (TPP) to form IAAld (Dewick, 2001). This intermediate can then form a byproduct named indole-3-ethanol, commonly referred to as tryptophol (TOL) or it can form IAA. Much like IPA, IAAld can be converted to TOL via an IAAld reductase and this reaction is also reversible (Dewick, 2001). A dehydrogenase activity will convert IAA from IAAld.

Fig. 1. Tryptophan-dependent enzymatic pathways in microbial IAA biosynthesis. TRP, tryptophan; TAM, tryptamine; IPA, indole-3-pyruvic acid; IAM, acetamide; N-TAM, N-hydroxyl tryptamine; ILA, indole-3-lactic acid; IAOx, indole-3-acetaldoxime; TOL, tryptophol; IAN, indole-3-acetonitrile; IAAld, indole-3-acetaldehyde; IAA, indole-3-acetic acid. Illustration inspired from Reineke et al. (2008) and Spaepen et al. (2007).


TRP
ILA
IPA
TAM
N-TAM
TOL
IAAld
IAOX
IAN
IAM
IAA

The TAM pathway is more complex as it involves more possible intermediates (Fig. 1). L-TRP first undergoes a decarboxylation reaction that depends on pyridoxal phosphate (PLP) to form TAM (Dewick, 2001). The TAM intermediate can then form N-hydroxyl tryptamine (N-TAM) or IAAld. TAM will be converted into IAAld by monoamine oxidase enzymes, a reaction that typically involves a flavin adenine dinucleotide (FAD) and molecular oxygen which transform the amine group into an aldehyde (Dewick, 2001). For the conversion of TAM to N-TAM, flavin mono-oxygenase like enzymes proceed in a hydroxylation reaction to form N-TAM (Dewick, 2001). This compound can then be oxidized to indole-3-acetaldoxime (IAOx), however IAOx can also form from L-TRP with cytochrome P450 enzymes that catalyze a monooxygenase reaction (Dewick, 2001). Once IAOx is formed it can undergo a deamination to form IAAld and the release of ammonia or it can form indole-3-acetonitrile (IAN) (Dewick, 2001). Nitrilase enzymes can catalyze the hydrolysis of the nitrile group of IAN to carboxylic acid and ammonia to form IAA or nitrilase hydratase can hydrolyse the nitrile group to an amide and form IAM which as mentioned earlier becomes IAA with IAM hydrolase (Dewick, 2001). In both TAM and the IPA pathway, IAAld will form IAA under the same dehydrogenase activity which catalyses the conversion of acetaldehyde to acetic acid.

1.5 The role of auxin in plant-pathogen interaction

In some cases the increased IAA levels in plants benefit both the microorganism and the plant. The biosynthesis of IAA from the microbial species; *A. brasilense* and *Bradyrhizobium sp*., nitrogen-fixing bacteria, and *A. niger*, a phosphate-solubilizing fungus, are beneficial to their host and promote plant growth (Malhotra and Srivastava, 2008,

Skorupska, 2010, Yadav et al., 2011). These species are found in the rhizosphere of their plant hosts, and it is believed that most of the auxin found at this region comes from the biosynthesis of IAA by these microorganisms, using tryptophan secreted by the roots. These studies suggest that auxin contributes to plant growth promoting activity. In the case of *A. brasilense*, genetic studies have determined that this species utilize the IPA pathway (Malhotra and Srivastava, 2008). Many other bacterial species, in a study that examined plant-associated bacteria, were found to have the ability to increase endogenous IAA in wheat (*Triticum aestivum*) and also promote growth (Ali et al., 2009). As mentioned earlier, auxin is also involved in plant defense and can have a role in SA repression as a defense response during infection. Therefore, auxin signalling can be important in plant defense against necrotrophic pathogens. However many pathogens have taken advantage of the plant's network of hormonal defense responses and manipulate it as a strategy to increase virulence and to promote disease (Spoel et al., 2008).

Species that are biotrophic and generate tumors or galls during infection have been commonly studied with respect to IAA biosynthesis and its involvement in major physiological changes in the host. *A. tumefaciens* and *U. maydis* represent both bacterial and fungal species, respectively, which cause tumors on the infected host. Infection from *A. tumefaciens* will cause crown galls to develop on the host once the transfer DNA (T-DNA) from the tumor-inducing (Ti) plasmid of this pathogen is inserted into the host genome (Veselov et al, 2003). It was found that the T-DNA carries two genes for auxin that are part of the IAM pathway. The integration and expression of these genes into the host is believed to be responsible for the tumor growth (Veselov et al, 2003). *U. maydis* is part of the

basidiomycete phylum and known as smut fungi; it also generates galls on its host plant, maize. This fungal pathogen has been the focus of many studies pertaining to the involvement of IAA in pathogenesis (Basse et al, 1996, Basse and Steinberg, 2004, Chung and Tzeng, 2004, Reineke et al, 2008). It has been found that elevated amounts of IAA were located in tumor tissue infected from *U. maydis*. Genetic studies have proposed that IAA biosynthesis occurs for this species through both IPA and TAM pathways (Reineke et al, 2008).

IAA can also be utilized by phytopathogens that are hemibiotrophic, that cause necrotic lesions or blights. In this case, the pathogen synthesizes auxin or manipulates plant auxin expression for its benefit and auxin is associated with virulence and disease. *P. syringae* is a hemibiotrophic bacterial plant pathogen and the causal agent of leaf spotting disease (Chen et al., 2007). During infection this pathogen causes diseases using the type III secretion system (T3SS) that involves the effector protein, AvrRpt2 (Chen et al., 2007). The role of AvrRpt2 was associated with altering host auxin biosynthesis as an infection strategy. At the site of infection, an increase in free IAA was found. Studies that examined the role of AvrRpt2 showed that without its activity, *P. syringae* was unable to grow and cause disease (Chen et al., 2004; Chen et al, 2007). Thus it was suggested that auxin promoted disease symptom development during infection. The other bacterial phytopathogen represented is *D. dadantii*. This species causes soft rot, wilt and blight on a variety of plant hosts (Yang et al., 2007). Its virulence involves a T3SS that is similar to that of *P. syringae*, however the involvement of auxin is different. It was found that *D. dadantii* possessed both iaaH and iaaM genes involved in the IAM pathway and was able to produce IAA (Yang et al., 2007).

In that study, it was suggested that IAA biosynthesis regulated T3SS and exoenzymes partially through a postrancriptional regulatory pathway.

N. crassa is an ascomycete, known as bread mold and one of the first fungal species to have been studied with respect to the effects of IAA (Nakamura et al., 1978). It was found that exogenous IAA had positive effects on conidial germination rate and promoted the elongation of germ tubes and young hyphae. Similar effects have been found for the ascomycete *S. cerevisae*, where IAA induced adhesion and filamentation (Prusty et al., 2004). This species is also capable of synthesizing IAA from tryptophan and carries genes homologous to the aldehyde dehydrogenase of *U. maydis,* as well as genes that function as Trp aminotransferase in the IPA pathway (Urrestarazu et al., 1992; Iraqui et al., 1998). *M. oryzae* is an ascomycete hemibiotrophic fungal pathogen that has been well studied as the causal agent of the rice blast disease (Tanaka et al., 2011). It was found that during the biotrophic phase, where the infective hyphae grew and invaded living rice tissue, a small amount of fungal IAA was biosynthesized. Following this phase, symptoms such as necrotic lesions will appear. This suggested that the response of the host tissue to the exogenous fungal auxin early in the infection may be directly involved in the intracellular hyphal infection and micromorphological changes in the host (Tanaka et al., 2011).

The IAM pathway is found to be utilized mostly by pathogenic bacteria, and is associated with the ability to build up large amounts of IAA in host plants for gall formation and control of free IAA level (Maor et al., 2004; Yang et al., 2007). Accordingly, phytopathogenic symptoms are mostly linked to the IAM pathway (Spaepen et al., 2007).

The use of the IPA pathway has been observed in both pathogenic and nonpathogenic bacteria, and is mostly linked to bacterial fitness in colonizing the rhizosphere. However, multiple opportunistic bacteria, including *P. agglomerans* strains, produce IAA exclusively through the IPA pathway and some nonpathogenic symbiotic bacteria, like *Rhizobium spp.*, synthesize IAA mainly through the IAM pathway (Spaepen et al., 2007). Some pathogens have the ability to produce IAA from only one pathway while in others two different pathways may be utilized. For example, the inactivation of the IPA pathway in *Azospirillum lipoferum* reduced IAA biosynthesis up to 90% (Prinsen et al., 1993; Spaepen et al., 2007). However fungal species like *Colletotrichum acutatum* are able to utilize both the IPA and IAM pathways, and *Ustilago maydis* both the IPA and TAM pathways (Maor et al., 2004, Chung and Tzeng, 2004, Reineke et al., 2008). *Colletrotrichum gloeosporioides sp aeschynomene* is another fungus, like *Fusarium graminearum*, that has a biotrophic phase early in infection and is then necrotrophic (Maor et al., 2004). This species produces IAM and IAA in plants during infection and it has been suggested that IAA production might be important during early stages of plant colonization (Maor et al., 2004).

1.6 Objectives

The molecular and genetic aspects of the biosynthesis of IAA in *F. graminearum* remain elusive. An investigation at this level will provide basic information pertaining to how IAA is biosynthesized by *F. graminearum* and may be useful for further studies focused on understanding the role of IAA in *F. graminearum*'s pathogenicity. This study aims at identifying the pathways and genes involved in the *F. graminearum* biosynthesis of IAA. To determine which enzymatic pathway(s) *F. graminearum* utilizes for IAA biosynthesis,

cultures will be treated with intermediates found in the most common microbial tryptophan-dependent pathways. The supernatant of these cultures will be analyzed to determine the presence and quantity of IAA, as well as other possible intermediates with techniques such as HPLC and LC-MS/MS. To identify genes involved in IAA biosynthesis, the *F. graminearum* genome will first be screened using bioinformatics and putative candidate genes will be proposed from a homologous BLASTp search from IAA genes of other microbial species. A microarray experiment will also be performed on treated cultures of *F.graminearum*. The time point and the treatment examined will be chosen based on the biochemical information obtained from the HPLC results. The differential gene expression from the microarrays, along the bioinformatic search will provide candidate genes. If time allows, these genes will be replaced in the *F.graminearum* genome creating knockouts and their function further assessed using the previous biochemistry techniques. Successful knockout will be tested to determine the role of IAA during *F. graminearum* infection. Ultimately, the characterization of pathways and genes utilized for the biosynthesis of IAA will contribute to the understanding of the involvement of the IAA hormone in the life of the pathogen *F.graminearum*.

Chapter 2 – Materials and Methods

2.1 Fusarium strains and culture conditions

F.graminearum wild-type DAOM 180378 was used in this study; however for the purpose of transgenic studies, the strain DAOM 233423 was utilized. Spores from both strains were kept on potato dextrose agar (PDA) plates with appropriate antibiotics. To collect the spores, sterile water was added to the PDA plate and the surface was gently scraped using the edge of a sterile microscope slide. The water with spores was filtered through 4 layers of cheesecloth (Fisher Healthcare, Houston, TX, USA) and was quantified using a hemocytometer. These spores were used to grow new spores, re-plated for storage or inoculated to liquid media to grow mycelia. To grow spores, 50 mL of CMC liquid media was inoculated with 20 000 spores/mL (Cappelli and Peterson, 1965) and kept at 170 rpm, 28ºC, in the dark for 24 hrs. Spores were also grown in these conditions by adding a plug, from a PDA plate with mycelial growth, to the CMC liquid media (Cappelli and Peterson, 1965). To store the spores, $1 \cdot 10^6$ of resuspended spores was spread over fresh PDA plates with appropriate antibiotics, exposed to UV light for 48hrs and stored at 4 degrees. In order to grow mycelia, $1 \cdot 10^6$ spores were inoculated into 50 mL of first stage medium for high level of mycotoxin production (Taylor et al., 2008) in 250 mL Erlenmeyer flasks, 200 rpm, 28ºC, in the dark for 72 hrs.

2.2 Feeding experiments

Mycelia from *F. graminearum* strain DAOM 180378 was grown in the first stage medium in the conditions mentioned above. The mycelia was homogenized in its medium, filtered through Miracloth (Calbiochem, Canada) and washed with 0.9% saline solution. The

mycelia was transferred to 50 mL of 2nd stage medium (Taylor et al., 2008), containing either 0.2 mM of TAM, IPA, IAM, or IAAld or 2 mM TRP and shaken at 200 rpm, at 28ºC in the dark. Controls included mycelia transferred to 2nd stage medium without the addition of any intermediate. Each treated-cultures and controls included three biological replicates for every time point of collection.

2.3 Biochemical analysis

The treated cultures from the feeding experiments were transferred to 50 mL Falcon tubes and centrifuged at 5000 rpm for 10 min. The supernatant was collected and filtered through 0.2 µm Nylon Syringe filters (Nalgene, Canada) and subjected to HPLC analysis and/or LC-MS/MS analysis. All of the compounds that were used as standards, including: IAA, TAM, IPA, IAAld, TRP, TOL and ILA were purchased from Sigma-Aldrich Co. LLC, Canada.

2.3.1 High Performance Liquid Chromatography analysis

Ten µl of the filtrate from each of the liquid cultures mentioned above was injected into an ATKA P-10 HPLC system (GE Healthcare, Canada) with a 5 micron C18 Hypersil Reverse Phase column (ThermoFisher Scientific Inc., Canada). The compounds were separated in a gradient of 85:15 to 25:75 water:methanol during 16 min at a flow rate of 1 mL/min. The column was then washed with 100% methanol for 5 min followed by 85:15 water:methanol for 10 min. Trifluoroacetic acid (0.1%) was added to both solvents. The eluted compounds were detected at 220 nM and the data was processed using the UNICORN software (GE Healthcare, Canada). Eluted compounds were quantified based on the area under the curve (mAU*min) in reference to standard curves.

2.3.2 Mass spectrometer analysis

Ten µl of filtrate from each of the liquid cultures mentioned above was injected into an LCMS-8030 triple quadrupole LC-MS/MS system (Shimadzu Scientific Instruments, US). The LC was equipped with a 100 mm in length, 3 microns C18 Hypersil column (ThermoFisher Scientific Inc., Canada). Solvents included 95:5 and 5:95 water:acetonitrile and 0.05% of formic acid was added to both solvents. The compounds were separated in a gradient of 5 to 60% acetonitrile over 25 min, at a flow rate of 0.3 mL/min and were detected at 210 and 245 nM. The data was recovered from the positive mass spectrometry data and analyzed using the Analyst software (AB ScienX, US). Background noise was subtracted from the extracted ion chromatogram for each eluted compound of interest. Saskoski-Gadlay, a smoothing algorithm, was applied to smooth the curve of the AU/UV*min chromatogram. Eluted compounds with a Noise to Signal ration of less than 4 were ignored. The area counts were used for quantification based on standard curves created from IAA and TOL purchased from Sigma-Aldrich Co. LLC, Canada.

2.4 Gene expression analysis

The mycelia from *F.graminearum* cultures that were either untreated or feed 0.2 mM IAAld, 0.2 mM TAM or 2 mM TRP were collected in biological triplicates at 6hrs to be part of a treated vs untreated gene expression profile comparison. The mycelium from each sample was immediately submerged into liquid nitrogen after being collected and filtered. The frozen mycelia were grounded using a mortar and pestle and stored at -80ºC until needed.

2.4.1 RNA extraction

The RNA from the grounded mycelia collected in the conditions above was extracted using TRIZOL reagent (Invitrogen, Life Technologies Co., Canada) and purified using RNeasy Mini Kit (Qiagen Science, Canada) and the RNA cleanup protocol which included a DNase treatment. The integrity of the RNA was examined before and after the purification with a 2100 Bioanalyzer (Agilent Technologies Inc., Canada) for the RNAs used for microarray analysis. The concentration of the RNA used for RT-qPCR, was measured using Ribogreen with the Ribogreen RNA Quantitation kit (Invitrogen, Life Technologies Co., Canada), fluorescence reading was performed by the MJ Research PTC-200 with Chromo 4 detector, DNA Engine Opticon 3 RT PCR Detection System (Bio-Rad, Canada).

2.4.2 Microarray hybridization and analysis

A custom-design *F. graminearum* microarray consisting of 13918 predicted genes that are represented by up to three different 60mer oligos and totaling 41388 test oligos, in addition to 1417 oligos representing spike in and negative controls (NCBI, GEO record# GPL11046) was used for the profiling experiments. That array was manufactured by Agilent Technologies Inc (Canada) using their 4x44k microarray slide format. For each treatment and control, three biological replicates each labeled with two dye-swapped technical replicates were hybridized to the custom array. The two dye swapped technical replicates each consisted of 500ng of total RNA that was labeled using the Two-color Quick Amp Labeling Kit (Agilent Technologies Inc., Canada) as described in the protocol (version 5.7, available online Agilent Technologies Inc., Canada), except that only half of the reagents were used in Step 2. Each array represented the hybridizations from both the treated

samples versus untreated (control) samples. The labeled product was purified using the RNeasy MinElute Cleanup Kit (Qiagen Science, Canada). The hybridization was performed overnight in a Robbins Scientific Model 400 oven with an Agilent Hybridization Oven Rotator (G2530-60029). The standard wash protocol was performed with an added final 10sec acetonitrile wash.

The slides were scanned using a GenePix 4200A scanner and the data was prepared with GenePix Pro 6 (Molecular Devices, USA). The data (.gpr file) was imported in Acuity 4.0 (Molecular Devices, USA) and normalized using the Lowess method (Dudoit et al., 2002; Yang et al., 2002) to obtain Lowess M log ratios and Lowess A values. Lowess M represented the log ratios between the values obtained for the treated sample versus the control sample in the hybridyzation. Lowess A consisted of the average of the log values for the signal intensity of the treated and untreated samples. An analysis dataset was created for each of the treatments. Hybridization features within the datasets that had Lowess A values above 7.5 in at least 2 of the 6 arrays were kept. Reverse-dye technical replicates were averaged after cy3 test columns were swapped to cy5. Spike-in control features were removed. The names of the enzymes involved in the biosynthesis of IAA from Tryptophan were used as keywords to search for the differential expression of relevant genes in these datasets.

2.4.3 Reverse transcribed quantitative PCR

The RNA was reverse transcribed into cDNA using the Ambion RetroScript kit (Applied Biosystems, Life Technologies Co., Canada). Primers were designed and optimized using

the programs PrimerQuest and OligoAnalyzer found on the Integrated DNA Technologies website (Integrated DNA Technologies Inc., Canada). The primers were synthesized by Sigma-Genosys (Sigma-Aldrich, Canada), resuspended to 100µM in 10 mM Tris buffer and diluted with sterile water to 10µM for use. The list of primers can be found in the Appendix, Table A1. Three fold dilution series performed on the cDNA of the experimental samples (treated cultures) was used to obtain standard curves of values 1:10 to 1:810. For each gene analysed, there was a standard curve, technical duplicates of each sample and biological triplicates at each time point for treated and control (non-treated) samples. The controls for each time point (non-treated samples) were added to each RT-qPCR plate as well as a non-template control sample and a non-reaction control (did not add reverse transcriptase during cDNA synthesis) were also performed with each RT-qPCR plate. The RT-qPCR was performed using the Brilliant SYBR Green qPCR Core Reagent Kit (Stratagene, Agilent Technologies Inc., Canada).

The fluorescence reading was obtained from the MJ Research PTC-200 with Chromo 4 detector, DNA Engine Opticon 3 RT PCR Detection System (Bio-Rad, Canada). The Opticon3 software was used to analyze the raw data. Quantification cycle (Ct) values were collected at threshold point, with PCR efficiency (E) ranging between 1.6 and 2.2 and the generated standard curve displaying an R^2 over 0.99. Ct values for two technical replicates were converted into relative expression levels ($E^{-\Delta Cq}$), normalized, rescaled then averaged. The relative expression levels were normalized using the Ct values from three reference genes, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; fg06257), β-Tubulin (fg09530) and Elongation Factor-1 (fg08811, see Appendix Table A1 for primer sequences).

For each gene, the value of the sample with the lowest averaged relative expression was used as a reference for the rescaling. Relative expression levels, their normalization and rescaling were calculated as described by Vandesompele et al. (2002).

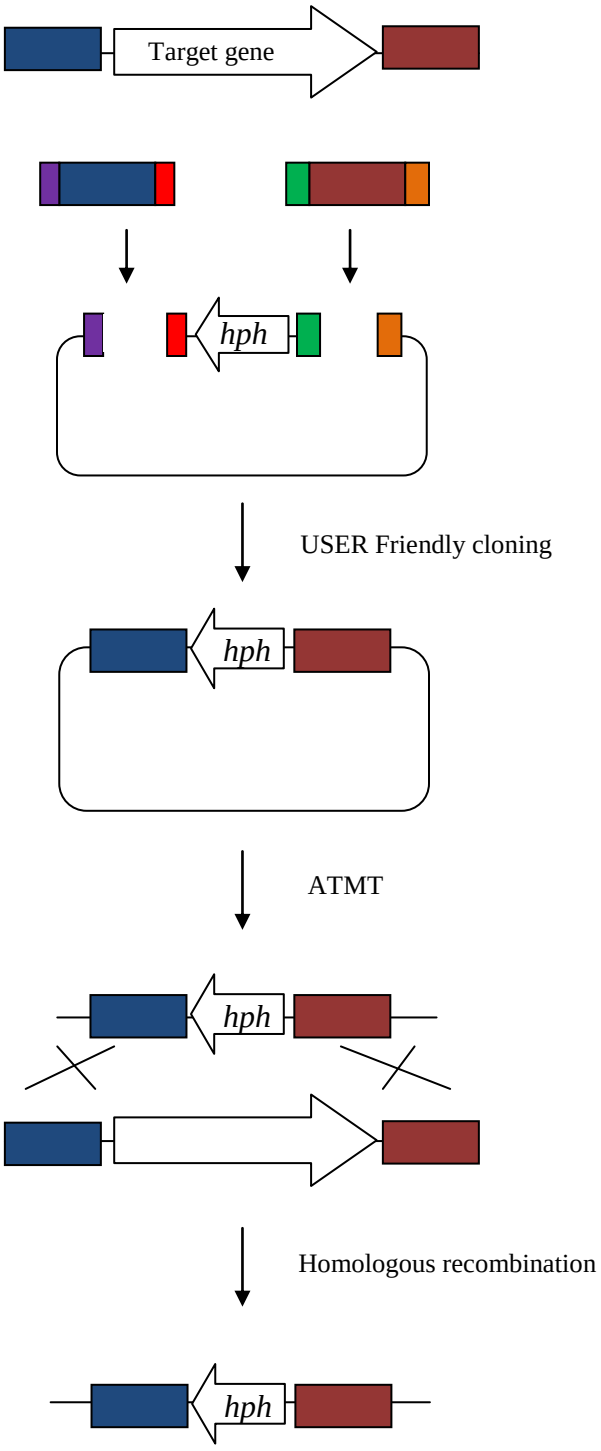
2.5 Generation of gene replacement construct and fungal transformation

The gene fg02296, a probable aldehyde dehydrogenase and fg08509, a probable methylcrotonyl-CoA carboxylase beta chain, were replaced in the *F. graminearum* genome. The *F. graminearum fg02296*Δ transgenic strain and *fg08509*Δ transgenic strain, used as a positive control for the transformation protocol, were generated based on the protocol from Frandsen et al. (2008). The replacement construct was generated using USER (uracil-specific excision reagent) Friendly cloning (New England Biolabs). This technique allowed for the annealing of 4 DNA sequences in 1 step, including the vector backbone pRF-HU2 (Frandsen et al., 2008), the selection marker hygromycin, and two homologous recombinant sequences framing the gene of interest, which were independently obtained based on sequences downloaded from the *Fusarium* Comparative Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/). The USER Friendly cloning process is illustrated in Fig. 2. The primers utilized for the amplification of the homologous recombinant sequences and the selection marker can be found in the Appendix, Table A2. The generated replacement construct was transformed into TOP10 competent *E.coli* cells. Hygromycin positive *E. coli* colonies were screened by PCR amplification directly on colony material and the correct plasmid was isolated using QIAprep Spin miniprep Kit (Qiagen Science, Canada). The identity of the clones was confirmed by sequencing using a LI-COR 4200 sequencing system (LI-COR, Lincon, NE). The plasmid DNA was

transformed into *Agrobacterium tumefaciens* LBA4404 by electroporation. *Agrobacterium tumefaciens*-mediated transformation (ATMT) was used to transform *F. graminearum* (Frandsen et al., 2008).

The transformed *F. graminearum* was selected on various media plates with appropriate antibiotics and the transformants were screened using fungal colony PCR. Single spore colonies were grown from positive transformants as well as negative transformants. The DNA from the transgenic strains as well as from the negative transformants was extracted using the e.Z.N.A, Fungal DNA Mini Kit (OMEGA bio-tek, USA). Primers targeting the gene of interest, hygromycin, and β-Tubulin as an internal control were used in PCRs to confirm the replacement of the gene of interest with hygromycin (Appendix, Fig. A1).

Fig. 2. Schematics of USER Friendly cloning and ATMT transformation. The USER (uracil-specific excision reagent) allows directional cloning of PCR products, by relying on vector-specific overhangs that contain a single 2-deoxyuridine nucleoside added to the primers used to amplify the sequences framing the target gene of interest. The replacement vector is constructed from the annealing of the vector, PCR products and selection marker in a one step process. *Agrobacterium tumefaciens*-mediated transformation (ATMT) can be utilized in fungal transformations where the target gene of interest is replaced by a selection marker using homologous recombination. Illustration inspired from Frandsen et al., 2008.


Target gene
hph
USER Friendly cloning
hph
ATMT
hph
Homologous recombination
hph

2.5.1 Assessment of *F.graminearum* mutant fitness

The fitness of *fg02296*Δ transgenic strain, including 3 independent single spore colonies, was compared to colonies that were not successfully transformed (negative transformants), also including 3 single spore colonies, as well as WT *F. graminearum*. Plugs were taken from the periphery of PDA plates covered in mycelia from each transgenic strain, negative transformant and WT. Each plug was placed in the center of a new PDA plate and mycelial growth was assessed by measuring new growth surrounding the plug. Two diameter measurements were taken in order to account for the non-perfect circular growth of the mycelia, the largest and smallest diameters amd an average was taken from these 2 diameters. Mycelia growth was also measured in liquid media, $1 \cdot 10^6$ spores were added to 1st stage liquid media and grown in the same conditions as in the feeding experiments. After 72 hrs, the mycelia was transferred to 2nd stage media and it was collected, filtered and weigh at each point of a time course of 40 min, 80 min, 120 min (2 hrs), 360 min (6 hrs), 720 (12 hrs), 1440 (24 hrs). Spore production was examined for the *fg02296*Δ transgenic strains, negative transformants, and WT *F. graminearum*. A plug, taken from the periphery of PDA plates with mycelia growth of each culture, was added to 50 mL of CMC medium in 250 mL flasks, shaken at 170 rpm and kept at 28ºC in the dark for 48 hrs. The number of spores was counted using a hemocytometer.

The production of IAA and TOL by the *fg02296*Δ transgenic strains and negative transformants were assessed with the same protocol as in section 2.3.2 – Mass spectrometer analysis**.** The protocol of section 2.2 – Feeding experiments, was applied here to the

*fg02296*Δ transgenic strain, negative transformant and WT with added intermediates TAM and IAAld.

Chapter 3 – Results

3.1 Identification of candidate genes in IAA biosynthesis

3.1.1 Screening of *F. graminearum* genome using keyword search

The Munich Information Center for Protein Sequences (MIPS) *Fusarium graminearum* Genome Database (FGDB) was used to screen for putative candidate genes involved in each enzymatic reaction within the proposed pathways for IAA biosynthesis (refer back to Fig. 1). The names of the enzymes in the reactions were used in keyword searches against the *F. graminearum* genome (refer back to section 1.4.1). There were a total of 14 enzymes that served as keywords and these generated an extensive putative list of 135 candidate genes (Appendix, Table A3). The total number of genes per enzyme was as low as 2, both in the case of amino acid decarboxylase and aminotransferase, and reached as high as 72, in the case of alcohol dehydrogenase. This genome-wide screening showed that putative genes were present for every enzymatic reaction, as well, based on the presence of these genes every proposed pathways remained potential pathways utilized by *F. graminearum* for the biosynthesis of IAA.

3.1.2 Predicted genes involved in IAA biosynthesis from selected microbial species

The list of putative *F. graminearum* candidate genes generated in the previous section was refined by using the protein sequence of genes involved in IAA biosynthesis of other relevant microorganisms to identify homolog genes in the *F. graminearum* genome. The identification of the auxin related genes from these microbial species relied on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and genes database. The KEGG Pathway database is generated manually from published materials, and maps for metabolism

and other cellular processes. In this case the Tryptophan metabolism was examined under Amino Acid Metabolism. KEGG genetic information pertaining to genomes and genes are generated from NCBI Reference Sequences (RefSeq) and other public resources.

A total of 10 microbial species were chosen, all of which were briefly described in section 1.5. Each species has been studied with respect to auxin and its effect on pathogenesis or plant-microbe interaction. Since fewer studies have focused on IAA biosynthesis and its genetics in fungi, bacterial species were also used in the search. These included, 5 bacterial species; *Pseudomonas syringae pv. tomato DC3000, Agrobacterium tumefaciens str. C58, Dickeya dadantii 3937(Erwinia chrysanthemi 3937), Bradyrhizobium sp. BTAi1, Azospirillum brasilense,* and 5 fungal species; *Saccharomycete cerevisiae, Magnaporthe oryzae, Ustilago maydis, Aspergillus niger* and *Neurospora crassa.* These phytopathogens represent a wide range of lifestyles and hosts and notably generate various symptoms. All three pathways are represented in both fungal and bacterial species, which is indicative of the role of IAA within these species.

Table 1 lists the genes predicted by KEGG pathways to be involved in IAA biosynthesis for the 10 species. This list contains a total of 37 enzymes that are relevant to the three proposed pathways, IAM, IPA and TAM. In the case of the IAM pathway, 3 bacterial genes were found for Trp monooxygenase and 8 bacterial and fungal genes were found for IAM hydrolase. Specific to the TAM pathway were 2 fungal genes found for Trp decarboxylase, 3 bacterial and fungal genes for amine oxidase and 5 bacterial and fungal genes for nitrilase.

Table 1. Predicted genes involved in the biosynthesis of IAA for various bacterial and fungal species. The genes were obtained using the KEGG tryptophan pathway. The reference column provides a citation about the use of auxin by the corresponding specie.

Species	IAA pathway	Gene	Accession (NCBI)	Primary host	Infection	Reference
Pseudomonas syringae						
pv. tomato DC3000	IAM	Trp monooxygenase	NP_790366.1	Tomato plant	Leaf spotting	Chen et al. 2007
	IAM	IAM hydrolase	NP_792235.2			
	TAM	Monoamine oxidase	NP_794806.1			
	-	IAAld dehydrogenase	NP_790575.1			
Agrobacterium tumefaciens str. C58	IAM	Trp monooxygenase	NP_396528.1	Dicotyledons	Gall formation	Veselov et al. 2003
	IAM	IAM hydrolase	NP_396527.1			
	-	IAAld dehydrogenase	NP_357204.2			
Dickeya dadantii 3937	IAM	Trp monooxygenase	YP_003881156.1	Broad	Rot/Wilt/Blight	Yang 2007
		IAM hydrolase	YP_003885004.1			
Bradyrhizobium sp. BTAi1	TAM	Nitrile hydratase	YP_001240056.1	Leguminous plants	Plant growth	Skorupska et al. 2010
	IPA	IPA decarboxylase	NC_009485.1			
	-	IAAld dehydrogenase	YP_001236508.1			
Azospirillum brasilense	IPA	IPA decarboxylase	ABF58692.1	Broad	Plant growth	Malhotra and Srivastava 2008
Saccharomycete cerevisiae	IAM/TAM	IAM hydrolase	NP_010528.1	Broad	Broad	Prusty et al. 2004
	IPA	Trp aminotransferase	NP_011313.1			
	-	IAAld dehydrogenase	NP_010996.1			
	IPA	IPA decarboxylase	NP_013235.1			
Magnaporthe oryzae	IPA	Trp aminotransferase	XP_001523024.1	Rice plant	Rice blast	Tanaka et al. 2011
	TAM	Amine oxidase	XP_364839.2			
	-	IAAld dehydrogenase	XP_368525.1			
	IAM/TAM	Nitrilase	XP_360737.2			
	IAM/TAM	IAM hydrolase	XP_365216.1			
Ustilago maydis	IPA	Trp aminotransferase	XP_757951.1	Maize plant	Gall formation	Reineke 2008
	-	IAAld dehydrogenase	XP_758655.1			
	TAM	Trp decarboxylase	XP_762230.1			
	IAM/TAM	Nitrilase	XP_759600.1			
	IAM/TAM	IAM hydrolase	XP_756158.1			
Aspergillus niger	IPA	Trp aminotransferase	XP_001399690.1	Broad	Plant growth	Yadav et al. 2011
	TAM	Amine oxidase	XP_001388623.1			
	-	IAAld dehydrogenase	XP_001392844.1			
	IAM/TAM	Nitrilase	XP_001391017.1			
	IAM/TAM	IAM hydrolase	XP_001391478.1			
Neurospora crassa	IAM/TAM	IAM hydrolase	XP_961990.1			

	IAM/TAM	Nitrilase	XP_963200.1	Broad	Red bread mold	Nakamura et al. 1978
	TAM	Trp decarboxylase	XP_961903.1			
	IPA	Trp aminotransferase	XP_958031.1			
	-	IAAld dehydrogenase	XP_956862.1			

In the case of the IPA pathway, 5 fungal genes were found for Trp aminotransferase and 3 bacterial and fungal genes were found for IPA decarboxylase. Two out of the 10 species did not have an IAAld dehydrogenase annotated, A. brasilense, as well as D. dadantii that use the IAM pathway.

3.1.3 Identification of *F. graminearum* candidate genes based on sequence similarity

The protein sequences of the 37 enzymes mentioned above were used in a BLASTp search provided by the FGDB (Table 2). The *F. graminearum* genes obtained from the BLASTp search in Table 2 were presented in a descending order based on their e-value. The highest bit-score or range of bit-score is also provided for the first 5 genes of the BLASTp results from each species. The score is a numerical value that represents the quality of the alignment between the query sequence and the database sequence segment. It is calculated based on a scoring system that involves a substitution matrix (Henikoff and Henikoff, 1992). The bit-score is a rescaled version of the score that is independent of the size of the search space and is useful to compare alignment scores from different searches (reviewed by Altschul et al., 1997). The overall range of the bit-scores was from 44 to 752.

The search for Trp monooxygenase identified 4 *F. graminearum* genes. Among these, fg17417 is the only gene that is annotated as being related to L-amino-acid oxidase. The genes fg11192 and fg16405 are annotated as hypothetical proteins. Lastly, fg04621 is related to monoamine oxidase N, which is found using an amine oxidase enzyme sequence from *P. syringae*, *Bradyrhizobium sp*. and *A. niger*. Thus in this BLASTp search no annotated gene was found for Trp monooxygenase in the *F. graminearum* genome.

Table 2. *F. graminearum* genes homologous to predicted genes involved in the biosynthesis of IAA in other microbial species. BLASTp searches were performed using the MIPS database. In the case where the BLASTp resulted in a list of more than ten putative candidate *F. graminearum* genes, only the top ten genes were kept based on the highest e-value. All genes are presented in a descending order and had BLASTp E-values smaller or equal to $10e^{-10}$.

Enzymes	*P. syringae*	*A. tumefaciens*	*D. dadantii*	*Brad. sp.*	*A. brasilense*	*S. cerevisiae*	*M. oryzea*	*U. maydis*	*A. niger*	*N.crassa*
Trp monooxygenase	fg11192*	fg11192*	fg11192*	-	-	-	-	-	-	-
	-	-	fg17417*	-	-	-	-	-	-	-
	-	-	fg16405*	-	-	-	-	-	-	-
			fg04621*							
Trp aminotransferase	-	-	-	-	-	fg01285[3]	fg01285[1]	fg01285[1]	fg01285[5]	fg01285[6]
	-	-	-	-	-	fg07188[1]	fg07188*	fg07188*	fg07188[1]	fg07188[1]
	-	-	-	-	-	fg04256*	fg04256*	fg04256*	fg04256*	fg04256*
Trp decarboxylase	-	-	-	-	-	-	-	fg05295[1]	-	fg05295[5]
	-	-	-	-	-	-	-	fg03391[1]	-	fg03391[1]
	-	-	-	-	-	-	-	fg03181[1]	-	fg03181[1]
	-	-	-	-	-	-	-	fg07023*	-	fg13141*
	-	-	-	-	-	-	-	fg08083*	-	fg07023*
	-	-	-	-	-	-	-	fg13141	-	-
IPA decarboxylase	-	-	-	fg09834[1]	fg10446[1]	fg09834[4]	-	-	-	-
	-	-	-	fg10446[1]	fg09834[1]	fg13946[3]	-	-	-	-
	-	-	-	fg13946[1]	fg13946[1]	fg10446[2]	-	-	-	-
	-	-	-	fg01086*	fg16351*	fg16351[2]	-	-	-	-
	-	-	-	fg16351*	fg01086*	fg01086*	-	-	-	-
Amine oxidase	fg16690[1]	-	-	fg16732[2]	-	-	fg05272[2]	-	fg05272[2]	-
	fg04621[1]	-	-	fg05272[1]	-	-	-	-	fg04621*	-
	fg09230[1]	-	-	fg01758[1]	-	-	-	-	fg01758*	-
	fg05272[1]	-	-	fg04621[1]	-	-	-	-	fg12647*	-
	-	-	-	fg10284[1]	-	-	-	-	fg16732*	-
IAAld dehydrogenase	fg02273[3]	fg02160[3]	-	fg00139[1]	-	fg00979[5]	fg09960[2]	fg00979[5]	fg00979[6]	fg00979[7]
	fg00979[3]	fg02273[3]	-	fg02160[1]	-	fg02160[4]	fg11542[1]	fg02273[4]	fg02160[5]	fg02160[5]
	fg04194[3]	fg00139[3]	-	fg11843[1]	-	fg02273[4]	fg04196[1]	fg02160[4]	fg02273[4]	fg02273[4]
	fg00139[3]	fg00979[3]	-	fg06752[1]	-	fg00139[4]	fg08596[1]	fg00139[4]	fg04194[4]	fg04194[4]
	fg02160[3]	fg04194[3]	-	fg02273[1]	-	fg04194[4]	fg03936[1]	fg04194[4]	fg00139[4]	fg00139[4]
	fg01759	fg05831	-	fg04194	-	fg01759	fg11843	fg05831	fg05831	fg05831
	fg05831	fg01759	-	fg11542	-	fg05831	fg10673	fg01759	fg02296	fg02296
	fg02392	fg02296	-	fg00979	-	fg02296	fg04670	fg02296	fg01759	fg01759
	fg02296	fg04196	-	fg03936	-	fg02392	fg02220	fg02392	fg02392	fg13865
	fg05375	fg11843	-	fg04196	-	fg06752	fg00718	fg13865	fg13865	fg02392
	fg02220	fg13865	-	fg05375	-	fg13865	fg02160	fg04196	fg04196	fg17538

Nitrilase	-	-	-	-	-	-	fg01698[1]	fg01698[1]	fg01698[3]	fg01698[3]
	-	-	-	-	-	-	fg06480[*]	fg06480[*]	fg06480[*]	fg06480[*]
	-	-	-	-	-	-	fg00051[*]	fg00051[*]	fg00051[*]	fg00051[*]
	-	-	-	-	-	-	-	fg11357[*]	fg05805[*]	fg05805[*]
IAM hydrolase	fg07505[2]	fg16646[*]	fg03177[1]	fg16271[2]	-	fg10318[2]	fg07505[6]	fg10318[3]	fg07505[6]	fg03963[5]
	fg16646[1]	fg03938[*]	fg09140[1]	fg03938[1]	-	fg09337[2]	fg16646[*]	fg08078[3]	fg16646[1]	fg09337[2]
	fg10913[*]	fg07505[*]	fg08078[*]	fg04236[1]	-	fg03963[2]	fg10913[*]	fg07612[2]	fg09140[*]	fg12442[2]
	fg03938[*]	fg10913[*]	fg07505[*]	fg10913[1]	-	fg12442[2]	fg03938[*]	fg12442[2]	fg03177[*]	fg08078[2]
	fg04236[*]	fg04236[*]	fg13871[*]	fg07505[1]	-	fg08078[2]	fg03177[*]	fg03963[2]	fg03938[*]	fg07612[2]
	fg04022	fg04022	fg16271	fg16646	-	fg15685	fg04236	fg09337	fg10913	fg10318
	fg07612	fg09140	fg03938	fg04022	-	fg07612	fg09140	fg03346	fg13871	fg15685
	fg08078	fg07612	fg07612	fg13871	-	fg03346	fg08078	fg15685	fg04236	fg03346
	fg13871	fg16271	fg10318	fg15685	-	fg03177	fg07612	fg16271	fg08078	fg09140
	fg03346	fg10318	fg15685	fg03177	-	fg07505	fg15685	fg09140	fg16271	fg03177

Bit-score
[*] 1-100
[1] 101-200
[2] 201-300
[3] 301-400
[4] 401-500
[5] 501-600
[6] 601-700
[7] 701-800

Accordingly the bit-scores of the 4 genes that were identified were very low ranging from 44 to 53 bit. The results for Trp aminotransferase were unanimous across the five fungal species. Both fg01285 and fg07188 are related to aromatic amino acid aminotransferase and fg04256 is a hypothetical protein. These genes scored highest from the *N. crassa* results, fg01285 scored 619 bits, fg07188 155 bits and fg04256 72 bits.

In the case of Trp decarboxylase, the same genes were obtained from the *U. maydis* and *N. crassa* results, except for one gene identified with the *U. maydis* BLASTp search, fg08083. Only two of these genes, fg05295 and fg03391, were related to aromatic-L-amino-acid decarboxylase, the other four genes were annotated as glutamic-acid decarboxylase. The highest score for fg05295 was 513 bits from *N. crassa* and the same score of 114 bits was found for fg03391 and fg03181 from *U. maydis* and *N. crassa*, respectively. Lower scores below 85 bits were found for the three other genes.

The search from IPA decarboxylase, interestingly for both bacterial and fungal species, presented the same homologous genes, all of which were annotated as pyruvate decarboxylase, except for fg01086, a probable acetolactate synthase. The highest scores for these genes came from *S.cerevisiae* and ranged from 208 to 475 bits excluding fg01086 that scored 68 bits, with fg09834 having the highest score. The gene that scored the highest from *Brad. sp* was also fg09834 with 160 bits and from *A. brasilense* it was fg10446 with 141 bits.

Amine oxidase had variation in the *F. graminearum* genes identified by BLASTp search across the species. The only common gene that was present for all four species is fg05272, related to amine oxidase. fg05272 is also the only gene that was obtained from *M. oryzae*, it had the same score from *A. niger* and the highest score with 209 bits. The three species, *P. syringae, Bradyrhizobium sp*. and *A. niger* had in common, fg04621, related to monoamine oxidase, for the amine oxidase BLASTp search, the alignment for this gene had a score that was highest for *P. syringae* at 180 bits. For *P. syringae*, two other genes were identified by the BLASTp search, fg16690, predicted to be a putative cytoplasmic structural protein with 184 bits, and fg09230, a conserved hypothetical protein that scored 148 bits. *Bradyrhizobium sp*. and *A. niger* shared fg01758, related to monoamine oxidase with scores of 46 bits and 60 bits respectively, and fg16732, a hypothetical protein with scores of 70 bits and 66 bits respectively. As well these two species each had another gene in their BLASTp results, for *Bradyrhizobium sp*. it was fg10248 related to amine oxidase activity with 53 bits and for *A. niger*, fg12647, a hypothetical protein scoring 52 bits.

The BLASTp search for IAAld dehydrogenase provided many homologous genes that were found across species and all are probable or related to aldehyde dehydrogenase. In particular, fg02160 was common to all species. The highest score for this gene came from *N. crassa* with 514 bits. fg02160 also scored relatively high among the other fungal species except in the case of *M. oryzea*, with a range from 467 to 500 bits. From the bacterial species the scores for fg02160 ranged from 177 to 326 bits. Most of the genes obtained from *M. oryzea* were different then the genes obtained from the other fungal species in the BLASTp search for IAAld dehydrogenase, as well the scores of these genes were relatively

low with the highest score at 295 bits for fg09960. Many genes including, fg00979, fg02273, fg04194 and fg00139 were present in all but one species, *M. oryzae*. Among these four genes, fg00979 had the highest scores ranging from 520 to 752 bits for the fungal species. The three other genes also had high scores all within 400 to 500 bits for these species. A few genes were not found for *Bradyrhizobium sp*. and *M. oryzae* but were present in all of the other species, these include: fg01759, fg02296 and fg05831. Other genes that were repeatedly found among the species were fg2392, fg13865, fg04196. The genes that were common in the bacterial species-only were fg11843 and fg05375. Interestingly the two very different bacterial and fungal species *Bradyrhizobium sp*. and *M. oryzae* have genes in their BLASTp results that are only shared between these two species, including, fg11542 and fg03936. Overall, *M. oryzae* had the least amount of genes that were found in common with the results from all species.

The nitrilase enzyme search generated a total of 5 annotated genes, related to nitrilase. The genes that were shared among the 4 fungal species are fg01698, fg06480, fg00051 with highest scores of 370 bits, 90 bits, 81 bits, respectively. The gene fg05805 was common to both *A. niger* and *N. crassa* and scored 54 bits from both species, while fg11357 a gene part of the *U. maydis* BLASTp results-only scored 57 bits.

The last enzyme that was part of the BLASTp search was IAM hydrolase (amidase). This search resulted in a total of 18 genes that were annotated as probable amidases. Unlike the IAAld dehydrogenase results, the order in the score for these genes was more scattered but some of the same genes did reappear across species. The highest scores from the fungal

species ranged from 238 to 657 bits and those from the bacterial species ranged from 89 to 236 bits. There was no gene that was present in all 9 species. There were 3 genes that reoccurred across 7 species, these included: fg07505, fg07612, fg08078. Four genes were present across 6 species; fg09140, fg15685, fg03177 appeared in 4 fungal species and 2 bacterial species, while fg03938 appeared in 2 fungal species and 4 bacterial species. There were 5 genes that were found across 5 species, these include fg06646, fg10913, fg04236 and fg16271, found in 3 bacterial and 2 fungal species, while fg10318 was found in 3 fungal and 2 bacterial species. Only 1 gene was found in the results across 4 species, but it was found in the majority of bacterial species, this was fg13871. Another 3 genes were found across 3 fungal species, these included: fg09337, fg03963, fg12442. Overall some variations in the gene results were found between the bacterial and fungal species, as well as in between the species within these two categories. This suggests that this enzyme may not be highly conserved or utilized for the biosynthesis of IAA across most of these species.

Overall the results of this search provided a number of homologous genes that were found across several species for all 8 enzymes. As well, many of the same genes were found across both bacterial and fungal species. These results suggest that some degree of conservation is present among the genes for the enzymes involved in the IAA biosynthesis.

3.2 Biosynthesis of IAA by *F. graminearum* in culture

The objective for this section was to identify the enzymatic pathway(s) used by *F. graminearum* for the biosynthesis of IAA. This was accomplished by treating *F. graminearum* cultures with distinct intermediates that are found within the different

pathways to then analyze the filtrates of these cultures for the presence of IAA (refer back to Fig. 1). *F. graminearum* cultures were treated with 0.2 mM of IAM, IPA, TAM, IAAld and 2 mM of TRP. The filtrates were analyzed by high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrophotometry in tandem (LC-MS/MS) to identify IAA along with other identifiable byproducts.

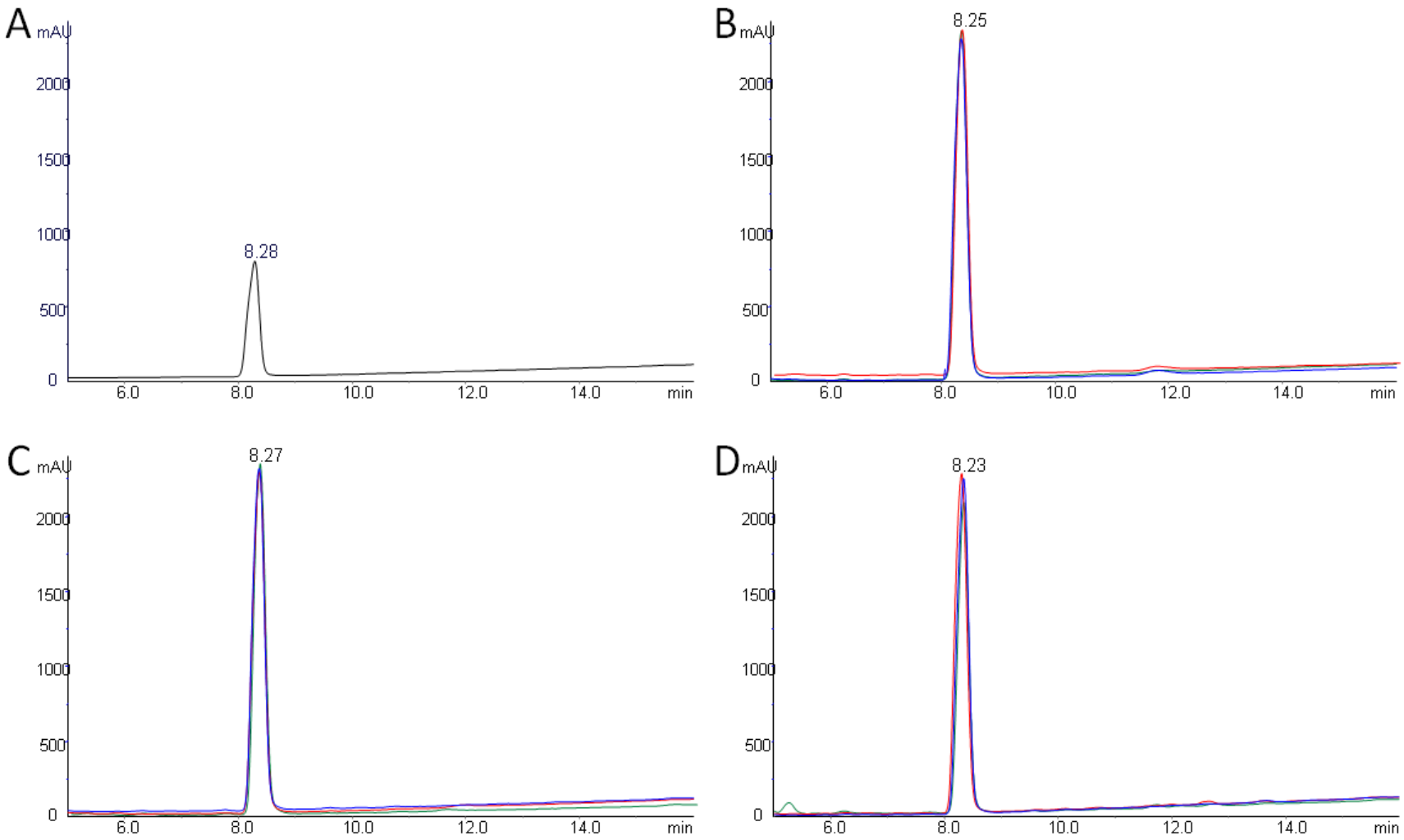
3.2.1 HPLC analysis of filtrates from treated *F. graminearum* cultures.

The filtrates from all of the treated cultures were first analyzed with HPLC. This technique was used as a scanning tool primarily to identify the presence of IAA or of other compounds based on HPLC retention time, by comparison with commercial standards of IAA and the intermediates in known IAA pathways. The retention time of IAA was around 10.7 minutes under our separation conditions; however it was later found that IAAld and the byproduct TOL co-eluted with IAA. Although this technique did not allow for the definitive identification of IAA, it provided preliminary results as to whether one or more of these three compounds were present in the filtrates. The HPLC results are represented by overlaid chromatograms of biological replicates for each treatment during a time course of 24 hrs (Fig. 3-7).

In filtrates from cultures treated with IAM (Fig.3), no enzymatic activity was observed. The chromatograms for the filtrates of the IAM-treated cultures at the 6hr, 12hr and 24hr time points showed the presence of only one peak, at the same retention time as the IAM standard and at an estimated concentration of 0.2mM. These results indicated that the IAM

intermediate was not metabolized by *F. graminearum* during a 24hr time course, suggesting that the IAM pathway was not used by *F. graminearum* for biosynthesis of IAA.

Fig. 3. HPLC chromatograms of the filtrates from IAM-treated *F. graminearum* cultures during a 24hr time course. A. Standard for IAM. Filtrate collected at B. 6hrs, C. 12hrs, D. 24hrs post treatment of 0.2mM IAM. Each panel consists of chromatogram overlays of three biological replicates represented by the blue, red and green colours.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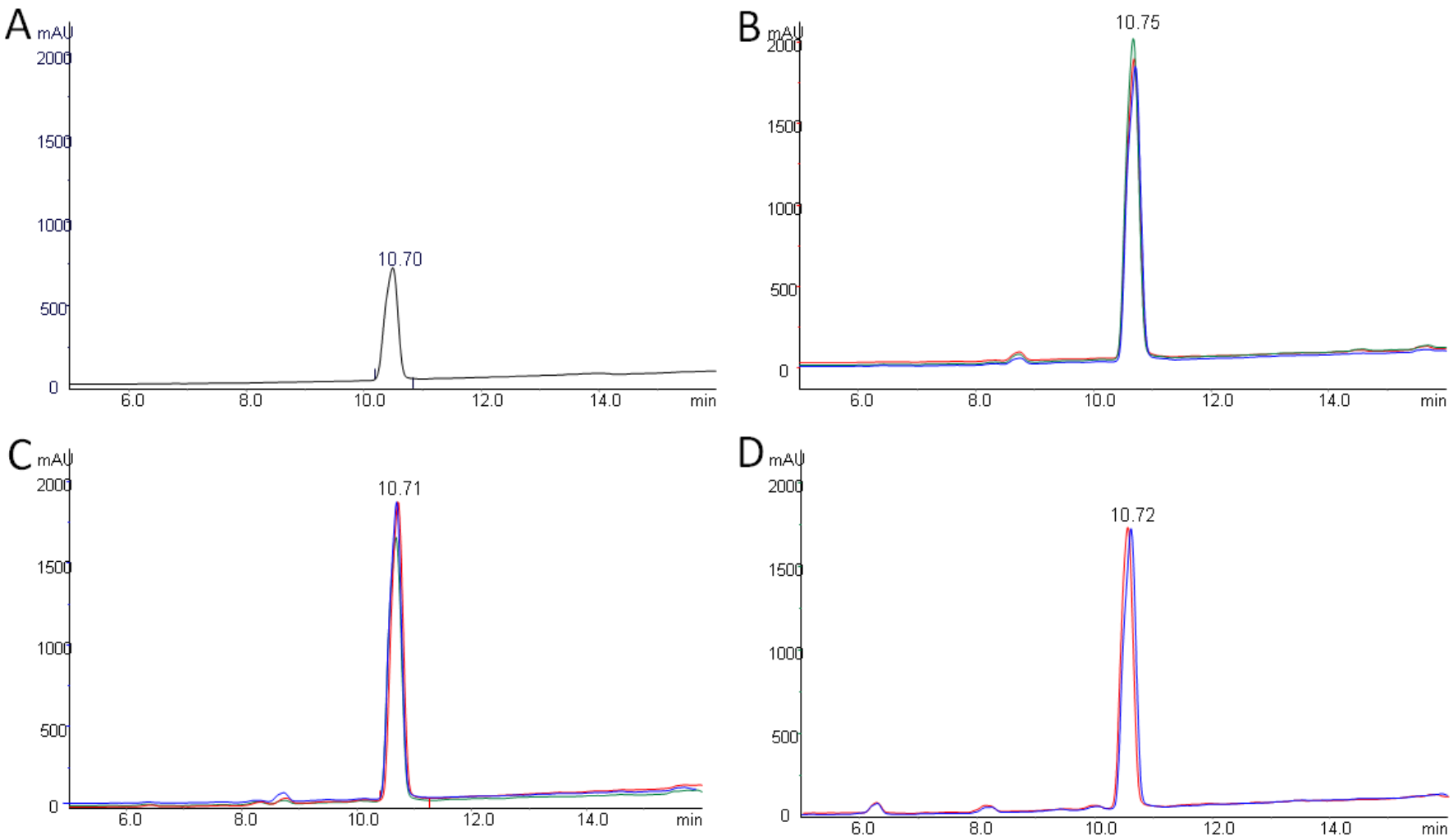
1500
1000
500
0
8.28
6.0
8.0
10.0
12.0
14.0
min
B
mAU
8.25
2000
1500
1000
500
0
6.0
8.0
10.0
12.0
14.0
min
C
mAU
8.27
2000
1500
1000
500
0
6.0
8.0
10.0
12.0
14.0
min
D
mAU
8.23
2000
1500
1000
500
0
6.0
8.0
10.0
12.0
14.0
min

Fig. 4. HPLC chromatograms of the filtrates from IAAld-treated *F.graminearum* cultures during a 24hr time course. A. Standard for IAAld. Filtrate collected at B. 6hrs, C. 12hrs, D. 24hrs post treatment of 0.2mM IAAld. Each panel consists of chromatogram overlays of three biological replicates represented by the blue, red and green colours.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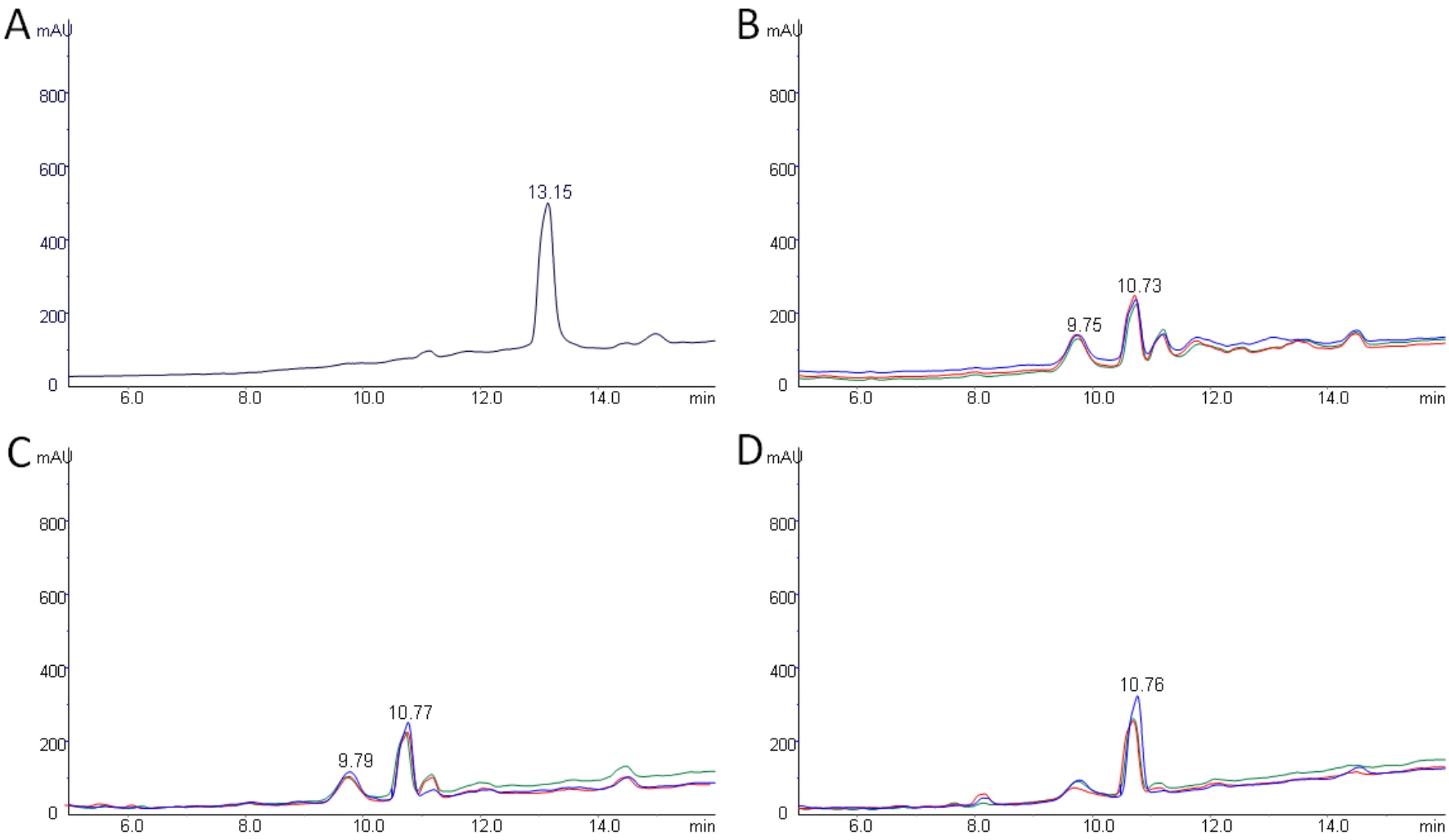
2000
1500
1000
500
0
10.70
6.0
8.0
10.0
12.0
14.0
min
B
mAU
2000
1500
1000
500
0
10.75
6.0
8.0
10.0
12.0
14.0
min
C
mAU
2000
1500
1000
500
0
10.71
6.0
8.0
10.0
12.0
14.0
min
D
mAU
2000
1500
1000
500
0
10.72
6.0
8.0
10.0
12.0
14.0
min

Fig. 5. HPLC chromatograms of the filtrates from IPA-treated *F.graminearum* cultures during a 24hr time course. A. Standard for IPA. Filtrate collected at B. 6hrs, C. 12hrs, D. 24hrs post treatment of 0.2mM IPA. Each panel consists of chromatogram overlays of three biological replicates represented by the blue, red and green colours.


A
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800
600
400
200
0
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6.0
8.0
10.0
12.0
14.0
min
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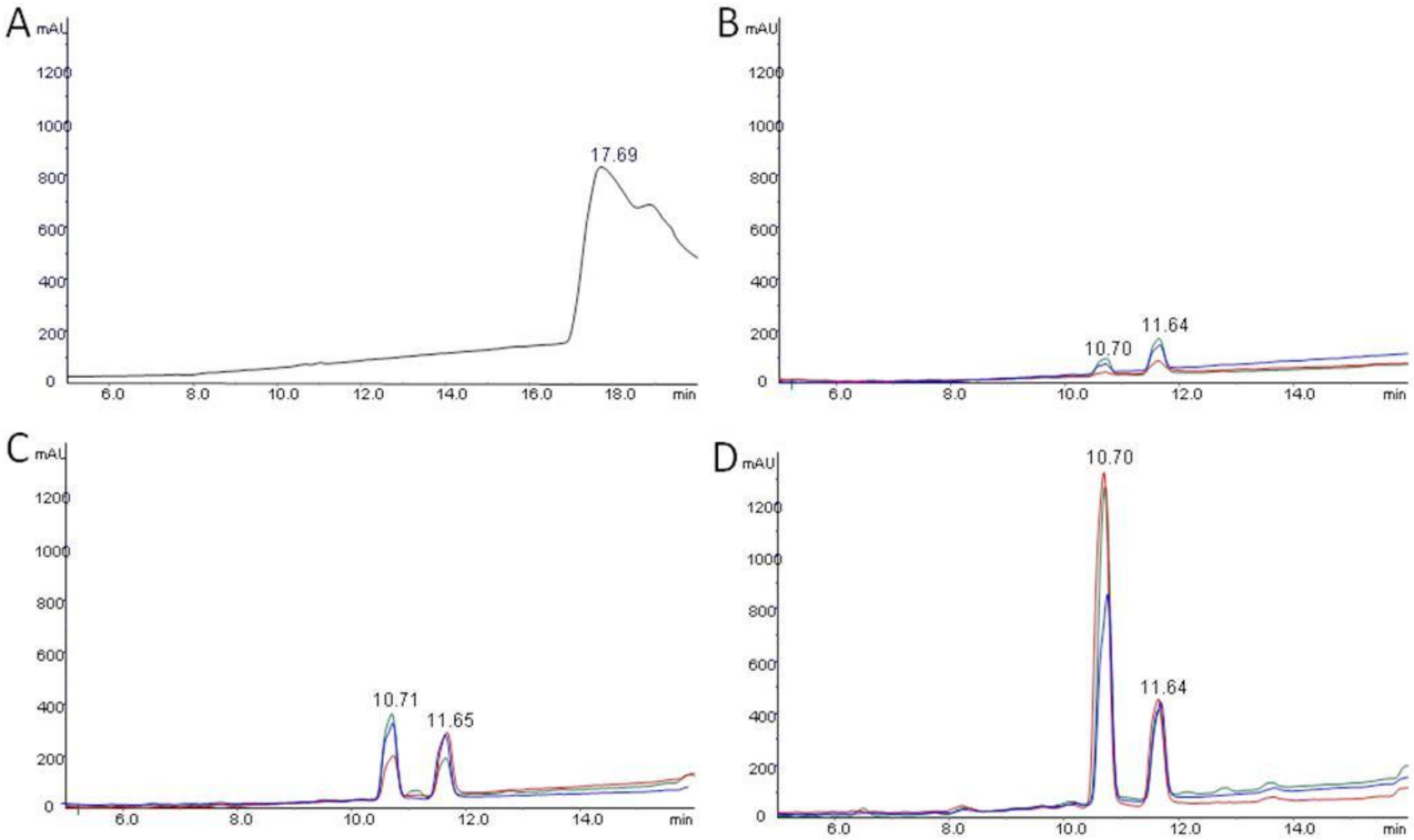
800
600
400
200
0
10.73
9.75
6.0
8.0
10.0
12.0
14.0
min
C
mAU
800
600
400
200
0
10.77
9.79
6.0
8.0
10.0
12.0
14.0
min
D
mAU
800
600
400
200
0
10.76
6.0
8.0
10.0
12.0
14.0
min

Fig. 6. HPLC chromatograms of the filtrates from TAM-treated *F.graminearum* cultures during a 24hr time course. A. Standard for TAM. Filtrate collected at B. 6hrs, C. 12hrs, D. 24hrs post treatment of 0.2mM TAM. Each panel consists of chromatogram overlays of three biological replicates represented by the blue, red and green colours.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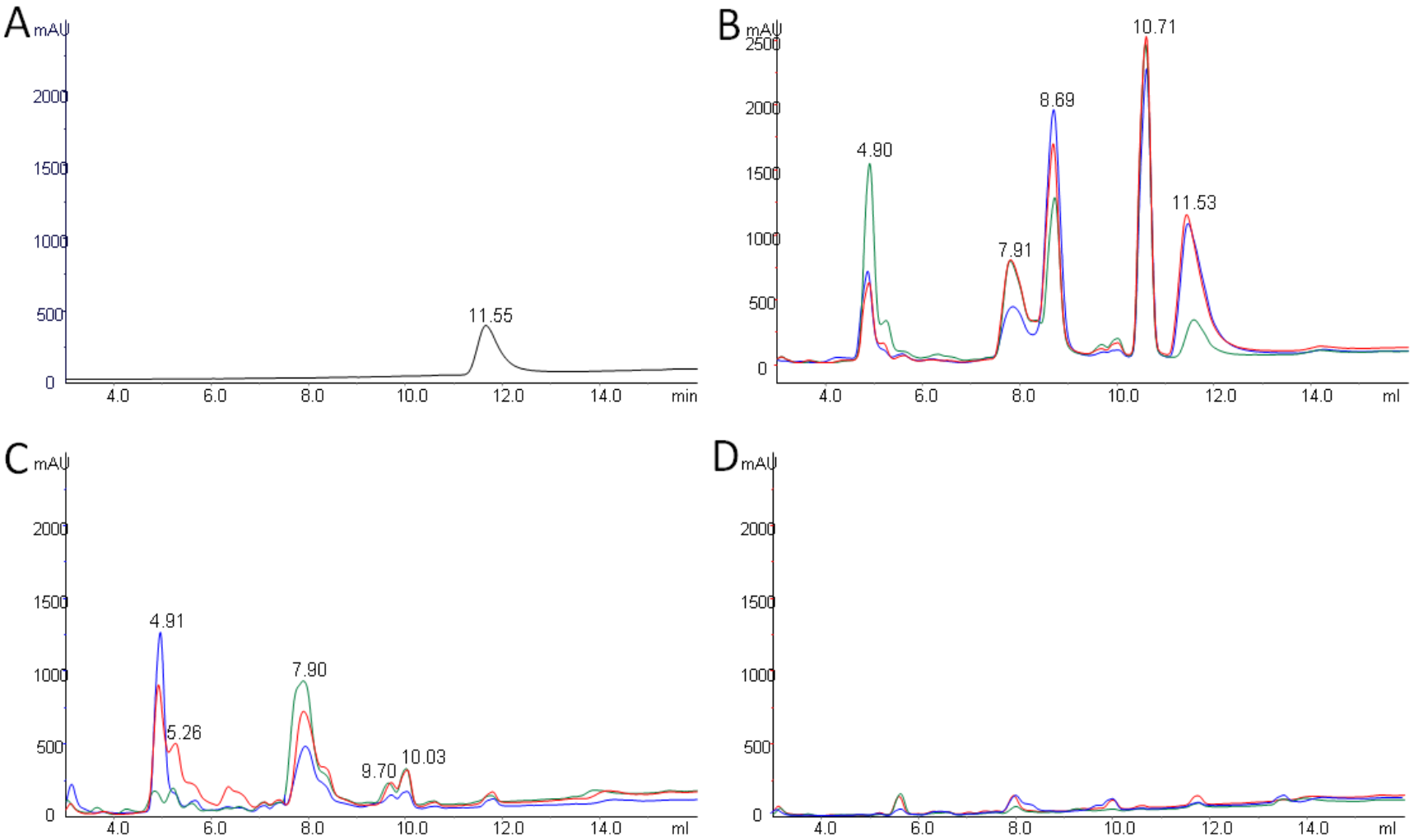
1200
1000
800
600
400
200
0
17.69
6.0
8.0
10.0
12.0
14.0
16.0
18.0
min
B
mAU
1200
1000
800
600
400
200
0
11.64
10.70
6.0
8.0
10.0
12.0
14.0
min
C
mAU
1200
1000
800
600
400
200
0
10.71
11.65
6.0
8.0
10.0
12.0
14.0
min
D
mAU
1200
1000
800
600
400
200
0
10.70
11.64
6.0
8.0
10.0
12.0
14.0
min

Fig. 7. HPLC chromatograms of the filtrates from TRP-treated *F.graminearum* cultures during a 24hr time course. A. Standard for TRP. Filtrate collected at B. 6hrs, C. 12hrs, D. 24hrs post treatment of 2mM TRP. Each panel consists of chromatogram overlays of three biological replicates represented by the blue, red and green colours.


A
mAU
2000
1500
1000
500
0
11.55
4.0
6.0
8.0
10.0
12.0
14.0
min
B
mAU
2500
2000
1500
1000
500
0
4.90
7.91
8.69
10.71
11.53
4.0
6.0
8.0
10.0
12.0
14.0
ml
C
mAU
2000
1500
1000
500
0
4.91
5.26
7.90
9.70
10.03
4.0
6.0
8.0
10.0
12.0
14.0
ml
D
mAU
2000
1500
1000
500
0
4.0
6.0
8.0
10.0
12.0
14.0
ml

The chromatograms for the filtrates of the cultures treated with IAAld (Fig.4) also showed only one peak, at 10.7min. However, as mentioned above, since the IAAld standard co-eluted with IAA as well as with TOL, the presence of IAA cannot be determined with this HPLC gradient. The filtrates from the IPA-treated cultures (Fig.5) showed two repeating peaks at retention times of 9.7 and 10.7 during the 24hr time course. Based on the retention time of the ILA standard, a byproduct, the peak at 9.7min could represent this compound and the peak at 10.7 could represent IAAld, TOL and/or IAA. This suggested that metabolic activity towards the biosynthesis of IAA had taken place and started at least within the first 6hrs after adding IPA to cultures. IPA itself was not observed in any of the filtrates at any point during the 24hr time course. The IPA standard, observed at 13.5min in our HPLC conditions, was very unstable in solution.

The chromatograms for the filtrates of TAM-treated cultures (Fig.6) also showed two peaks, with retention times of 10.7 and 11.6min. The earlier peak at 10.7min may represent IAAld, TOL and/or IAA; however the peak at 11.6min remained unknown and did not correspond to any of our standards. During the 24hr time course, the area under the curve for these two peaks increased and at 24hrs there was a large relative increase for the peak at 10.7min. The added TAM compound was also detected, although it was outside of the HPLC working gradient and seen as a broad peak at 17min. Over time, the peak representing TAM decreased in intensity (data not shown). These results suggested that metabolic activity had occurred within 6hrs after adding TAM and that the biosynthesis of IAAld, TOL and/or IAA increased considerably between 12 and 24hrs.

The filtrates of the TRP-treated cultures (Fig.7) showed the most HPLC-eluted compounds in the first 12hrs of treatment. Among the five peaks that were found at 6hrs, three were unidentified while the other two represented IAAld/TOL/IAA and TRP, at 10.7 and 11.5min, respectively. At 12hrs, the peaks had lower retention time and intensity, and the working gradient did not seem to be optimal for the compounds present. A small peak at 9.7min could represent ILA, however the peak was not clearly separated and its identity remained elusive. TRP was no longer detected in the 12 and 24hr samples. At 24hrs, no peaks were detected above a background of 100mAU. Overall this data suggested that TRP treatment promoted enzymatic activity towards the biosynthesis of IAAld/TOL/IAA within 6hrs of treatment. The disappearance at 24hrs of the compounds observed in the early time points could be due to chemical instability or to additional conversion of those compounds; however, since most of these compounds are unknown, it is difficult to hypothesize on what occurred in this environment as time progressed.

These preliminary results provided an additional observation, referring to the large variation between the biological replicates in some treatments. These differences were particularly noticeable in the filtrates from TAM- and TRP-treated cultures. These variations should be carefully taken into consideration in later experiments and analysis of results. The HPLC results were useful to determine which added intermediates promoted auxin producing conditions; however they lacked in the identification of compounds, particularly for IAA. Therefore LC-MS/MS was used next with the purpose of identifying and quantifying the amount of IAA present in the filtrates.

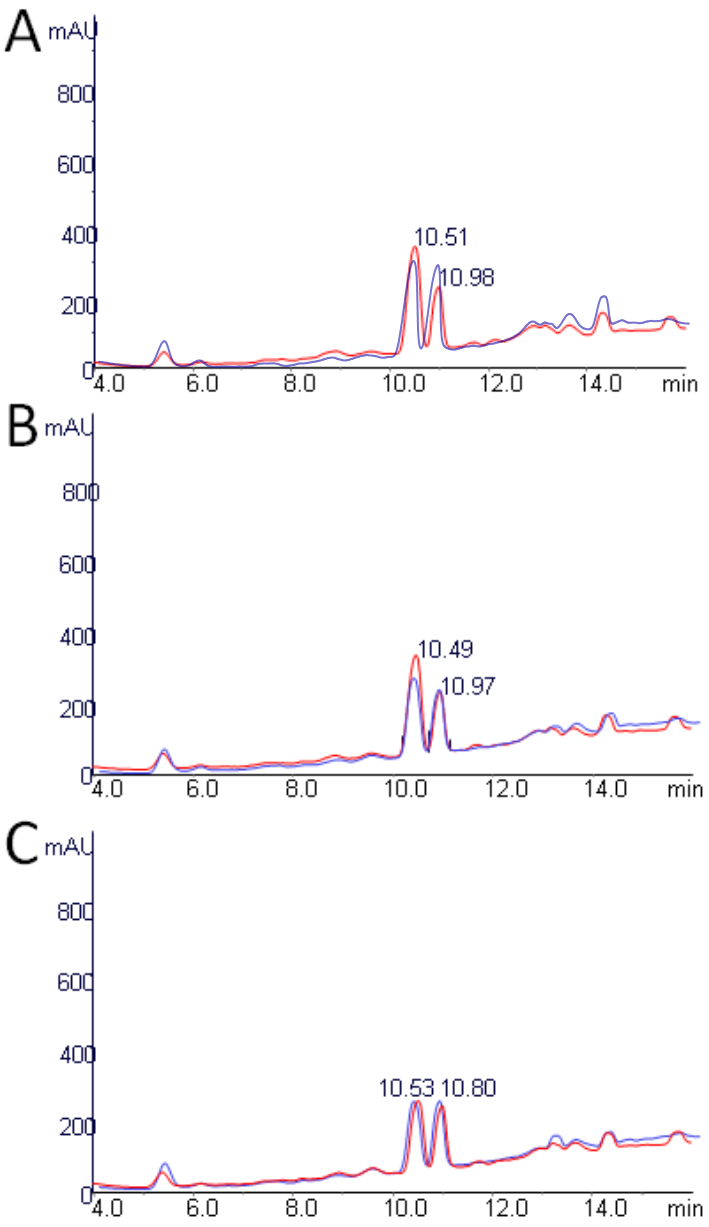
3.2.2 LC-MS/MS analysis of filtrates from treated *F. graminearum* cultures.

The standards for the intermediates that were analyzed by HPLC were also all identifiable by mass with LC-MS/MS, with the exception of IPA because of its instability (Appendix, Fig. A2). The byproducts such as ILA and TOL were also identified by mass using the same protocol (Appendix, Fig. A3).

One issue that needed to be addressed in this section was the possibility for the intermediates to convert non-enzymatically to IAA. This was reported in previous studies to occur with IPA but it was also important to test all of the intermediates to determine their stability in media and to eliminate the possibility of any spontaneous conversion to IAA (Wildman et al 1947, Kaper & Veldstra, 1958, Kawaguchi & Syōno, 1996). Therefore all of the intermediates were tested in media-only (ie without *F. graminearum*) for the presence of IAA after incubation. Preliminary analysis by HPLC (Fig.8) showed two peaks, neither one being IPA, in IPA-treated media. LC-MS/MS confirmed that IPA could spontaneously convert to IAA (Fig. 9). In media treated with the other intermediates, the added intermediates were identified after incubation and no IAA was detected.

The presence of TOL was also tested and not detected in any of the treated media. In the IPA-treated media, a global average of 17.5 µM IAA was detected, representing 8.75% of the added IPA converting non-enzymatically to IAA. The average concentration of IAA was not found to be significantly different between time points during the 24 hr time course, thus the rate of spontaneous conversion was not time dependent (Fig. 9).

Fig. 8. HPLC chromatograms of IPA-treated media-only during a 48hr time course. Second-stage media was treated with 0.2mM of IPA. The media was collected at A. 6hrs, B.24hrs, C.48hrs, post treatment. Two replicated are represented by the blue and red profiles.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400
200
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6.0
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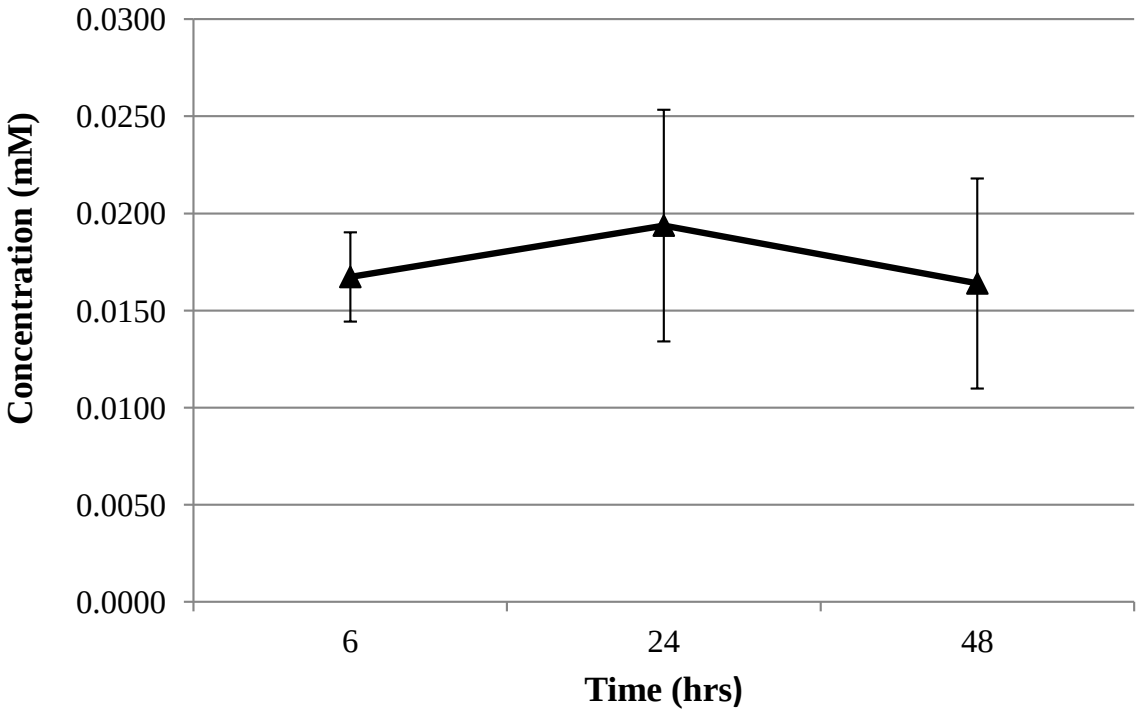
10.0
12.0
14.0
min
B
mAU
800
600
400
200
0
10.49
10.97
4.0
6.0
8.0
10.0
12.0
14.0
min
C
mAU
800
600
400
200
0
10.53 10.80
4.0
6.0
8.0
10.0
12.0
14.0
min

Fig. 9. Concentration of non-enzymatically converted IAA from added IPA to media during a 48hr time course. Second-stage media was treated with 0.2mM of IPA. The data presented is a mean ±standard deviation, n=2.


0.0300
0.0250
0.0200
0.0150
0.0100
0.0050
0.0000
Concentration (mM)
6
24
48
Time (hrs)

LC-MS/MS was performed on filtrates from each culture treated with an intermediate. The data obtained was analyzed for the presence of the intermediates: IAM, TRP, TAM, IPA and IAAld. The presence of IAA and TOL, from the LC-MS/MS data, was also identified and their concentrations were quantified based on standard curves. In the TRP-treated cultures, surprisingly no IAA was detected above the signal to noise ratio for any of the filtrates (Table 3). However some weak peaks, below the signal to noise ratio, were detected for IAA at 6 and 12hrs. The added TRP was present at 6hrs, not detected above signal to noise ratio in the filtrates collected at 12hrs and was absent at 24hrs, which is in accordance with the HPLC data (refer back to Fig. 7). Although only a very weak concentration of IAA was detected, this data shows that metabolic activity was taking place in the cultures as the concentration of TRP decreased over time. The presence of the other intermediates was also examined and none of them were detected in the filtrates of TRP-treated cultures. The lack of a larger concentration for either IAAld/TOL/IAA at 6hrs does not coincide with the presence of a noticeable peak representing IAAld/TOL/IAA, observed with HPLC (refer back to Fig. 7B).

Table 3. Concentration (µM) of IAA and TOL in treated *F. graminearum* cultures during a 24hr time course analyzed with LC-MS/MS. The data presented is a mean, n=3, <min; below minimum value for detection.

	Time (Hrs), concentration (µM)					
	6		12		24	
Treatment	IAA	TOL	IAA	TOL	IAA	TOL
IAM	-	-	-	-	-	-
TRP	-	-	-	-	-	-
IPA	9.4±1.0	<min	4.4±1.8	<min	2.9±0.5	2.5±0.8
TAM	-	-	<min*	-	18±13	-
IAAld	12±3.8	3.5±3.2	28±8.4	<min	35±6.2	<min

- not detected
* 2 biological replicates

The presence of IAA was detected in the cultures treated with IPA (Table 3). The average IAA concentration decreased over time, ranging from 9.4µM at 6hrs, to 4.4µM at 12hrs and 2.9µM at 24hrs. Although IPA can convert non-enzymatically to IAA, the *F. graminearum* culture filtrates also contained TOL which was not observed in absence of the fungus. The concentration of TOL was below the limit of quantification in the 6 and 12hr samples; however it increased to 2.5µM at 24hrs. The presence of TOL signifies that some metabolic activity did take place in these treated cultures. However, it remains elusive as to how much of the IAA measured was biosynthesized by the fungus versus simply converted non-enzymatically since the average concentration of IAA was lower in these filtrates than when the intermediate was added to media-only. No other intermediates were found in the filtrates of the IPA-treated cultures.

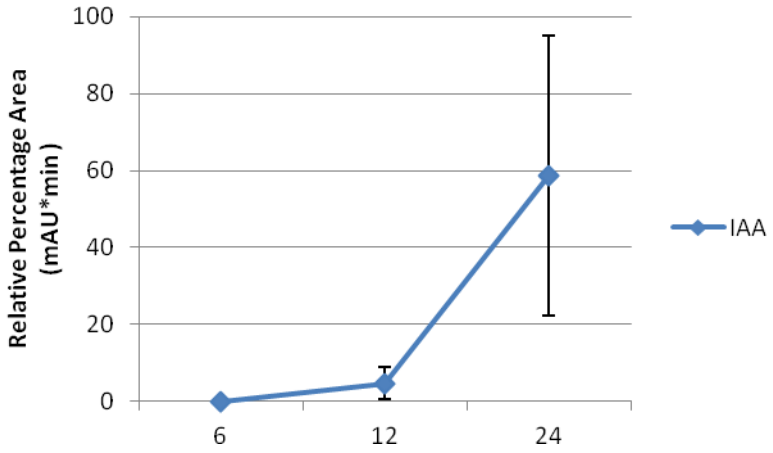
In the TAM treated cultures, IAA was detected at 12hrs and 24hrs (Table 3). At the 12hr time point, the concentration of IAA was below the limit for quantification and IAA was only detected in two biological replicates. At 24hrs, an increase to 18µM IAA was detected. The added TAM was also detected at both 6 and 12hrs; however the detection of TAM in the 24hr time point was variable: in one replicate TAM was detected, in the second the peak for TAM was below the signal to noise ratio and TAM was not detected in the third replicate. This variability might be reflected in the large standard deviation that was found in the concentration of IAA at 24hrs. No other IAA intermediates were found in these filtrates. The absence of TOL suggest that by adding TAM to cultures, IAA is metabolized differently than by adding IPA or IAAld to promote auxin biosynthesis.

IAA was detected in all of the time points in cultures treated with IAAld. As expected, these cultures generated the highest concentrations of IAA within 24hrs. The added IAAld was not detected in any of the filtrates. The concentration of IAA increased over time from 12µM at 6hrs, 28µM at 12hrs and 35µM at 24hrs which represents a conversion of 17.5% of the added 0.2mM of IAAld to IAA. TOL was also detected in these filtrates and decreased over time but it was only quantifiable for 6hrs at 3.5µM. No other intermediates were found in these filtrates.

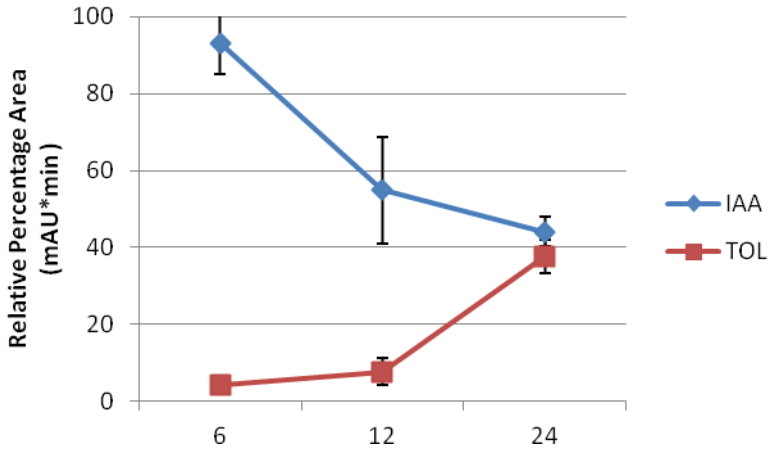
To compare the values and trends for IAA and TOL in the IPA-, TAM-, and IAAld-treated cultures, the original values from the UV detector (Area*min) were converted into a relative percentage for each intermediate (Fig.10). This allowed for detected values that were lower than the limit of the standard curve which was 0.003µM for IAA and 0.005µM for TOL to be represented graphically, which was important particularly for TOL. In TAM-treated cultures (Fig. 10A) the average value for IAA between 12-24hrs increased by approximately 60%. In the IPA-treated cultures (Fig. 10B), the amount of IAA went down while TOL values in these filtrates increased slightly between 6 and 12hrs and by approximately 30% from 12 to 24hrs. The amount of IAA that may have accumulated from IPA via a spontaneous conversion versus an enzymatic one could not be defined. However it was shown that when IPA was added to media-only the conversion of IPA to IAA was relatively equal at the different time points (refer back to Fig. 8-9). In IAAld-treated cultures (Fig.10 C), IAA values increased as TOL values decreased overtime. At 6hrs, the average value for IAA was approximately 20% larger than the value for TOL. This average value increases to 60% at 12hrs and 80% at 24hrs.

Fig. 10. Relative percentage areas (mAU*min) of IAA and TOL in treated cultures during a 24hr time course. Cultures were treated with **A**. TAM, **B**. IPA and **C**. IAAld. Values from LC-MS/MS data for areas (mAU*min) of both IAA and TOL were converted into a percentage relative to the highest value between these 2 compounds for each treatment. The data presented is a mean, n=3, except for the TAM 12 hr sample where n=2.

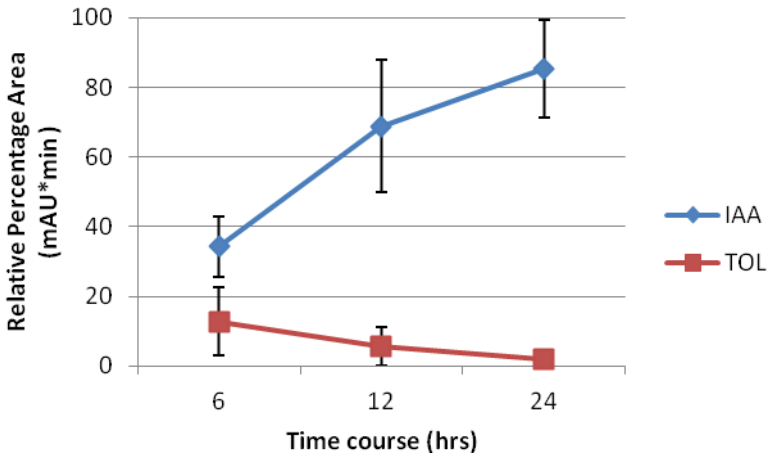
A.TAM



B.IPA



C.IAAld



Overall, the results in this section show that TRP, IPA, TAM and IAAld promoted metabolic activity in *F. graminearum* leading to the biosynthesis of IAA or indolic compounds such as TOL.

3.3 Gene expression in IAA producing conditions

Candidate genes with enzymatic activity in IAA biosynthesis were identified using microarrays, by comparing global expression profiling between untreated cultures and cultures treated with auxin precursor compounds and by searching for genes with differential expression. In these conditions up-regulated genes represented genes that may have a role in IAA biosynthesis. RNA was isolated from *F. graminearum* cultures that were grown in media treated with IAAld (0.2mM), TAM (0.2mM) or TRP (2mM). Three biological replicates for each treatment were collected at a single time point of 6hrs; the time point was selected based on the HPLC profiles of the supernatant of IAAld-, TAM- and TRP-treated cultures. Hybridizations in the microarrays were performed on treated vs untreated (control) cultures. The data obtained from the hybridizations was exported to the Acuity software, which was used to normalize it and convert it into a log2 function. This data was then converted into mean values from the three biological replicates of *F. graminearum* cultures grown for each of the three treatments: IAAld, TAM and TRP and was screened for genes with an expression ratio of at least 2 between the treated and untreated samples, representing 4 fold in differential expression.

Analysis of the microarray expression data generated a list of 222 up-regulated genes and 55 down-regulated genes from the three treatments (Fig. 11). In the *F. graminearum* cultures

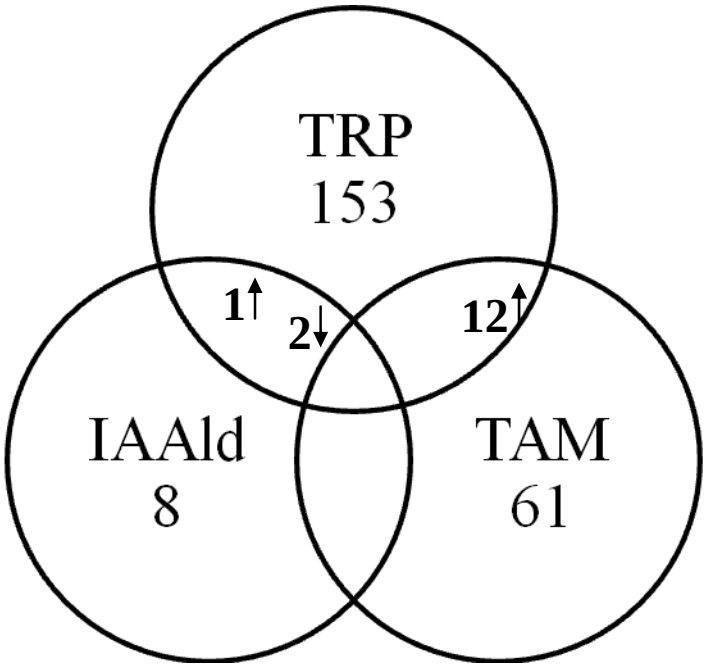
treated with IAAld, 8 genes were up-regulated and 2 were down-regulated when compared to untreated (control) cultures. With the TAM treatment, 61 genes were up-regulated when compared to the control. In the case of the TRP treated cultures, 153 genes were up-regulated and 54 were down-regulated. A total of 104 of the 222 up-regulated genes were annotated as conserved hypothetical or hypothetical proteins.

Similarly, 23 of the 54 down-regulated genes were annotated as conserved hypothetical or hypothetical proteins. None of the genes, annotated as conserved hypothetical or hypothetical proteins, that were found in either the up-regulated or down-regulated genes from the microarray data were found among the BLASTp results in section 3.1.

Of the 8 genes that were found up-regulated in the IAAld-treated cultures, five were annotated as conserved hypothetical or hypothetical proteins (Table 4). The most up-regulated gene in this group was fg02953, a conserved hypothetical protein, with a change of 152 folds. There were 3 genes whose function was annotated, including: fg10506 related to monocarboxylate transporter 2, fg04317 related to multidrug resistant protein and fg02417 related to integral membrane protein. All 3 of these genes are membrane proteins that are involved in transport of various molecules and compounds across the plasma membrane. In particular, monocarboxylate transporter 2 is involved in the catalysis of the transport of monocarboxylates, including acetate. Of the two genes down-regulated in the IAAld treatment, fg12103 was related to epoxide hydrolase and fg08157 to a conserved hypothetical protein (Table 4). None of the up-regulated and down-regulated genes in the IAAld treatment had a predicted function that could be associated with IAA biosynthesis.

Fig. 11. Venn diagrams of up- and down-regulated genes from *F. graminearum* cultures grown in TRP-, TAM- and IAAld-treated media. **A**. Up-regulated genes from the three treatments, and common genes between treatments (relative to up-regulated genes of cultures grown in TRP). **B**. Down-regulated genes from the three treatments, and common genes between treatments (relative to down-regulated genes of cultures grown in TRP). Arrows, up: up-regulated, down: down-regulated.

A



B

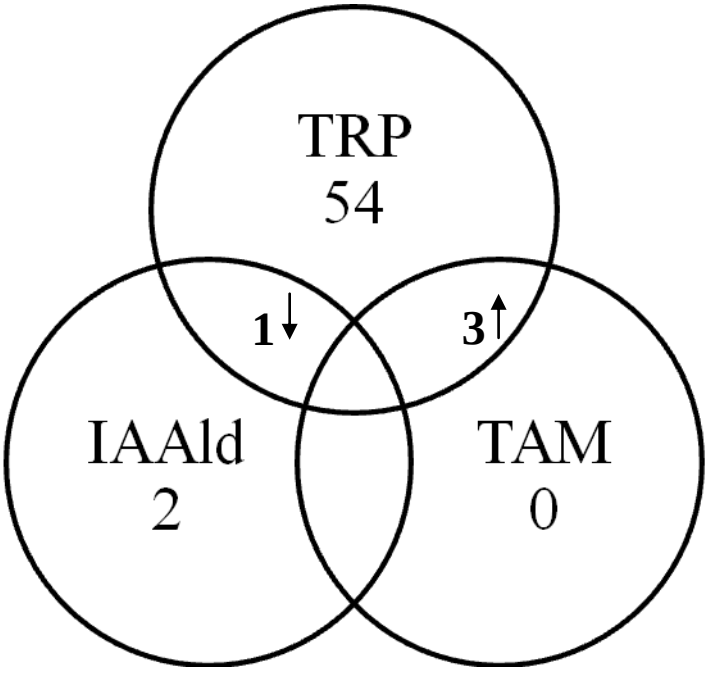


Table 4. Microarray profiling of gene expression in *F. graminearum* cultures treated with indole-3-acetaldehyde, IAAld. Up-regulated genes were selected as genes with ≥ 4 fold difference and down-regulated genes with ≤ -4 fold difference between treated and untreated samples. The values represent means of 3 biological replicates ± standard deviation.

Annotation	Description	Mean	SD
fg02953	conserved hypothetical protein	152.0	1.5
fg01060	conserved hypothetical protein	20.1	2.6
fg10506*	related to monocarboxylate transporter 2	19.7	3.3
fg05059	conserved hypothetical protein	9.0	1.6
fg02417	related to integral membrane protein	7.3	1.4
fg04317**	related to multidrug resistant protein	5.9	1.6
fg10505	conserved hypothetical protein	5.8	2.0
fgd355-500	conserved hypothetical protein	4.4	1.4
fg12103*	related to epoxide hydrolase	-6.5	1.3
fg08157*	conserved hypothetical protein	-4.3	3.2

*In common with up-regulated TRP results
**In common with down-regulated TRP results

Analysis of the microarray data from the TAM-treated cultures identified a total of 61 up-regulated genes, with only 31 of these that were functionally annotated (Table 5). There were no down-regulated genes above the 4-fold cut off line that were present in this group of results. Many of the up-regulated genes are predicted to be involved in transmembrane transport, such as various permeases, or in various enzymatic activities such as oxidation, hydrolysis and transamination. There were also a few genes with predicted functions that were compatible with the expected enzymatic reactions in IAA biosynthesis (Fig. 12). These genes were labeled as candidate genes and included the most up-regulated gene in the microarray experiment: fg02296, a probable aldehyde dehydrogenase, with a 278 fold increase. This candidate gene is likely to be involved in the reaction of IAAld to IAA. The gene fg04621, related to monoamine oxidase N, was also found to be up-regulated 28 folds; it could catalyze the enzymatic reaction converting TAM to IAAld. As well, the gene fg11492, related to flavin-containing monooxygenase, was up-regulated by 5 folds; it could be involved in converting TAM to N-TAM.

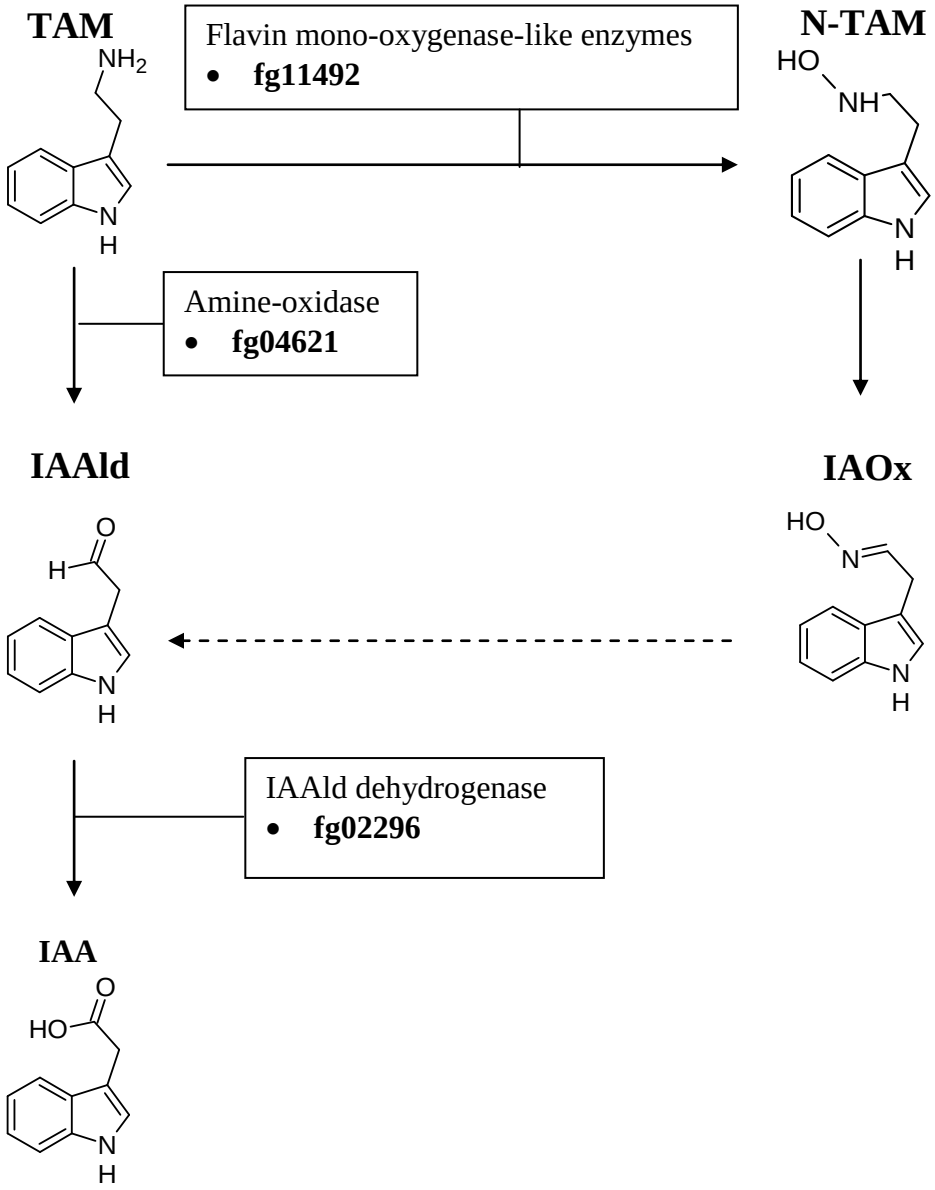
Table 5. Microarray profiling of gene expression in *F. graminearum* cultures treated with tryptamine, TAM. Up-regulated genes were selected as genes with ≥ 4 fold difference and down-regulated genes with ≤ -4 fold difference between treated and untreated samples. The values represent means of 3 biological replicates ± standard deviation.

Annotation	Description	Mean	SD
fg02296**	probable aldehyde dehydrogenase	278.4	1.4
fg12170	probable ABC1 transport protein	98.0	1.4
fg02278	related to HNM1 - Choline permease	55.5	1.5
fg02279	related to peroxisomal amine oxidase (copper-containing)	50.0	1.1
fg07903**	conserved hypothetical protein	29.8	4.0
fg06449	probable fumarylacetoacetate hydrolase	28.7	1.6
fg04621	related to monoamine oxidase N	27.8	1.5
fg09067	conserved hypothetical protein	22.9	2.2
fg04371	conserved hypothetical protein	17.1	1.3
fg01389	related to transaminase type I	13.7	3.7
fg06046	conserved hypothetical protein	13.5	2.4
fgd465-500*	hypothetical protein	13.5	6.7
fg01389	related to transaminase type I	13.0	3.6
fg03016	conserved hypothetical protein	12.5	3.0
fg03772*	related to aminotriazole resistance protein	10.4	1.9
fgd194-80	conserved hypothetical protein	9.8	2.4
fg07592	conserved hypothetical protein	9.8	3.5
fg06381	conserved hypothetical protein	9.4	2.9
fg11073	related to aminopeptidase	9.4	4.7
fg00028**	probable metalloprotease MEP1	9.4	2.1
fg03015	related to protein MCH2 (monocarboxylate permease homolog)	8.6	2.5
fg12196	conserved hypothetical protein	8.1	2.4
fg12226	related to UDPglucose 4-epimerase	7.9	2.6
fg04449	conserved hypothetical protein	7.7	1.8
fg04468*	related to neutral amino acid permease	7.6	1.3
fg07862	conserved hypothetical protein	7.0	2.6
fg10363	conserved hypothetical protein	6.9	1.4
fg03368	conserved hypothetical protein	6.9	4.2
fg07804	related to benzoate 4-monooxygenase cytochrome P450	6.8	4.5
fg06537	probable neutral amino acid permease	6.5	1.3
fg01773*	conserved hypothetical protein	6.4	2.6
fg04593	related to para-hydroxybenzoate polyprenyltransferase precursor	6.2	2.0
fg04993	conserved hypothetical protein	6.2	4.0
fg11412*	conserved hypothetical protein	6.1	2.1
fg02119	related to YER185w, Rta1p	5.7	2.0
fg03351	conserved hypothetical protein	5.7	2.2
fg04627*	related to nik-1 protein (Os-1p protein)	5.6	1.4
fg09566	conserved hypothetical protein	5.6	3.2
fgd185-1060*	probable lactose regulatory protein	5.5	2.4

fg08219	conserved hypothetical protein	5.4	1.9
fg03149	conserved hypothetical protein	5.4	2.2
fg11492	related to flavin-containing monooxygenase	5.4	1.9
fg11324	conserved hypothetical protein	5.4	1.3
fg00195	related to monocarboxylate transporter 2	5.2	3.5
fg08398	probable glucosamine-6-phosphate isomerase	5.2	3.1
fg04012*	related to NADH oxidase	5.1	2.6
fg02467	related to isopenicillin N epimerase	5.0	2.5
fg08232	conserved hypothetical protein	5.0	2.0
fg08641	conserved hypothetical protein	5.0	2.4
fg11311*	conserved hypothetical protein	4.7	2.8
fg08396	related to N-acetylglucosamine-6-phosphate deacetylase	4.6	2.3
fg01596	related to SPR1 - exo-1,3-beta-glucanase precursor	4.6	1.4
fg11111	related to HNM1 - Choline permease	4.5	1.6
fg03032	related to vacuolar membrane protein HMT1	4.4	2.7
fg03990	conserved hypothetical protein	4.4	2.6
fg10433*	conserved hypothetical protein	4.3	3.2
fg01818	related to bacterial leucyl aminopeptidase	4.3	1.9
fg03397*	conserved hypothetical protein	4.2	2.7
fg06433	conserved hypothetical protein	4.2	1.6
fg13349	conserved hypothetical protein	4.1	1.7
fg03595*	related to ADP-ribosylation factor	4.1	1.6

*Shared with up-regulated TRP results
**Shared with down-regulated TRP results

Fig. 12. Up-regulated genes from *F .graminearum* cultures grown in TAM-treated media. Illustration of TAM pathways for the biosynthesis of IAA from the TAM intermediate. The boxes include the type of enzyme needed for the reaction depicted by the arrow and the related up-regulated genes in bold.


TAM
NH2
N
H
Flavin mono-oxygenase-like enzymes
• fg11492
N-TAM
HO
NH
N
H
Amine-oxidase
• fg04621
IAAld
O
H
N
H
IAOx
HO
N
N
H
IAAld dehydrogenase
• fg02296
IAA
O
HO
N
H

The total number of genes affected by the TRP treatment was much larger than those affected by the IAAld and TAM treatments, which was expected since TRP is the precursor of many more various compounds and processes. Of the 153 up-regulated genes, 69 were annotated as hypothetical or conserved hypothetical proteins (Table 6). The most up-regulated gene in these results was fg09061, related to 5-carboxyvanillate decarboxylase, up-regulated by 643 folds. This gene is likely involved in the degradation of lignin which is part of the carbon cycle. The many other genes that were found represent an array of functions such as hydrolysis, oxidation, reduction, hydroxylation, transcriptional regulation, transmembrane transport. One gene could be involved in a peripheral reaction conducting to IAA biosynthesis via in the conversion of TOL to IAAld; it is fgd166-420, related to ADH2 alcohol dehydrogenase II. Among the 54 down-regulated genes 22 were annotated as conserved hypothetical or hypothetical proteins (Table 6). The most down-regulated gene was fg02681 related to alpha-1,6-mannosyltransferase HOC1 which functions as a glycosyltransferase. Interestingly, some of the genes affected by the TRP treatment were also affected in both the IAAld and TAM treatments.

Table 6. Microarray profiling of gene expression in *F. graminearum* cultures treated with tryptophan, TRP. Up-regulated genes were selected as genes with ≥ 4 fold difference and down-regulated genes with ≤ -4 fold difference between treated and untreated samples. The values represent means of 3 biological replicates ± standard deviation.

Annotation	Description	Mean	SD
fg09061	related to 5-carboxyvanillate decarboxylase	643.1	2.7
fg04828	related to tryptophan 2,3 dioxygenase	178.7	1.4
fg12194	related to phenol hydroxylase	166.3	1.8
fg09063	related to salicylate 1-monooxygenase	124.3	1.9
fg11347	probable hydroxyquinol-1,2-dioxygenase	119.8	1.5
fg03893	conserved hypothetical protein	88.6	5.3
fg10434	related to glutathione S-transferase GST-6.0	76.1	1.8
fg08588	conserved hypothetical protein	73.5	2.1
fg08079	probable benzoate 4-monooxygenase cytochrome P450	64.3	1.2
fg04616	conserved hypothetical protein	63.0	1.2
fg03667	related to hydroxyquinol-1,2-dioxygenase	57.5	2.0
fg06540	conserved hypothetical protein	56.2	2.0
fg04845	conserved hypothetical protein	56.1	2.0
fg03666	conserved hypothetical protein	54.5	1.9
fg03772*	related to aminotriazole resistance protein	54.1	1.6
fgd160-1200	conserved hypothetical protein	50.9	2.4
fg09062	conserved hypothetical protein	45.0	1.7
fg11132	related to M.verrucaria cyanamide hydratase	44.2	4.0
fg06465	related to haloacetate dehalogenase H-1	41.0	3.2
fg10433*	conserved hypothetical protein	39.5	1.5
fg03657	related to salicylate 1-monooxygenase	37.5	3.1
fg03914	probable ycaC, hydrolase of unknown specificity	36.5	1.6
fg11311*	conserved hypothetical protein	35.8	1.4
fg00172	related to glutathione transferase omega 1	30.7	2.2
fg01773*	conserved hypothetical protein	30.0	5.0
fg07888	related to arylamine N-acetyltransferase 2	29.3	1.5
fg07991	conserved hypothetical protein	28.7	1.1
fg12049	conserved hypothetical protein	27.9	2.1
fg01866	conserved hypothetical protein	27.2	1.7
fg12103***	related to epoxide hydrolase	25.7	1.7
fg04829	related to kynureninase	25.1	1.6
fg00828	related to 7alpha-cephem-methoxylase P8 chain	24.6	2.9
fg02210	conserved hypothetical protein	23.8	1.4
fg02000	related to URE2 - nitrogen catabolite repression regulator	23.4	2.8
fg13141	probable 1,4-Benzoquinone reductase	23.3	1.1
fg05168	related to pirin	22.3	1.6
fg12330	conserved hypothetical protein	22.1	3.7
fg12857	hypothetical protein	21.7	1.2
fg11040	probable glutathione S-transferase	21.2	1.4
fg03175	related to quinone reductase	20.9	2.4
fg10971	conserved hypothetical protein	19.7	2.4
fg03040	conserved hypothetical protein	19.1	2.3
fg05942	related to phenylcoumaran benzylic ether	18.8	2.4

	reductase		
fg07557	related to transcription co-repressor GAL80	17.3	2.1
fg10497	conserved hypothetical protein	17.0	2.1
fg09684	related to flavin oxidoreductase	16.9	1.3
fg00118	related to neutral amino acid permease	16.2	1.9
fg04012*	related to NADH oxidase	16.0	3.8
fg01772	conserved hypothetical protein	14.4	1.9
fgd166-420	related to ADH2 - alcohol dehydrogenase II	14.3	1.6
fgd465-500*	hypothetical protein	13.9	3.2
fg13368	hypothetical protein	13.7	1.6
fg03717	conserved hypothetical protein	13.7	1.6
fg08157***	conserved hypothetical protein	13.5	1.8
fg04468*	related to neutral amino acid permease	13.4	1.4
fg02545	conserved hypothetical protein	13.4	2.4
fg11412*	conserved hypothetical protein	12.8	1.9
fg08452	probable RIB3 - 3,4-dihydroxy-2-butanone 4-phosphate synthase	12.3	2.0
fg03942	conserved hypothetical protein	12.2	2.9
fg09372	conserved hypothetical protein	11.8	3.5
fgd197-560	hypothetical protein	11.5	1.5
fg07378	conserved hypothetical protein	11.2	2.5
fg08077	related to flavin oxidoreductase	11.0	1.5
fg09863	related to acyl-CoA cholesterol acyltransferase	10.9	1.3
fg10429	related to transcriptional activator CMR1	10.9	1.1
fg04377	conserved hypothetical protein	10.8	1.9
fg09677	conserved hypothetical protein	10.7	2.1
fg11312	hypothetical protein	10.3	1.1
fg12200	probable ACC deaminase	10.1	1.8
fg03595*	related to ADP-ribosylation factor	10.1	1.2
fg08078	related to general amidase	10.0	1.4
fg06528	related to flavin oxidoreductase	10.0	2.0
fg08229	related to monocarboxylate transporter	9.9	1.5
fg03774	related to 7alpha-cephem-methoxylase P8 chain	9.8	2.2
fg11375	related to fluconazole resistance protein (FLU1)	9.7	3.5
fgd194-240	related to nitrogen metabolic regulation protein nmr	9.6	1.6
fg07249	related to C.carbonum toxD gene	9.4	1.2
fg10506**	related to monocarboxylate transporter 2	9.1	2.1
fg02431	related to transcriptional regulator	9.0	1.5
fg04568	related to SIS2 protein (cycle-specific gene control)	9.0	1.2
fg03151	related to integral membrane protein	8.8	1.1
fg00249	conserved hypothetical protein	8.6	2.4
fg07832	related to CCC1 protein (involved in calcium homeostasis)	8.5	1.3
fgd160-1190	related to transcriptional activator Mut3p	8.3	1.3

fg07708	conserved hypothetical protein	8.1	1.4
fg11381	related to integral membrane protein	7.9	1.4
fg02037	conserved hypothetical protein	7.8	1.8
fg08269	conserved hypothetical protein	7.6	1.8
fg10442	conserved hypothetical protein	7.4	1.2
fg03451	conserved hypothetical protein	7.4	1.3
fg01531	probable CYB2 - lactate dehydrogenase cytochrome b2	7.4	2.3
fg04627*	related to nik-1 protein (Os-1p protein)	7.1	1.4
fg06401	conserved hypothetical protein	7.1	1.1
fg05772	conserved hypothetical protein	7.0	1.5
fg10153	conserved hypothetical protein	6.9	1.3
fg09806	conserved hypothetical protein	6.9	2.5
fg00226	related to multidrug resistant protein	6.8	2.7
fg02485	conserved hypothetical protein	6.7	2.2
fg02127	conserved hypothetical protein	6.7	1.2
fg03941	related to bifunctional 4-hydroxyphenylacetate degradation enzyme	6.7	1.8
fg11295	related to enoyl-CoA hydratase precursor, mitochondrial	6.6	1.8
fg10974	conserved hypothetical protein	6.6	1.4
fg09348	conserved hypothetical protein	6.4	2.4
fg02201	conserved hypothetical protein	6.4	1.4
fg03397*		6.2	1.6
fg03710	related to NADH oxidase	6.2	2.0
fg08453	probable COQ3 - enzyme of ubiquinone (coenzyme Q) biosynthesis	6.2	1.5
fg05467	related to dTDP-glucose 4,6-dehydratase	6.1	1.6
fg12965	probable catalase isozyme P	5.9	1.3
fg10124	probable NADPH quinone oxidoreductase homolog PIG3	5.9	1.9
fg06343	conserved hypothetical protein	5.8	2.2
fgd323-800	conserved hypothetical protein	5.8	1.6
fg10537	related to D-amino acid oxidase	5.8	1.6
fgd237-250	hypothetical protein	5.8	1.6
fg03563	related to LSB3 - possible role in the regulation of actin cytoskeletal organization	5.8	2.1
fg02997	conserved hypothetical protein	5.7	1.4
fg10809	conserved hypothetical protein	5.7	1.8
fg09570	conserved hypothetical protein	5.5	1.4
fg04830	related to positive regulator of PUT (proline utilization) genes	5.5	2.1
fg09701	related to major facilitator MirA	5.5	1.7
fg10430	conserved hypothetical protein	5.4	1.4
fg03970	conserved hypothetical protein	5.3	2.0
fg09991	related to gamma-glutamylcysteine synthetase	5.2	2.0

fg07880	conserved hypothetical protein	5.1	1.3
fg11062	conserved hypothetical protein	5.0	1.6
fg11308	hypothetical protein	4.9	2.0
fg05190	conserved hypothetical protein	4.9	1.3
fg06553	related to xylulose-5-phosphate/fructose-6-phosphate phosphoketolase	4.9	1.4
fg12529	related to arginase	4.8	1.2
fg03940	conserved hypothetical protein	4.8	1.3
fg08123	conserved hypothetical protein	4.7	1.5
fgd311-90	related to maleylacetate reductase	4.7	1.4
fg05401	related to beta-1,3-glucanase	4.7	1.4
fg00773	related to copper transport protein	4.5	1.2
fg00171	conserved hypothetical protein	4.5	1.3
fg07960	related to YTP1	4.5	1.1
fgd448-30	conserved hypothetical protein	4.5	1.2
fgd185-1060*	probable lactose regulatory protein	4.4	1.0
fg07943	conserved hypothetical protein	4.4	1.3
fg04124	probable LYS7 - copper chaperone for superoxide dismutase Sod1p	4.4	1.6
fg04817	related to serine protease	4.4	1.2
fg12945	conserved hypothetical protein	4.3	1.2
fgd367-510	conserved hypothetical protein	4.3	1.2
fg05921	related to 24-dehydrocholesterol reductase precursor	4.3	1.6
fg02263	conserved hypothetical protein	4.3	2.0
fg10374	related to putative fatty acid desaturase (mld)	4.2	2.3
fg09820	related to cysteine dioxygenase type I	4.1	1.5
fg02120	hypothetical protein	4.1	1.6
fg03237	related to integral membrane protein	4.1	1.3
fg12788	conserved hypothetical protein	4.1	1.7
fg13455	conserved hypothetical protein	4.1	1.1
fg10854	related to antioxidant protein and metal homeostasis factor	4.0	1.2
fg10152	conserved hypothetical protein	4.0	1.1
fg02681	related to alpha-1,6-mannosyltransferase HOC1	-19.1	1.4
fg00028*	probable metalloprotease MEP1	-15.2	4.0
fg03163	related to monooxigenase	-12.7	1.0
fg04745	related to antifungal protein	-10.7	1.2
fg03123	conserved hypothetical protein	-9.9	2.3
fg11528	related to monophenol monooxygenase (tyrosinase)	-9.8	1.7
fg09119	conserved hypothetical protein	-9.8	1.9
fg09042	probable formamidase	-9.1	1.6
fg08295	conserved hypothetical protein	-8.4	2.1
fg11164	probable trypsin precursor	-8.3	1.1

fgd132-760	conserved hypothetical protein	-8.3	2.2
fg02296*	probable aldehyde dehydrogenase	-8.0	N/A
fg03816	probable lactonohydrolase	-6.6	1.3
fg05660	related to Mx protein	-6.5	1.4
fg12371	probable endopeptidase K	-6.4	2.6
fg09044	conserved hypothetical protein	-6.3	1.2
fg01988	related to monophenol monooxygenase (tyrosinase)	-6.2	2.1
fg12145	related to 3-hydroxybutyryl-CoA dehydratase	-5.9	1.7
fg02920	related to ROT2 - glucosidase II, catalytic subunit	-5.8	2.4
fg12146	conserved hypothetical protein	-5.7	1.2
fg02828	conserved hypothetical protein	-5.6	1.6
fg11498	related to pisatin demethylase (cytochrome P450)	-5.6	2.4
fg10595	related to alkaline protease (oryzin)	-5.5	1.6
fg11099	conserved hypothetical protein	-5.4	1.5
fg03985	probable carnitine transporter	-5.3	1.6
fgd147-160	conserved hypothetical protein	-5.2	1.4
fg13202	conserved hypothetical protein	-5.1	1.9
fg02982	related to pisatin demethylase / cytochrome P450 monooxygenase	-5.1	1.4
fg07901	conserved hypothetical protein	-5.1	1.3
fg12394	probable neutral amino acid permease	-5.1	2.8
fg04801	conserved hypothetical protein	-4.9	2.4
fg05836	related to membrane protein, peroxisomal	-4.9	1.1
fg02174	conserved hypothetical protein	-4.8	1.2
fg02615	related to O-methyltransferase	-4.7	1.6
fg03778	related to neutral amino acid permease	-4.7	1.2
fg09045	related to stage V sporulation protein K	-4.7	1.3
fg04317**	related to multidrug resistant protein	-4.7	5.5
fg02255	conserved hypothetical protein	-4.6	3.2
fg11578	related to acetylxylan esterase	-4.6	1.0
fg03911	conserved hypothetical protein	-4.6	1.7
fg04941	conserved hypothetical protein	-4.6	1.6
fg09048	related to adenosylmethionine-8-amino-7-oxononanoate aminotransferase	-4.5	1.8
fg07903*	conserved hypothetical protein	-4.5	N/A
fg05622	related to trehalase precursor	-4.5	1.6
fg11577	conserved hypothetical protein	-4.4	1.5
fg03700	related to O-methylsterigmatocystin oxidoreductase	-4.3	1.3
fg10935	related to A.gambiae ATP-binding-cassette protein	-4.2	1.3
fg07896	trichothecene 3-O-acetyltransferase	-4.2	1.1
fg02365	conserved hypothetical protein	-4.1	1.2
fg07822	conserved hypothetical protein	-4.1	1.9
fgd346-10	probable GUT1 - glycerol kinase	-4.1	1.6

fg10471	related to helicase-like transcription factor protein	-4.1	1.2
fgd473-630	conserved hypothetical protein	-4.0	1.2
fg01304	conserved hypothetical protein	-4.0	1.8

*Shared with up-regulated TAM results
** Shared with up-regulated IAAld results
***Shared with down-regulated IAAld results.

The IAAld and TRP results shared 4 differentially expressed genes (Fig. 11 A, B). From the IAAld results, the up-regulated fg10506 gene, related to monocarboxylate transporter 2, and the 2 down-regulated genes, fg12103, related to epoxide hydrolase, and fg08157, a conserved hypothetical protein, were found among the upregulated genes in the TRP results (Table 4, 6). As well the IAAld-up-regulated fg04317 gene, related to multidrug resistant protein, was also found among the down-regulated genes in the TRP results (Table 4, 6). In the case of common up-regulated genes found between the TAM and TRP results, a total of 12 genes were found (Fig. 11A). These include, 6 genes functionally annotated for transmembrane transport, lactose regulation, development and germination, and 6 genes annotated as conserved hypothetical protein (Table 5, 6). A few up-regulated genes from the TAM results also coincided with the down-regulated TRP results (Fig. 11B). Interestingly this included the gene, fg02296, which as mentioned earlier was the most up-regulated gene in the TAM results (Table 5). The genes fg07903, a conserved hypothetical protein, and fg00028, a probable metalloprotease MEP1, were also found in both up-regulated TAM and down-regulated TRP microarray results (Table 5, 6). Although the TAM and IAAld results did not have any genes in common, some genes with the same annotation or function were found up-regulated in both of these groups. These include, genes related to monocarboxylate transporter 2, and genes related to a resistance protein (Table 4, 5).

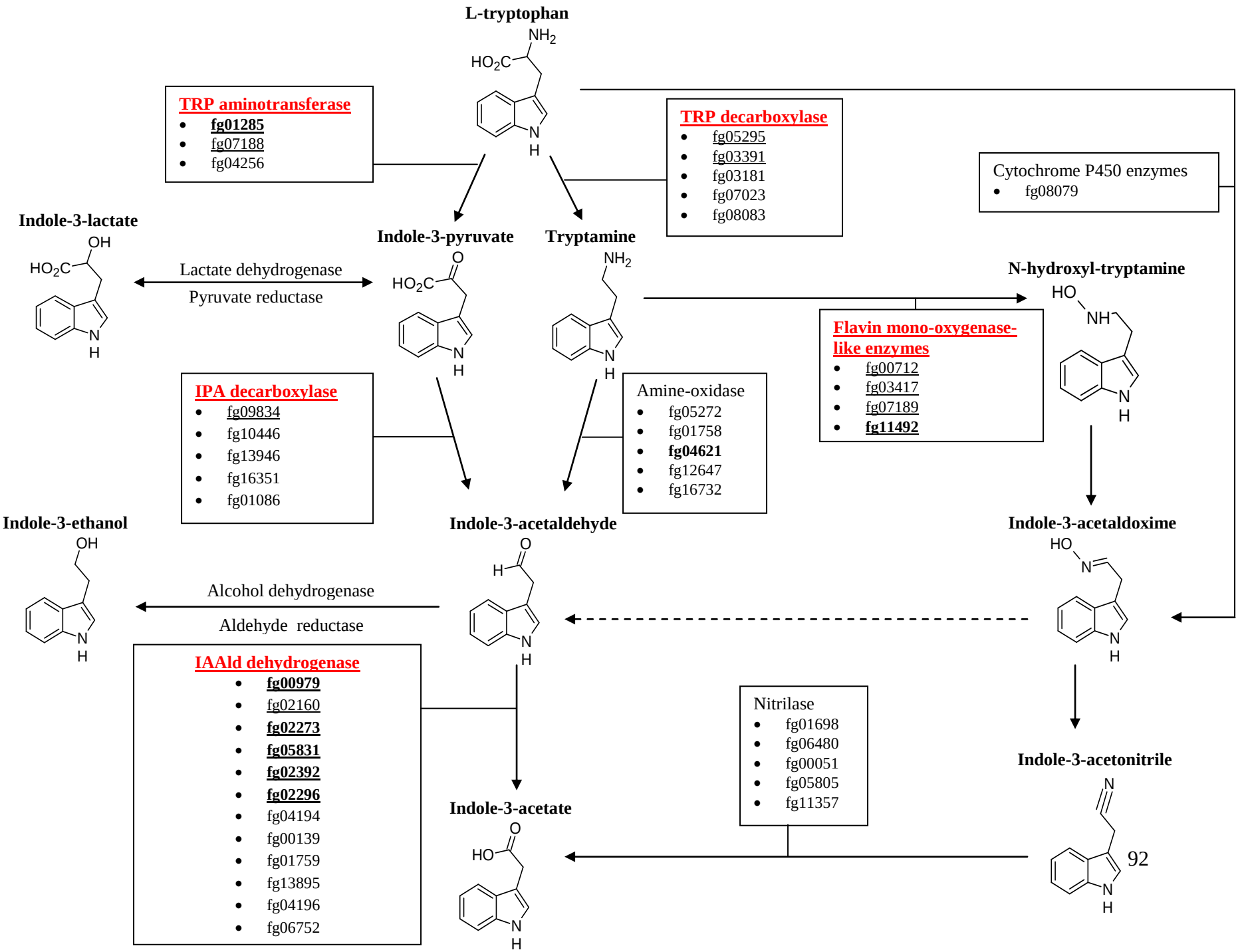
3.3.1 RT-qPCR of candidate genes

Three up-regulated genes were identified as potential candidates enzymes for IAA biosynthesis, based on the microarray data from the TAM treatment, including: fg02296,

fg04621 and fg11492. However the microarray data from both the IAAld and TRP treatment did not provide any candidate genes, suggesting that the time point at which the cultures were collected for the microarray experiment may have been inappropriate. To address that possibility, cultures were collected at new time points of 2, 4 and 6 hrs and quantitative PCR was used to determine the expression of 15 candidate genes. The candidate genes, listed in Fig. 13 for each enzymatic reaction, were chosen based on both the microarray experiment and the homology BLASTp search (refer back to section 3.1.3, Table 2). Due to restraints on time and resources, only some of the candidate genes for 5 enzymes were focused on. The final group of genes was chosen based on their close homology to the corresponding genes in other fungal species, using the BLASTp scores, as well as which enzymatic reaction they are involved in, favoring the TAM pathway. The chosen genes for RT-qPCR are underlined in Fig. 13.

Only *F. graminearum* cultures treated with TRP and the controls were used for the analysis of expression of Trp aminotransferase and Trp decarboxylase. Three genes were listed as candidate genes for Trp aminotransferase: fg01285, fg07188 and fg04256. RT-qPCR was performed on fg01285 and fg07188, and only fg01285 was found up-regulated at 2hrs by 4.2 folds (Table 7). The candidate genes for Trp decarboxylase included: fg05295, fg03391, fg03181, fg07023, fg08083 and fg13141. The 2 genes that were used for RT-qPCR were fg05295 and fg03391, and neither one showed up-regulation during the 2, 4 and 6hr timeline.

Fig. 13. Schematic of the proposed candidate genes for enzymatic reactions in the biosynthesis of IAA by *F.graminearum*. The underlined candidate genes were analysed by RT-qPCR. The canditate genes that were up-regulated in microarray or RT-qPCR analysis, or in both, were bolded.


L-tryptophan
TRP aminotransferase
fg01285
fg07188
fg04256
TRP decarboxylase
fg05295
fg03391
fg03181
fg07023
fg08083
Cytochrome P450 enzymes
fg08079
Indole-3-lactate
Lactate dehydrogenase
Pyruvate reductase
Indole-3-pyruvate
Tryptamine
N-hydroxyl-tryptamine
Flavin mono-oxygenase-like enzymes
fg00712
fg03417
fg07189
fg11492
IPA decarboxylase
fg09834
fg10446
fg13946
fg16351
fg01086
Amine-oxidase
fg05272
fg01758
fg04621
fg12647
fg16732
Indole-3-ethanol
Indole-3-acetaldehyde
Indole-3-acetaldoxime
Alcohol dehydrogenase
Aldehyde reductase
IAAld dehydrogenase
fg00979
fg02160
fg02273
fg05831
fg02392
fg02296
fg04194
fg00139
fg01759
fg13895
fg04196
fg06752
Nitrilase
fg01698
fg06480
fg00051
fg05805
fg11357
Indole-3-acetonitrile
Indole-3-acetate

The expression of the candidate gene fg09834, among a list including 4 other genes (fg10446, fg13946, fg16351 and fg01086) was analyzed for the enzyme pyruvate decarboxylase in both TRP- and IPA-treated cultures. There was no up-regulation found for fg09834 between the two treatments. Four genes were tested in TRP- and TAM-treated cultures for the flavin mono-oxygenase like enzymes, these genes included: fg00712, fg03417, fg07189 and fg11492. The gene fg11492 was found up-regulated in the TAM-treated cultures by microarray analysis, and differential expression was confirmed by RT-qPCR, with an increase of 118 folds relative to the untreated cultures (Table 7). However the up-regulation was found at 2hrs in the samples analyzed by RT-qPCR rather than at the 6 hr time point as in the samples used for the microarray experiment. The level of expression for fg11492 at 6hrs in the TRP-treated cultures was also found up-regulated by 2 folds by RT-qPCR, which was however below the set level of detection for the microarray results. The expression level of the other genes for this enzyme was similar in treated and untreated cultures.

Table 7. Relative gene expression levels of candidate genes from RT-qPCR of treated *F.graminearum* cultures. Cultures were treated with 2mM TRP or 0.2mM TAM, IPA or IAAld. Genes were normalized using three reference genes: β-tubulin, actin and GAPDH. Relative expression is represented by a mean of three biological replicates.

		Timeline				
Gene annotation	**Treatment**	40min	80min	120min	4hr	6hr
fg01285	TRP			*		
fg11492	TRP					*
	TAM	**	***	****		
fg00979	TRP			*	*	
	TAM					*
	IPA				*	
fg02273	TRP					*
	TAM		**	**		
	IPA			*		
	IAAld	*				
fg02296	TAM	*****	*****	*****	*****	*****
fg02392	TAM	*				
fg05831	TRP				*	
	TAM		*			
	IAAld	*				

*	2≤5 fold
**	5<50 fold
***	50≤150 fold
****	150<500 fold
*****	500≤ fold

The genes used in the RT-qPCR for the enzyme of aldehyde dehydrogenase included a total of 6 out of 23 candidate genes. These included, fg00979, fg02160, fg02273, fg02296, fg05831 and fg2392, and were tested in TRP-, TAM-, IPA- and IAAld-treated cultures (Table 7). The only candidate gene for this enzyme that was found up-regulated in the microarray results was fg02296, and only in the TAM-treated cultures. Using RT-qPCR, the level of expression for fg02296 was found to be more than 1000 fold higher in TAM-treated vs untreated cultures at all three time points (2, 4 and 6 hrs), and was not up-regulated in the other treatments. The gene fg00979 was found up-regulated in the TRP-treated samples by 2 folds at 2hrs and increased to 4 folds at 4hrs however it was not up-regulated at 6hrs. This gene was also found up-regulated for TAM- and IPA-treated cultures by 2 folds at 6 hrs and 4 hrs respectively. The gene fg02273 was up-regulated in TRP-, TAM- and IPA-treated cultures. In TAM and IPA treatments, this gene was up-regulated by 47 folds and 3.5 folds respectively, at 2hrs. In TRP-treated cultures, fg02273 was found up-regulated by 3.9 folds at 6hrs. At the 4hr time point, fg05831 was up-regulated in TRP-treated cultures by 2.1 folds and in the IAAld ones by 2.1 folds at 40 min. The level of expression for the genes fg02160 and fg02392 did not change in any of the treatments for the time points of 2, 4 and 6hrs.

The up-regulated levels of expression for some of the genes tested by RT-qPCR at the 2hr time point suggested that an earlier timeline may be needed to establish a timeframe of when these genes were up-regulated and possibly discover candidate genes that may be turned on before 2hrs for IAA biosynthesis. A new experiment with a timeline of 40min, 80min and 120min was performed and the samples used for RT-qPCR analysis of a subset of the

candidate genes. The six candidate genes that were tested in the earlier timeline included five aldehyde dehydrogenase (fg00979, fg02273, fg02296, fg02392, fg05831) and one flavin mono-oxygenase-like gene (fg11492); the results are summarized in Table 8. The gene fg00979 was not found up-regulated in the earlier time points. The gene fg02273, that was found up-regulated by 47 folds at 2hrs in TAM-treated cultures, was also found up-regulated by 19 folds at 80min. This gene also showed a higher level of expression by 2.5 folds in IAAld-treated cultures at 40min. In the TAM-treatment only, the gene fg02296 was found at the same level of expression at all time points including 40min, 80min, 2hrs, 4hrs and 6hrs and 1000 folds above the level of expression of untreated cultures. Also in the TAM-treated cultures, fg02392 was found up-regulated by 4.5 folds at 40min and fg05831 by 4.9 folds at 80min. In the case of fg11492, since the enzymatic reaction for this gene occurs before IAAld, only TAM-treated cultures were utilized for RT-qPCR. In these cultures, fg11492 showed an up-regulation of 118 folds at 2hrs, and in the earlier time points this gene was up-regulated by 26.5 folds at 40min and increased to 168 folds at 80min.

Overall, the RT-qPCR analysis of IAA candidate genes revealed that 7 genes were up-regulated in one or more treatments. Although most often these genes could be found in TAM-treated cultures, up-regulated genes were found for enzymatic reactions taken place in both IPA and TAM pathways. The gene fg02296 was the most up-regulated candidate gene for IAA biosynthesis, as tested by RT-qPCR and in the microarray.

3.4 Generation of IAA knockout using USER friendly cloning

Among the up-regulated candidate genes in auxin-inducing conditions found in the previous section 3.3, one of these genes was chosen to further determine its function in IAA biosynthesis. The gene fg02296 was inactivated by replacing it with the selective marker hygromycin phosphotransferase using the USER cloning technique and *Agrobacterium tumefaciens*-mediated transformation (Frandsen et al., 2008). fg02296 was chosen to be replaced, based on its highest expression level in auxin producing conditions from both the microarray and RT-qPCR results. fg02296 was also an ideal candidate gene since it coded for a probable aldehyde dehydrogenase, potentially involved in the last and common enzymatic reaction in the pathways leading to the final product, IAA.

A first attempt to produce a deleted mutant of fg02296 (*fg02296*Δ) in the *F. graminearum* strain DAOM 180378 was deemed unsuccessful since only one positive colony was recovered from the hygromycin-only selection on DFM media. In addition, the *F. graminearum* colony did not appear as expected large white colonies. Another *F. graminearum* strain, DAOM 233423, known to produce successful *Fusarium* transformations using the *Agrobacterium* transformation protocol, was tested. As a positive control for the transformation procedure, another gene that was previously successfully replaced in DAOM 233423, fg08509 (a probable methylcrotonyl-CoA carboxylase beta chain, mitochondrial precursor), was used at the same time as fg02296. The appearance of the transformed *F. graminearum* colonies for *fg02296*Δ in DAOM 233423 was identical to that observed with DAOM180378; however they differed from the colonies of *fg08509*Δ. The colonies of *fg02296*Δ appeared as very small and scarce white colonies and a yellowish

film produced from the culture covered the black filter paper onto which the transformation took place. At the same time, the *fg08509*Δ colonies grew as expected, as large white colonies on the black filter paper. The small colonies from *fg02296*Δ were still tested for the presence of the hygromycin-resistance gene (*hph*) using a PCR assay. Hygromycin-positive colonies were identified and three independent ones were chosen to produce single spore colonies; in addition, three independent hygromycin-negative colonies were chosen, for use as transformation controls in future experiments.

3.4.1 Analysis of knockout fitness and production of IAA

The growth of mycelia and the production of spores were observed to assess the fitness of the *fg02296*Δ transformants. The measurements for the growth of mycelia were recorded for 5 days and no significant difference between the growth of the *fg02296*Δ transformants, negative transformants and WT *F. graminearum* was found (Fig. 14). The initial growth differences observed at transformation time disappeared once cultures were grown onto PDA plates with appropriate antibiotics. The range of the average measurements among the cultures were: at Day 1, from 10-13.5mm; at Day 2, from 20-26.5mm; at Day 3, from 37.5-48.5mm; Day 4, from 60.5-69mm; and Day 5, from 80.5-90mm. There were also no significant difference in the number of spores produced between the *fg02296*Δ transformants, negative transformants and WT *F. graminearum* (Fig. 15). The average number of spores produced was $18\pm3\cdot10^4$ spore/mL by the wild-type strain, $16\pm2\cdot10^4$ spore/mL by the three negative transformants, and $19\pm3\cdot10^4$ spore/mL by the three *fg02296*Δ transformants.

Fig. 14. Mycelial growth for fg02296Δ, negative transformants and WT *F. graminearum* over 5 days. Mycelial growth was assessed by taking the mean of 2 diameter measurements from new growth surrounding a plug with mycelia on a PDA plate. Data is represented as a mean of three biological replicates ± standard deviation for the wild-type, -ve transformants and fg02296Δ transformants. The mean also combines the mean values for the three independent -ve transformants: TRF4, TRF5 and TRF6, or for the three independent fg02296Δ transformants: KO1, KO2, and KO3.

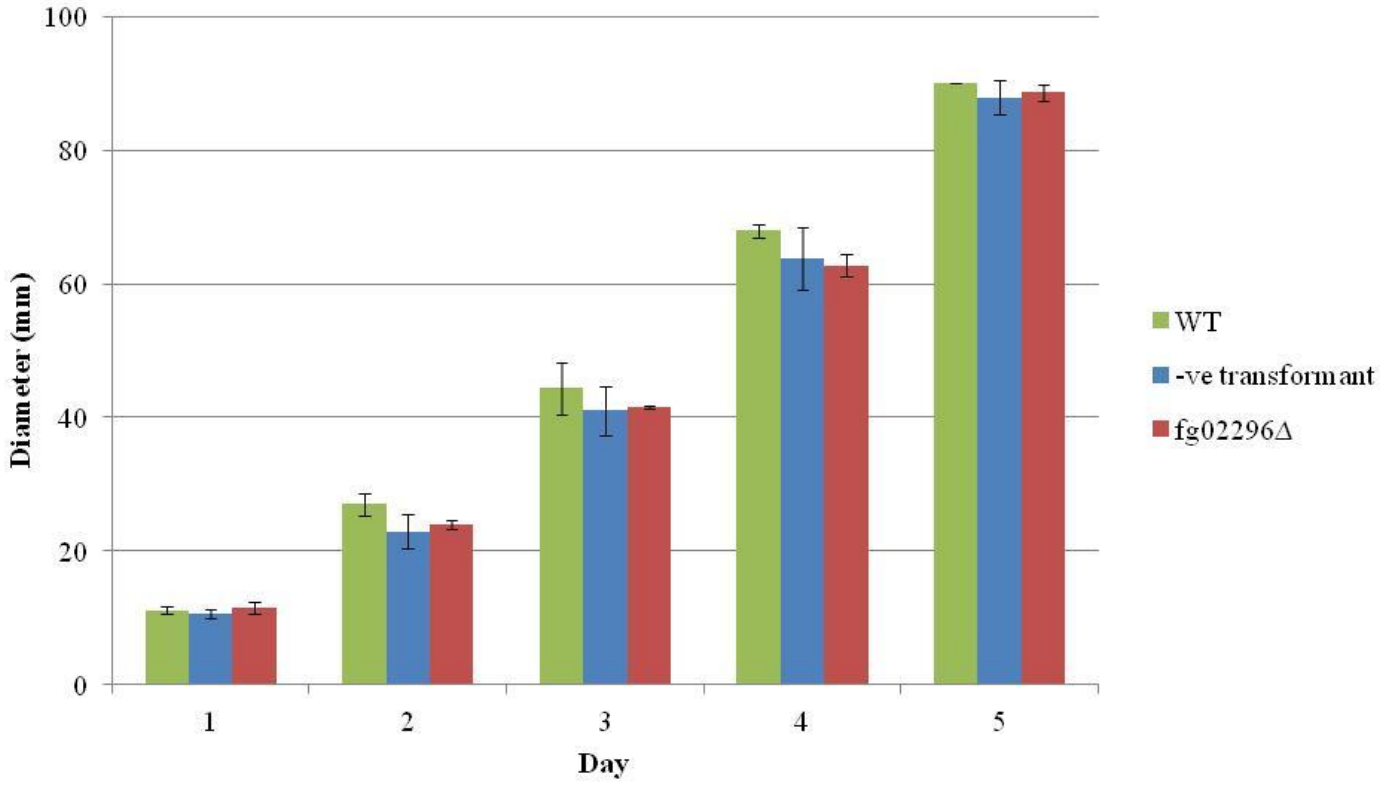
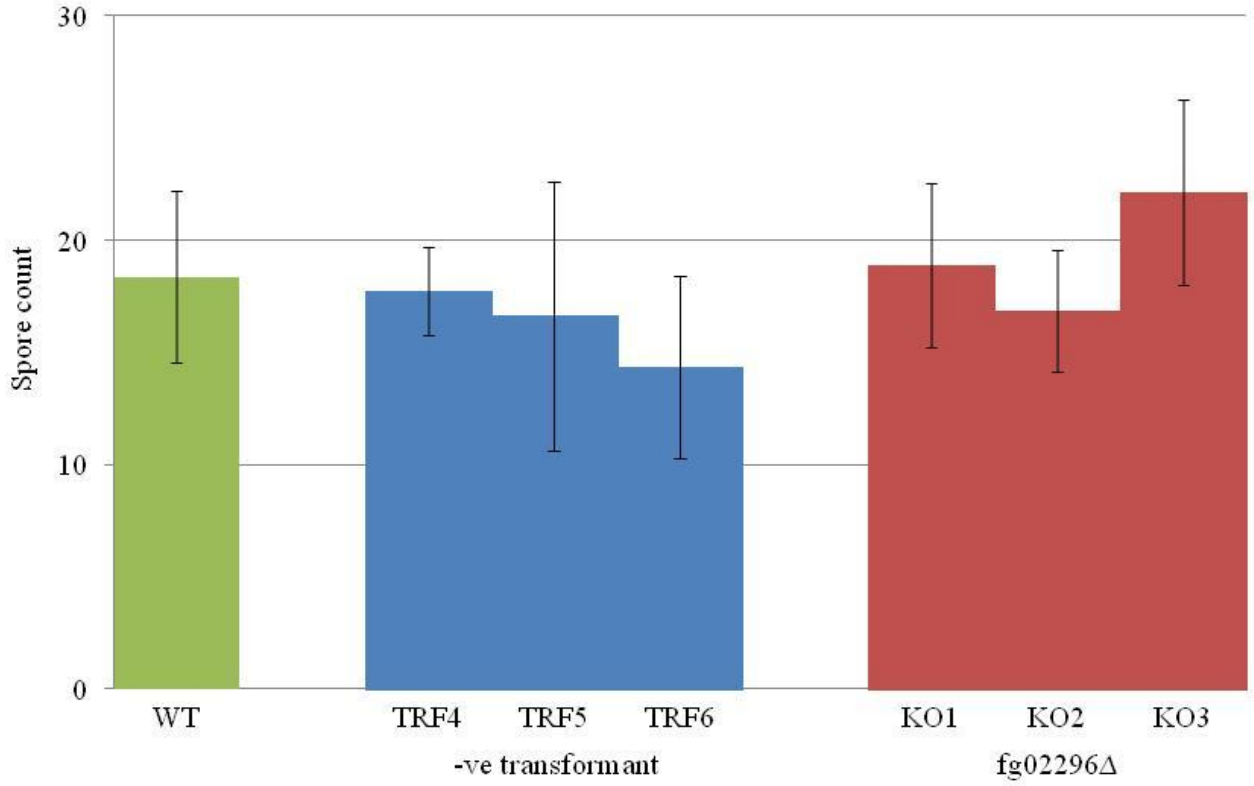
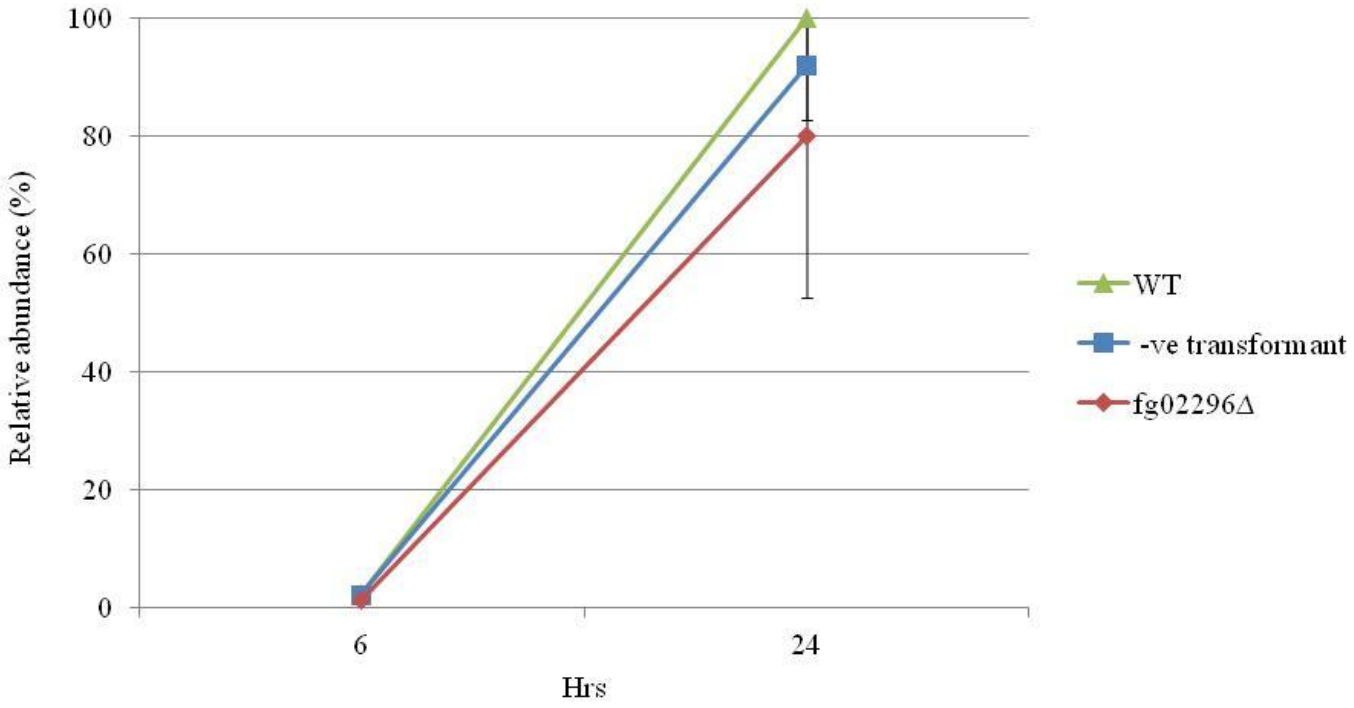

100
80
60
40
20
0
Diameter (mm)
1
2
3
4
5
Day
WT
-ve transformant
fg02296Δ

Fig. 15. Spore count for WT *F. graminearum*, -ve and fg02296 Δ transformants. A plug taken from the periphery of PDA plates with mycelia growth of each strain was added to CMC medium. The number of spores produced was counted using a haemocytometer. Three negative transformants: TRF4, TRF5 and TRF6, and three fg02296Δ transformants: KO1, KO2, and KO3 were compared. Data is represented as a mean of two biological replicates ± standard deviation.


30
20
10
0
Spore count
WT
TRF4
TRF5
TRF6
KO1
KO2
KO3
-ve transformant
fg02296Δ

The production of IAA and TOL by the transformant strains was examined by LC-MS/MS. Cultures that were feed TAM were examined at 6, 12 and 24 hrs and those that were feed IAAld were examined at 40, 80, 120 and 720 mins (6 hrs). As in section 3.2, the concentrations for IAA and TOL were calculated from the LC-MS/MS data based on standard curves. The concentrations of IAA and TOL were converted into a relative value and further converted into a percentage in order to compare the data. For the TAM feed cultures, IAA increased between 6 and 24 hrs by approximately 98% for WT, 90±9 % for the –ve transformants, and 80±27 % for the *fg02296*Δ transformants (Fig. 16). Due to the large standard variation of the values from the –ve and *fg02296*Δ tranformants, no significant difference was found in the concentration of IAA produced. TOL was not found in any of the TAM feed cultures.

Fig. 16. Relative abundance (%) of IAA produced from WT *F. graminearum,* -ve and fg02296Δ transformants feed with TAM, during a 24hr time course. The concentrations of IAA were obtained using an LC-MS/MS, converted into a relative abundance between WT *F. graminearum*, -ve and fg02296Δ transformants and further converted into a percentage. The data represent the mean ±standard deviation for three independent –ve transformant isolates: TRF4, TRF5, TRF6, and three independent fg02296Δ isolates: KO1, KO2, KO3.

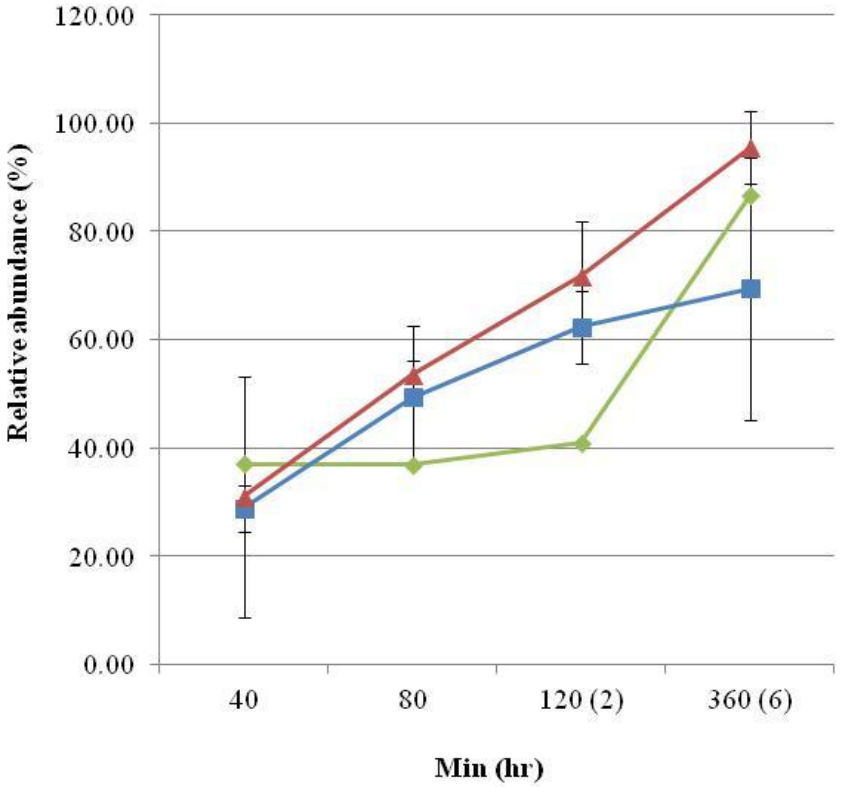

100
80
60
40
20
0
Relative abundance (%)
6
24
Hrs
WT
-ve transformant
fg02296Δ

In the cultures that were feed IAAld, the relative percentage value for IAA was under 20% for the WT, and for the isolates of the negative and *fg02296*Δ transformants (Fig. 17B). The IAA values for the WT decreased between 40 to 80 min, increased between 80mins to 2hrs and plateau between 2 to 6 hrs. The averaged IAA values from the –ve transformant increased by approximately 10% from 40 min to 6 hrs, with a significant increase in IAA found between 2 and 6 hrs. For the *fg02296*Δ transformants, averaged IAA values increased by approximately 15% from 40 min to 6 hrs. Again, a significant increase in IAA concentration was found between 2 and 6hrs. No significant difference was found between the IAA values for the –ve and *fg02296*Δ transformants.

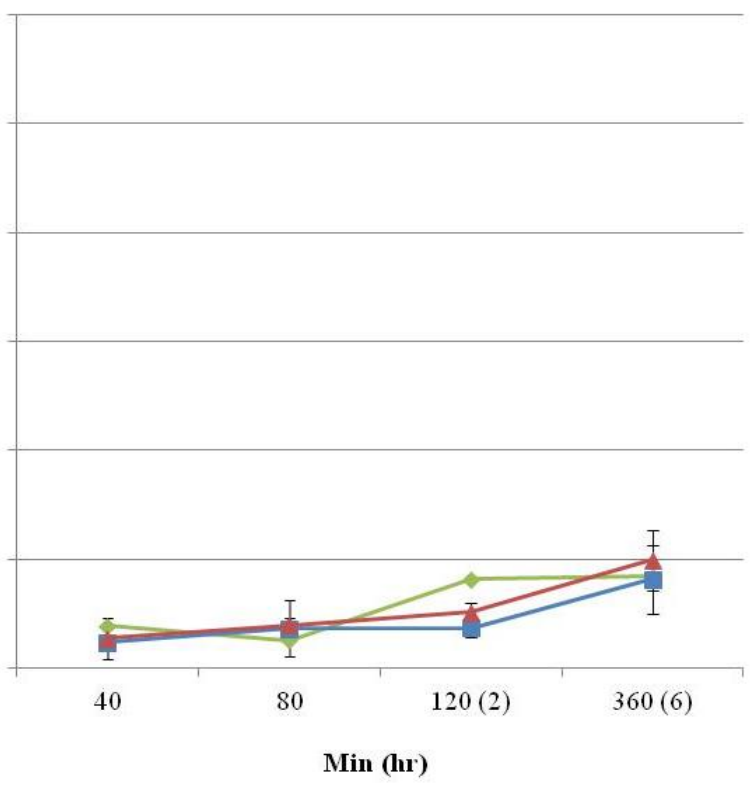
TOL was found in higher averaged concentrations than IAA for the WT, -ve and *fg02296*Δ transformants at all time points analysed (Fig. 17A). For the WT cultures, the TOL concentration varied from around 40% at 40 mins and 86% at 6 hrs. The values for TOL in the –ve transformants included 29±4 % at 40mins, 49±13 % at 80mins, 62±6 % at 2hrs, and 69±24 % at 6hrs. Thus a significant increase was found between 40 to 80mins only. TOL in the *fg02296*Δ transformants included 31±22 % at 40mins, 53±3 % at 80 mins, 72±10 % at 2hrs, and 96±7 % at 6hrs. A significant increase in the concentration of TOL was therefore found between 80min, 2hrs and 6hrs. No significant difference was found between the –ve and *fg02296*Δ transformants in the concentration of TOL at any time point.

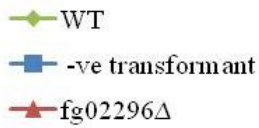
Fig. 17. Relative abundance (%) of TOL and IAA produced by WT *F. graminearum*, -ve and fg02296Δ transformants feed with IAAld, during a 6hr time course. The concentrations of TOL and IAA were obtained using an LC-MS/MS, converted into a relative abundance between WT *F. graminearum*, -ve and fg02296Δ transformants and further converted into a percentage. A. Data for the TOL LC-MS/MS analysis. B. Data for the IAA LC-MS/MS analysis. The data represent the mean ±standard deviation for three independent –ve transformant isolates: TRF4, TRF5, TRF6, and three independent fg02296Δ isolates: KO1, KO2, KO3.

A


120.00
100.00
80.00
60.00
40.00
20.00
0.00
Relative abundance (%)
40
80
120 (2)
360 (6)
Min (hr)

B


40
80
120 (2)
360 (6)
Min (hr)


WT
-ve transformant
fg02296Δ

Chapter 4 – Discussion

In order to gain a better understanding of the biosynthesis of IAA in *F. graminearum*, the potential pathways and genes involved were investigated. Bioinformatic tools were first used to screen the *F. graminearum* genome and generate candidate genes based on their homology to IAA genes of other relevant microbial species. The pathways were assessed by treating *F. graminearum* cultures with pathway specific intermediates and analyzing the filtrates of these cultures for IAA production. In particular the IAM pathway was found not be utilized by *F. graminearum*. Cultures were grown in auxin producing conditions to study differential gene expression. One gene in particular coding for an aldehyde dehydrogenase enzyme was found highly up-regulated and was chosen for replacement to create a mutant strain. The fitness of the *fg02296*Δ mutant strain as well as its ability to biosynthesize IAA was assessed, unfortunately the fitness level and more importantly the ability to produce IAA was not affected.

The keyword search provided a preliminary screening of the *F. graminearum* genome for genes annotated as enzymes with a general function that would be involved in the three proposed pathways for the biosynthesis of IAA. This screening was informative for some enzymes of a more specific function such as the aminotransferase where only two genes were found, as well as the amino acid decarboxylase however it was less informative for enzymes that are involved in many various metabolic processes such as aldehyde dehydrogenase.

The genes that were found annotated with the appropriate enzymatic activity in the screening of the *F. graminearum* genome did not necessarily code for enzymes that had a role in IAA biosynthesis. This was evident in the case of monooxygenase in the enzymatic reaction converting tryptophan to indole-3-acetamide. The search provided 8 genes for monooxygene or related/probable monooxygenase enzymes however this enzyme was not found in the homologous search. The missing putative genes of the IAM pathway, IAM monooxygenase and IAM hydrolase, in *F. graminearum* was also confirmed by Tsavkelova et al. (2012). Tsavkelova et al. (2012) performed BLASTp searches with the tryptophan monooxygenase (IaaM) and the indole-3-acetamide hydrolase (IaaH) protein sequences from the bacterial species *A. tumefaciens* and *P. syringae* in order to find homologs in *Fusarium* species. Interestingly, the genes for the IAM enzymes were found in four *Fusarium* species that belong to the *G. fujikuroi* species complex (GFC) including*: F.verticillioides, F.oxysporum, F. proliferatum* and *F. fujikuroi* and was missing in *F. graminearum* and in *F. solani*. This suggested that the genes for the IAM enzymes were acquired by the GFC possibly from a horizontal gene transfer after its divergence from the other *Fusarium* species. However, the presence of IAA biosynthesis genes does not imply a functional gene; in the case of *F.oxysporum* and *F. fujikuroi,* the IAM pathway is inactive due to mutations (Tsavkelova et al., 2012).

Genes that are involved in IAA biosynthesis have been found to be conserved for example, in bacteria, IAM genes in particular were found to be highly conserved among species (Patten and Glick, 1996; Tsakelova et al., 2012). In fungal species some degree of sequence similarity have also been found among other IAA genes; for example between the aromatic

amino acid aminotransferase from *S. cerevisiae* (ARO8 and ARO9) and *U. maydis* (tam1 and tam2), which functions in the reaction of TRP to IPA (Reineke et al., 2008; Zuther et al,. 2008). In our BLASTp search some *F. graminearum* homologs were found with high alignment scores (bit-scores) and were also repeatedly found between the BLASTp results from the different species. Interestingly, the *F. graminearum* genome contained the homolog gene fg01285, related to ARO8 – aromatic amino acid aminotransferase, suggesting that the IPA pathway may be utilized for the biosynthesis of IAA. Overall this search provided many genes annotated for enzymes that could be involved in IAA biosynthesis, and most importantly revealed the absence of homolog genes for the TRP monooxygenase needed for the IAM pathway.

F. graminearum cultures that were fed the IAM compound did not produce any IAA. This could have resulted from a lack of IAM uptake or that IAM was not metabolized by the fungus. Since the genes involved in the IAM pathway were missing from *F. graminearum*, it was determined that IAM was not metabolized and not involved in the biosynthesis of IAA. When cultures were treated with TRP, IPA or TAM, a peak that could have represented IAA was found using HPLC with all three substrates; however there was some difficulty separating the compounds of TOL, IAAld and IAA using this technique. The solution was to analyze the filtrates using LC-MS/MS. Unfortunately the results from the HPLC and LC-MS/MS did not correspond and the concentrations of IAA, TOL or IAAld were higher using HPLC for detection than with LC-MS/MS. Degradation of indolic compounds overtime, found to occur in vitro, can explain this discrepancy (Grambow and Langerbeck-Schwich, 1983). This can also explain the identification of TRP and TAM

within the time course by HPLC while within the same filtrates these compounds were not found using LC-MS/MS.

The expected concentration of IAA produced by phytopathogenic fungi in culture is dependent on the concentration of the added substrate as well as the type of substrate added. For example the concentration of IAA was found to be proportional to the concentration of tryptophan added as substrate within a species (Chung and Tzeng, 2004). Among different studies, the initial concentration of intermediates added to cultures is varied for example added tryptophan can be seen to range from 1mM to 5mM (Chung and Tzeng, 2003, Maor et al., 2004, Reineke et al., 2008). The concentration of IAA produced overtime is also dependent on the various intermediates added as substrates, for example cultures treated with IAAld produced higher IAA concentration than IPA overtime (Chung and Tzeng, 2004). Most importantly the concentration of IAA biosynthesized was also found to be species dependent (Chung et Tzeng, 2003). Overall the IAA concentration produced in culture from pathogens was found to range from 2-1500µm (Porter et al., 1985, Yamada et al., 1990, Sosa-Morales et al., 1997, Robinson et al., 1998, Chung and Tzeng, 2004, Tanaka et al., 2003, 2011). The IAA concentration in this study quantified by LC-MS/MS was within this range with TAM, IPA and IAAld as substrates however no IAA was identified with TRP as substrate and for all treatments the HPLC results did not coincide with LC-MS/MS as mentioned earlier. Even with these difficulties some important information was able to be extrapolated from the LC-MS/MS results.

Although it was found that IPA non-enzymatically converted to IAA, the presence of TOL within these filtrates showed that IPA was metabolized by *F. graminearum*. Percival et al. (1973), analyzed the effect of IAA on the enzyme TOL-oxidase which catalyzes the oxidation of TOL to form IAAld, and found that at high concentrations, IAA had an inhibitory effect on this enzyme (Percival et al., 1973). This suggested that this TOL-oxidase may have a regulatory role in IAA biosynthesis and that TOL would be an important by-product in the formation of IAA. In the filtrates that were treated with IPA, non-enzymatic conversion of IPA to IAA could have had a role in the higher concentration of IAA seen at the 6hr time point as such the IAA levels could have contributed to the formation of TOL seen over time. However in IAAld treated cultures the level of IAA increases while the levels of TOL do not significantly change. This suggests that when IAAld is the substrate, IAA is efficiently formed as seen by the high concentration at 6 hrs. The significance of IAA/TOL ratio when IAAld is the substrate will be discussed later. When TAM was added to cultures, IAA was biosynthesized but TOL was not. This suggests that the TAM pathway utilizes another path to IAA such as IAN to form IAA in the last reaction. However during the differential gene expression study, the IAAld-dehydrogenase gene fg02296 in particular was very highly up-regulated with TAM-treated cultures and so IAAld would need to be part of the pathway utilized to form IAA. TOL may also be a by-product more specific to the IPA pathway and thus would reach detectable levels under IPA conditions only. Further analysis of the intermediates present in the filtrates of TAM-treated cultures would need to be done to clarify.

It is not surprising that F. graminearum was able to metabolize both IPA and TAM for the biosynthesis of IAA. As mentioned earlier, more than one pathway is often found within a microbial or plant species (reviewed by Spaepen et al., 2007). Among bacterial, fungal and plant species, all three pathways have been identified, although IAM genes, which are more commonly found in bacteria have been identified in the Colletotrichum and Fusarium genus (Robinson et al., 1998, Chung et al., 2003, Tsavkelova et al., 2012). However IPA and TAM remain the most common pathways in the biosynthesis of IAA for fungal species.

In the differential gene expression study, many genes were up-regulated as a result of the treatments of TRP, IAAld and TAM, in particular genes that are involved in transmembrane transport. The transport system for IAA in plant is quite well established, the flux of auxin into plant cells can either result from membrane diffusion of the protonated form (IAAH), or by the action of proton-driven auxin influx carrier system of the anionic form (IAA-). The formation of IAA within plants involves a complex system of transport inhibition proteins and ubiquitination of repressor proteins activating gene expression (reviewed by Woodward and Bartel, 2005). The anionic form is found within the plant cell and need a carrier-mediated transport to exit (Delbarre et al., 1996; Spaepen et al., 2007). IAA influx and efflux facilitators such as AUX1, pGlycoprotein/multiple drug resistance (PGP/MDR; ABC transporter) and PIN proteins have been identified in Arabidopsis. Homology for these transporters between plants and bacteria has been found (reviewed by Spaepen et al., 2007). Interestingly the gene fg12170, a probable ABC1 transport protein, was found up-regulated 98 folds in TAM treated cultures. As well, several MDR proteins and monocarboxylate transporter proteins that could be involved in IAA efflux were found up-regulated in the

treatments with TRP, TAM and IAAld. The specificity of these transport proteins would need to be characterized to understand and determine their functional role. In particular among the genes that were shared between TRP and TAM was fg02296. This gene was up-regulated with TAM and down-regulated with TRP. This could suggest a level of IAA regulation at the indole-3-aldehyde dehydrogenase reaction from IAAld to IAA at least in the TAM-treated cultures.

RT-qPCR was performed on list of candidate genes compiled from the gene expression results and the bioinformatic search. An earlier timeline was chosen due to the high level of fg02296 expression in the TAM results which lead to suggest that the 6hr timepoint may have been too late to find up-regulated IAA genes from cultures treated with TRP and IAAld as well as genes involved in earlier reactions within the pathways. With RT-qPCR up-regulated genes were found for cultures treated with TRP and IPA suggesting that the IPA pathway was utilized. As well some genes were found up-regulated at 6hr in cultures that were treated with TRP that were not found up-regulated in the microarray, however the level of differential expression is low and this may attest to the level of sensitivity of RT-qPCR. On the other hand the gene fg11492 found up-regulated in the microarray was not found up-regulated by RT-qPCR. Unfortunately some differences in the level of IAA produced were found between experiments when working with *F.graminearum* which could explain this discrepancy. Overall when TAM was the added substrate, it produced the most conclusive results. Genes involved in the conversion of TAM to N-TAM and of IAAld to IAA were found highly regulated.

Interestingly results from a *F.graminearum* gene expression study performed on infected wheat heads collected at 2dpi and 4dpi, showed many up-regulated genes (≤4 fold) that could have possible roles in IAA biosynthesis (unpublished, provided by Linda Harris, ECORC, Agriculture Canada). These genes included: related to ARO8 – aromatic amino acid aminotransferase, probable lactate dehydrogenase, related to flavin-containing monooxygenase, probable cytochrome P450 monooxygenase, related to monoamine oxidase, probable pyruvate dehydrogenase, related to aldehyde reductase and several probable aldehyde dehydrogenases. Some *F. graminearum* genes were found in common between the list of up-regulated genes at 2dpi from the infected wheat and those of the treated cultures from our study. In particular from the IAAld-treated cultures 2 out of the 8 up-regulated genes were shared, from the TAM-treated cultures 20 out of the 61 upregulated genes were shared and from the TRP-treated cultures, 40 out of the 153 up-regulated genes were shared and 34 out of the 54 down-regulated genes were shared. In particular from the candidate genes presented in Fig. 15 (section 3.3.1), the following were found up-regulated in *F. graminaerum* infected wheat at 2dpi: fg01285 (TRP aminotransferase), fg05295, fg08083 (TRP decarboxylase), fg00712, fg03717 (Flavin mono-oxygenase-like enzymes), fg09834 (IPA decarboxylase) and fg00979, fg02160, fg02273, fg05831, fg02392, fg02296, fg04196 (IAAld dehydrogenase).

In a study that looked at the growth and behavior of *F. graminearum* during infection, it was found that within the first 2dpi, *F. graminearum* behaved like a biotroph, little mycotoxins were produced, no cell death occurred at the advancing infection front and only intercellular colonisation took place (Bushnell et al., 2003, Brown et al., 2010). Similar stages during

infection were also found in the rice pathogen, *M. oryzae* (Tanaka et al., 2011). The role of IAA in pathogenesis is complex; however, for most pathogens when it is involved during infection, it creates an imbalance in the host plant which is beneficial for the invading organism. The role of IAA in *F. graminearum* pathogenesis is still elusive; however this information leads to suggest a possible role for IAA in the initial stage of infection.

The gene fg02296, probable aldehyde dehydrogenase was chosen to be silenced based on its highest expression level in auxin producing conditions. The location of this enzyme in the IAA biosynthesis pathways was ideal to generate an IAA mutant strain since it includes both TAM and IPA pathways. Mutants deficient in tryptophan-dependent IAA biosynthesis were also initially generated in *U. maydis* and *S. cerevisiea* by replacing indole-3-acetaldehyde dehydrogenase genes (Basse et al., 1996, Reineke et al., 2008, Rao et al., 2010). Basse et al., 1996, identified two indole-3-acetaldehyde dehydrogenase enzymes (Iad1 and Iad2) in the *U. maydis* genome with the ability to convert IAAld to IAA. A Δ*iad1* mutant strain was created by gene replacement and showed a 100-fold reduction in the conversion of IAAld to IAA compared with WT (Basse et al., 1996). A Δ*iad1*Δ*iad2* mutant strain was later created by Reineke et al., 2008, which showed a complete block in the conversion of IAAld to IAA. To assess role of indole-3-pyruvaic acid in the biosynthesis of IAA by *U. maydis*, a mutant strain was created by replacing two identified aromatic amino acid aminotransferase genes (tam1 and tam2) in the *iad1*Δ*iad2* mutant strain. The *iad1*Δ*iad2* Δ*tam1*Δ*tam2* mutant showed a significant reduction in the conversion of TRP to IAA (Reineke et al., 2008). These results demonstrated that IPA is able to be utilised by *U. maydis* for the biosynthesis of IAA and ultimately provided information on the role of IAA during infection.

Unfortunately the transformation was unsuccessful in producing a mutant strain deficient in IAA. The fitness of *fg02296*Δ strain was not effect by the replacement of the gene. The levels of IAA were not significantly different between the -ve transformant and mutant strain with either TAM or IAAld as substrates. In particular when cultures are treated with IAAld, the level of TOL exceeds the level IAA produced. However the amount of TOL in comparison to IAA produced was found to be species and even strain specific as well the ratio can vary under different aerobic conditions (Basse et al., 1996, Furukawa et al., 1996). In IAA mutant strains, the level of TOL is often increased from the WT strain since the replaced gene is often indole-3-acetaldehyde dehydrogenase blocking the conversion of IAAld to IAA (Basse et al., 1996, Reineke et al., 2008). Although the IAA and the TOL abundance found in the WT was in the same range as in the –ve transformant, the values should have been more similar. Differences between experiments could explain this discrepancy since the WT was treated with IAAld in a different experiments although under the same culture conditions.

4.1 Concluding Remarks

Overall the results of this study have provided some pertinent information on the biosynthesis of IAA in *F. graminearum* in culture. This study has created a good platform for future projects focused on IAA and its potential involvement in pathogenesis. The role of IAA produced by *F. graminearum* remains elusive; additional genetic work and the creation of a non-producing IAA mutant strain would be needed to determine if IAA is part of the infection strategy of this species to render the host more susceptible and promote

pathogenesis. This information would contribute to the understanding of the involvement of IAA as part of the complex plant-microbe interactions during infection.

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APPENDIX

Table A1. Primers used for RT-qPCR. The primers were stored at 100µM and diluted to 10µM for use.

Accession #	Name	Sequence
fg01285	tamA-c-3F	AGCGAAGTATCGTCGATATAGA
fg01285	tamA-c-3R	GCATCTGCTACACCTGAGAATATG
fg07188	tamB-b-2F	CATGGATATTGACGGCAGAG
fg07188	tamB-b-2R	CTCGGCAATCCTTGTAAGAC
fg05295	Decarbox-3F	CTTCTCACCACCTTTGACTG
fg05295	Decarbox-3R	CAGTCGCGGTAATCAGTAAC
fg03391	Decarbox-3F	GGGAAATAATCAGTCCAGCG
fg03391	Decarbox-3R	CAACTCTACCTCGCACTTTC
fg09834	Decarbox-1F	GGAATCTGATCTCCTTCTCACC
fg09834	Decarbox-1R	GTATGTGGAGTATCGGACCTTG
fg00712	Flavmonox-2F	GAGAGTGCCAAAGATCAAGC
fg00712	Flavmonox-2R	CCTCTTCCACTCCTCATAATCG
fg03417	Flavmonox-2F	CTCCATTTCGACATCACACC
fg03417	Flavmonox-2R	GAGTTATCACCCACTCATCTCC
fg07189	Flavmonox-1F	GATATCAGGCATCCTACCC
fg07189	Flavmonox-1R	CTCCTTCTCGCTTCTCTAGTTC
fg11492	Flavmonox-3F	GTGGGAACTAGATAGAGAGAGC
fg11492	Flavmonox-3R	CCTGAAATCTGGAAGAACCC
fg00979	iadA-b-2F	GGTGTCTTCAACCTTGTCTC
fg00979	iadA-b-2R	CAATGACGGTAGAACCAGTG
fg02160	iadB-c-3F	CAAGTTCTCCACTGACGAGGA

fg02160	iadB-c-3R	CGCGACCAATGCCTGAA
fg02273	iadC-b-2F	GGTGTATGGACAAGTCATCG
fg02273	iadC-b-2R	AAGGTAGAGGATTGAGAGGG
fg05831	Aldehydro-3F	GGTCCTGTTATCAGCTCTACTC
fg05831	Aldehydro-3R	CCGTGCAAGAGTTCAATTCC
fg02392	iadD-a-1F	GTCATCAAGACAGAAGAGGAGG
fg02392	iadD-a-1R	CCT ACG TAA CCA GAG TTG AGC
fg02296	Aldehydro-3F	GTTTAGCACCGAAGAAGAGG
fg02296	Aldehydro-3R	GTAACTTGTCCACTCTCAAGC
fg09530	bTUB-a-1F	GTTGATCTCCAAGATCCGTG
fg09530	bTUB-a-1R	CATGCAAATGTCGTAGAGGG
fg08811	EF1-b-2F	CCTCCAGGATGTCTACAAGA
fg08811	EF1-b-2R	CTCAACGGACTTGACTTCAG
fg06257	GAPDH -2F2	TGACTTGACTGTTCGCCTCGAGAA
fg06257	GAPDH -2R2	ATGGAGGAGTTGGTGTTGCCGTTA

Table A2. Primers used in USER friendly cloning and confirmation of transgenic strains.

Name	Sequence
fg02296.1000down.F	GGCATTAAUTTGTTCCTGGGTCGTGAACTGAGA
fg02296.1000down.R	GGTCTTAAUTGATCTTTGAGGCCAACGGACAGA
fg02296.1000up.F	GGGTTTAAUAACTTGGAGCTATCCACTGGGCTT
fg02296.1000up.R	GGACTTAAUACTGCGGCTGGGTCATATGAATTG
hygromycin F- RVR	AGATATCATTTGTGTACGCCCGACAG
hygromycin R- Gentup	ATTCTACCCAAGCATCGATAT
fg02296 1F	GGTCAATCTCTACACATCCCTC
fg02296 3R	GTAACTTGTCCACTCTCAAGC
fg08509 seq1	AGGGTTCGTACGCTTGCC
fg08509 seq2	CGATGTGCTTCTGCCTGG
geneticin F2	GAAGCACTTGTCCAGGGAC
geneticin R2	GACCGACCTGTCCGGTGCCC
fg09530 bTUB-a-1F	GTTGATCTCCAAGATCCGTG
fg09530 bTUB-b-2R	GAACTCCATCTCATCCATACCC

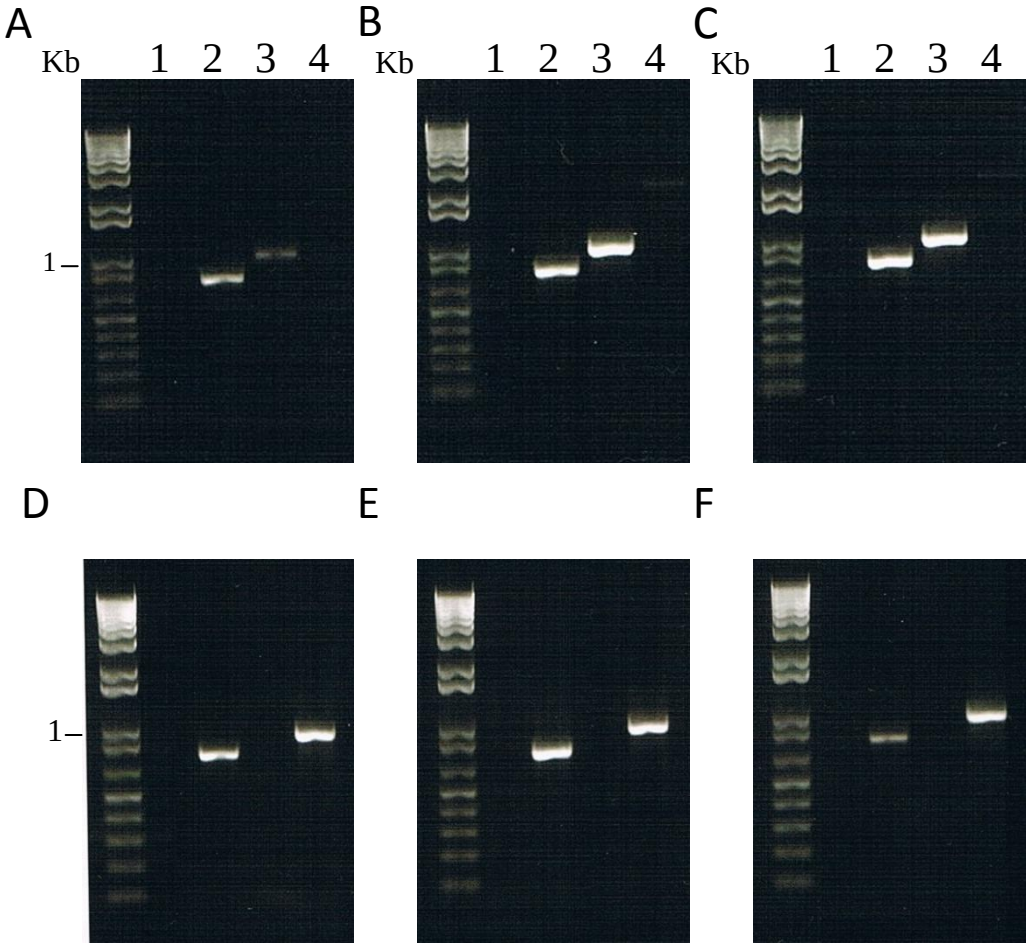

Fig. A1. Gel electrophoresis for the confirmation of fg02296Δ transgenic strains and negative transformants. Single spore colonies of fg02296Δ transgenic strains, **A**=1**, B**=2**, C**=3. Single spore colonies of negative transformants, **D**=4, **E**=5, **F**=6. 1-negative control, 2-β-tubulin (774bp), 3-hygromycin (944), 4-fg02296 (937bp).

Table A3. Screening of the annotated *F. graminearum* genome for putative candidate genes involved in IAA biosynthesis by keyword search in the MIPS database.

Pathway	Reaction	Enzyme	Accession	Description
IAM	TRP --> IAM	monooxygenase	fg00759	related to monooxygenase
			fg02138	probable monooxygenase
			fg03531	monooxygenase
			fg03548	monooxygenase
			fg04720	related to monooxygenase
			fg05683	related to monooxygenase
			fg07003	related to monooxygenase
			fg11298	related to monooxygenase
IPA	TRP --> IPA	Aminotransferase	fg01285	related to ARO8 - aromatic amino acid aminotransferase I
			fg07188	related to aromatic amino acid aminotransferase I
	ILA --> IPA	Lactate dehydrogenase	fg00990	related to dehydrogenase/reductase
			fg00145	probable L-lactate dehydrogenase (cytochrome)
			fg01027	related to L-lactate dehydrogenase
			fg01531	probable CYB2 - lactate dehydrogenase cytochrome b2
			fg01812	probable CYB2 - lactate dehydrogenase cytochrome b2
			fg04988	related to L-lactate dehydrogenase (cytochrome)
			fg05328	probable L-lactate dehydrogenase (cytochrome)
			fg10444	related to L-lactate dehydrogenase
			fg16220	probable L-lactate dehydrogenase (cytochrome)
			fg16565	related to CYB2 - Lactate dehydrogenase cytochrome b2
	IPA --> ILA	Pyruvate reductase	fg00078	related to aldo/keto reductase
			fg00990	related to dehydrogenase/reductase
			fg01586	related to oxidoreductase
			fg03186	related to oxidoreductase
			fg03224	reductase
			fg03309	related to oxidoreductase
			fg03818	related to reductases
			fg03819	related to reductases
			fg06240	related to aldo-keto reductase YPR1
			fg08283	related to reductases
			fg11399	related to oxidoreductase

			fg12331	related to oxidoreductase
			fg12488	related to reductases
			fg15719	related to oxidoreductase
			fg16205	related to oxidoreductase
	IPA --> IAAld	Pyruvate decarboxylase	fg09834	probable pyruvate decarboxylase
			fg10446	related to PDC5 - pyruvate decarboxylase, isozyme 2
			fg13946	related to pyruvate decarboxylase
			fg16351	probable PDC1 - pyruvate decarboxylase, isozyme 1
TAM	TRP --> TAM	L-amino-acid decarboxylase	fg03391	related to aromatic-L-amino-acid decarboxylase
			fg05295	related to aromatic-L-amino-acid decarboxylase
	TAM --> IAAld	Monoamine oxidase	fg01758	related to monoamine oxidase N
			fg04621	related to monoamine oxidase N
			fg12647	probable monoamine oxidase N
			fg05272	related to amine oxidase [flavin-containing] B
	TAM --> N-TAM	Flavin monooxygenase	fg00712	related to flavin-containing monooxygenase
			fg03417	related to flavin-containing monooxygenase
			fg07189	related to flavin-containing monooxygenase
			fg11492	related to flavin-containing monooxygenase
			fg13822	related to flavin-containing monooxygenase
			fg11010	related to flavin-containing monooxygenase
			fg01741	related to flavin-containing monooxygenase
			fg07685	related to flavin-containing monooxygenase
			fg11974	probable flavin-containing monooxygenase
			fg13875	related to flavin-containing monooxygenase
			fg17444	related to flavin-containing monooxygenase
	IAN --> IAA	Nitrilase	fg00051	related to aliphatic nitrilase
			fg00821	probable nitrilase
			fg01698	related to nitrilases, putative pseudogene
			fg05805	related to aliphatic nitrilase
			fg06480	related to aliphatic nitrilase
			fg10250	probable nitrilase (NIT3)
			fg11357	related to aliphatic nitrilase
IAOx	TRP --> IAOx	Cytochrome P450 monooxygenase	fg00071	TRI1 cytochrome P450 monooxygenase
			fg01868	related to cytochrome P450 monooxygenase (lovA)
			fg02117	related to cytochrome P450 monooxygenase (lovA)
			fg02672	probable cytochrome P450 monooxygenase (lovA)

			fg04717	probable cytochrome P450 monooxygenase (lovA)
			fg08187	related to cytochrome P450 monooxygenase (lovA)
			fg13259	related to cytochrome P450 monooxygenase
			fg13259	related to cytochrome P450 monooxygenase
			fg17400	related to cytochrome P450 monooxygenase
			fg17486	probable cytochrome P450 monooxygenase (lovA)
Non-specific	IAM --> IAA	Amidohydrolase	fg12069	related to amidohydrolase AmhX
			fg06613	related to amidohydrolase AmhX
			fg03856	related to amidohydrolase AmhX
			fg02121	related to amidohydrolase AmhX
			fg02305	related to amidohydrolase AmhX
			fg01713	related to amidohydrolase family protein
	IAAld --> Tol	Aldehyde reductase	fg01476	related to aldehyde reductase II
			fg01742	related to aflatoxin B1 aldehyde reductase
			fg02751	probable aldehyde reductase 6
			fg08941	probable aldehyde reductase
			fg11217	related to aldehyde reductase II
	Tol --> IAAld	Alcohol dehydrogenase	fg00177	related to short-chain alcohol dehydrogenase
			fg00231	related to alcohol dehydrogenase, class C
			fg00236	related to alcohol dehydrogenase
			fg00578	related to short-chain alcohol dehydrogenase
			fg01686	related to alcohol dehydrogenase, class C
			fg01794	related to short-chain alcohol dehydrogenase
			fg01809	related to aryl-alcohol dehydrogenases
			fg02034	probable alcohol dehydrogenase I - ADH1
			fg02165	related to ADH7- NADP(H)-dependent alcohol dehydrogenase
			fg02266	related to short-chain alcohol dehydrogenase
			fg02291	related to ADH5 - alcohol dehydrogenase V
			fg03041	related to NADP-dependent alcohol dehydrogenase
			fg03935	related to short-chain alcohol dehydrogenase
			fg06202	related to alcohol dehydrogenase
			fg06518	related to short-chain alcohol dehydrogenase
			fg06598	related to alcohol dehydrogenase I - ADH1
			fg06619	related to alcohol dehydrogenase
			fg07318	related to ADH5 - alcohol dehydrogenase V
			fg07987	related to alcohol dehydrogenase II

		fg08065	related to aryl-alcohol dehydrogenases
		fg08066	related to ADH3 - alcohol dehydrogenase III
		fg08816	related to short-chain alcohol dehydrogenase
		fg08980	probable alcohol dehydrogenase (NADP+)
		fg09028	related to short-chain alcohol dehydrogenase
		fg09088	related to peroxisomal short-chain alcohol dehydrogenase
		fg09245	related to ADH4 - alcohol dehydrogenase IV
		fg09364	related to ALCOHOL DEHYDROGENASE I - ADH1
		fg09882	related to short-chain alcohol dehydrogenase
		fg10200	probable alcohol dehydrogenase (FDH1)
		fg10855	probable ALCOHOL DEHYDROGENASE I - ADH1
		fg11022	related to short-chain alcohol dehydrogenase
		fg11387	related to zinc-containing long-chain alcohol dehydrogenase
		fg11556	related to short-chain alcohol dehydrogenase
		fg11557	related to alcohol dehydrogenase
		fg12365	related to aldehyde-alcohol dehydrogenase
		fg12368	related to ADH2 - alcohol dehydrogenase II
		fg12386	related to ADH3 - alcohol dehydrogenase III
		fg16840	related to ADH2 - Alcohol dehydrogenase II
		fg17564	probable alcohol dehydrogenase
IAAld --> IAA	Aldehyde dehydrogenase	fg00139	probable aldehyde dehydrogenase
		fg00718	related to aldehyde dehydrogenase
		fg00979	probable aldehyde dehydrogenase
		fg01759	probable aldehyde dehydrogenase
		fg02160	probable aldehyde dehydrogenase
		fg02220	related to aldehyde dehydrogenase (NAD+), mitochondrial
		fg02273	probable aldehyde dehydrogenase
		fg02296	probable aldehyde dehydrogenase
		fg02392	probable aldehyde dehydrogenase
		fg04194	probable aldehyde dehydrogenase
		fg04670	related to aldehyde dehydrogenase
		fg05375	related to aldehyde dehydrogenase
		fg05831	probable aldehyde dehydrogenase
		fg09762	related to aldehyde dehydrogenase
		fg09960	related to aldehyde dehydrogenase [NAD(P)]
		fg11542	related to aldehyde dehydrogenase (NAD+), mitochondrial

		fg12120	related to aldehyde dehydrogenase
		fg13865	related to aldehyde dehydrogenase
		fg16899	related to aldehyde dehydrogenase (NAD+), mitochondrial
		fg17538	probable aldehyde dehydrogenase

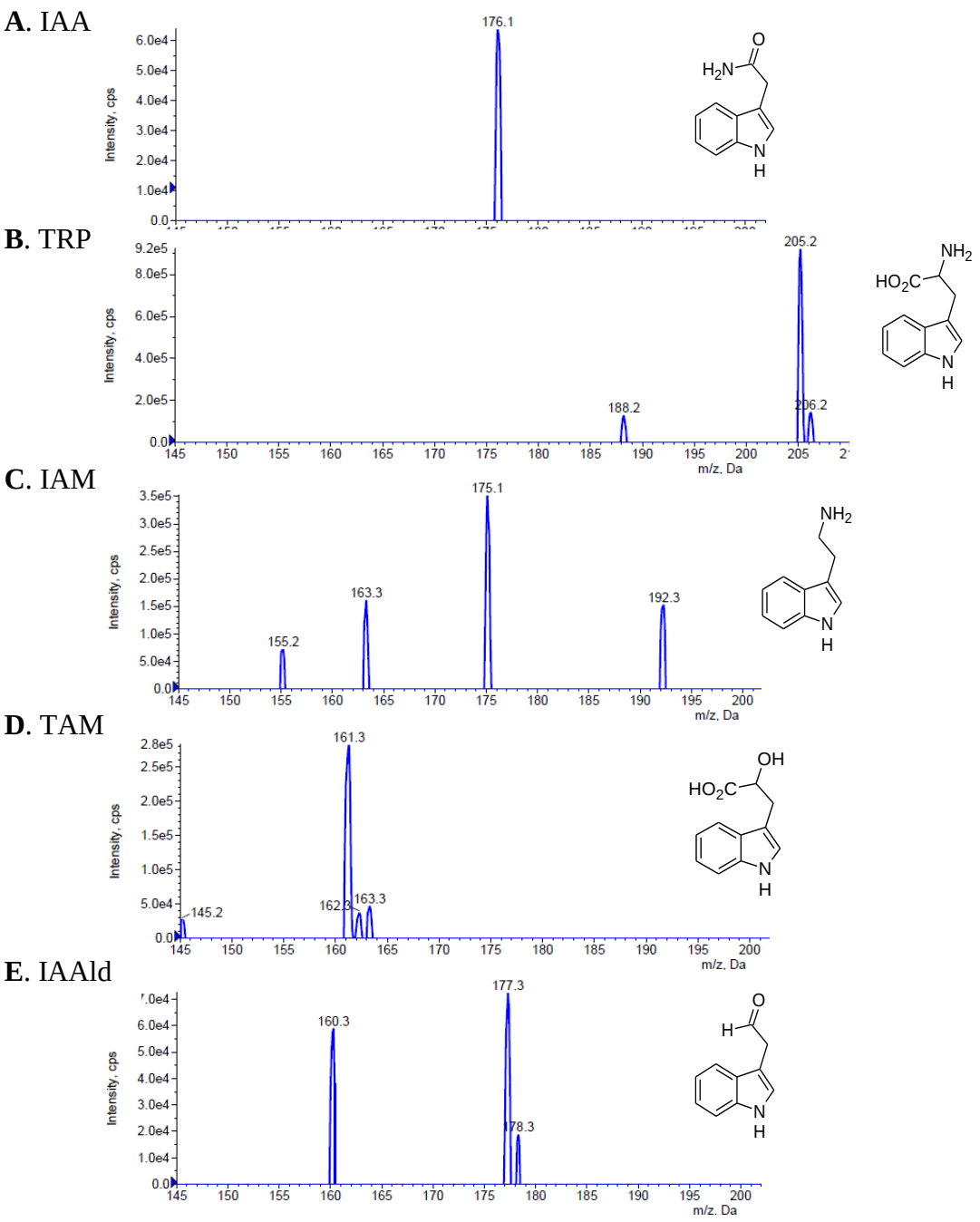

Fig. A2. Mass spectrophotometer chromatograms of compounds involved in IAA biosynthesis. The respective mass for the protonated compounds are; **A**. IAA, 176; **B**. TRP, 205; **C**. IAM, 175; **D**. TAM, 161; **E**. IAAld, 160.

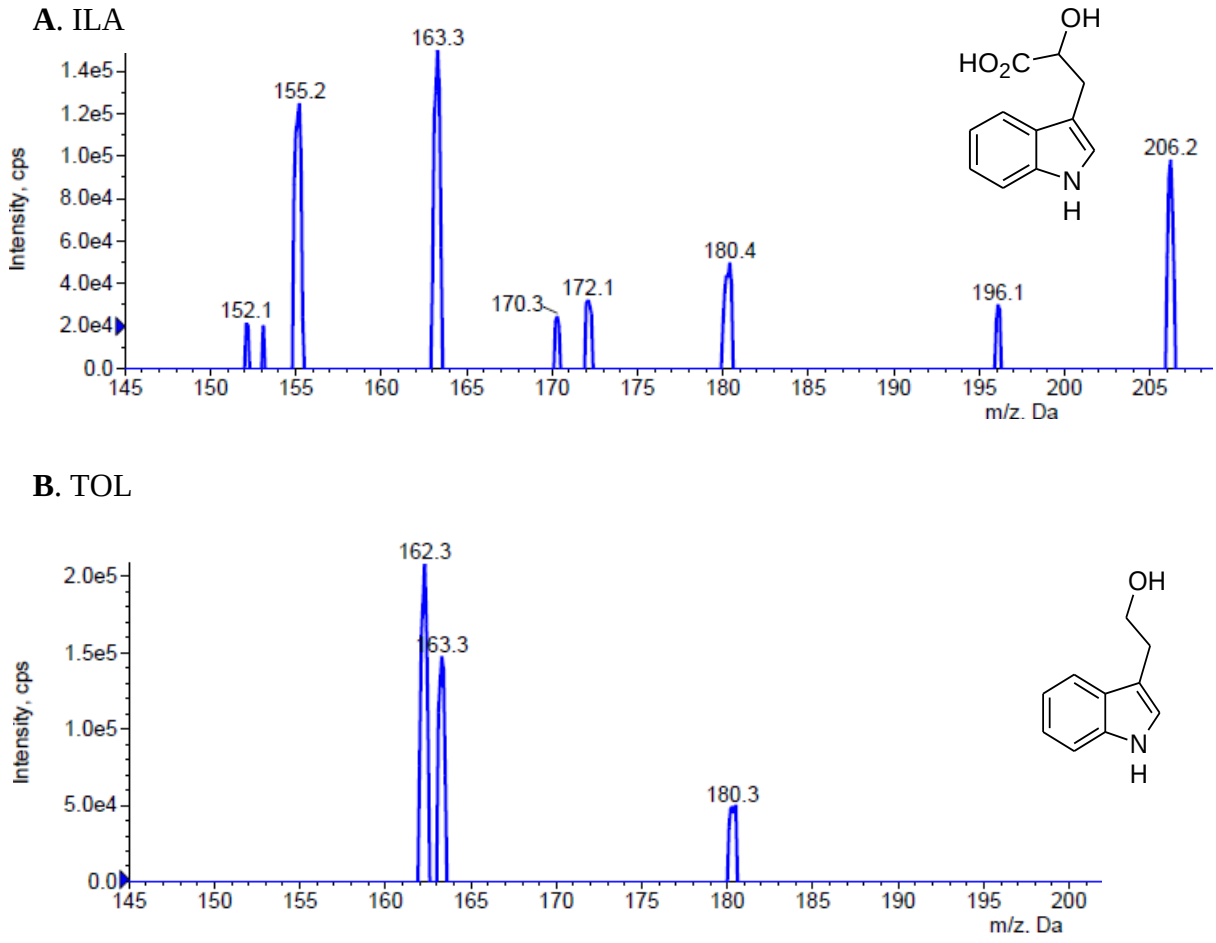

Fig. A3. Mass spectrophotometer chromatograms of IAA biosynthesis byproducts. The respective mass for the protonated by products are; **A**. ILA, 206; **B**. TOL, 162.