

CRISPR-Cas9 Mediated Gene Editing of Secondary Metabolite Gene Clusters in *Fusarium graminearum*

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Abstract

Fusarium graminearum is responsible for causing Fusarium head blight in cereals and maize imposing a significant impact in Canadian agriculture. While a handful of secondary metabolites produced by *F. graminearum* are recognized as contributors to disease virulence, the functions of numerous molecular products arising from biosynthetic gene clusters expressed during infection remain undiscovered. Presented here are the results of CRISPR-Cas9 mediated gene-deletion experiments disrupting core biosynthetic genes from four biosynthetic gene clusters with reported *in-planta* transcription: C08, C16, C13 and C70. Both wheat head infection assays and coleoptile infection assays were used to evaluate the pathology phenotypes of transformant strains illustrating potential links between C16 and pathogenicity. Culture medium screening experiments using transformant strains were profiled by UHPLC-HRMS and targeted MS2 experiments to confirm the associated secondary metabolite products and attempt to identify unknown secondary metabolites of the biosynthetic gene clusters. While C08 secondary metabolite remained elusive, confirmation of C16 secondary metabolites led to hypotheses regarding their potential connections to the inhibition of plant immune response and untargeted secondary metabolite profiling of the C13/C70 transformant strains suggests that this BGC may have significant implications for global secondary metabolite production.

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List of Abbreviations

15-ADON	15-Acetyldeoxynivalenol
3 ANX	7- α Hydroxy, 15-Deacetylcalonectrin
3-ADON	3-Acetyldeoxynivalenol
A	Adenylation
ACP/PP	Acyl Carrier Protein
AT	Acyltransferase
BGC	Biosynthetic Gene Cluster
C	Condensation
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DH	Dehydratase
DMAPP	Dimethylallyl Diphosphate
DON	Deoxynivalenol
DPI	Days Post-Inoculation
FDDP	Fungal Decalin-Containing Diterpenoid Pyrone
FHB	Fusarium Head Blight
FPP	Farnesyl Diphosphate
GGPP	Geranylgeranyl Pyrophosphate
GOI	Gene of Interest
GPP	Geranyl Diphosphate
HPI	Hours Post-Inoculation
HRMS	High Resolution Mass Spectrometry
IPP	Isopentyl Diphosphate
KR	Ketoreductase
KS	β -Ketoacyl Synthase
KW	Kruskal Wallis
MT	Methyltransferase
NMR	Nuclear Magnetic Resonance
NRP	Non-ribosomal Peptide
NRPS	Non-ribosomal Peptide Synthases
OE	Over-Expressor
PAM	Protospacer Adjacent Motif
PAMP	Pathogen Associated Molecular Patterns
PKS	Polyketide synthases

POI	Point of Inoculation
PTI	Pathogen Triggered Immunity
QE	QExactive
RNP	Ribonuclear Protein
SM	Secondary Metabolite
T	Thiolation
TC	Terpene Cyclase
TE	Thioesterase
TF	Transcription Factor
TS	Terpene Synthases
UPLC	Ultra Performance Liquid Chromatography
WMW	Wilcoxon Mann Whitney
WT	Wildtype
XIC	Extracted Ion Chromatogram

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Chapter 1 Introduction

1.1 Fusarium Head Blight and Economic Importance in Canada

Fusarium head blight (FHB), also known as scab, is a fungal disease globally effecting small grain cereal crops, the most notable being wheat, barley and corn (McMullen et al., 2012; Fernando et al., 2021). In Canada, FHB is caused by several *Fusarium* species including: *F. graminearum*, *F. avenaceum*, *F. poae*, *F. sporotrichoides*, *F. culmorum*; with *Fusarium graminearum* imposing the greatest etiological impact in Canadian agriculture, as determined by isolation frequency and detrimental effects on grain quality and yield (Dexter et al., 1996; McMullen et al., 1997; Paul et al., 2010; Chen et al., 2019).

Accompanying the loss in revenue to agricultural producers due to decreased yields, *F. graminearum* also contaminates grains with mycotoxins, the most notable of which being deoxynivalenol (DON) and its associated derivatives which pose serious health concerns for both animals and humans (Health Canada, 2020). As a result, economic losses are compounded, deriving from the reduced grain grade or rejected sale/export due to stringent regulation of permissible DON levels nationally and internationally in grain destined for human consumption and animal feeds (Dean et al., 2012). FHB occurrences in wheat caused an estimated \$3 billion USD in losses across Canada and the United States during the 1990s (Windels 2000). In addition, epidemic years of FHB continue to occur, with 2016 being one of the worst on record for Canada. This epidemic alone resulted in an estimated \$1 billion CAD in losses (Chin et al., 2023).

1.2 *Fusarium graminearum* Disease Cycle

Fusarium graminearum overwinters saprophytically on crop residues from plants that were infected from the previous year. When weather conditions become wet, warm, and humid, *F. graminearum* ascomatal perithecia (the sexual stage of the fungus) forcibly discharge sexual ascospores into the air where they are spread by wind, rain and insects and act as the primary inoculum of infection in wheat heads (Trail et al., 2002; Leplat et al., 2013). In addition to ascospores, asexual macroconidia also impact short distance distribution via splash dispersal (Figure 1) (Trail, 2013).

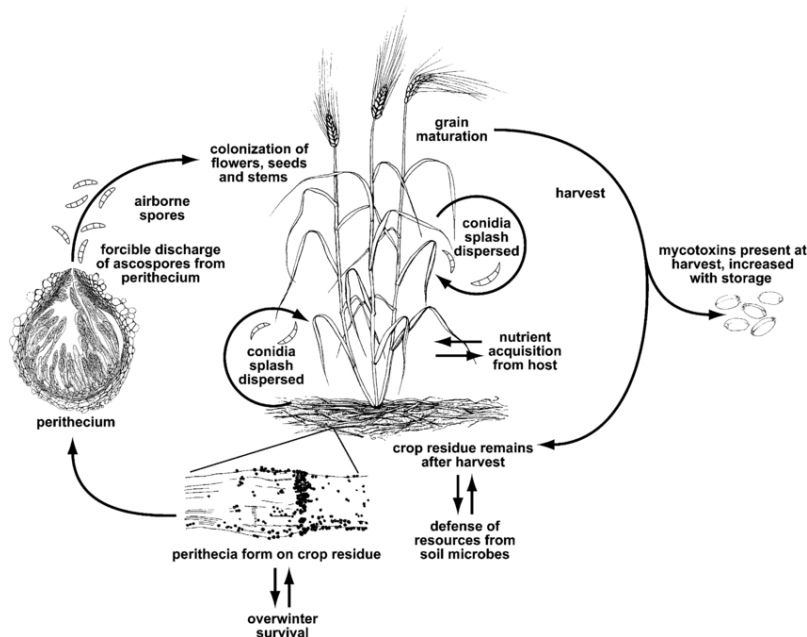


Figure 1: Overview of the *Fusarium graminearum* disease cycle (Trail, 2009)

The infection cycle begins when conidia or ascospores come into contact with plant tissues such as florets and extruded anthers during anthesis of host crops, which is thought to be the primary site of infection. Upon contact, spores germinate and extend elongating hyphae across the plant surface. Hyphae that elongate on the exterior surfaces of the glumes and florets gain internal access into the host through susceptible sites such as stomata or exposed anthers. Non-direct

entry of plant tissues is also thought to occur through the formation of lobed hyphal structures (or cushions) that are also thought to serve as a mechanism of epidermal cell penetration (Pritsch et al., 2000; Leonard and Bushnell, 2003). Once inside plant tissues, the fungus spreads from spikelet to spikelet via vascular bundles (xylem, pith) in the rachis. After a brief biotrophic phase, once fungal hyphae are established in host cells, the fungus transitions into a necrotrophic stage of growth in which internal colonization is hastened - leading to host cell death and bleaching of tissue.

1.3 *Fusarium graminearum* Secondary Metabolite Gene Clusters

The *F. graminearum* genome is predicted to contain 76 secondary metabolite or biosynthetic gene clusters (BGCs). Sieber et al., 2014 identified 67 of these gene clusters (denoted C01-C67) through prediction algorithms searching for genomic regions enriched in genes with predicted secondary metabolism related enzymatic functions. Of these, 20 gene clusters were demonstrated to exhibit strong expression correlation *in-planta*. The additional nine gene clusters were later identified by Adpressa et al., 2019 using a metabolomics guided approach in which they used a gene deletion strain to remove repressive histone modifications, thus promoting gene cluster transcription. Of the 76 identified clusters described, only 21 are associated with known biosynthetic products (Table 1) suggesting that the chemical potential of *F. graminearum* is much greater than currently understood. Many *F. graminearum* secondary metabolite gene clusters, including those described by Sieber and Adpressa, contain genes encoding for one or more of three classes of core biosynthetic enzymes: non-ribosomal peptide synthases (NRPSs), polyketide synthases (PKSs) and terpene synthases (TSs).

Table 1: List of 21 *F. graminearum* BGC identified by Sieber et al., 2014 and Adpressa et al., 2019 with known SM products.

BGC	Core Biosynthetic Gene(s)	Associated SM	Function	Reference
C02	NRPS8, NPS-Like FAS	Gramillin A, B	Host specific virulence factor in maize	Bahadoor et al., 2018
C13	PKS12	Aurofusarin	Red Pigment	Malz et al., 2005
C15	PKS4, PKS13, NRPS15	Zearalenone	Powerful xenoestrogen in mammals	Kim et al., 2005
C16	PKS15	FDDP D/E, Gramiketide A/B	Unknown	Tsukada et al., 2020; Seidl et al., 2022
C18	PKS14	Orcinol, Orsellinic acid		Jorgensen et al., 2014
C21	NRPS6	Triacetylfulvarin		Oide et al., 2006
C23	TS	Trichothecene	Virulence factor, mycotoxin	Proctor et al., 2005
C24	STC4	Fusariumdiene, Fusagramineol		Bian et al., 2018
C26	PKS8	Gibepyrone, Prolipyrone		Westphal et al., 2018
C28	DTC1	Carotenoids		Jin et al., 2010
C33	NRPS2	Ferricrocin	Siderophore	Tobiasen et al., 2007
C42	PKS10	Fusarins (fusarin A, C, D)		Gaffoor et al., 2005
C47	PKS6, NRPS7	Fusaristatin A		Sørensen et al., 2014
C48	STC5, NPS-like	<i>Tricinolonoic acid</i> , <i>Tricinonoic acid</i> , <i>Tricindiol</i> , <i>Tricinolone</i>		Adpressa et al., 2019
C49	N/A	Butenolide		Harris et al., 2007
C53	PKS3	perithecial pigment, Bostrycoidins		Frandsen et al., 2016
C59	STC9	Culmorin	Antifungal, phytotoxic at high concentrations	McCormick et al., 2010
C60	NRPS-like, PKS9	Fusarielin		Sorensen et al., 2012
C63	NRPS1	Malonichrome	Siderophore	Oide et al., 2014
C64	NRPS5, NRPS9	Fusaotaxin	Host specific virulence factor in wheat	Jia et al., 2019
C66	NRPS14	Chrysogine	Yellow pigment	Wollenberg et al., 2017
C70	NRPS4	Fusahexin	Potential association with membrane hydrophobicity	Westphal et al., 2021

1.3.1 Non-Ribosomal Peptide Synthases

Non-ribosomal peptide synthetases (NRPS) are multienzyme complexes capable of catalyzing the formation of amide bonds between amino acids to form non-ribosomal peptide (NRP) products (Süssmuth & Mainz, 2017). The *F. graminearum* genome contains 19 identified NRPS genes as well as a number of NRPS-like genes, some of which are associated with the production of several secondary metabolites including fusaoctaxins, gramillins, fusahexin, ferricrocin and malonichrome. NRP synthesis is modular and involves a series of repeating steps by three core catalytic domains of the NRPS. These domains are the adenylation (A), thiolation (T) and condensation (C) domain, typically found in a C-A-T order where each module incorporates a single amino acid – or in some cases other carboxylic acids (Kleinkauf and von Döhren, 1990). The A domain is responsible for the ATP-dependent activation of the carboxylate group of the incorporated amino acid creating a highly reactive amino acyl adenylate. The A domains are responsible for the specificity of the amino acid incorporated. The adenylate reacts with the thiol of the phosphopantetheine arm attached to a conserved residue of the T domain resulting in the formation of a thioester derivative. The C domain is then responsible for catalyzing the peptide bond formation between the amino acyl thioester attached to the T domain and that of the preceding module (Challis and Naismith, 2004). Thioesterase (TE) domains are often located at the C-terminus of NRPSs and are associated with peptide release from the synthase via hydrolysis, aminolysis or cyclization. In addition to the described core domains, modules often contain additional epimerase domains or methyltransferase domains further contributing to structural complexity of the associated metabolites products.

1.3.2 Polyketide Synthases

Polyketides are another class of fungal secondary metabolites. Composed of ketide chains, polyketides are synthesized from acetyl-CoA and malonyl-CoA subunits (Hansen et al., 2012). Fungal PKSs have a modular architecture and contain three fundamental domains: a β -ketoacyl synthase (KS), an acyltransferase (AT), and an acyl carrier protein (ACP or PP). The AT domain is responsible for the recruitment of the molecular subunits, the KS domain then catalyzes the condensation reaction of the growing chain, while the ACP facilitates assembly of the polyketide via transference of starter units, extender units, and acyl intermediates to the correct catalytic domain. In addition to these core domains, PKS modules may also have ketoreductase domains (KR), dehydratase domains (DH), methyltransferase domains (MT) and thioesterase domains (TE). KR domains act to reduce newly formed ketones to alcohols while also determining the stereochemistry of the bond. The DH domains can act on these newly formed alcohols by eliminating water to make an alkene. MT domains also promote derivatization of these alcohols by using S-adenosyl methionine to methylate them. Finally, the TE domain is responsible for releasing the polyketide from the megasynthase by breaking the thioester linkage. The *F. graminearum* genome contains 15 identified PKS genes. Examples of some known PKS secondary metabolites include aurofusarin, zearalenone, orcinol, and the fusarins.

1.3.3 Terpene Synthases

Terpene/terpenoid molecules are a third structural class of molecules, representing the most numerous and structurally diverse family of natural products (Rudolf and Chang., 2020). Terpene metabolites are synthesized from the C₅ isoprene units, dimethylallyl pyrophosphate (DMAPP) and isopentyl pyrophosphate (IPP), which are either used directly to alkylate other

metabolite products or combined to form linear isoprene chains: geranyl pyrophosphate (GPP - C10), farnesyl pyrophosphate (FPP - C15), and geranylgeranyl pyrophosphate (GGPP - C20). Biosynthetic gene clusters encoding a TS often also contain a gene encoding for a terpene cyclase (TC) which is responsible for the structural diversification through unique cyclization of these linear isoprene chains. TCs initiate catalysis by cleaving off the pyrophosphate group of the isoprene chain, generating a reactive allylic carbocation and subsequently facilitate a number of reaction cascades with high fidelity until the final product is achieved (González-Hernández et al., 2023). TS products are even further diversified in the presence of prenyltransferase genes which can catalyze the addition of PKS products, amino acids or additional isoprene chains (Greenhagen et al., 2001). The most thoroughly investigated TS metabolite family of molecules produced by *F. graminearum* is the sesquiterpene trichothecenes.

1.3.4 Other Biosynthetic Genes

In addition to the core biosynthetic enzymes, BGCs often also contain genes that encode “tailoring” enzymes, such as P450 monooxygenases, short chain dehydrogenases/reductases, FAD dependent oxidoreductases or methyltransferases, which are responsible for modifying the molecule produced by the core synthase/cyclase enzymes. Furthermore, some BGCs contain genes that encode putative membrane transporters, which are necessary for the efflux of metabolites from the cell in some cases. Many BGCs also contain genes that encode transcription factors (TFs) that regulate their activity.

1.4 Secondary Metabolites with Phytotoxic Effects

Some of the identified secondary metabolites produced by *F. graminearum* act as virulence or pathogenicity factors due to their ability to delay or confound aspects of the plant immune

response – resulting in ingress and infiltration of the fungus within the plant host (Rauwane et al., 2020). Some SM virulence/pathogenicity factors have been classified as host specific, aiding in infection only within specific crop types (Bahadoor et al., 2019, Tang et al., 2021) .

Siderophores are the first of the virulence factors to be expressed by *F. graminearum* during the infection processes (Haas et al., 2008). Siderophores exhibit strong iron-chelating properties and are believed to play a crucial role in facilitating iron uptake by the fungi during host plant invasion. Iron acquisition is essential for aerobic metabolism in fungi. In *F. graminearum*, three siderophores have been identified; fusarinine C, ferricrocin and malonichrome, all of which are products of three different NRPSs (NRPS 6, 2 and 1 respectively).

The trichothecenes are one of the most well described class of pathogenicity factors as they act as non-specific pathogenicity factors. The terpene synthase Tri5 synthesizes the trichodiene carbon skeleton for these molecules with *F. graminearum* producing three major tricothecene end products, 15-acetyldeoxynivalenol (15-ADON), 3-acetyldeoxynivalenol (3-ADON), and 7- α hydroxy, 15-deacetylcalonecitrin (3ANX) (Crippin et al., 2019). Jansen et al., 2005 demonstrated that *F. graminearum* mutants lacking *Tri5* (and ultimately DON production) could not infect wheat heads beyond the inoculated kernel indicating it is significant factor in virulence.

Structural analysis by Garreau et al., 2014 suggests that DON is able to interfere with the peptidyl transferase center of the large ribosomal subunit thereby preventing protein translation in Eukaryotes, including host plants.

In addition to the trichothecenes, gramillin A and B are examples of other *F. graminearum* SMs with virulence function, first identified by Bahadoor et al., 2018. Gramillins A and B demonstrated a host specific effect on virulence within maize stalk infection. However, their

mechanism(s) of action is not yet known. Both gramillin metabolites are products of the NRPS8 located within the C02 gene cluster.

Another recently described SM virulence factor produced *F. graminearum* are the fusaoctaxins. Jia et al., 2019 first identified and named the product fusaoctaxin A, a linear octapeptide synthesised by both NRPS5 and NRPS9 (C64). They determined that fusaoctaxin A is a host specific virulence factor in wheat that facilitates *F. graminearum* infection by aiding in the cell-to-cell migration through inhibition of the host defence responses, such as the formation of papillae-like cell wall callose dispositions and closure of the cell plasmodesmata. Tang et al., 2021 later identified a second and more abundant biosynthetic product of the NRPS5/9 genes; fusaoctaxin B which differs only in the N-terminal amino acid incorporation by NRPS5 in which fusaoctaxin A receives a GABA(γ -amino butyl acid) and B a GAA (guanidinoacetic acid). Additionally, Tang et al. determined that the two virulence factors differ in host cell response however the mechanisms in which they are involved is still unclear.

1.5 Cryptic Activation of BGC

As mentioned, many of the biosynthetic gene clusters of *F. graminearum* have yet to be associated with secondary metabolite products. However, there is also a large overlap between BGCs with unknown metabolite products that have demonstrated gene expression within *in-planta* infection assays. Table 2 summarizes the results found by Harris et al., 2016 from transcriptome data from *in-planta* infection trials which clearly demonstrates the large number of BGCs expressed *in-planta* that lack known products. The metabolite products derived from the expression of these gene clusters pose a compelling area of study as the metabolites have an increased plausibility in having specific functions associated with *F. graminearum* infection. A

variety of different experimental approaches can be employed to elucidate the molecular structure of these metabolites as well as to estimate an association with fungal growth or pathogenicity. However, molecular elucidation is hindered in many laboratory conditions, as transcription of many of the *F. graminearum* BGCs are either silent or expressed at very low levels *in-vitro* (Gaffoor et al., 2005; Sieber et al. 2014). This is because only a limited number of SMs are constitutively expressed; transcription of the remaining SMs genes is tightly regulated. Expression of these SM BGCs are often under the influence of global regulators, such as AreA which is highly expressed when preferred nitrogen sources are limiting or velvet complex proteins which are expressed in the presence of light (Jiang et al., 2011; Giese et al., 2013). As a result, *in-vitro* experiments often do not sufficiently mimic environmental conditions to induce the expression of all secondary metabolite BGCs encoded for the genome. Additionally, unknown detected metabolites may prove difficult to link back to genomic sequences due to complex synthesis and lack of appropriate algorithms for the analysis of A domains of fungal biosynthetic genes making prediction of potential products from BGCs difficult.

Table 2: Gene loci and their reported expression intensities on maize, barley and wheat as reported by Harris et al. (2016). Associated BGC and end products are listed demonstrating many in-plant upregulated BGCs with unknown products

Gene loci	GC	End Product	Gene	Harris et al., 2016 <i>In planta</i> expression		
				Maize	Barley	Wheat
FGSG_15673	C02	Gramallin A/B	NRPS8	++	nd	+
FGSG_15676	C06		NRPS19	nd	+	+
FGSG_15872			NRPS16	nd	+	++
FGSG_01738	C07		TS	nd	+	+
FGSG_01790	C08		PKS11	nd	+	++
FGSG_02324	C13	Aurofusarin	PKS12; AUR1	+	nd	+
FGSG_04588	C16		PKS15	+	+	+
FGSG_04591			TS	+	+	+
FGSG_03964	C18		PKS14	+	+	+
FGSG_03747	C21	Fusarinine	NRPS6	++	+	++
FGSG_03537	C23	Trichothecenes	TRI5	++	++	++
FGSG_03340	C26		PKS8	++	+	+
FGSG_04694	C31		PKS2	+	nd	+
FGSG_05372	C33	Ferrocrocine	NRPS2	+	+	++
FGSG_17677	C34		PKS5	nd	+	++
FGSG_06507	C38		NRPS10	+	+	++
FGSG_07798	C42	Fusarin C	PKS10	nd	+	+
FGSG_08795	C44		PKS7	+	+	++
FGSG_08208	C47	Fusaristatin A	PKS6	nd	nd	+
FGSG_17168	C53	*Perithecial Pigment	PKS3; PGL1	nd	+	nd
FGSG_10397	C59	Culmorin	CLM1	++	++	++
FGSG_17386	C61		NRPS18	+	+	+
FGSG_17387	C61		PKS1	+	nd	+
FGSG_11026	C63	Malinochrome	NRPS1	nd	+	++
FGSG_10990	C64	Fusaoctaxin A/B	NRPS9	++	+	++
FGSG_17487			NRPS5	++	+	++
FGSG_11395	C66		NRPS14	+	+	++
FGSG_02315	C70		NRPS4	++	++	++
FGSG_10523	C71		NRPS3	nd	+	++
FGSG_03245	C72		NRPS11	+	+	++
FGSG_17574	C73		NRPS12	nd	++	++

Often, linking SM production with a given BGC involves genetic manipulation and the generation of mutant strains, such as the development of transformant strains, in which genes of interest (GOIs) are removed or replaced from the fungal genome of interest. However disrupting GOIs alone may still prove difficult in the detection of associated metabolites, especially when the resulting SM product is unknown, or SM discovery is hampered due to the lack of *in-vitro*

expression. Manipulation of gene expression through the generation of over-expressor (OE) strains, in which GOIs are appended with a constitutive promoter to ensure gene expression have been used as a method to attempt to avoid this *in-vitro* bias (Westphal et al., 2018; Westphal et al., 2021). Although the term OE is most commonly used in the literature, the term is somewhat of a misnomer, rather constitutive expression typically is the result of promoter insertion, however, to be consistent with literature usage the term OE will be used in this thesis.

There are particular nuances in gene regulation and expression that can hamper the effectiveness of generating OE strains for SM discovery. For example, the generation of OE strains needs to take into account epigenetic regulation of gene transcription, meaning that for BGCs under strict epigenetic control, the inhibition of gene expression may still persist in OE strains. To circumvent the issue of epigenetic regulation, the generation of global SM overproducing strains have been attempted through the deletion of genes responsible for chromatin remodeling. This has been completed alone (as single gene deletions) to evaluate global changes in SM production, as well as in combination with deletion of GOIs to try and synthetically simulate chromatin structure in *in vitro* conditions (Connolly et al., 2013; Seidl et al., 2022). The generation of complex heterologous recombinant strains in which native gene clusters of interest are integrated into a new host genome have also been used to try and elucidate BGC SM products (Nielsen et al., 2019; Tsukada et al., 2020).

1.6 CRISPR Cas-9

CRISPR-Cas9, or Clustered Regularly Interspaced Short Palindromic Repeats mediated gene editing has quickly gained popularity as a method for the generation of transformant strains. The use of CRISPR techniques is largely due to its relative ease and efficiency as well as the high

level of gene editing precision relative to other gene editing platforms (Abdallah et al., 2017).

CRISPR was first identified as a mechanism of bacterial immunity against viral infections

(Figure 2).

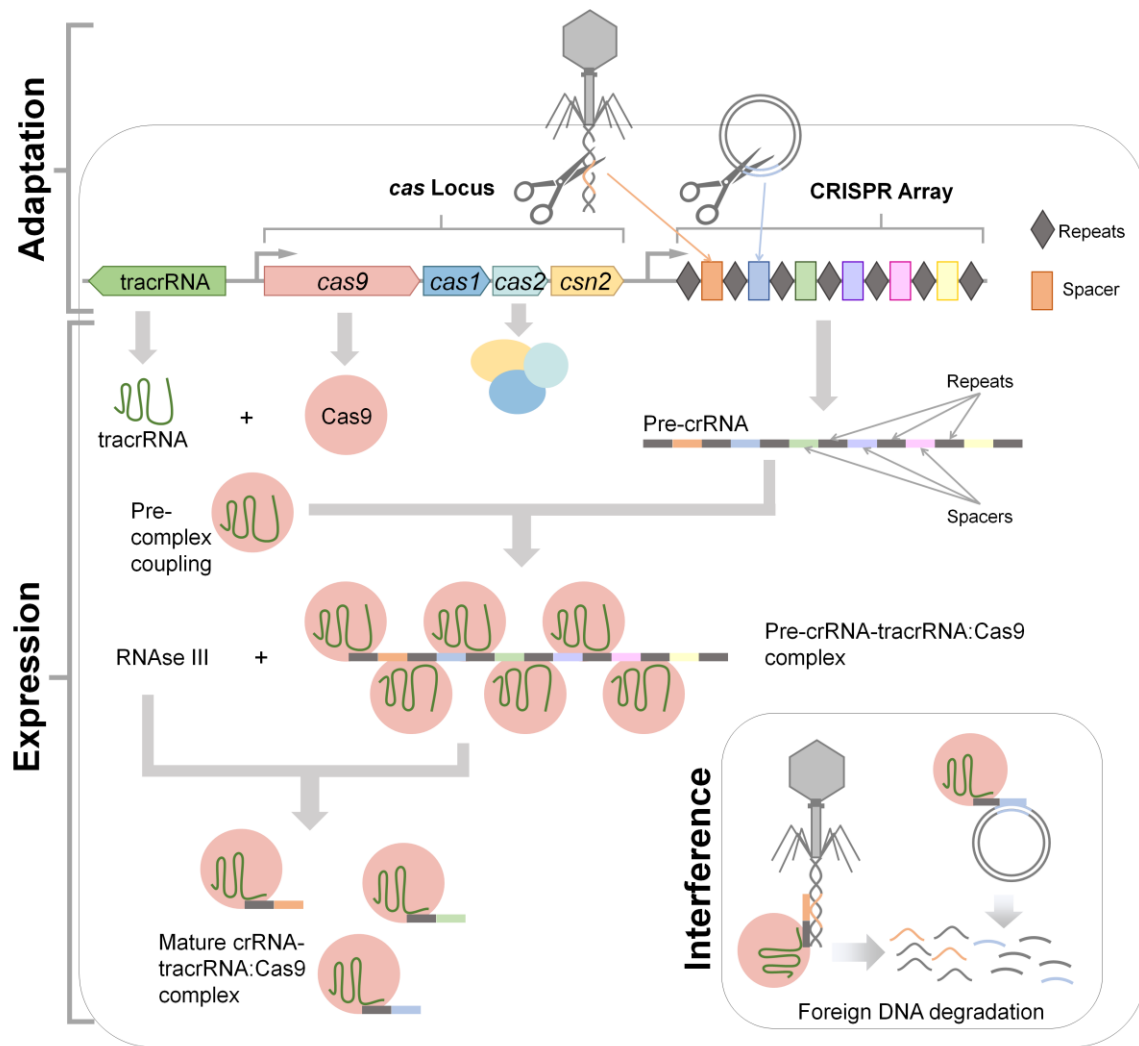


Figure 2: CRISPR-Cas9 mediated immunity demonstrating the three phases: Adaptation, in which foreign DNA is incorporated into the CRISPR array, Expression in which mature crRNA-*tracrRNA*:Cas9 complexes are assembled, and Interference, in which foreign DNA is degraded.

The CRISPR Cas-9 mediated immunity occurs in three phases: Adaptation (spacer acquisition), Expression (crRNA biogenesis) and Interference (cleavage of target DNA or RNA) (Marraffini,

2015; Barrangou et al., 2014). In the adaptation phase, foreign DNA is fragmented and incorporated into the CRISPR locus amongst a series of short palindromic repeats known as the CRISPR array. The incorporated viral DNA fragments are known as spacer sequences.

In the expression phase, the CRISPR array is transcribed into pre-crRNA, which includes both the CRISPR repeats and the incorporated fragments of viral DNA (spacers). Also transcribed are the trans-activating CRISPR RNA (tracrRNA) and the cas operon, within which the Cas-9 protein is translated and directly associated with the CRISPR immunity (Burstein et al., 2017).

The tracrRNA and the Cas-9 proteins then associate to form a pre-complex coupling. The formation of the complex allows for the complementary base pairing of the tracrRNA to the palindromic repeats of the pre-crRNA resulting in the generation of the pre-crRNA:tracrRNA:Cas-9 complex. The pre-crRNA:tracrRNA:Cas-9 complex is then cleaved by RNaseIII resulting in the production of mature crRNA:tracrRNA:Cas-9 complexes, each containing a unique spacer fragment corresponding to the incorporated viral DNA. These mature complexes scan for viral DNA looking for protospacer adjacent motif (PAM) sequences and regions complementary to the fragmented viral DNA originally incorporated into the CRISPR array. The PAM sequence (NGG) prevents the degradation of the CRISPR array (from the bacterial genome) as the absence of a PAM sequence prevents binding of the Cas-9 protein.

The final phase of CRISPR immunity, interference (sometimes referred to as targeting), involves the induction of double stranded DNA breaks and viral genome degradation by the Cas-9 proteins directed by the crRNA(spacer) sequences. These double stranded breaks will occur in the RuvC and HNH Domains of the Cas-9 proteins generating breaks 3bp upstream from the PAM sequence (Figure 3).

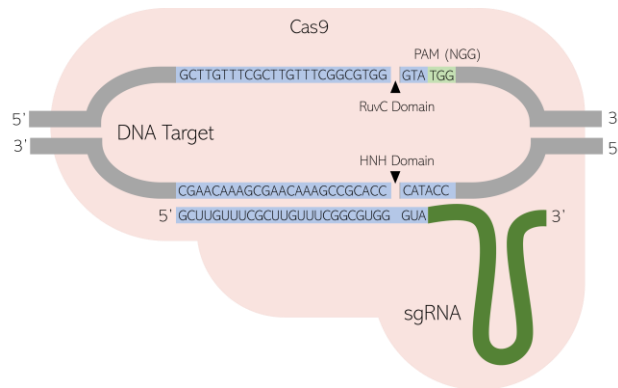


Figure 3: Overview of CRISPR-Cas9 mediated DNA cleavage by RNP

1.6.1 CRISPR Cas-9 as Tool in Editing Fungal Systems

Since its discovery, researchers have been working to understand and utilize the bacterial immune response for genetic engineering applications by using the innate CRISPR sequence specificity to mediate DNA degradation of select sequences in other organisms. TracrRNA, (transactivating CRISPR RNA) - analogous to native spacer sequences, are coupled with custom crRNAs designed specifically for genomic regions of interest to form sgRNAs (single guide RNAs). These sgRNAs are then combined with the Cas-9 enzymes and are used to implement double stranded DNA breaks in specific locations, followed by native cell repair mechanisms (non-homologous end joining, homology directed repair etc.) (Figure 4).

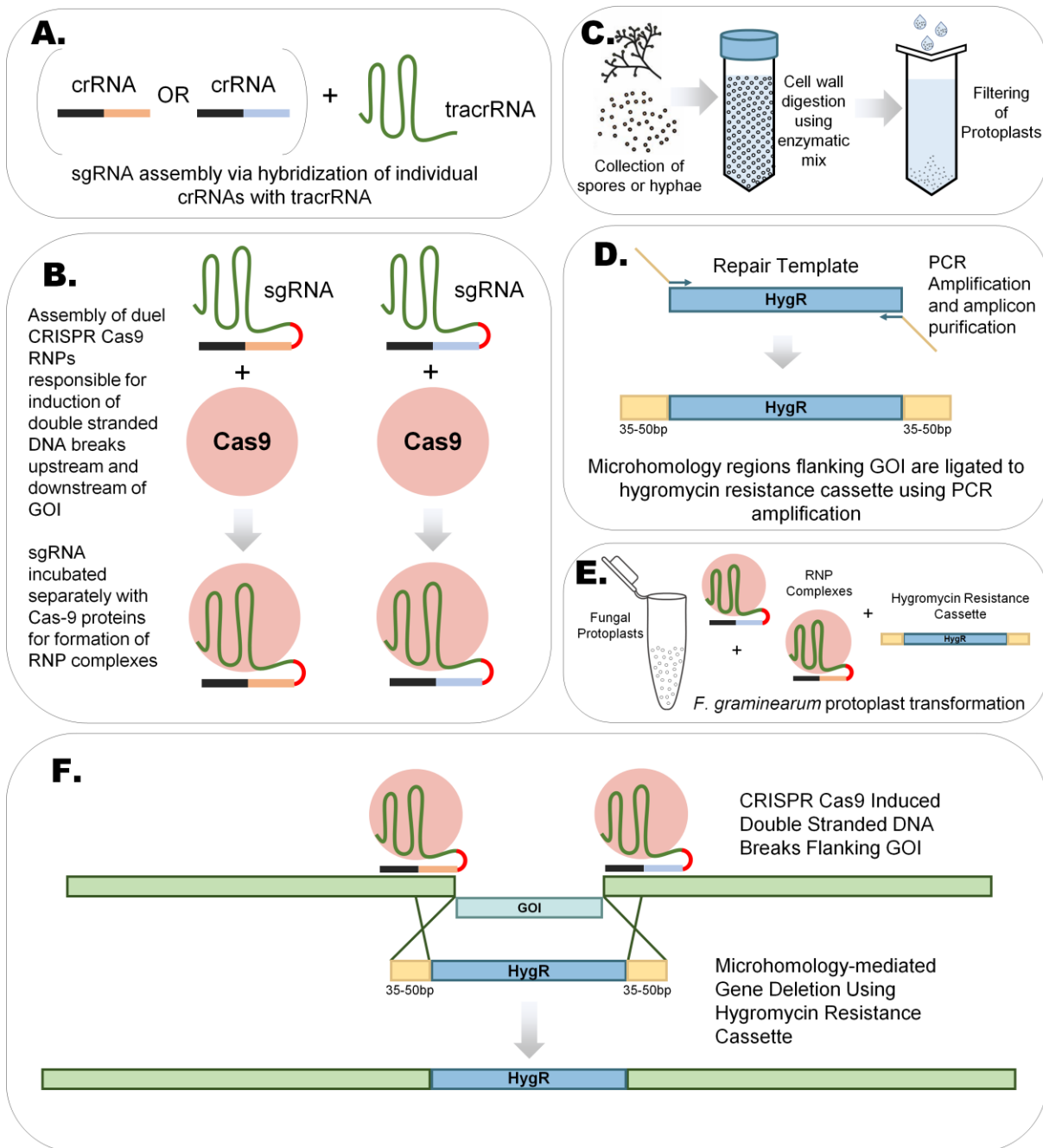


Figure 4: Overview of microhomology mediated gene deletion coupled with in-vitro assembled dual Cas-9 RNP cleavage. (A) Assembly of sgRNA duplexes by hybridization of downstream and upstream crRNAs separately with commercial tracrRNA in equimolar amounts. (B) Generation of Cas-9 RNP complexes by incubation of separate sgRNA's with Cas-9 protein followed by combination of corresponding upstream and downstream RNPs. (C) Isolation of fungal protoplasts via enzymatic digestion of cell wall and filtration. (D) Repair template amplification of HygB cassette through high fidelity PCR amplification using primers with microhomology regions for directed target of GOIs. (E) Assembly of transformation reaction by combination of fungal protoplasts, dual RNPs and HygB repair template. (F) Dual Cas-9 RNP cleavage upstream and downstream of GOI, resulting in complete removal of the coding sequence and replacement with HygB repair template mediated by microhomology regions and directed repair mechanisms. Graphic inspired by Abdallah et al., 2017.

Although several strategies have been established for the delivery of the CRISPR-Cas9 system into filamentous fungi, this thesis will focus on the use of ribonuclear protein (RNP) in which sgRNAs and Cas-9 enzymes are assembled *in-vitro* with cellular delivery via PEG-mediated transformation with hygromycin resistance as a transformation reporter.

1.6.2 Uses of CRISPR on *Fusarium graminearum*

Many implementations of CRISPR with *F. graminearum* are ‘proof of concept’ reporting, in which genes with known function were deleted or ‘knocked out’ and phenotypic analysis performed to confirm the success of the deletion strategy. Gardiner and Kazan, 2018, were one of the first researchers to use CRISPR successfully in *F. graminearum*, where the *Tri5* and *FgOs1* genes were knocked out, generating easily measurable phenotypes in plant infection and fungicide resistance assays respectively. Several reports have also used PKS12, known to produce the pigments rubrofusarin and aurofusarin in CRISPR proof of concepts experiments (Lee et al., 2022). The relative novelty of CRISPR accounts for the limited number of research publications on its use in *F. graminearum* gene editing. Despite CRISPR being a relatively new tool, gene editing is not a new concept, and the approach of deleting GOIs for the confirmation of SMs and their functions is routine (Jansen et al., 2005; Gaffoor et al., 2005; Bahadoor et al., 2018; Jia et al., 2019). The ease with which CRISPR allows for precise modification in genetic material suggests the potential for a significant increase in its utilization in the future.

1.7 Biosynthetic Gene Clusters of Interest

The research intent of this thesis was to transgenic strains by deleting core biosynthetic genes within four gene clusters identified in the *F. graminearum* genome (BGCs: C08, C16, C13/70) and inserting a hygromycin resistance marker. Transformant strains were then screened to

determine if they demonstrated variation in pathogenic phenotype relative to the wildtype (WT) strain using both mature wheat head assays and wheat coleoptile assays. Following this analysis, an attempt was made to discern the secondary metabolite products produced by these core biosynthetic genes through the use of untargeted metabolomic profiling of WT to transformant strains. The four selected biosynthetic gene clusters were reported to be transcribed by *F. graminearum* during infection in wheat. Prior to the start of this project the C08, C16 and C70 clusters had unknown associated SM products, however during the course of the thesis research structures from the C16 and C70 gene cluster were identified and published by other research groups.

1.7.1 BGC C08

The C08 BGC has not yet been linked to any known secondary metabolites. Additionally, *in-planta* expression has been detected from genes within this BGC. The decision to investigate the C08 gene cluster was driven by the presence of two core biosynthetic genes; a PKS and a TS, indicating the potential biosynthetic product may be a hybrid or meroterpenoid molecule. Figure 5 displays the C08 BGC (fungiSMASH output) while Table 3 indicates the protein signatures of each gene within the outlined region. PKS11, a reducing polyketide synthase, the TS, synthesizing a GGPP, and a potential transcription factor were selected as targets for gene deletion.

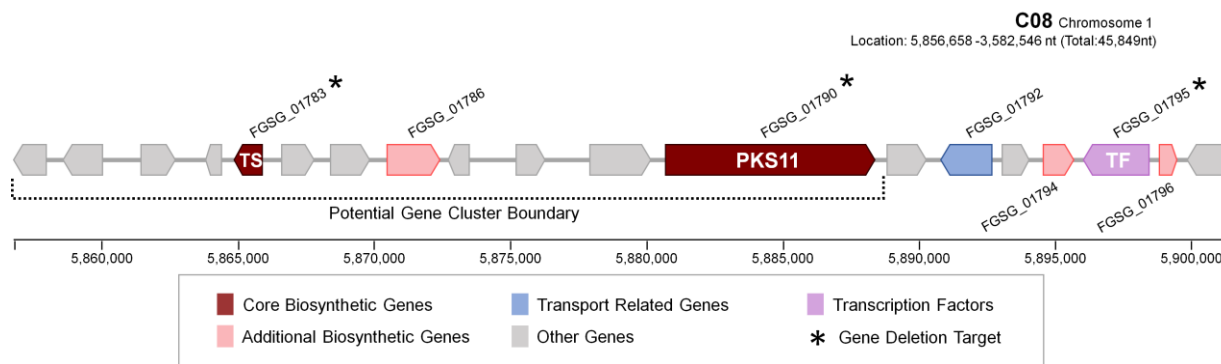


Figure 5: C08 BGC as reported by fungiSMASH. Targets for fungal transformation are indicated (*)

Table 3: Genes within the C08 BGC including their associated protein signatures. Bolded genes indicate targets for fungal transformation.

Gene Cluster	LocusID	Gene Name	Protein Signature
C08	FGSG_01779	-	Hypothetical protein
	FGSG_01780	-	Hypothetical protein
	FGSG_01781	-	Choline dehydrogenase
	FGSG_01782	-	Organic hydroperoxide resistance
	FGSG_01783	-	Geranylgeranyl pyrophosphate synthetase
	FGSG_01784	-	Cral trio
	FGSG_01785	-	Malic acid transport
	FGSG_01786	-	Ent-kaurene oxidase
	FGSG_01787	-	Hypothetical protein
	FGSG_01788	-	Hypothetical protein
	FGSG_01789	-	Ferric reductase transmembrane component 4
	FGSG_01790	PKS11	Reducing Polyketide synthase
	FGSG_01791	-	Immune-responsive 1
	FGSG_01792	-	Mfs transporter
	FGSG_01793	-	Short-chain dehydrogenase reductase
	FGSG_01794	-	Related to short-chain alcohol dehydrogenase
	FGSG_01795	-	Transcription Factor
	FGSG_01796	-	Related to 4-carboxymuconolactone decarboxylase
	FGSG_01797	-	Muconate cycloisomerase

Interestingly, cluster analysis suggests that the C08 BGC boundaries likely lie within the

FGSG_01781 – FGSG_01790 gene regions as indicated in Figure 5. This gene region is

conserved across the fungal strains within the *Fusarium sambucinum* species complex (species closely related to *F. graminearum*). Genes between the FGSG_01791 and FGSG_01798 gene loci are often observed without the neighboring PKS11 and TS in other *Fusaria* suggesting that TS and PKS products may not be associated with genes within this locus. Included in this potential ‘out of cluster’ region is the potential transcription factor (FGSG_01795) and a transport related gene (FGSG_01792). At present, it is unclear whether or not these two regions have any association to one another.

1.7.2 BGC C16

Prior to the start of this thesis, the products of the C16 BGC were unknown, since then two papers have been published describing the potential associated mass features (Tsukada et al., 2020 and Seidl et al., 2022). Tsukada (2020) implemented heterologous biosynthesis technique in which they used conserved early pathway genes from *Aspergillus oryzae* as well as tailoring genes specific to *F. graminearum* and other fungi. Tsukada’s intent was to try and reveal new SMs with pharmaceutical implications. Their heterologous ‘native’ *Fusarium* gene cluster lead to the discovery of 3 new molecules coined FDDP (fungal decalin-containing diterpenoid pyrones) B, D and E that could potentially be produced by *F. graminearum*. However, FDDP biosynthesis has not yet been demonstrated within non-genetically modified strains of *Fusarium*. Seidl (2022) also identified potential mass features associated with the C16 BGC using a multi gene deletion approach in order to identify end products associated with the BGCs polyketide synthase (PKS15). Through their experimentation they correlate two mass features to PKS15 and designated them gramiketide A and gramiketide B. However, they did not provide any supporting

structural information that would enable us to compare their identified structures to the FDDP molecules identified by Tsukada et al.

The C16 BGC contains both a non-reducing PKS (PKS15) and a geranylgeranyl diphosphate synthase (TS) which together with the terpene cyclase and prenyltransferase provide the structural backbone for Tsukada et al.'s described meroterpenoid FDDP molecules. As such, both synthases (PKS15 and TS) were selected as targets for gene deletion. Additionally, a hypothetical 5.6kb gene containing WD40 repeats motif was also selected for investigation. WD40 motifs typically act as sites for protein-protein interactions and have been associated with signalling cascades. Figure 6 displays the C16 BGC (fungiSMASH output) while Table 4 indicates the protein signatures of each gene within the outlined region.

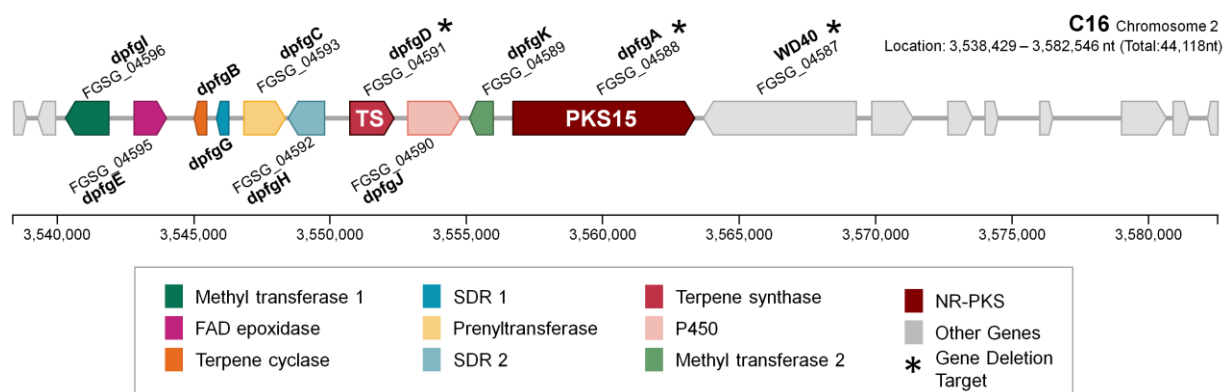


Figure 6: C16 BGC as reported by fungiSMASH. Targets for fungal transformation are indicated (*).

Table 4: Genes within the C16 BGC including their associated protein signatures and historically referenced gene names. Bolded genes indicate targets for fungal transformation. Red indicating the gene not associated with synthesis of known C16 products.

Gene Cluster	LocusID	Gene Name(s)	Protein Signature
C16	FGSG_04596	dgfgI	O-methyltransferase
	FGSG_04595	dpfgE	Flavin-containing monooxygenase
	FGSG_12222	dpfgB	Terpene cyclase
	FGSG_12223	dpfgG	Short-chain dehydrogenase/reductase
	FGSG_04593	dpfgC	Prenyltransferase
	FGSG_04592	dpfgH	Short-chain dehydrogenase/reductase
	FGSG_04591	dpfgD / TS	Geranylgeranyl diphosphate synthase
	FGSG_04590	dpfgJ	Cytochrome p450
	FGSG_04589	dpfgK	O-methyltransferase
	FGSG_04588	dpfgA / PKS15 / PLSP1	Non-reducing polyketide synthase
	FGSG_04587	-	WD40

1.7.3 BGC C13/70

The *F. graminearum* genome demonstrate the co-localization of the C13 and C70 BGCs. This co-localization raises questions as to whether or not the BGCs are co-expressed or governed by the same regulatory mechanism or if there is an evolutionary advantage in having these two clusters adjacent to one another. Figure 7 demonstrates the two gene cluster proximity with the C13 gene cluster outlined in black. The C13 gene cluster has been thoroughly characterized, and numerous associated products are well-documented, including rubrofusarin, aurofusarin, and fuscofusarin, all of which are linked to mycelial pigmentation. Prior to the start of this thesis, products associated with the C70 gene cluster were unknown. Since, Westphal et al., 2020 has identified the 5 module NRPS4 product as a cyclic hexapeptide in which they coined fusahexin. In addition to fusahexin, they also identified three additional mass features of identical m/z, two of which are assumed to be linearized products of the cyclic peptide and a final unknown.

NRPS4 was chosen as a prime candidate for targeted gene deletion within the scope of this research.

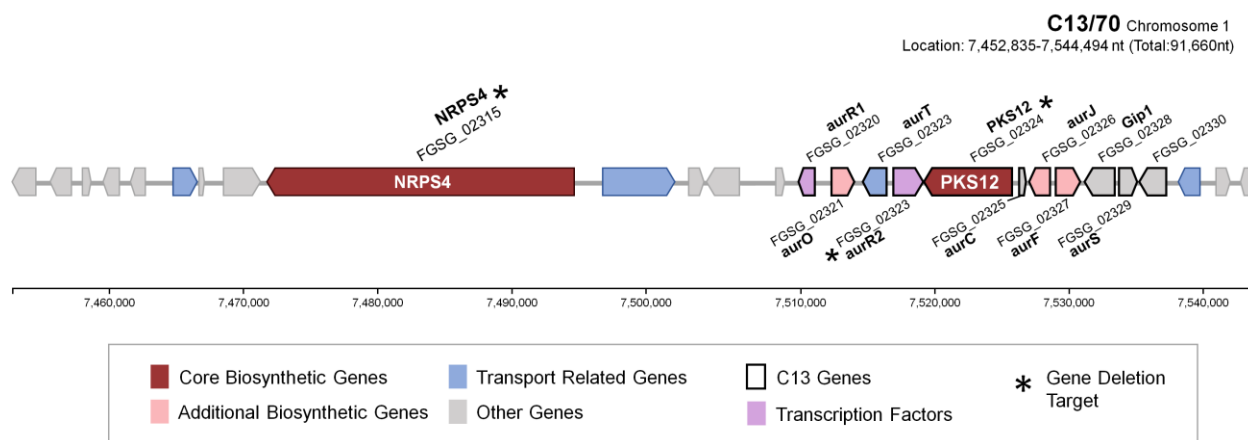


Figure 7: C13/C70 BGC as reported by fungiSMASH. Targets for fungal transformation are indicated (*)

Table 5: Genes within the C13/C70 BGC including their associated protein signatures and historically referenced gene names. Bolded genes indicate targets for fungal transformation.

Gene Cluster	LocusID	Gene Name	Protein Signature
C13	FGSG_02320	aurR1	Transcription Factor
	FGSG_02321	aurO	FAD linked oxidoreductase
	FGSG_02322	aurT	MFS Transporter
	FGSG_02323	aurR2	Transcription Factor
	FGSG_02324	PKS12	Nonreducing Polyketide synthase
	FGSG_02325	aurC	Hypothetical Dehydratase
	FGSG_02326	aurJ	O-methyltransferase
	FGSG_02327	aurF	Flavin-containing monooxygenase
	FGSG_02328	Gip1	Oxidase
	FGSG_02329	aurS	Hypothetical faxilin domain family protein
	FGSG_02330	-	L-ascorbate oxidase
C70	FGSG_02319	-	Hypothetical protein
	FGSG_02318	-	Kinsase light chain 1
	FGSG_02317	-	Hypothetical protein
	FGSG_02316	-	Metal resistance ycf1
	FGSG_02315	NRPS4	Nonribosomal peptide synthase

	FGSG_02314	-	Related to beta-mannosidase
	FGSG_02313	-	Hypothetical protein
	FGSG_02312	-	maltose permease

The C13 gene cluster contains a nonreducing PKS (PKS12) as well as two transcription factors AurR1 (FGSG_02320) and AurR2(FGSG_02323). AurR1 was identified as directly regulating the biosynthesis of PKS12 (Kim et al., 2006; Malz et al., 2005). AurR2 is thought to act as a co-regulator of the PKS12 gene cluster and is thought to be associated with increase synthesis of downstream pathway genes (Frandsen et al., 2006). The C70 BGC does not encode a putative transcription factor of its own which is why in addition to the NRPS4 both the PKS12 and the AurR2 transcription factor were selected for investigation.

1.8 Thesis Outline

In this thesis, CRISPR-Cas9 mediated gene deletions of select biosynthetic genes in *F. graminearum* was attempted to identify associated secondary metabolite products of the targeted BGCs. Additionally, pathology experiments were undergone to determine if the GOIs could be correlated with a pathogenicity phenotype. Chapter 2 focuses on the generation of GOI deletion mutants and comparison of generated strains to that of the wild type (WT) in two different *in-planta* experiments using wheat. Disease phenotypes were evaluated using both wheat head infection assays and coleoptile infection assays. Chapter 3 focuses on an evaluation of the metabolomes of the generated transformants strains for confirmation of putative metabolite products. Previously identified secondary metabolite products from the C13 and C70 BGCs are demonstrated to have a large metabolomic variation between transformant and WT strains in an untargeted analysis. Putative metabolites from C16 BGC were also confirmed as well as

potential additional unknown molecular family constituents that have yet to be structurally elucidated from *F. graminearum*. The final chapter of the thesis attempts to rationalize the findings from the two experiments.

1.9 References

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Chapter 2 Assessing Associations Between Targeted Gene Clusters and Plant Pathology

2.1 Introduction

Fusarium graminearum affects a variety of cereal crops, including wheat and barley, and is responsible for causing significant economic losses in agricultural industries worldwide. The pathogenicity of *F. graminearum* is complex and multifaceted, involving several factors that contribute to its ability to infect and colonize plant hosts. One of these factors is the production of secondary metabolites, such as mycotoxins and other natural products effecting host immunity, which play crucial roles in the pathogenicity of the fungus. The implication that these secondary metabolite mycotoxins act as pathogenicity factors are significant, as they not only contribute to the virulence of the disease but also pose serious health risks to humans and animals that consume contaminated crops. Therefore, understanding the mechanisms underlying the *F. graminearum* pathogenicity and the role of secondary metabolites in its virulence is crucial for developing effective strategies to mitigate Fusarium head blight (FHB) and reduce the risk of mycotoxin contamination in food and feed.

Schroeder and Christensen (1963) described host plant resistance to FHB in two distinct forms: Type I resistance as referring to resistance to the initial infection, and Type II resistance as the prevention of hyphal spread by the pathogen in the host. Since then, other types of pathogen resistance mechanisms have been described such as resistance to kernel infection (Mesterházy 1995), tolerance to FHB (Mesterházy 1989) and resistance to toxins (Miller et al., 1985). However, methods used to evaluate resistance are often associated with the classical Type I and II resistance definitions. Measurements attempting to quantify the spread of fusarium head blight

symptoms are used as a reliable method to assess for crop cultivar resistance (Bai and Shaner, 1996; Schroeder and Christensen, 1963; Wang and Miller, 1988). These same methods have since been used to assess and characterize the pathogenicity of fungal inoculants (with specific relevance to the use of transformant strains) to monitor relative changes in visual disease symptoms often using highly susceptible crop cultivars in attempt to elucidate the role of specific fungal pathogenicity/virulence factors as compared between mutant and WT strains (Desjardins et al., 2000; Desjardins et al., 2004, Gaffoor et al., 2005; Bonner et al., Kumar et al., Maré et al., 2022).

The inoculum procedure used to evaluate fungal spread within the wheat head involves the introduction of fungal inoculum (both macroconidia and ascospores yield similar infection results (Wang and Miller 1988)) to a central spikelet at early anthesis. Post inoculation, plants are typically incubated at temperatures around 25°C with high humidity for the first 2-3 days either via continuous or intermediate dew periods (Wang and Miller, 1988; Bai and Shaner, 1996). Type II resistance is typically measured by observations of disease development at frequent intervals providing data on the relative spread of the disease, provided that plants are protected from additional inoculation events (Mesterházy 1995). Symptoms of FHB that are often measured include infection of the spikelets and rachis as well as bleaching of spikelets (Figure 8). The infection phenotype produced by *F. graminearum* is characterized by discoloration of tissue, with glumes of spikelets or rachis transitioning to a brownish colour that often appears amorphous in shape (Trail, 2009). This infection symptom results from necrosis of plant cells by the invading fungi. Infection of the spikelet and rachis are demonstrated in Figure 8-A and B respectively with arrows indicated the infection symptoms. Figure 8-C demonstrates the bleaching phenotype of an infected wheat head with the bleached region being indicated by

the white bar. Bleaching of tissues is a result of the fungal biomass colonizing the xylem and pith resulting in the prevention of water and other nutrients from being transported up the wheat head (Trail, 2009). As a result, spikelets will transition in colour from green to a yellow-beige and become more ridged and brittle relative to those that are unbleached.



Figure 8: Infection phenotype of *Fusarium graminearum* in wheat (Roblin) demonstrating A. infected spikelets, B. Infection of the rachis and C. bleaching as measured in wheat head infection assays.

In addition to infection trials using mature wheat, recently coleoptile infection assays have been developed as a method of high throughput screening of fungal transformants specifically to observe the effect on virulence (Jia et al., 2017). The coleoptile is the outermost layer of the seedling shoot and provides a protective surface for the underlying first leaf as depicted in Figure 9-A. Coleoptiles are composed of nearly identical cells to those infected during seedling blight which is why it was selected as a favorable model (Zhang et al., 2012). In terms of turnaround time, coleoptile infection assays are much more efficient relative to wheat head assays, as the assay may be completed within 14 days, in contrast, wheat head infection assays require a 6–7-

week growth period to reach the optimal infection period (mid anthesis). Additionally, coleoptile infection assays occupy significantly less physical space within a growth cabinet allowing for higher replication within the same area relative to wheat head infection assays. Figure 9-B demonstrates the optimized experimental set up for the completion of the coleoptile infection assays with germination of seeds and subsequent inoculation occurring within in 2mL tubes.

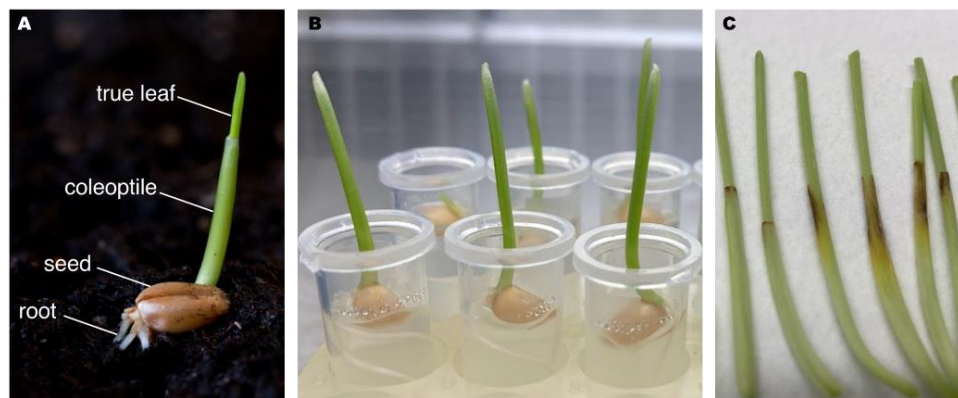


Figure 9: (A) Coleoptile anatomy (Patrice A. Salomé) and (B) optimized coleoptile growth set up. (C) Necrotic lesions measured as assessment for pathogenicity

The coleoptile assay involves the inoculation of wounded coleoptiles with a suspension of macroconidia. Within 10 hours post inoculation (hpi) *F. graminearum* spores germinate on the coleoptile epidermis with germ tube growth directed towards the wounded coleoptile end or stomata opening. Hyphae that enter through wounds rapidly elongate inside the coleoptiles. By 170 to 240 hpi lesions appear and often abundant fungal growth can be observed on the coleoptile surface, and in some instances, sporulation is observed. To evaluate the pathogenicity of the generated strains, the necrotic lesions on the coleoptiles are measured and characterized. Figure 9-C demonstrates some disease symptoms of infected coleoptiles showing the clear

necrotic dark brown regions stemming from the wound site migrating down the coleoptile.

Yellow-brown regions can also be seen within some of the coleoptiles which is thought to be early-stage necrosis.

When selecting an appropriate assay to use when assessing virulence/pathogenicity factors associated with FHB, it is important to take into consideration that secondary metabolite production from *F. graminearum* varies between coleoptile and wheat head infection strategies (Zhang et al., 2012). Most notably, in coleoptile assays a clear lack of expression of genes involved in the biosynthesis of DON or zearalenone in *F. graminearum* occurs, while expression of aurofusarin demonstrated significant increase (Zhang et al., 2012). A degree of overlap in *F. graminearum* gene expression has been observed between the two infection assays, but this overlap is not complete which is likely due to the increased diversity in tissue types within mature wheat heads as compared to coleoptiles (Zhang et al., 2012). A complementary approach using both assay techniques is recommended when studying the effect of secondary metabolite virulence factors upon FHB pathology. For example, Zhang et al. (2012) also noted that a reduced virulence phenotype in coleoptile assays when comparing *F. graminearum* WT and mutant NRPS9 strains, an enzyme required for the synthesis of fusaoctaxin, but no reduction in virulence in florets indicating both assays may yield to differing conclusions if completed alone. As such, pathogenicity testing of the transformant strains generated by in this portion of the thesis was completed using both mature wheat head infection assays and coleoptile infection assays.

In this chapter, nine transformant *F. graminearum* strains were generated using CRISPR-Cas9 gene editing in which the GOI was replaced with a hygromycin resistance cassette. The selected

nine genes pertain to four BGCs and were selected for analysis due to their reported upregulation *in-planta*. This led to the hypothesis that the BGC associated secondary metabolite products could have potential involvement with the infection process. All four of these gene clusters contain core biosynthetic genes which were primarily the targets selected for transformation. Table 6 contains selected BGCs and their putative protein annotations as well as the designation for which each of the transformants will be referred to throughout this thesis. To investigate their impact on virulence and pathogenic potential, both wheat head infection assays and coleoptile infection assays were carried out using transformant and WT strain on the highly susceptible wheat cultivar Roblin. These experiments allowed for the comparison of disease phenotype and virulence of the generated transformant strains relative to the WT strain, shedding light on the potential contribution of the targeted gene clusters to the pathogenicity and virulence of *F. graminearum*.

Table 6 Genes selected for CRISPR Cas-9 mediated disruption.

BGC	LocusID	Gene Name	Transformant	Protein Signature
C08	FGSG_01783	-	Δ TS	Geranylgeranyl pyrophosphate synthetase
	FGSG_01790	PKS11	Δ PKS11	Reducing Polyketide synthase
	FGSG_01795	-	Δ TF	Transcription Factor
C16	FGSG_04591	dpfgD	Δ TS	Geranylgeranyl diphosphate synthase
	FGSG_04588	dpfgA /PKS15 / PLSP1	Δ PKS15	Nonreducing polyketide synthase
	FGSG_04587	-	Δ WD40	WD40
C13	FGSG_02323	aurR2	Δ TF	Transcription Factor
	FGSG_02324	PKS12	Δ PKS12	Nonreducing Polyketide synthase
C70	FGSG_02315	NRPS4	Δ NRPS4	Nonribosomal peptide synthase

2.2 Materials and Methods

2.2.1 Generation and Validation of Transformant Strains

2.2.1.1 Spore Generation

The wildtype (WT) *F. graminearum* strain selected for experimentation was DAOMC233423. Growth medium formulations and buffers used are provided in Appendix-A section 2.63-2.65 To generate macroconidia, a sampling straw was used to transfer six actively growing SNA culture plugs into a 250 mL Erlenmeyer flask containing 50 mL of CMC media before incubation at 28 °C shaking at 160 rpm for 4 days. Cultures were then filtered through cheese cloth and macroconidia were pelleted by centrifugation for 10 min at $4100 \times g$ before the CMC supernatant was removed and replaced with sterile MQ water. Macroconidia were then centrifuged again, and the supernatant was again decanted out and replaced with additional sterile MQ water. This washing process was completed three more times. Macroconidia were then quantified using a haemocytometer.

2.2.1.2 Generation of Fungal Protoplasts

A 200 mL volume of Littman Oxgall liquid medium was inoculated with 10^8 *F. graminearum* macroconidia, incubated at 19 °C and shaken at 160 rpm for 15 hours, after which germling mycelium was harvested using a cell strainer (100 µm mesh size) and rinsed once with sterile water and once with 1.2 M KCl buffer. Resulting germling mycelia were transferred into a 125 mL Erlenmeyer flask containing protoplasting buffer, composed of 1.2 M KCl, 500 mg Driselase, 200 mg lysing enzymes from an *Aspergillus sp.*, 200 mg Yatalase per 20 mL and incubated at 30°C, shaking at 80 rpm. The protoplasting buffer was prepared 45 min before use and filtered through a 0.45 µm filter prior to use. Progression of protoplast formation was

monitored every 30 min from a 10 μ L aliquot via visualization under a microscope for a total incubation time of 1 hour and 15 min at which point the majority of mycelium was observed to be digested. The resulting protoplasts were diluted with an additional 20 mL of 1.2 M KCl buffer, filtered through a 20 μ m cell strainer and centrifuged at $4100 \times g$ for 6 min at 4 °C. The pellet was resuspended and washed several times with a series of buffers (once with 20 mL of cold 1.2 M KCl buffer and twice with 20 mL of cold STC buffer (1.2 M D-sorbitol, 10 mM Tris-HCl, pH 8.0, 50 mM CaCl_2)), each followed by a centrifugation step. The final washed pellet was resuspended in cold STC buffer, and the protoplasts were quantified using a haemocytometer before being diluted to a final concentration of 2×10^8 protoplasts/mL in cold STC buffer. A 7% volume of dimethyl sulfoxide (DMSO) was then added, and the protoplast solution was transferred into 400 μ L aliquots in sterile 2 mL Eppendorf vials and stored at -20 °C for 24 hours before transfer to -80 °C.

2.2.1.3 PCR Amplification of HDR-HygB Repair Template

A hygromycin B expression cassette (HygB) was used as a selectable marker for the Cas9-mediated gene deletion. A 1395 bp region spanning the 364 bp pTrpC and 947 bp hygromycin sequences were amplified from the pRF-HU2 vector (Appendix-A Figure 1) using primers with the addition of 35-50 bp microhomology regions specific to each intended gene deletion (Appendix-A Table 1). Amplification was completed using Phusion® High-Fidelity DNA polymerase (NEB, cat#M05305) and HF Phusion buffer following the manufacturer's amplification protocol with minor modification to incorporate a touchdown start to increase primer specificity. Elongation temperatures started at 67 °C decreasing 1 °C per cycle for a total of 10 cycles before following recommended 56 °C elongations. Initially, a single 50 μ L reaction was completed for each of the primer sets in order to evaluate amplification efficacy. Once

amplicon size was confirmed, additional PCR reactions (~10 reactions) were completed to obtain the desired 6-8 µg template quantity. Amplicons were then cleaned using the PureLink® PCR Purification Kit (K3100-01) following manufacturer's protocol and a 1 µL aliquot of the cleaned template was then run on an agarose gel to confirm quality (Appendix-A Figure 2). Template quantity was determined using a Nanodrop spectrophotometer.

2.2.1.4 In-Vitro assembly of Cas9-gRNA Ribonucleoprotein Complexes

Cas-9 ribonucleoproteins (RNPs), composed of gene specific crRNA (Appendix-A Table 2), tracrRNA, and the Cas-9 protein, were assembled *in vitro* using Alt-R-CRISPR-Cas-9 components commercially available from Integrated DNA Technologies Inc. (Coralville, Iowa). To ensure deletion of the entire coding sequences of the desired genes, crRNAs were designed to target the protospacer sequence of both the 5' UTR and 3' UTR thereby implementing a dual Cas-9-gRNA system. Generation of gRNAs was completed by hybridization of crRNA to tracrRNA by mixing equal molar amounts followed by a dilution to 33 µM with the nuclease-free duplex buffer. gRNAs were then placed in a thermocycler for 5 min at 95 °C for denaturation and then cooled to room temperature to allow for hybridization. Each gRNA was then used individually in the generation of a Cas-9 RNP complex by combining 1.5 µL with 0.75 µL of Cas-9 nuclease (1 µg/µL) and 11 µL of Cas-9 working buffer (20 mM HEPES, 150 mM KCl, pH 7.5) and incubating at room temperature for 10 min to allow for RNP complex formation before combining to a final volume of 26.5 µL and incubating for 5 min at room temperature before use in *F. graminearum* protoplast transformation.

2.2.1.5 F. graminearum Protoplast Transformation

For the transformation of fungal protoplasts, 200 μL of the 2×10^8 protoplasts/mL aliquots were transferred into a 2 mL tube along with 26.5 μL of RNP complex, 6-8 μg of the purified repair template and 25 μL of a PEG (4000)- CaCl_2 buffer (60% w/v PEG 4000, 50 mM $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 450 mM Tris-HCl, pH 7.5) followed by incubation on ice for 1 hour. Afterward, an additional 1.5 mL of the PEG (4000)- CaCl_2 was added and incubated for 20 min at room temperature. The mixture was then transferred to a 50 mL culture tube followed by the addition of 250 μL of STC buffer, and 3 mL of TB3 media and incubated at a 45 ° angle for 18 hrs at 25 °C, shaking at 80 rpm. Regenerated fungal protoplasts were then combined with 20 mL of molten TB3 media containing 100 mg/L hygromycin B at volumes of 300, 500 and 1000 μL and poured into sterile petri plates and incubated at 28 °C until the appearance of resistant colonies (5-6 days).

2.2.1.6 Strain Purification and Identification of Transformants

Six putative transformants (hygromycin resistant colonies) for each gene disruption target, were transferred to PDA containing 150 mg/L hygromycin B and incubated in the dark at 28 °C for 3 days to allow for fungal biomass growth. A culture explant was used to inoculate a 50 mL culture tube containing 10 mL of CMC media and incubated at 28 °C shaking at 160 rpm at a 45 ° angle for 3 days to induce conidiation. Resulting cultures were then filtered through cheese cloth to separate the mycelium from the macroconidia. Conidia were pelleted from the filtrate by centrifugation for 10 min at 4100 rpm and subjected to several washes with sterile water and centrifugation steps to remove traces of the CMC medium and resulting conidia were diluted to a final concentration of 1×10^3 spores/mL. A 50 μL aliquot of each spore stock was then plated on PDA plates (150 mg/L hygromycin B) and spores were dispersed with a cell spreader and plates

were incubated in the dark at 28°C for 24 hours to reconfirm retention of hygromycin resistance via germination of macroconidia. Three single fungal colonies (originating from a single germinating macroconidium) were then transferred to individual PDA plates supplemented with 100 mg/L hygromycin B to allow for growth of fungal biomass and isolation of axenic cultures. Mycelium captured by the cheese cloth from original CMC cultures was transferred to a 2 mL screwcap tube containing a single ¼” ceramic bead and 2 mm zirconium oxide beads for gDNA extraction using the E.Z.N.A.® Plant DNA Kit following manufacturer’s protocol, with the initial cell lysis completed using MP Biomedicals™ FastPrep-24™ Classic Instrument at maximum speed for 3 intervals of 20 seconds, resting on ice for 2 min between runs. Extracted gDNA was diluted to 5 ng/μL and stored at -20 °C. PCR reactions were performed using amplification primers corresponding to the inserted hygromycin cassette as a positive control, and internal primers corresponding to the intended gene deletion (Appendix-A Table 3). Single colonies isolated from positively screened putative transformations (i.e., failed to PCR amplify the gene of interest (GOI) and amplified hygromycin resistance sequences) were then screened a final time repeating DNA isolation and amplification protocol described above. A selection of 2-3 transformant strains that demonstrated amplification of the hygromycin gene, as well as a lack of amplification of their respective GOI, were retained for further study. These axenic purified strains were cultured in 10 mL SMYA media using a single homogenized fungal plug for 7 days at 28 °C shaking at 160 rpm. Glycerol stocks of the strains were then made by combining 600 μL of the SMYA culture with 600 μL of sterilized 30 % glycerol and cryopreserved at -80 °C for long term storage. Strains were also inoculated on SNA plates and allowed to grow at 28 °C in the dark until confluent. Plates were then transferred into 4 °C for short term storage.

2.2.1.7 Southern Blot Analysis

Southern Blot analysis was carried out using published methods (Gebbie, 2014). The WT control and a single transformant strain for each of the GOIs was selected and cultured in S1M medium, shaking at 28 °C and 170 rpm for 5 days and mycelium was then harvested via vacuum filtration and ground in liquid nitrogen using a mortar and pestle prior to gDNA isolation using a Illustra Nucleon Phytopure Genomic DNA Extraction Kit (GE Healthcare UK Limited, Buckinghamshire, UK) following the manufacture's protocol. For enzymatic digestion, 20 µg of gDNA from each of the strains was then digested with 5 µL of digestion enzyme (20 units/µL) and 20 µL of cut smart (10X) buffer (New England BioLabs, Ipswich, Boston) and brought up to a final volume of 200 µL with MilliQ water. Samples were left to digest at 37 °C for 6 hours before spiking with an additional 5 µL of enzyme and an additional 16 hour incubation. DNA was then precipitated with cold ethanol:sodium acetate (pH 5.2) (20:1; v:v) at -20 °C for 30 min followed by centrifugation at $17000 \times g$ for 30 min at 4 °C before washing with cold 70% ethanol. DNA pellets were resuspended in 12 µL TE buffer and transferred to Nylon membranes by the capillary elution method (Gebbie, 2014). Prior to elution, DNA was depurinated and denatured *in situ* via 10-minute washes with respective buffers, followed by a water wash and neutralization solution to neutralize the pH prior to transfer.

Post-transfer, the Nylon membrane was rinsed in 5× SCC buffer before UV crosslinking of the ssDNA to the membrane. The membrane was then transferred to hybridization tubes with 12 mL of DIG Easy Hyb (Roche® Life Sciences, Penzberg, Germany) and placed in a hybridization oven at 42 °C for 5 hours. Prior to the end of prehybridization, the hybridization probe was prepared by boiling for 10 min before transferring to ice and then being added to an additional 12 mL of DIS Easy Hyb solution at a concentration 10 ng/mL. After 5 hours of prehybridization, the

solution was decanted off and the probe solution was filtered through a 0.45 µm syringe and added directly to the hybridization bottle. Hybridization was completed overnight (for 16 hours) at 42 °C. The probe was removed, and the membrane was washed 3 times with buffers increasing in stringency: first and second wash for 15 min (at 30 °C) and a third wash for 30 min (at 62 °C). Post washes, the membrane was transferred into a Pyrex container and gently shaken in a detection wash buffer for 2 min at room temperature and removed into a detection buffer containing blocking reagent (Roche® Life Sciences, Penzberg, Germany) and incubated for 1 hour before transfer into an additional detection buffer containing anti-DIG-AP (Roche® Life Sciences, Penzberg, Germany) antibody at a dilution of 1:10000 where it was incubated for 30 min. Post incubation, the membrane was rinsed 3 times for 15 min each time with the detection wash buffer followed by equilibration buffer for 5 min. To visualize the DNA bands, lumigen CPSD (Roche® Life Sciences, Penzberg, Germany) was applied directly into the membrane at a 1/100 dilution and incubated for 30 min in the dark. The membrane was then transferred to a transilluminator for visualization.

2.2.1.8 Phenotype Assessment of Transformant Strains

To obtain a superficial look into potential effects that the gene disruptions might have had on colony phenotype, a single SNA plug was placed on the center of a ½ PDA plate and a ½ PDA plate with 100 mg/L hygromycin from each of the strains. Cultures were incubated at 28 °C for 5 days, at which point the WT strain had reached confluence. Isolates were then photographed, and general features were evaluated.

2.2.2 Wheat Head infection Assays

2.2.2.1 Plant Growth

Susceptible spring wheat cv. Roblin seeds were surfaced sterilized for 10 minutes with a 30% bleach solution followed by two rounds of washing with sterile water before being transferred to a large Petri dish with sterile water soaked Whatman filter paper and left to germinate in the dark at room temperature for 48 hours. Germlings were then transplanted into 6 inch round fiber pots filled with a planting medium composed of 66 % black earth, 33% Pro-Mix BX M and 1% lime for a total of 4 seeds per pot. Potted seedlings were grown under both LED and fluorescent lighting with a 16 hour photoperiod and a temperature cycle of 18 °C days and 16 °C nights until week five where day temperatures were increased to 20 °C and nights increased to 18 °C. A 20-20-20 (nitrogen, phosphorous, potassium) fertilization was applied to pots once weekly. Plants were grown to mid-anthesis (6-7 weeks) at which point *F. graminearum* inoculations were performed. Figure 10-B and C demonstrating a wheat head at mid anthesis as indicated by the protrusion of yellow anthers (a) from the lemma (le) and palea (p).

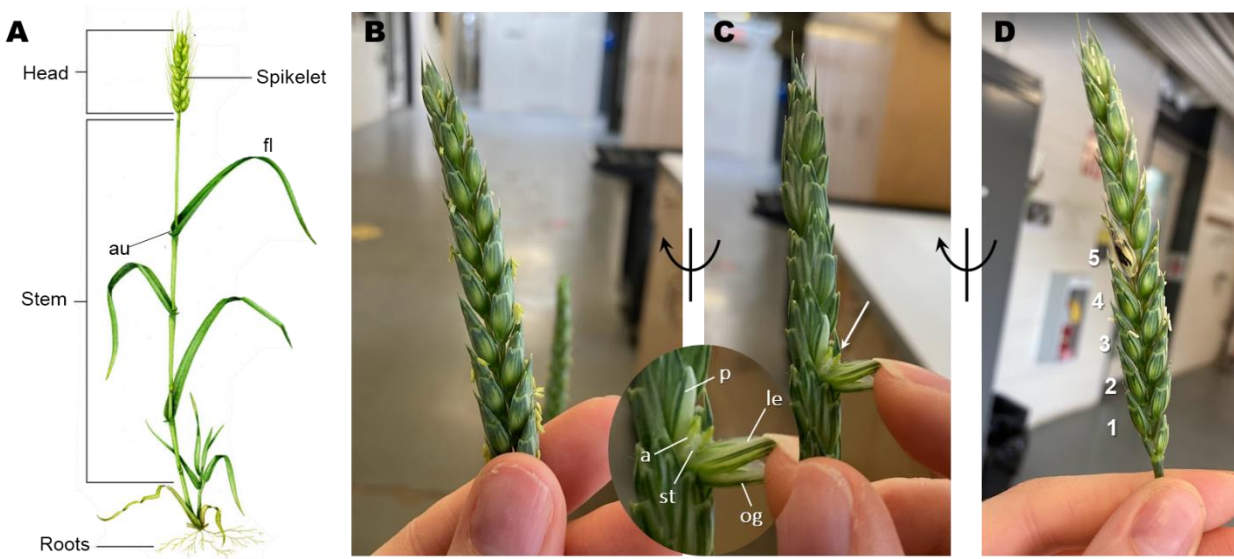


Figure 10: Anatomy of wheat showing (A). general features of the wheat stalk (B) Wheat head at anthesis with yellow protruding anthers indicating readiness for inoculation. (C) anthers (a) and stigma (st) of floret visible between palea (p) and lemma (le). (D) 3 day old inoculated wheat head.

2.2.2.2 Inoculation of Wheat Heads

When 4+ heads from a given pot were at anthesis concurrently, the pots were selected for inoculation. Pots were then allocated in a manner that balances the total number of heads among the strains, ensuring all strains had the same number of heads for the experiment. This was repeated until all pots were inoculated over the course of several days.

Wheat heads were inoculated on the 5th spikelet on the short side of the head as demonstrated in Figure 10-D; inoculated spikelets were marked for ease of identification. Both fertile florets on a single spikelet were point inoculated between the palea and lemma (Figure 10-C) with 1×10^3 macroconidia from either WT or transformant strains. Mock inoculations with sterile water were also completed, to be used as a control. Post inoculation, the plants were returned to the growth chambers with overhead misting for 30 seconds every hour for 48 hours, to help promote fungal infection, and growth temperatures were then increased to 24 °C days and 18 °C nights.

Phenotypic scoring was completed 5, 9, 12 and 14 days post inoculation (dpi). On the 14th dpi heads were also harvested and placed in a -80°C for later metabolomics analysis.

2.2.2.3 Scoring and Data Transformation

Three metrics of fungal infection were scored at each of the time points: bleaching, infection of spikelets and downward infection of the rachis. All scoring values were expressed as a percentage value. For the infected spikelets, percentages were calculated as [number of infected spikelets/total number of spikelets]. Similarly, infection of the rachis was scored by [number of downward rachis' infected/9] where 9 is the total number of possible rachi between the point of inoculation (POI) and the stalk. However, bleaching was scored on a pseudo-total percentage where all spikelets above the POI were considered to be 50% regardless of the actual number above the POI. Scoring was executed this way in part due to the nature of *F. graminearum* infection, where bleaching above the POI occurs often all at once and relatively quickly (~5dpi). This is a result of fungal biomass laterally entering and colonizing the xylem and pith (responsible for distributing water and nutrients upwards in the plant) of the rachis and chocking out the spikelets above the poi (Trail, 2009). This coupled with the large variability in the total number of spikelets (~13-22) across all wheat heads is why a pseudo-total is a more effective measure of scoring overall bleaching.

2.2.2.4 Analysis of Scoring

Score data was then imported into R Studio where it was plotted using the R package ggplot2 (Wickham, 2016)). Statistical significance of measurement comparisons were calculated and plotted using ggstatsplot (Patil, 2021). Kruskal-Wallis (KW) and Wilcoxon Mann-Whitney (WMW) tests were selected due to the non-parametric and ordinal nature of the data. It is

important to note, that the analysis of the individual experimental replicates has a reduced accuracy of p-value calculations due to the smaller sampling sizes, prohibiting the WMW from breaking ties appropriately. However, for the C16 mutants, combined experiments yielded high enough replicate numbers for calculations to be completed as intended.

2.2.3 Coleoptile Infection Assays

Coleoptile optimization procedures are described in Appendix-A section 2.6.3. Roblin seeds were surfaced sterilized (as described above) before being transferred to 2 mL collection tubes filled with 1.7 mL of 0.25% water agar. The tubes were then transferred into a plexi glass container with Whatman cellulose filter paper rounds saturated with 10 mL of sterile MilliQ water lining the bottom of the container. Lids were then secured using binder clips and containers were placed into an incubator to be grown in the dark with a temperature cycle of 22 °C days and 22 °C nights. After 72 hours, when coleoptiles were approximately an inch in length and the first leaves had not yet emerged, containers were moved into a biosafety cabinet for inoculation. Seeds with obvious contamination or coleoptiles of insufficient length were discarded. Coleoptiles were cut with sterile scissors; care was taken as to not cut the first leaf and cut coleoptile ends were inoculated with 2000 macroconidia of one of experimental strains (WT or transformant strains). Mock inoculations with sterile MilliQ water were also completed as negative controls. Containers were then returned to the incubators where they were grown under both LED and fluorescent lighting with a 16 hour photoperiod and a temperature cycle of 22 °C days and 22 °C nights. At 4 dpi, coleoptiles were scored by measuring the longest point of lesion length of the necrotic tissue with a caliper.

2.3 Results

2.3.1 Generation and Verification of Transformant Strains

2.3.1.1 Designing sgRNA Protospacers for Deletion of GOIs

In order to evaluate potential pathogenic effects pertaining to the secondary metabolite products of the BCGs of interest, targeted gene disruption was performed on 9 individual genes spanning 4 gene clusters C08, C16, C70 and C13. Multiple transformants were selected for each GOI by apparent hygromycin resistance and PCR validation (Appendix-A Table 4; Appendix-A Figures 3-11).

2.3.1.2 Southern Blot Analysis

To confirm correct incorporation of the HygB repair template was completed and that recombination resulted in the cassette's insertion into the targeted gene locus, Southern blot analysis was performed on a single purified transformant from each of the genes of interest. DNA digestion was completed on extracted gDNA from each of the transformant strains as well as a negative control WT strain. Enzymes selected for the DNA digest were selected for each of the respective transformants based on their respective GOI with the intention to isolate the inserted hygromycin cassette within a 4-10kb fragment. This allowed for the band to be visualized within the limits of the ladder. Enzymes selected for each of the respective transformants as well as their expected fragment size are specified in Figure 11-C. Following the digestion, DNA was run on an agarose gel with samples spanning two different gels arranged by the enzyme used in digestion. This was followed by DNA hybridization with a HygB specific probe with results depicted in Figure 11-A-B. Regarding Figure 11-A and B, observed bands indicate the length of resulting DNA fragments with the incorporated HygB cassette. All

transformants contained HygB cassettes of the expected size as determined by comparison of each samples band size following their respective digestions relative to the ladder and the calculated predicted size. Each of the WT strain digests lacked DNA bands indicating the expected absence of the HygB cassette.

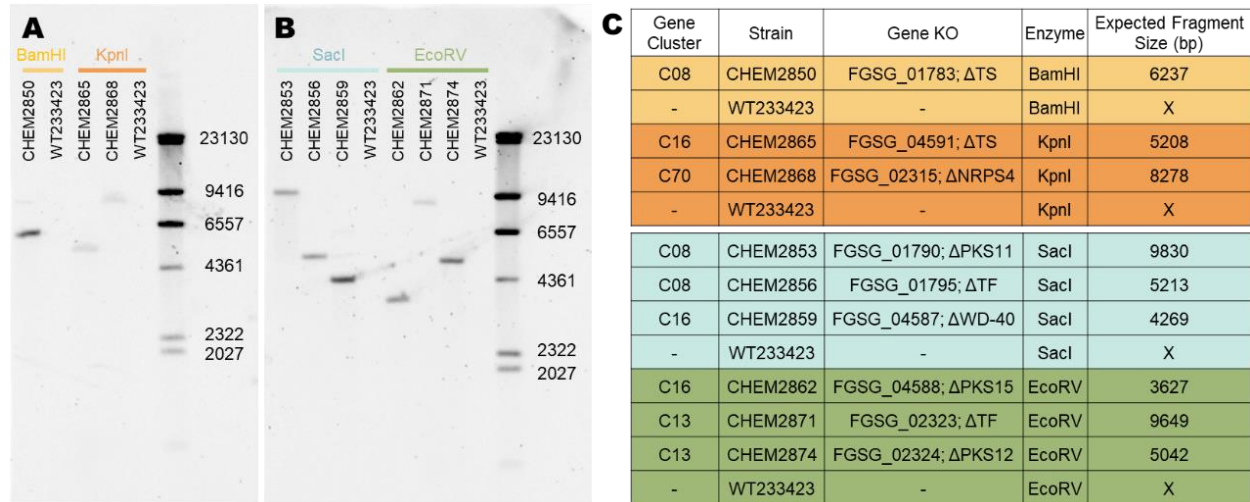


Figure 11: Southern blot analysis of select transformants with hybridization probe specific of the HygB gene. Strains were organized by the enzyme used in the gDNA digest (A) BamHI and KpnI and (B) SacI and EcoRV. (C) Table indicating the strains and their corresponding enzyme as well as the expected sized of the bands for each transformant.

2.3.1.3 Phenotype Assessments

All generated transformant strains were grown on ½ PDA and ½ PDA with hygromycin to compare phenotypes of the cultures as well as to demonstrate hygromycin resistance (Figures 12-14). The majority of the resulting culture phenotypes of WT and transformant strains grown on ½ PDA were found to be comparable in terms of growth diameter and colony appearance. All transformant strains demonstrated growth on medium supplemented with hygromycin further demonstrating success of HygB incorporation. For the C08 BGC (Figure 12), transformant lines

(Δ TS, Δ PKS11, Δ TF) produce floccose colonies that grew to confluency (or near confluency) and had a pinkish hue of the colony center (as observed for the WT strain) on $\frac{1}{2}$ PDA. Colonies of the C08 transformant lines on $\frac{1}{2}$ PDA supplemented with hygromycin were restricted in growth compared to non-hygromycin supplemented colonies; however, colony phenotypes between the transformant lines (Δ TS, Δ PKS11, Δ TF) were comparable between each other, while no growth was observed on $\frac{1}{2}$ PDA + hygromycin for the WT strain.

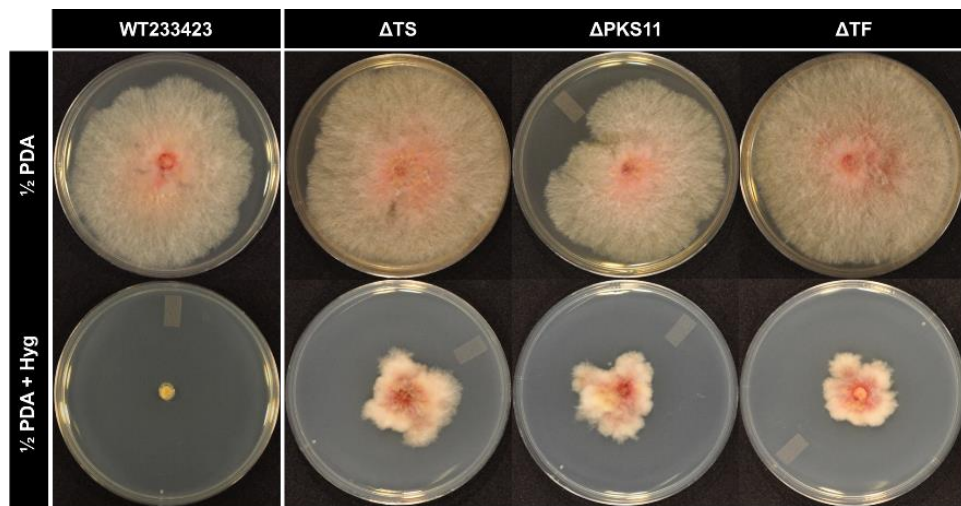


Figure 12: Phenotypes of respective transformants from the C08 gene cluster cultured on $\frac{1}{2}$ PDA (top) and $\frac{1}{2}$ PDA supplemented with 100mg/L hygromycin (bottom).

For the C16 BGC, colony phenotypes of all three transformant strains (Δ WD40, Δ PKS11 and Δ TS) resembled the WT strain when grown under the $\frac{1}{2}$ PDA condition (Figure 13). However, when cultured on $\frac{1}{2}$ PDA supplemented with hygromycin, the C16 Δ TS transformant strain displayed a noticeably constricted growth phenotype (with prominent yellow/brown pigmentation) relative to the other transformant strains (Δ WD40, Δ PKS11). The C16 Δ TS

transformant phenotype presented in Figure 13 was observed for all of the ΔTS transformant colonies generated in this study (Appendix-A Figure 12).

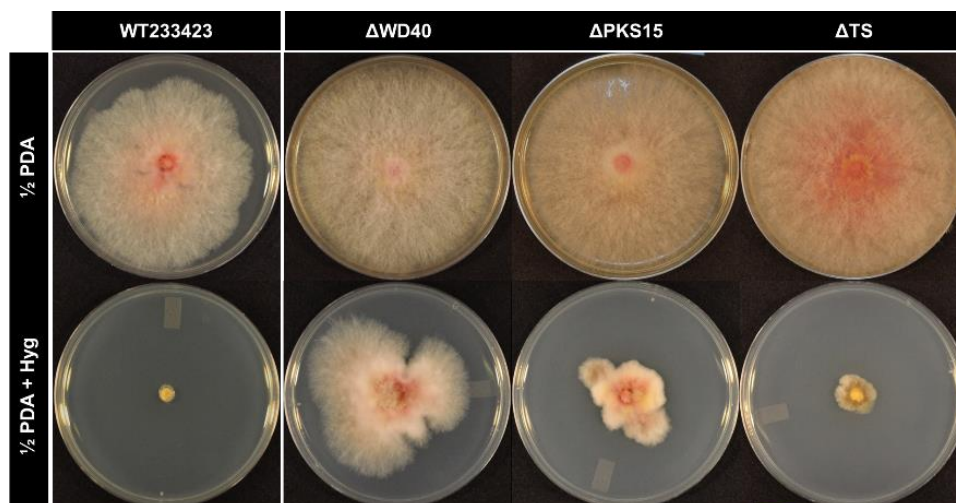


Figure 13: Phenotypes of respective transformants from the C16 gene cluster cultured on $\frac{1}{2}$ PDA (top) and $\frac{1}{2}$ PDA supplemented with 100mg/L hygromycin (bottom).

From the C13 BGC, on $\frac{1}{2}$ PDA media, cultures of the $\Delta PKS12$ transformants presented an albino phenotype (Figure 14). The red pigmentation observed from *F. graminearum* in culture is characteristic of the production of the secondary metabolite aurofusarin, the polyketide product of the C13 BGC. The absence of pigmentation in the $\Delta PKS12$ transformant strain, as compared to the WT, provides further proof of the success of the intended disruption of *PKS12*. From the C70 BCG, the $\Delta NRPS4$ transformant strain resulted in a colony with restricted growth and irregular margins relative to the more consistent growth seen within WT strain when cultured on $\frac{1}{2}$ PDA. All transformant lines for the C13/C70 BGC were able to grow on $\frac{1}{2}$ PDA supplemented with hygromycin, with comparable colony phenotypes (taking into consideration the lack of aurofusarin production in the $\Delta PKS12$ transformant).

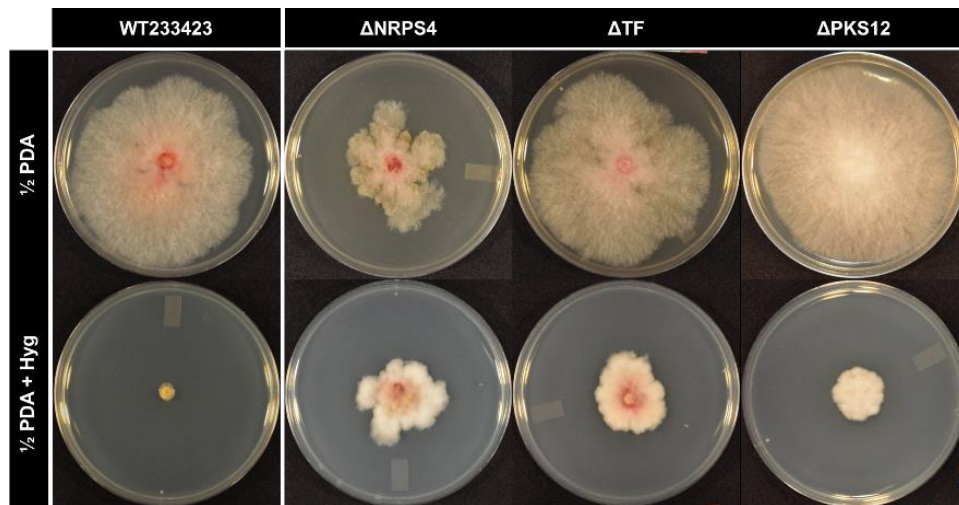


Figure 14: Phenotypes of respective transformants from the C70/C13 gene cluster cultured on 1/2 PDA (top) and 1/2 PDA supplemented with 100mg/L hygromycin (bottom).

2.3.2 In-Planta infection Assays

2.3.2.1 Wheat head infection Assays

Pathology testing of the *F. graminearum* WT and all of the transformant strains was carried out using the susceptible wheat cultivar Roblin by point inoculation. Phenotypic scoring of each infection was based on bleaching, infected spikelets, and downward rachis infection at 5, 9, 12 and 14 dpi. Two rounds of wheat head infection assays were included in the analysis. The first round of sampling included inoculations of all 9 transformant strains, WT233423 and water mock inoculations (n = 20). The second round consisted of only transformant stains belonging to the C16 gene cluster (ΔWD-40, ΔPKS15 and ΔTS) as well as WT233423 and water mock inoculations (n = 48). Data from both experimental rounds for the C16 gene cluster were combined for data analysis (total n = 68 replicates).

With regards to the attribute of bleaching, by the 5th dpi, wheat heads inoculated with the WT strain typically demonstrated bleaching within all spikelets apical to and including the POI

resulting in approximate pseudo-percentage score of 50% bleaching. However, the jump from 0% -to- 50% bleaching occurred rapidly as a result of use of the pseudo metric scoring as bleaching above the POI occurs almost simultaneously following infiltration of the fungi from the inoculated spikelet into the rachis resulting in the colonization xylem and pith preventing the transportation of water and nutrients. Bleaching of spikelets then persisted downward from the POI consecutively. The rate of bleaching is best observed within the day 9-14 scoring dates as this measure is more consistent within bleaching inferior to the POI. Bleaching typically occurred at a relatively consistent rate following the initial bleaching above the POI. By the 14th dpi, the wheat heads were either completely bleached or almost completely bleached (~90-100%).

The phenotype of infected rachis in and inferior direction from the POI consistently demonstrated a score of 100% infection by 12dpi as brown necrotic lesions could be seen through all downward nodes in wheat heads infected with the WT strain. Some wheat heads even displayed visible necrosis within the stem adjacent to the first spikelet; however, this was not included in the scoring metric. Similar to bleaching, rachis infection occurred consecutively from adjacent nodes.

Contrary to the consecutive symptoms of fungal invasion displayed by both bleaching and rachis infection phenotypes, the observation of “infected spikelets” was more sporadic in occurrence. The appearance of necrotic lesions within spikelets were overall less abundant and often only seen within one of the florets of each spikelet. Infected spikelets never presented below the point of the terminal infected rachis, indicating lateral colonization and symptoms of necrosis within adjacent spikelets may be slower to develop in appearance than the symptoms of rachis infection.

The percentage of infected spikelets was typically around 50% by 14dpi within WT inoculated wheat heads.

All tested transformant strains from the C08 (Δ TS, Δ PKS11, Δ TF; Figure 15) and the C13/C70 (Δ NRPS4, Δ TF, Δ PKS12; Figure 16) gene clusters provided similar pathogenicity phenotypes to those displayed by the WT strain within the measured criteria as demonstrated by the Figure 15 and 16 box violin plots. P-values obtained from the Kruskal-Wallis tests comparing the WT infection phenotypes to those of the C08 and C13/70 transformants demonstrated no statistically significant deviation (>0.05).

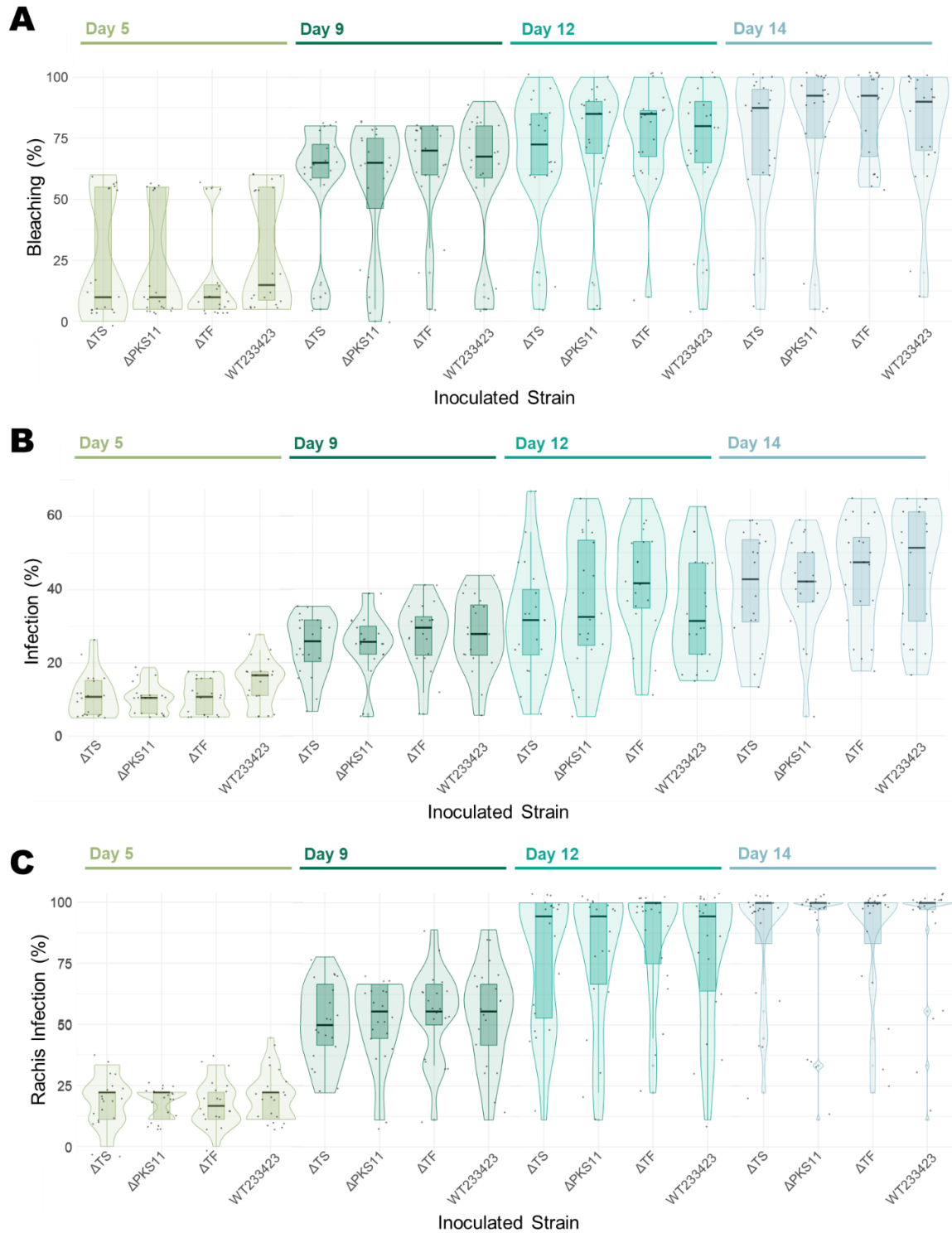


Figure 15: Box violin plots of wheat head infection scores (n= 20) from C08 transformant strains. (A) pseudo % of the overall number of bleached spikelets with spikelets above the point of infection representing 50% infection. (B) % of spikelets infected demonstrating necrotic lesions. (C) % of infected rachis nodes from the point of infection downwards

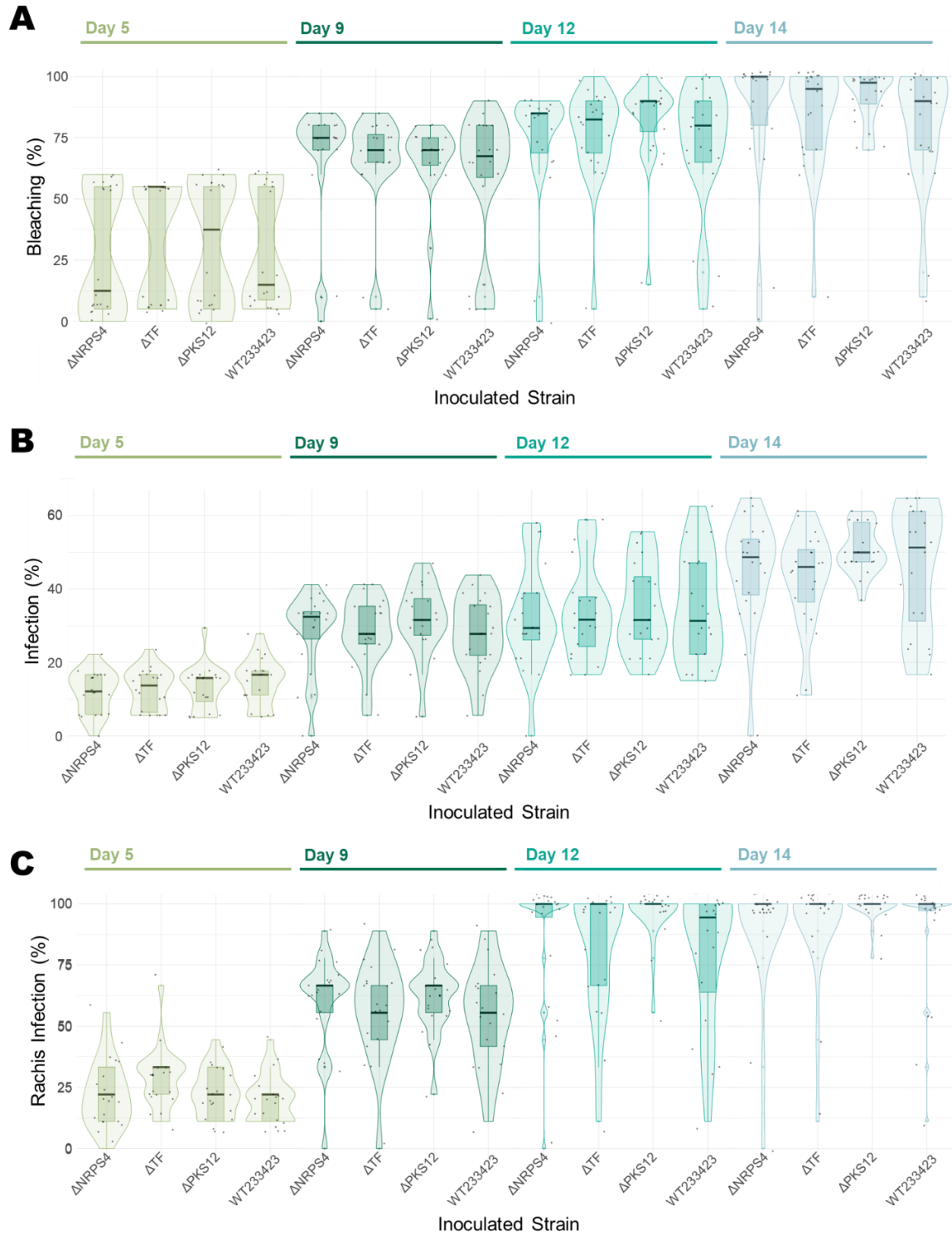


Figure 16: Box violin plots of wheat head infection scores (n= 20) from C13/C70 transformant strains. (A) pseudo % of the overall number of bleached spikelets with spikelets above the point of infection representing 50% infection. (B) % of spikelets infected demonstrating necrotic lesions. (C) % of infected rachis nodes from the point of infection downwards.

For the BGC C16 transformant lines (as compared to WT), box violin comparison of the disease progression scores demonstrates a consistent statistical reduction in the infection by the C16 Δ TS transformant strain across all phenotypic categories (Figure 17 and 18). Lower disease scores indicative of a slower infection rate are observed as early as 5 dpi and persist through to the final time point of 14 dpi. Wilcoxon Mann-Whitney (WMW) tests performed between the C16 Δ TS transformant and WT strains yielded p-values below 0.01 for each of the phenotypic categories across all time points with the exception of % spikelet infection at 12 dpi. Onset of infection and the progression of disease symptoms were not halted by the absence of the TS product; however, the rate of FHB infection does appear to be delayed compared to WT. Interestingly, both the other transformant strains of the C16 BGC (Δ PKS15 and Δ WD40) did not show a significant deviation from the WT in any of the measured phenotypic traits.



Figure 17: 14dpi In-planta phenotypes observed by C16 transformant strains from wheat head infection assays relative to the WT strain infection and water mock inoculations.

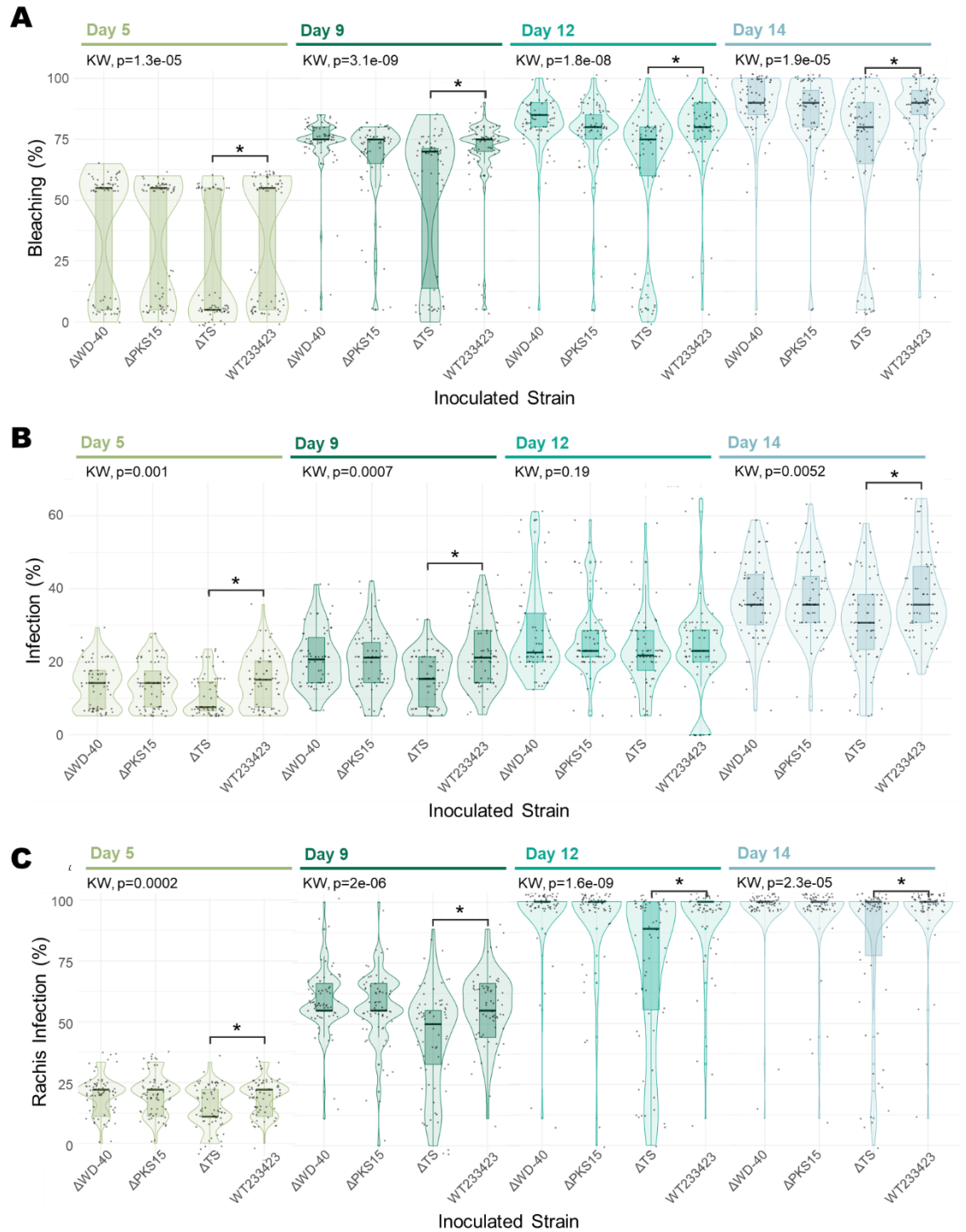


Figure 18: Box violin plots of wheat head infection scores (n= 68) from C16 transformant strains. (A) pseudo % of the overall number of bleached spikelets with spikelets above the point of infection representing 50% infection. (B) % of spikelets infected demonstrating necrotic lesions. (C) % of infected rachis nodes from the point of infection downwards. (*) indicates WMW scores <0.05.

2.3.2.2 Coleoptile Infection Assays

Phenotypic results from the coleoptile infection assays demonstrated much greater variability relative to the wheat head infection assay. This is illustrated in the WT strain for Figure 19-B in which infection symptoms can be seen ranging from full coleoptile necrosis to a complete lack of a necrotic lesion. As a result of this high variability, there is not sufficient replication to demonstrate a statistical significance between any of the transformant strains relative to the WT. Generated box violins plots (Figure 19-A) do demonstrate trends within the results that provide good candidates for further investigation. Transformants from the C08 gene cluster display results similar to those of the WT, in which the high variability in lesion length is still apparent and the average is pretty consistent with that of the WT. The C16 transformants displayed a similar trend to that observed within the wheat head infection assay, in which the Δ WD-40 and Δ PKS15 phenotypes were consistent with the WT strain and Δ TS presents a reduction in the average lesion length. Fewer coleoptiles inoculated with the C16 Δ TS transformant strain had necrotic lesions spanning the full coleoptile length, further suggesting a potential roll of the C16 BGC in pathogenicity.

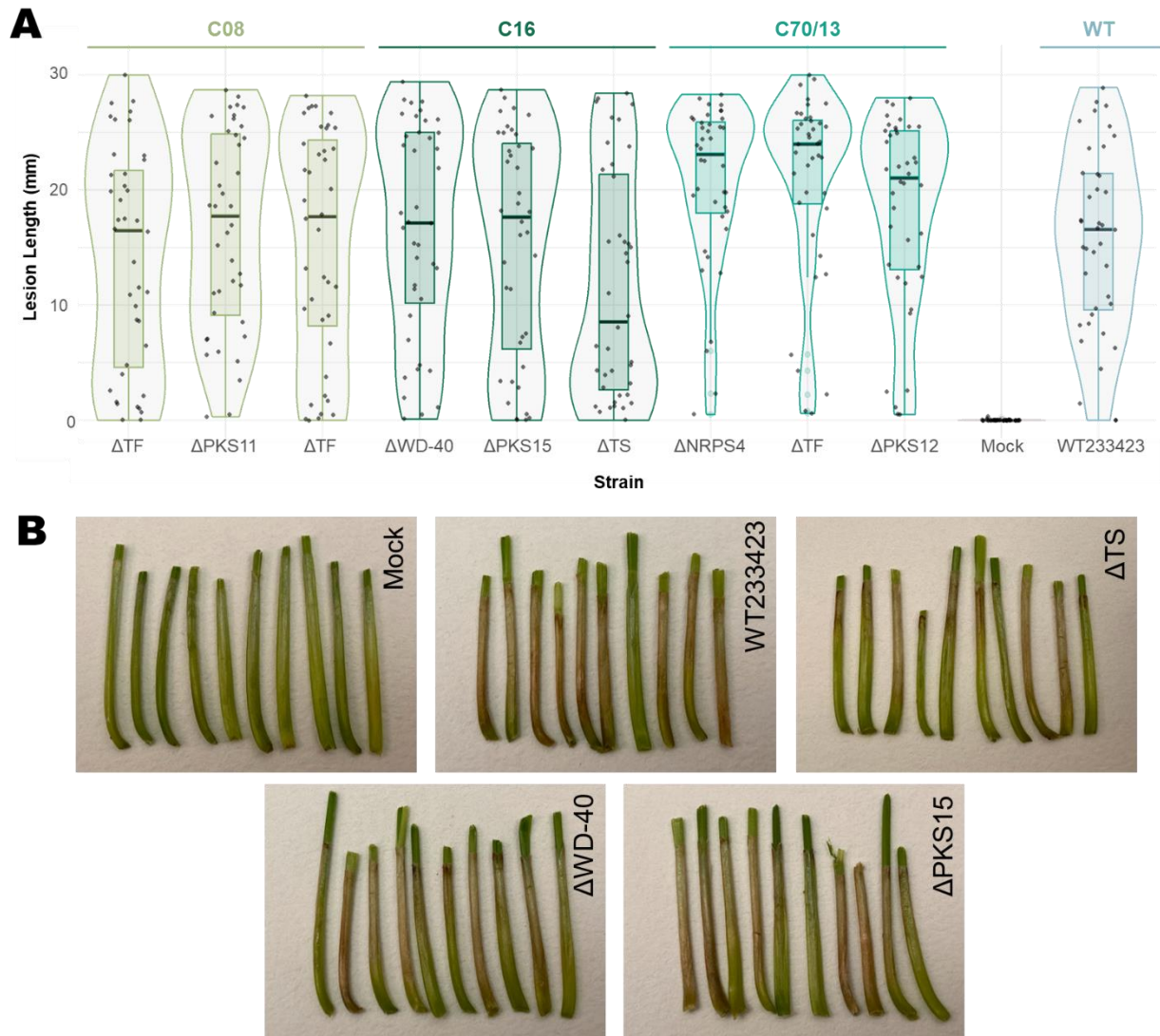


Figure 19: (A) Box violin plots comparing coleoptile infection (n=40) necrotic lesion lengths (mm) of transformant strains to WT strain. Violin plots were used to demonstrate the non-normal distribution of the data. (B) 4dpi in-planta phenotypes observed by the C16 transformant strains.

Alternatively, transformants within the C13/C70 all displayed an increase in the overall lesion lengths relative to the WT. This was unique to the coleoptile assay as wheat head infection assays displayed no change in phenotype among the C13/70 transformants. Replication was insufficient to determine if this increase resulted from the high variability of this assay, preventing accurate validation of the phenotype.

2.4 Discussion

2.4.1 Characterization of C08 Transformants

Transformant strains pertaining to the C08 BGC (Δ TS, Δ PKS11 and Δ TF) all produced phenotypes that do not deviate from those of the WT strain. Both the *in-planta* wheat head infection assays and coleoptile infection assays suggest that the secondary metabolites associated with these selected GOIs do not play a significant role in the pathogenicity of *F. graminearum* in wheat. Several reports of the expression of PKS11 *in-planta* have been made (Güldener et al., 2006; Lysøe et al., 2011; Harris et al., 2016). Güldener (2006) and Lysøe (2011) both isolated RNA from inoculated plant tissues and analyzed expression of PKS and NRPS genes using a *F. graminearum* Affymetric GeneChip designed by the former. Güldener (2006) completed the expression analysis using spray inoculated Morex malting barley (*Hordeum vulgare*) while Lysøe (2011) completed point inoculated *Triticum aestivum* wheat. Both researchers reported an increase in the expression of PKS11 during pathogenicity testing with *F. graminearum*, Lysøe (2011) reporting an increase 96hpi and Güldener (2006) 144hpi respectively. Harris (2016) also completed expression analysis of the PKS11 gene using a MicroArray of their own design. They demonstrated expression of PKS11 in both wheat cv. Roblin and barley cv challenged with *F. graminearum*. Unlike wheat and barley, Harris et al. (2016) reported a lack of PKS11 gene expression in *F. graminearum* challenged maize plants.

Gaffoor et al. (2005) previously performed a gene deletion of PKS11 and evaluated the pathogenicity of the transformant strain relative to that of their *F. graminearum* WT. The analysis was conducted using wheat cv. Wheaton and produced results consistent with our wheat cv. Roblin infection assays. Specifically, their generated Δ PKS11 strains exhibited a phenotype that

remained unchanged from the WT. In addition to the infection assays, Gaffoor (2005) also reported that the Δ PKS11 strains do not affect perithecius production or function in *F. graminearum*, as perithecia were able to form and discharge ascospores.

Experiments completed by Boedi et al. (2016) provide insight into a potential theory as to why *in-planta* gene expression of PKS11 is observed, however with no phenotypic effect in terms of disease symptoms were observed for Δ PKS11 strains. Boedi (2016) evaluated gene expression under both saprophytic and pathogenetic conditions in wheat cv. Remus. They report that PKS11 expression as being within the 35th percentile of gene expression levels within saprophytic conditions suggesting that SM production is more abundant on a non-living host. Observations of PKS11 gene expression within the pathogenic type experiments completed by Gldener (2006) and Lyse (2011) Harris and (2016) could all be a result of the fungal metabolism switching over from a biotrophic to a necrotrophic state (similar to a more saprophytic response) as the host begins dying. This theory correlates to the delayed onset of gene expression (typically 144 hpi) when compared to the expression patterns of other well known pathogenicity factors like DON for which gene expression is observed around 24-48 hpi.

Reports on gene expression for the C08 BGC are generally centered around PKS11, as it is the main biosynthetic gene within the cluster. Previous expression patterns as well as phenotypes observed within this study suggest that the C08 PKS11 gene is not significantly associated with the pathogenicity of wheat despite the reported *in-planta* expression. Our findings regarding the C08 Δ TS and C08 Δ TF within this gene region also suggest they are not likely associated with wheat pathogenicity.

2.4.2 Characterization of C16 Transformants

The transformant strains derived from the C16 BGC yielded intriguing outcomes during plant infection assays. Both Δ PKS15 and Δ WD40 strains exhibited phenotypes that remained consistent with the WT strain. In contrast, the Δ TS strain displayed a noticeable reduction in the rate of plant infection compared to the WT strain across all measured aspects of FHB infection, bleaching, rachis infection and spikelet infection.

Reports of in-planta expression of C16-associated genes were documented by Harris et al. (2016), who noted the presence of expression for both PKS15 and TS across all three tested crop types (maize, barley cv. Encore, and wheat cv. Roblin). Similarly, Gldener et al. (2006) and Lyse et al. (2011) also identified gene expression of PKS15 on barley cv. Morex and wheat cv. Wheaton respectively. Both Gldener (2006) and Lyse (2011) demonstrated early onset of transcription occurring around 48 hpi. This early onset is consistent with the onset of their reported expression of other pathogenicity factors (fusaoctaxin). Media studies by Gaffoor et al. (2005) exclusively observed the expression of PKS15 (then referred to as PLSP1) in inoculated wheat heads. Gaffoor analyzed gene expression on fifteen distinct *in-vitro* culture conditions however, only observed expression in wheat assays suggesting that the expression of the PKS15 gene may be highly regulated.

Boedi et al., 2016 conducted a comparative study on gene expression during both pathogenic and saprophytic infections of wheat cv. Remus. Their investigation revealed that ten genes within the C16 gene cluster exhibited exclusive induction by plant signals during pathogenesis. Among these genes were FGSG_04588 (PKS15) and FGSG_04591 (TS), indicating their potential association with defensive responses. Intriguingly, three genes from the C16 cluster, including

FGSG_04589, FGSG_04591 (TS), and FGSG_04592, were expressed among the 65% of the most highly expressed genes under pathogenic conditions. Furthermore, the study reported that the FGSG_04587 (WD40) gene displayed expression exclusively under saprophytic conditions, suggesting it may have a distinct function unrelated to that of PKS15 and TS within the C16 cluster.

The sole studies exploring the potential link between the C16 gene cluster and pathogenicity, apart from transcriptomic analyses, were conducted by Gaffoor et al. (2005) and Seidl et al. (2022). In their research, both investigators generated single-gene deletion mutants of Δ PKS15 and independently observed consistent infection phenotypes to the WT strain. This was consistent with the observed phenotype of our Δ PKS15 strain. However, no additional analyses were undertaken by either Gaffoor et al. or Seidl et al. to compare the impact of the TS on pathogenicity.

2.4.3 Characterization of C13/C70 Transformants

Wheat head infection assays pertaining to C13/C70 transformant strains (Δ NRPS4, Δ TF, Δ PKS12) all yielded phenotypes that did not deviate from the WT. This observation is consistent with the result of previous pathogenicity assays completed by Malz et al. (2005) and Westphal et al. (2021) with their own *F. graminearum* transformant lines. Using C13 Δ PKS12 transformant strains, Malz et al., 2005 completed assessments for barley stem blight on cv. Alexis, and wheat head infection assays using summer wheat cv. Nandu as compared to their WT, in which they found differential effect on pathogenicity. Disruption of the C13 *AurR2* gene (Δ TF) in *F. graminearum* has not been previously applied to pathogenicity studies; no deviation from the WT phenotype was observed in wheat head trials in this thesis. This result is unsurprising

considering a similar result was observed from the Δ PKS12 transformant strain and the fact that the TF AurR2 has been previously correlated to aurofusarin production. Using C70 transformant strains, Westphal et al. (2021) completed pathogenicity experiments using USU-Apogee full-dwarf hard red spring wheat (*Triticum aestivum* cv) and demonstrated that there was no significant effect on infection in their Δ NRPS4 strain as compared to WT. They did however demonstrate that there was a reduction in the infection symptoms observed in trials using their OE:NRPS4 strain. They hypothesised that the observed effect was a result of malformed conidia that were observed from cultures of their OE strain.

In coleoptile infection assays, all three C13/C70 BGC transformant strains produced consistently longer lesion lengths relative to those of the WT. This may be contradictory to phenotypes seen in the wheat head pathogenicity trials. Differences in pathological response of transformant *F. graminearum* lines in coleoptile vs wheat head challenge has been observed previously by other researchers. Zhang et al., 2012 demonstrated a similar phenomenon within infection phenotypes of fusaoctatin deletion strains, in which no alteration of the disease phenotype as compared to WT was observed in wheat head infection assays, but a reduced infection phenotype was identified in the coleoptile assays. This biological variation is original rationale as to why both assays were used to screen the transformant strains. However, the results of the coleoptile experiments performed are not statistically supported due to the large variability in biological response inherent in the coleoptile assay; therefore follow up experiments involving additional replicates are required to confirm the observed phenotype trend.

2.5 References

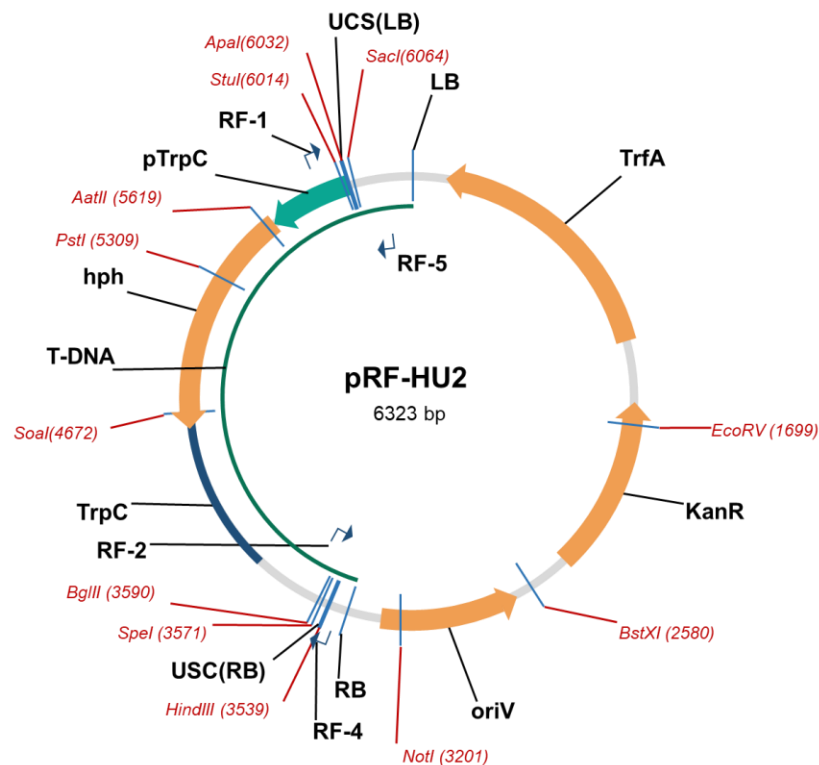
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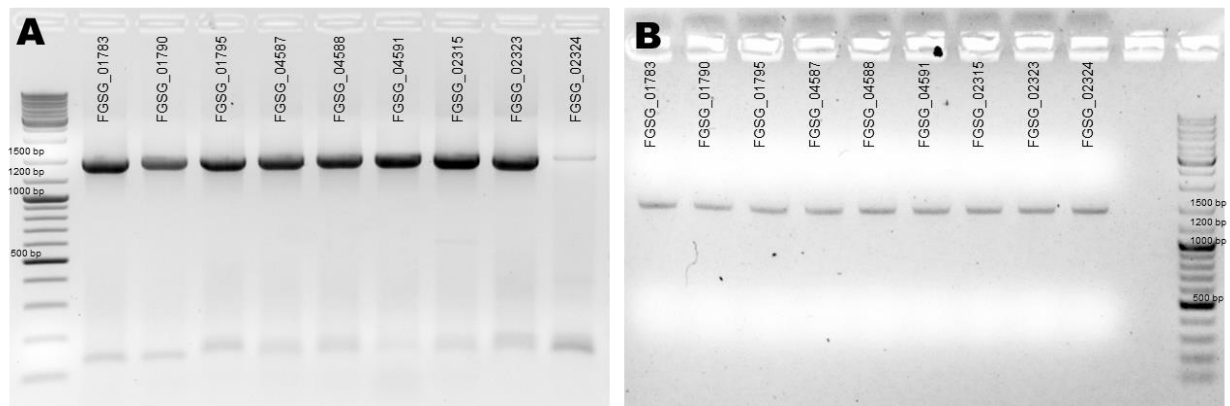
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2.6 Appendix-A

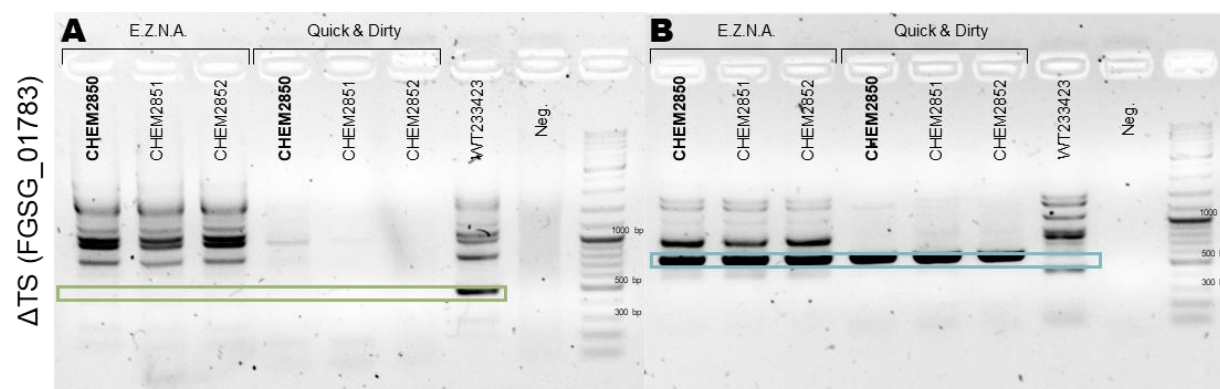
2.6.1 Appendix-A Figures



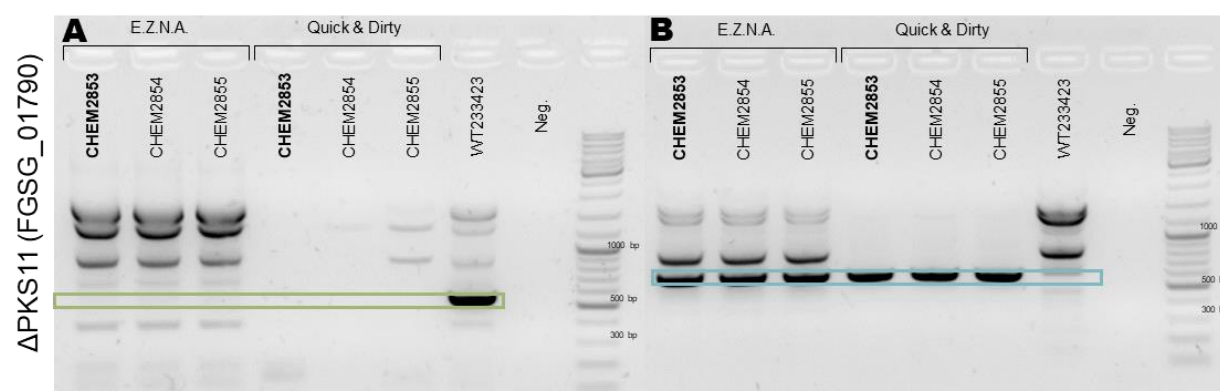
Appendix-A Figure 1: pRF-HU2 vector map used in construction of Hygromycin repair templates.



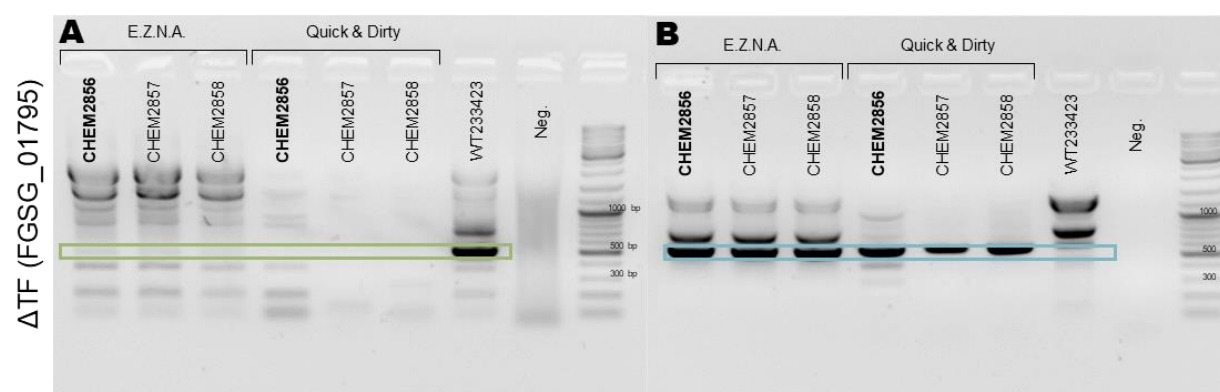
Appendix-A Figure 2: Constructed HDR templates with amplified hygromycin resistance gene with appended 45-50 bp of microhomology corresponding to each of the associated GOI's. (A) Repair templates as amplified from pRFHU2 vector (B) Repair templates post clean-up.



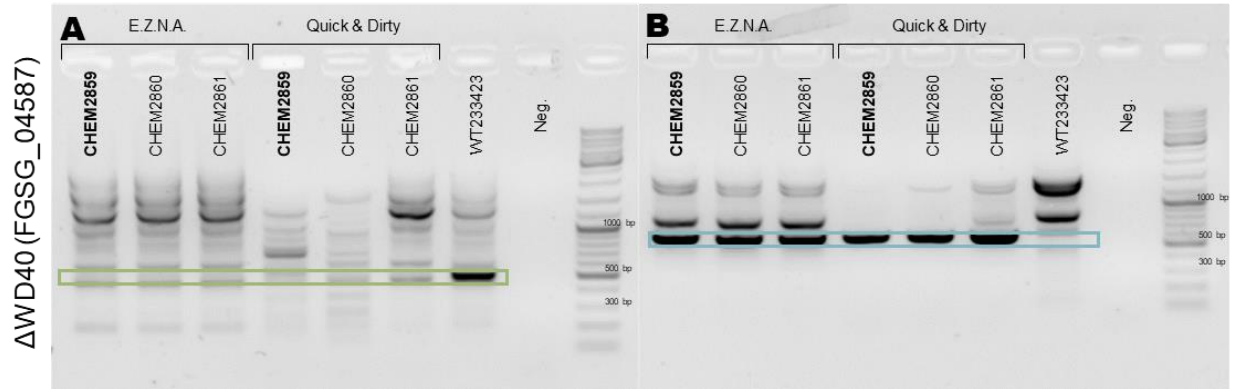
Appendix-A Figure 3: PCR validation of FGSG_01783 transformant strains by (A) absence of amplification of the GOI as determined using primers internal to the GOI and (B) presence of the hygromycin gene. Bolded strain indicates the strain used phenotype analysis and southern blot analysis.



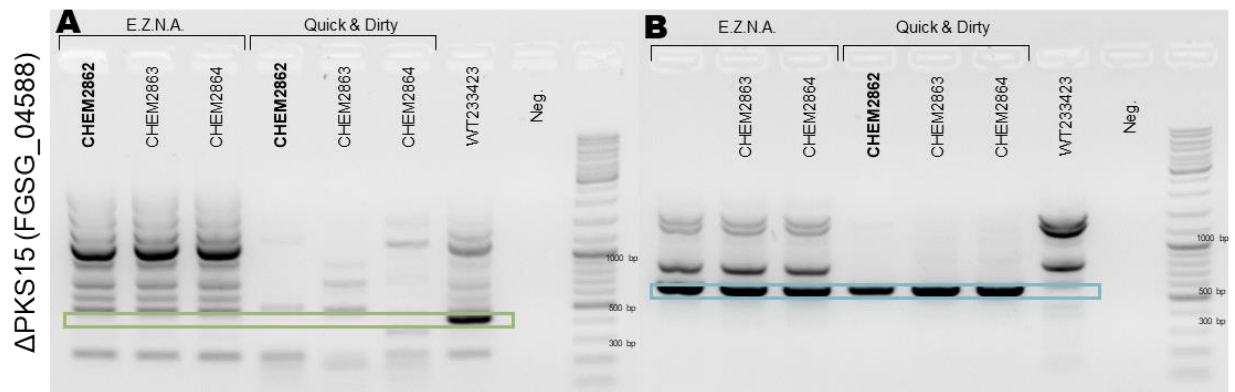
Appendix-A Figure 4: PCR validation of FGSG_01790 transformant strains by (A) absence of amplification of the GOI as determined using primers internal to the GOI and (B) presence of the hygromycin gene. Bolded strain indicates the strain used phenotype analysis and southern blot analysis.



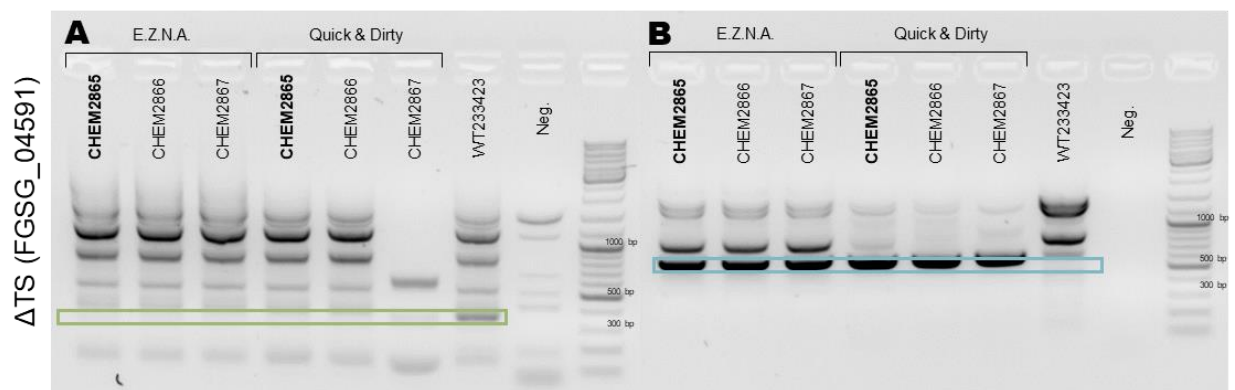
Appendix-A Figure 5: PCR validation of FGSG_01795 transformant strains by (A) absence of amplification of the GOI as determined using primers internal to the GOI and (B) presence of the hygromycin gene. Bolded strain indicates the strain used phenotype analysis and southern blot analysis.



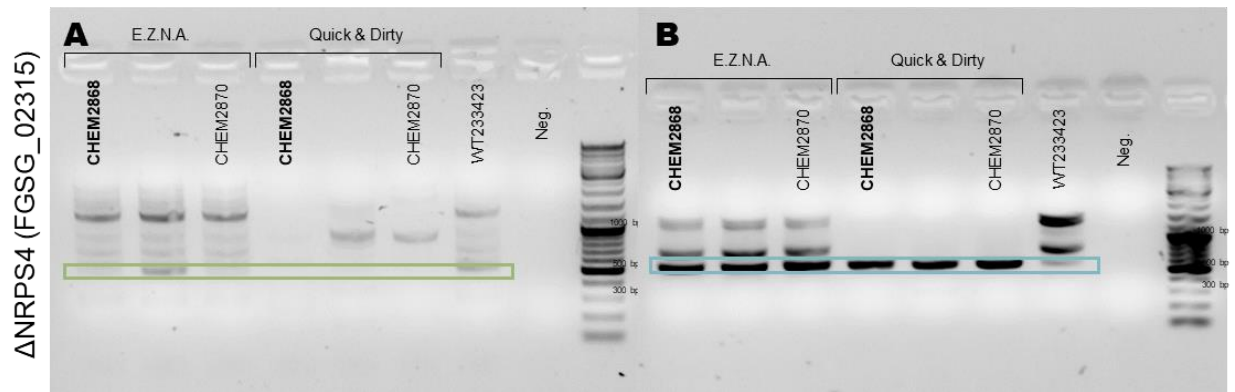
Appendix-A Figure 6: PCR validation of FGSG_04587 transformant strains by (A) absence of amplification of the GOI as determined using primers internal to the GOI and (B) presence of the hygromycin gene. Bolded strain indicates the strain used phenotype analysis and southern blot analysis.



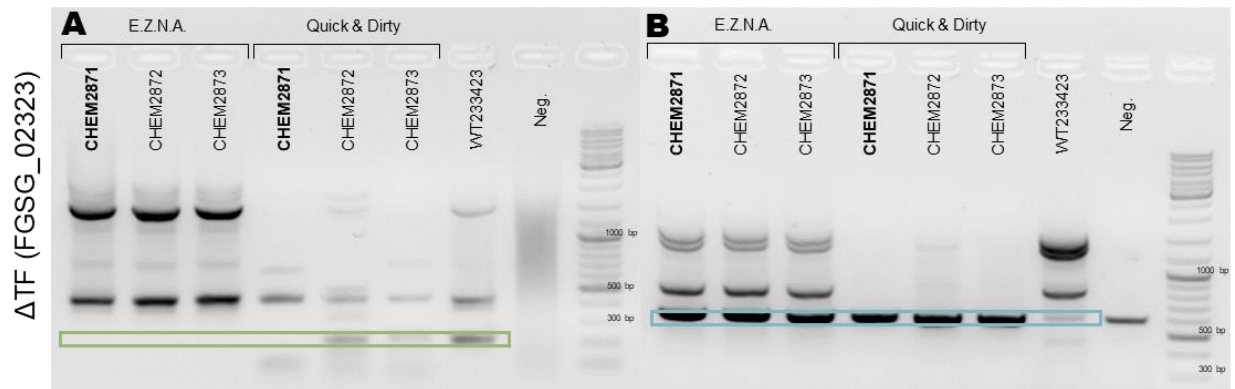
Appendix-A Figure 7: PCR validation of FGSG_04588 transformant strains by A) absence of amplification of the GOI as determined using primers internal to the GOI and B) presence of the hygromycin gene. Bolded strain indicates the strain used phenotype analysis and southern blot analysis.



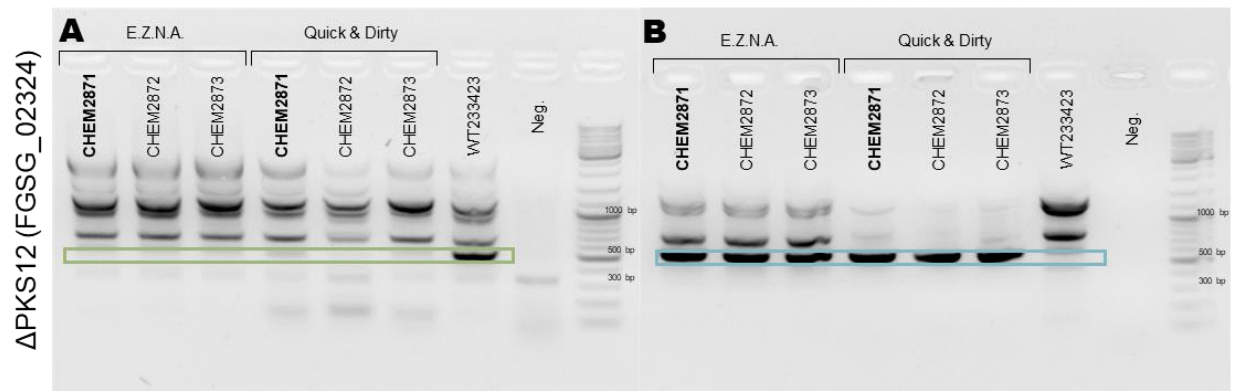
Appendix-A Figure 8: PCR validation of FGSG_04591 transformant strains by (A) absence of amplification of the GOI as determined using primers internal to the GOI and (B) presence of the hygromycin gene. Bolded strain indicates the strain used phenotype analysis and southern blot analysis.



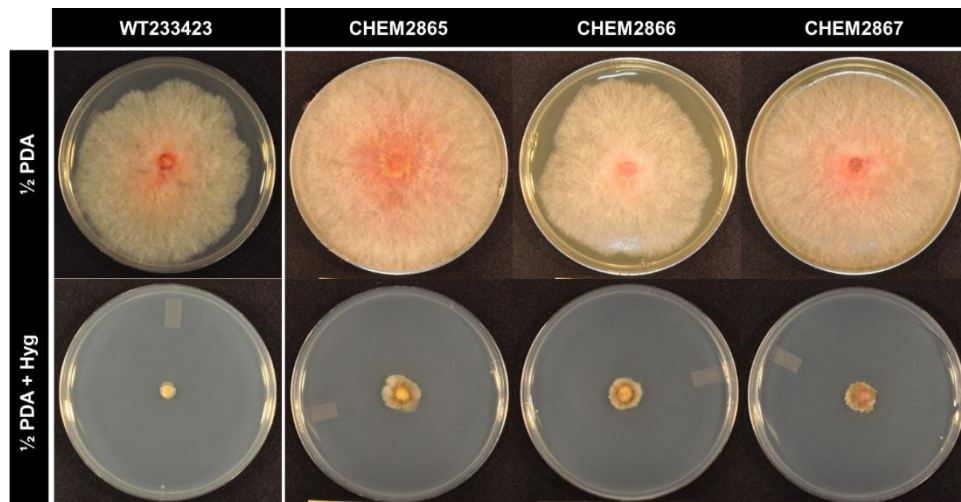
Appendix-A Figure 9: PCR validation of FGSG_02315 transformant strains by (A) absence of amplification of the GOI as determined using primers internal to the GOI and (B) presence of the hygromycin gene. Bolded strain indicates the strain used phenotype analysis and southern blot analysis.



Appendix-A Figure 10: PCR validation of FGSG_02323 transformant strains by (A) absence of amplification of the GOI as determined using primers internal to the GOI and (B) presence of the hygromycin gene. Bolded strain indicates the strain used phenotype analysis and southern blot analysis.



Appendix-A Figure 11: PCR validation of FGSG_02324 transformant strains by (A) absence of amplification of the GOI as determined using primers internal to the GOI and (B) presence of the hygromycin gene. Bolded strain indicates the strain used phenotype analysis and southern blot analysis.



Appendix-A Figure 12: Phenotypes displayed by all C16 Δ TS transformant strains demonstrating retardation of growth on hygromycin supplemented media formulation by all transformation events.

2.6.2 Appendix-A Tables

Appendix-A Table 1: Primers used in the amplification of hygromycin repair cassettes. Sequences in yellow and green represent regions homologous to the pRF-HU2 vector downstream and upstream to the Hyg resistance gene respectively. Sequenced in black are homologous to the *F. graminearum* genome adjacent to intended Cas-9 cut sites.

Gene Cluster	Location	Primer Name	Primer Sequence 5'-3'
FGSG_01783	DO	FGSG_01783hygMicroDO	CAAGTACGTCACCATGAGCCTGAGACTTAGTGGTGTATACCAACTAT AAACTATTCCCTTGCCCTCGGAC
	UP	FGSG_01783hygMicroUP	GCTAGACTAGACGATTGACTCGTGCCAGAGGCTAGGGTATGGAATGC CCGTGACAGAGAAGATGATATTGA
FGSG_01790	DO	FGSG_01790hygMicroDO	CTTGCTTTCTAGTGGTGGTTATGTGTACGCTTATAGCCCGTGGCCAG GCTCTATTCCCTTGCCCTCGGAC
	UP	FGSG_01790hygMicroUP	GCCGGCAATCAGTCTCACATAAGAACATAAGGGTATATTTGACCACCT CCTTCGACAGAGAAGATGATATTGA
FGSG_01795	DO	FGSG_01795hygMicroDO	CTCCATTCCATAGATCATGTCAATCTTTTTATGTAAATCAACAGA TCTATTCCCTTGCCCTCGGAC
	UP	FGSG_01795hygMicroUP	GTAATTGTGCCTCCTCGTCAACCTTGCCGCAATCTCCCCGCATCCT CCGTGACAGAGAAGATGATATTGA
FGSG_04587	DO	FGSG_04587hygMicroDO	GTTAATCAATCTTACAAAGCGAGTATGTAAGTCTGCAAGTTCATACG CTATTCCCTTGCCCTCGGAC
	UP	FGSG_04587hygMicroUP	CGGTTTCCCGTTTCCCGTCAAACTCCGCGCTCCATCCGAAGGACAG AAATCGACAGAGAAGATGATATTGA
FGSG_04588	DO	FGSG_04588hygMicroDO	CACCACAATTAATCCCGTCAGGTTACAGCTCTGTGGTCTAGCTAAA CGCTATTCCCTTGCCCTCGGAC
	UP	FGSG_04588hygMicroUP	CCAAATACGAATAATATAGTAAAAGAATGTGATTAGTCTGCATTGCTT CCGTGATGACAGAGAAGATGATATTGA
FGSG_04591	DO	FGSG_04591hygMicroDO	CGTCCGATGATGGGTCCGTAGAACAGCAGCTACTCAGGGTGTCTCTT TATCTATTCCCTTGCCCTCGGAC
	UP	FGSG_04591hygMicroUP	GAGACCGTTTAGTTGACATATCTTGTATATTCTAATAGTGGTGTCTTAT CGTCGACAGAGAAGATGATATTGA
FGSG_02315	DO	FGSG_02315hygMicroDO	AATAAAATGGTAAATATCAGGAAAACATAACTAAAAACACGCCCGAC AACTATTCCCTTGCCCTCGGAC
	UP	FGSG_02315hygMicroUP	GTCCGTTTCAACTGCGTTCCGCGTTCTTTTTGCAAAGTACAACATC GAATCGACAGAGAAGATGATATTGA
FGSG_02323	DO	FGSG_02323hygMicroDO	GTTTGTATTTCTTTGTCTTTCTCATCTTTGAGATAGAAGCAGCAAGATT GCTATTCCCTTGCCCTCGGAC
	UP	FGSG_02323hygMicroUP	CATCTTATCCGAGGTACACAAACAAAAGAACCTGTCCGCTTACTGATG CGTCGACAGAGAAGATGATATTGA
FGFG_02324	DO	FGFG_02324hygMicroDO	CATGGCGACTGGGGAGGAACCTTCTGGGTATGATGAAGGGCGTTGAT GTAGACCTATTCCCTTGCCCTCGGAC
	UP	FGFG_02324hygMicroUP	GTCATCAACATACCAACTCCTATATATAGGGGATTGTGCTTCATTACC TCTTCGACAGAGAAGATGATATTGA

Appendix-A Table 2: crRNA sequences for each GOI used in the assembly of RNPs and generation of transformant strains.

Gene	crRNA Name	Length	Sequence
FGSG_01783	01783gRNA-down1	36	/AITS1/rArGrArCrGrUrCrArCrArGrGrArArCrGrGrArGrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AITS2/
	01783gRNA-up1	36	/AITS1/rGrCrUrArGrGrGrUrArUrGrGrArArUrGrCrCrGrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AITS2/
FGSG_01790	01790gRNA-down1	36	/AITS1/rGrUrArCrUrGrUrCrGrCrCrArUrCrUrCrCrGrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AITS2/
	01790gRNA-up1	36	/AITS1/rGrGrUrArUrArUrUrGrArCrCrArCrCrUrCrCrUrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AITS2/
FGSG_01795	01795gRNA-down1	36	/AITS1/rArCrCrGrCrArArCrCrArUrGrUrCrUrCrUrCrUrArGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AITS2/
	01795gRNA-up1	36	/AITS1/rArArArUrCrUrCrCrCrGrCrArUrCrCrUrCrCrGrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AITS2/
FGSG_04587	04587gRNA-down1	36	/AITS1/rGrGrGrCrUrUrCrArGrArArArUrGrUrCrUrCrArGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AITS2/
	04587gRNA-up1	36	/AITS1/rCrUrCrCrArUrCrCrGrArArGrGrArCrArGrArArGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AITS2/
FGSG_04588	04588gRNA-down1	36	/AITS1/rGrArArCrGrGrGrArUrArCrCrArGrUrCrUrGrGrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AITS2/
	04588gRNA-up1	36	/AITS1/rArUrArGrUrCrUrGrCrArUrUrGrCrUrUrCrCrGrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AITS2/
FGSG_04591	04591gRNA-down1	36	/AITS1/rGrGrArUrGrGrArArCrCrGrUrGrUrGrArCrUrCrUrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AITS2/
	04591gRNA-up1	36	/AITS1/rGrUrArArUrArGrUrGrGrUrGrUrCrUrUrArUrCrGrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AITS2/
FGSG_02315	02315gRNA-down1	36	/AITS1/rArCrUrArArArArArCrArCrGrCrCrGrArCrArGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AITS2/
	02315gRNA-up1	36	/AITS1/rUrUrCrGrUrCrGrUrCrGrUrGrArUrArCrArGrUrUrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AITS2/
FGSG_02323	02323gRNA-down1	36	/AITS1/rGrArGrUrGrArUrUrArUrArArGrArArUrGrArGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AITS2/
	02323gRNA-up1	36	/AITS1/rGrCrUrGrUrCrCrGrCrUrUrArCrUrGrArUrGrCrGrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AITS2/
FGSG_02324	02324gRNA-down1	36	/AITS1/rGrGrArArGrGrGrCrGrUrUrGrArUrGrUrArGrArCrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AITS2/
	02324gRNA-up1	36	/AITS1/rGrArArCrUrCrCrArGrUrArGrUrUrGrUrUrArCrGrUrUrUrUrArGrArGrCrUrArUrGrCrU/AITS2/

Appendix-A Table 3: Internal primers used for the confirmation of the absence of the GOI in the generated transformant strains. (*) Indicates the primers used in the confirmation of the hygromycin insertion.

Gene	Direction	Primer Name	Nt Count	Tm (°C)	Primer Sequence 5' - 3'	Amplicon Size (bp)
FGSG_01783	F1	FGSG_01783I ntF1	20	57.1	TTCGGAGGCTGCAATCA CAT	479
	R1	FGSG_01783I ntR1	20	57.2	GCAGCCATCTAGAAGCA CCT	
FGSG_01790	F1	FGSG_01790I ntF1	20	57	AGCGCAGGACAATCTGT TGA	543
	R1	FGSG_01790I ntR1	20	57.2	GGA CTGGCTGGATTAGC ACA	
FGSG_01795	F1	FGSG_01795I ntF1	20	57.1	GGCGCTACTATTCCGAG CTT	530
	R1	FGSG_01795I ntR1	20	55.6	AATCGTCCACTGTACCTC GT	
FGSG_04587	F1	FGSG_04587I ntF1	20	58.1	TCATGGGTAGGGAGGAC AGG	508
	R1	FGSG_04587I ntR1	20	57.3	CCCGTCAACTGAGGGTT GAA	
FGSG_04588	F1	FGSG_04588I ntF1	20	56.9	CCATCAGTACGTCCGCT CAA	442
	R1	FGSG_04588I ntR1	20	57.1	CTCTGGGCCATATCCGT CAC	
FGSG_04591	F1	FGSG_04591I ntF1	20	57.3	TGGGCATGATTGGACTG GAG	350
	R1	FGSG_04591I ntR1	20	57.7	GTATGACCTGAGCTGCT GGG	
FGSG_02315	F1	FGSG_02315I ntF1	20	57.5	TTCTCTCCGAAAACCTGC CG	545
	R1	FGSG_02315I ntR1	20	57.1	GAGCTGGCTTTGGTTGA AGC	
FGSG_02323	F1	FGSG_02323I ntF1	21	54.5	TGTCGAAGAGTATGTCTT TGC	207
	R1	FGSG_02323I ntR1	20	57.2	CAGTCCTTGTTTCGCAGA GGT	
FGSG_02324	F1	FGSG_02324I ntF1	20	57.1	TTACTGACCTTGCTCCAG GC	500
	R1	FGSG_02324I ntR1	20	56.8	CTTGATACCACAATGCG GCG	
Hyg*	F1	Hyg588U	23	61.3	AGCTGCGCCGATGGTTT CTACAA	588
	R1	Hyg588L	22	62.3	GCGCGTCTGCTGCTCCA TACAA	

Appendix-A Table 4: Tables of transformant strains isolated for each of the GOIs. Bolded strain identifiers indicate strains used in southern blot analysis.

Gene Cluster	Locus ID	Transformant	Isolated Strain Identifier
C08	FGSG_01783	Δ TS	CHEM2850
			CHEM2851
			CHEM2852
	FGSG_01790	Δ PKS11	CHEM2853
			CHEM2854
			CHEM2855
	FGSG_01795	Δ TF	CHEM2856
			CHEM2857
			CHEM2858
C16	FGSG_04587	Δ WD-40	CHEM2859
			CHEM2860
			CHEM2861
	FGSG_04588	Δ PKS15	CHEM2862
			CHEM2863
			CHEM2864
	FGSG_04591	Δ TS	CHEM2865
			CHEM2866
			CHEM2867
C70/C13	FGSG_02315	Δ NRPS4	CHEM2868
			CHEM2870
	FGSG_02323	Δ TF	CHEM2871
			CHEM2872
			CHEM2873
	FGSG_02324	Δ PKS12	CHEM2874
			CHEM2875
			CHEM2876

2.6.3 Optimization of Coleoptile Assays

Several rounds of optimization were completed in order to address problems that arose within the assay. Initial experiments were completed within a high humidity cabinet, with growth of multiple coleoptiles on petri dishes with Whatman soaked water. In these conditions, the humidity was observed to be too low to achieve adequate infection and root ends often turned brown due to dehydration even when additional water was added daily. Also the lack of closed

system resulted in contamination from the cabinet environment. To increase the humidity and reduce external contaminants the plexi glass containers were introduced testing different volumes of added water. To combat the dehydration of root ends and eliminate the need for manual watering, attempts to grow coleoptiles on 1/2PDA media and 1% water agar were made. While roots were no longer showing dehydration under these conditions, contamination innate to seeds was still persistent and easily spread laterally via root to root contact and contact with the media. An increase in small root hairs (likely due to lower accessibility to water) of also made coleoptiles more difficult to handle coleoptiles. To address these issues, it was determined that single coleoptile grown contained to single vessel would be the optimal for prevention of contamination spread. In order to accomplish this while minimizing the amount of space required, roots needed to be influenced to grow downward. Several vessels were tested containing varying concentrations of water agar, where it was determined that a 2mL collection tube with 1.7mL of 0.25% water agar was effective in both downward root growth (thereby eliminating cross contamination), and providing enough water to be self sustainable without additional watering required. The final modification to the original protocol was to have the initial growth period, prior to inoculation, be executed in the dark. This alteration was made while observing that the distance between coleoptile end and first leaf was increased when grown without an initial photoperiod. This makes sense, as the coleoptile is a protective sheath for the first leaf, and emergence of the first leaf does not typically occur until after the emergence of the coleoptile through the soil, meaning there may be an association with light and accelerated growth of the first leaf.

2.6.4 Media Formulations

Composition of SNA Medium

KH ₂ PO ₄	1g
KNO ₃	1g
MgSO ₄ ·7H ₂ O	0.5g
KCl	0.5g
Glucose	0.2g
Sucrose	0.2g
Agar	20g
MilliQ Water	to 1.0 L

Composition of CMC Medium

Carboxymethylcellulose	15g
NH ₄ NO ₃	1g
KH ₂ PO ₄	1g
MgSO ₄ ·7H ₂ O	0.5g
Yeast Extract	1g
MilliQ Water	to 1.0L

Composition of Littman Oxgall Medium

Oxgall	15.0g
Dextrose	10.0g
Peptone	10.0g
MilliQ Water	to 1.0L

Composition of Liquid TB3 Medium

Yeast Extract	3.0g
Casamino Acids	3.0g
Sucrose	200.0g
MilliQ Water	to 1.0L

Composition of Molten TB3 Medium

Yeast Extract	3.0g
Casamino Acids	3.0g
Sucrose	200.0g
LMP Agarose	7.0g
MilliQ Water	to 1.0L

Composition of PDA Medium

Difco Potato Dextrose Agar	39.0g
MilliQ Water	to 1.0L

Composition of SIM Medium

NH ₄ Cl	3g
MgSO ₄ ·7H ₂ O	2g
FeSO ₄ ·7H ₂ O	0.2g
KH ₂ PO ₄	2g
Peptone	2g
Yeast Extract	2g
Malt Extract	2g
Glucose	20g
MilliQ Water	to 1.0L

Composition of SMYA medium

Peptone	10.0g
Maltose	40.0g
Yeast Extract	10.0g
Agar	1.0g
MilliQ Water	to 1.0L

2.6.5 Reagent Formulations used in Generation of CRISPR Mutants

Composition of STC Buffer

Sorbitol	1.2M
Tris-HCl, pH 8.0	10mM
CaCl ₂	50mM

Composition of Cas-9 Working Budder

HEPES	20mM
KCl	150mM
Adjust to pH 7.5	

Composition of PEG(4000)-CaCl₂ Buffer

PEG(4000)	60% (w/v)
CaCl ₂	50Mm
Tris-HCl, pH 7.5	450Mm

2.6.6 Reagents used in Southern Blot Analysis

Depurination Solution

HCl	0.25M
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Denaturation Solution

NaOH	0.5M
NaCl	1.5M

Neutralization Solution

Tris-Cl	0.5M
NaCl	1.5M

Adjust to pH 7.2

20X SSC

NaCl	3M
Tri-sodiumcitrate dihydrate	0.26M

Wash Buffer 1

SSC	6X
SDS	0.5%

Wash Buffer 2

SSC	1X
SDS	0.5%

Wash Buffer 3

SSC	0.1X
SDS	1%

Detection Wash Buffer

Maleic Acid	0.1M
NaCl	0.15M

Adjust to pH 7.5

Tween20	0.3%
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Detection Buffer

Maleic Acid	0.1M
NaCl	0.15M

Adjust to pH 7.5

Blocking Reagent	1%
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Equilibration Buffer

Tris-Cl (pH9.5)	100mM
NaCl	100mM

Adjust to pH 9.5

Chapter 3 Metabolomic Analysis of Transformant Strains

3.1 Introduction

Untargeted metabolomic analysis is a powerful tool used to capture a broad snapshot of the metabolic state of a biological system, providing a global view of the molecular changes occurring in response to a particular perturbation, such as an environmental stress (Witte and Overy, 2022). Untargeted metabolomics involves the use of high-throughput analytical platforms, such as mass spectrometry, to generate complex datasets containing information on hundreds to thousands of metabolites (Schrimpe-Rutledge et al., 2016; Hautbergue et al., 2018). The resulting data can then be analyzed using statistical and chemo-informatics tools to identify potential biomarkers, elucidate metabolic pathways, and gain insights into the underlying biology of a system. In this chapter, an untargeted metabolomics approach was used to compare chemical profiles of the *F. graminearum* WT strain used in Chapter 2, under different culture conditions as well as to compare the WT and transformant strains to try and identify SMs that correlate to each GOI.

Prior to the start of this thesis in 2020, gene cluster products for both the C16 and C70 BGC were unknown (not published in the literature). However since then, several reports have come out identifying SMs associated to core biosynthetic genes within each of the two clusters. Regarding the C16 BGC, Tsukada et al. (2020) and Seidl et al. (2022) both have identified mass features that may correspond to products of the C16 BGC in *F. graminearum*. Several of the core C16 BGC genes are found to occur in multiple fungi and Tsukada et al. (2020) heterologously expressed these conserved early pathway genes in *Aspergillus oryzae* as well as tailoring genes specific to the C16 BGC in *F. graminearum*. From their analysis, they identified and elucidated

the structures three SMs that could potentially be produced by native *F. graminearum* cultures; FDDP B (m/z 443.3159 $[M+H]^+$), FDDP D (m/z 503.3003 $[M+H]^+$) and FDDP E (m/z 487.3054 $[M+H]^+$). More recently, Seidl et al. (2022) identified two mass features associated with the PKS15 gene using multi-gene deletion strains. Using a Δ PKS15 strain with a triple mutant background (Δ tri5, Δ pks4, 13 and Δ kmt6) they were able to identify -but not structurally elucidate – two mass features correlated to the PKS15 gene expression; gramiketide A (m/z 521.3109 $[M+H]^+$) and gramiketide B (m/z 503.3003 $[M+H]^+$). Seidl et al.'s reported gramiketide B and Tsukada et al.'s FDDP D both share m/z ratios, however it is unclear at present if both are in reference to the same SM or different molecules that share the same molecular formula.

The following proposed C16 biosynthesis pathway (Figure 20) is based on the report of Tsukada et al., 2020 (Figure 20). Early shared pathway stages include pyrone (by DpfgA, analogous PKS15/FGSG_04588) and GGPP (by DpfgD, analogous TS/FGSG_04591) synthesis were initially elucidated by Kato et al., 2016 who were focused on related molecules in the sublutinol family in other fungi. They also determined that synthesis of the linear pyrone diterpenoid is catalyzed by the prenyltransferase-encoding DpfgC (FGSG_04593), followed by the formation of the decalin core by epoxidation of the C₁₀-C₁₁ by FAD-dependent oxidoreductase DpfgE (FGSG_04595) and cyclization catalyzed by the terpene cyclase DpfgB (FGSG_12222). Tsukada et al., 2020 corroborated these early synthesis steps as well as elucidated that DpfgG (FGSG_12223) catalyzed the oxidation of the 8S hydroxyl group to a ketone and DpfgH (FGSG_04592) reduces the ketone to a 8R hydroxyl group to form higginsianin B. From here DpfgI (FGSG_04596) catalyzed the formation of the methylester on the pyrone moiety producing FDDP B. Oxidation by DpfgJ (FGSG_04590) of C₂₆ to a primary alcohol as well as a

three-step oxidation at the C₂₇, generates the carboxylic acid of which DpfgK (FGSG_04589) is then responsible for methylation of the carboxylic acid generating FDDP D. Additional hydrolysis of the C₂₆ primary alcohol by DpfgJ (FGSG_04590) results in the production of FDDP E.

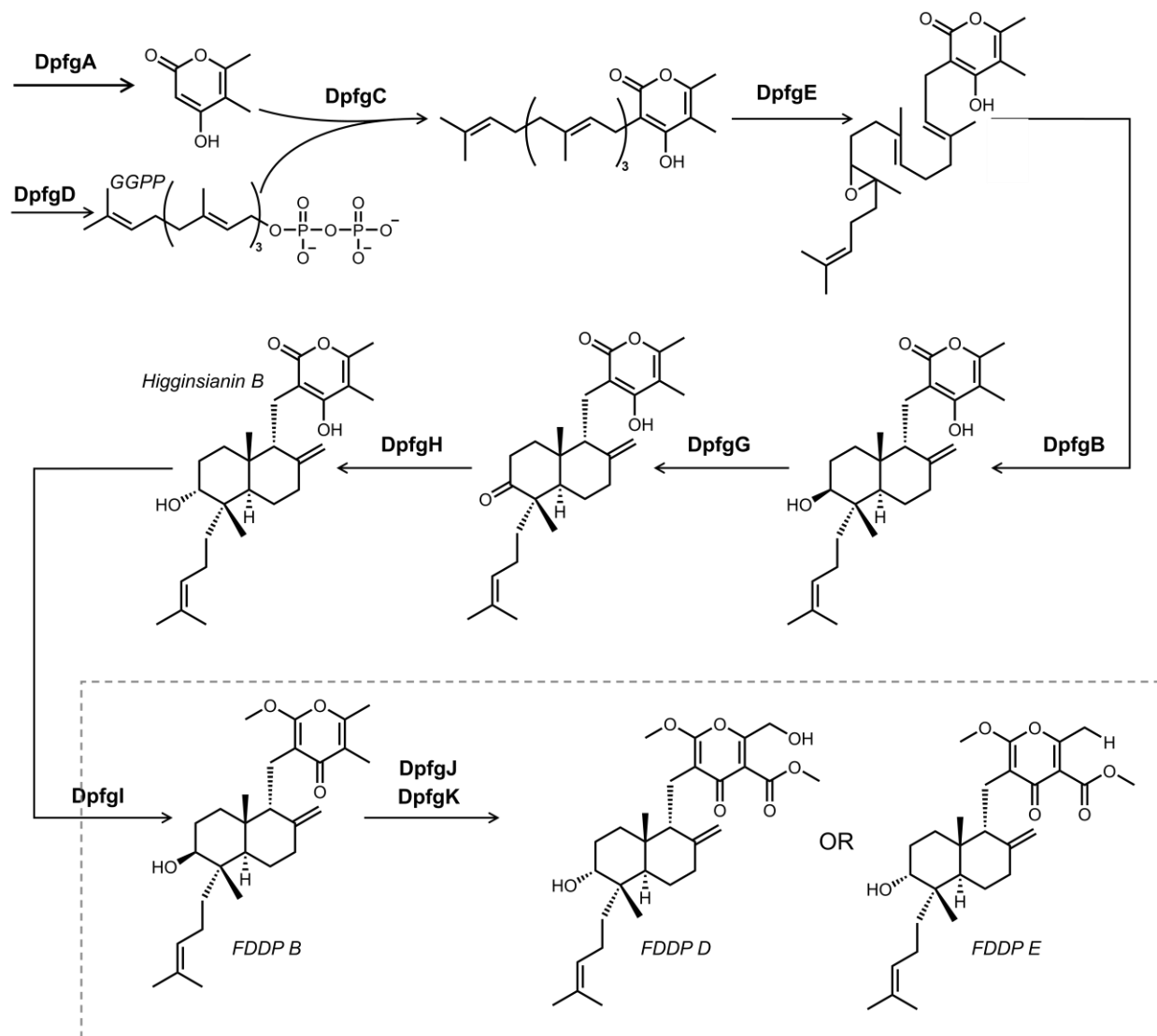


Figure 20: Biosynthetic steps towards final pathways end products of the C16 BGC designated as 'fungal decalin-containing diterpenoid pyrones' or FDDPs (Tsukada et al., 2020)

The second BGC for which an unknown product was subsequently published since the start of this thesis was C70. The identification and structural elucidation the C70 BGC product was determined by Westphals et al. (2021). They identified the NRPS4 product as a cyclic hexapeptide in they named fusahexin (m/z 607.3819 $[M+H]^+$). In addition to fusahexin, they also identified three additional mass features of identical m/z , two of which are assumed to be linearized products of the cyclic peptide and a third unknown. The proposed biosynthesis of a cyclized hexapeptide involving a 5 modular NRPS4 protein arises as the fifth (terminal) module incorporates two leucine units to generate the hexapeptide (Figure 21). The cyclization stage in which forms the bond between the proline and threonine residues remains unknown.

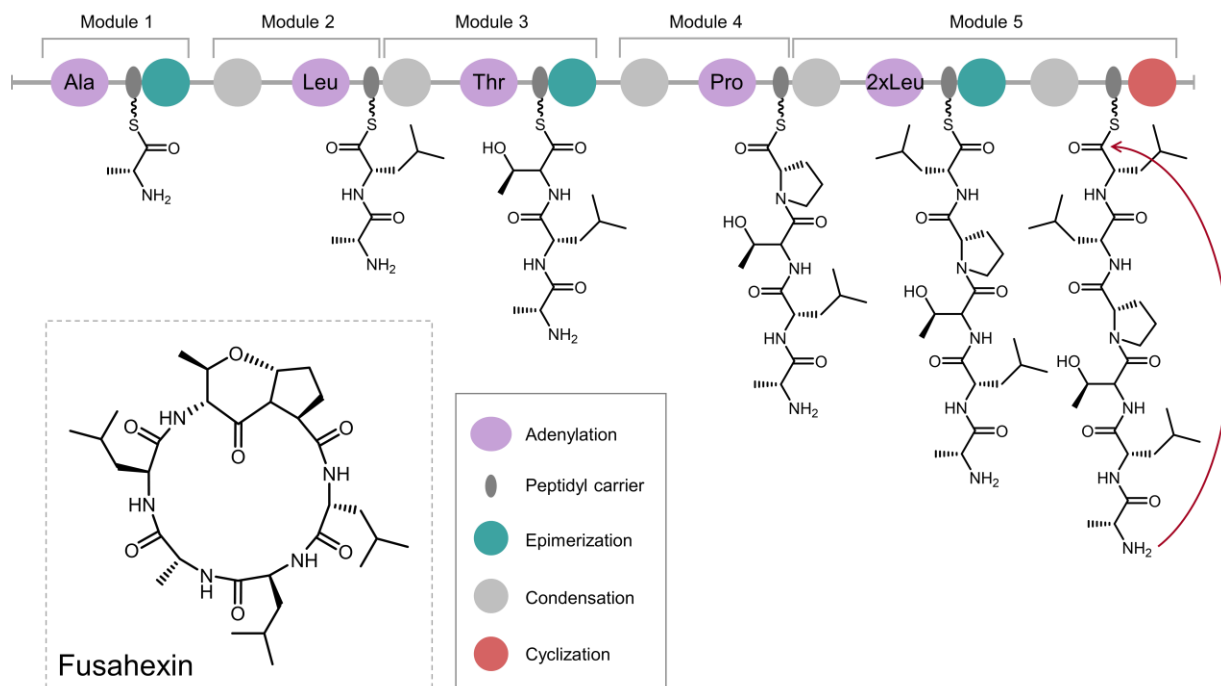


Figure 21: Proposed biosynthetic steps towards final pathway end product, fusahexin, of the C70 BGC as determined by Westphals et al. (2020)

The BGC C13 is directly adjacent to the C70 cluster. The metabolite products associated with the C13 BGC were first identified by Ashley et al. (1937); however their association with the C13 BGC was not made until 2005 by Malz et al., who correlated the production of rubrofusarin (m/z 273.0757 $[M+H]^+$), aurofusarin (m/z 571.0871 $[M+H]^+$) and fuscofusarin (m/z 557.1986 $[M+H]^+$) to the C13 BGC. The full biosynthesis pathway of the C13 BGC has yet to be fully elucidated. However, theoretical models have been posed by Frandsen et al. (2006) and Malz et al. (2005) (Figure 22). PKS12 provides the molecular backbone by catalysing the condensation of 1 acetyl-CoA and 6 malonyl-CoA units. The linear heptapeptide is then folded into nor-rubrofusarin by the Claisen cyclase domain of PKS12. AurJ catalyzes the conversion of nor-rubrofusarin to rubrofusarin, the first primary product, by an O-methylation of the C₅-OH group to C₅-O-CH₃. The downstream biosynthetic steps are still relatively unclear. AurF, Gip1 and AurO are all required for the synthesis of aurofusarin from rubrofusarin, however the order of reactions is debated. The monooxygenase AurF and laccase GIP1 are both capable in catalyzing the dimerization reaction or the oxidation of C₆, while AurO is thought to be responsible for the C₆ oxidation to a ketone group.

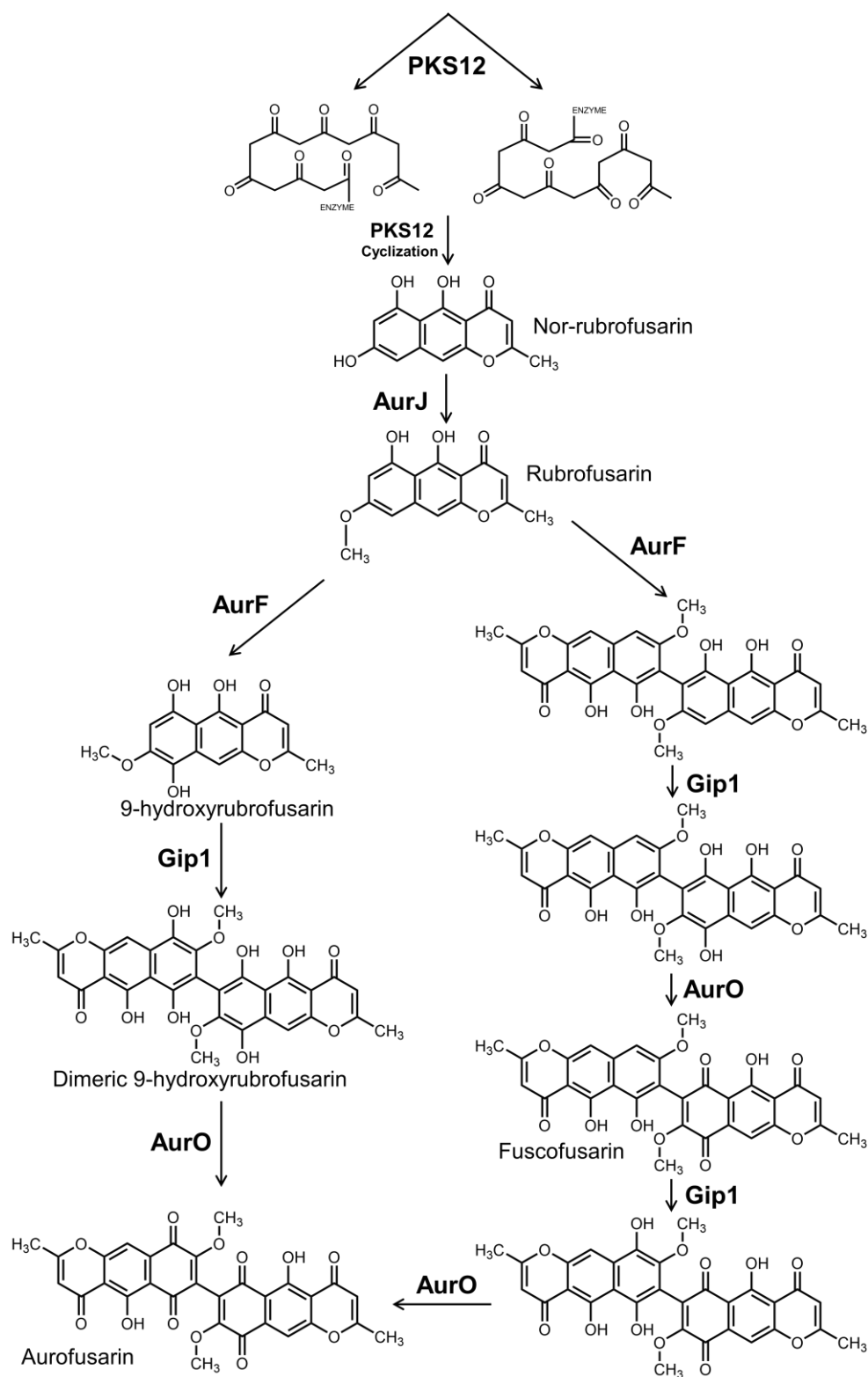


Figure 22: Proposed biosynthetic steps towards final pathways end products of the C13 BGC

In this chapter, an untargeted metabolomics approach is implemented to compare chemical profiles of the *F. graminearum* WT and transformant strains grown under different culture conditions to try and identify SMs that correlate to the GOIs. The first experiment in this chapter includes a broad media screening of the WT strain which is used to try and identify any of the predicted mass features produced by the selected BGCs. Additionally, the broad media screen is used to provide candidate media types to use for screening of transformants with unknown metabolomic products. Following this screening, wheat head tissues generated from pathogenicity trials carried out in Chapter 2 are extracted and profiled and metabolomic experiments were carried out for the comparison of C16 and C13/70 BGC transformants to the WT strain for the confirmation of *in-planta* production of putative metabolite products.

3.2 Materials and Methods

3.2.1 Metabolomic Data Acquisition

Extracts order was randomized, and chemical profiling was completed using a Thermo Ultimate 3000 (ultra-high-performance liquid chromatography (UPLC)) coupled to Thermo LTQ Orbitrap XL (high resolution mass spectrometer (HRMS) and UltiMate Corona VeoRS charged aerosol detector (Thermo Fisher Scientific Inc, Waltham, MS, USA). Chromatography was performed on a Phenomenex C18 Kinetex column (50 mm x 2.1 mm ID, 1.7 μ m) with a flow rate of 0.35 mL/min, running a gradient of H₂O (+ 0.1% formic acid) and ACN (+0.1% formic acid): starting at 5% ACN increasing to 95% CAN by 4.5 min and held at 95% ACN until 8.0 mins until returning to 5% ACN by 9 mins and holding to allow the column to equilibrate to starting conditions by 10 mins. The HRMS was operated in ESI+ mode (with a 100–2000 *m/z*

range) using the following parameters: sheath gas (40), auxiliary gas (5), sweep gas (2), spray voltage (4.2kV), capillary temperature (320 °C), capillary voltage (35 V), and tube lens (100 V). MassWorksTM software (v5.0.0, Cerno Bioscience) was used to improve spectral accuracy and confirm the molecular formulas of annotated ions. The sCLIPS searches were performed in dynamic analysis mode with elements C, H, N, O allowances set at minimum 1 and maximum 100. Charge was specified as 1, mass tolerance was set to 5 ppm and the profile mass range was -1.00 to 3.50 Da.

MSⁿ fragmentation was performed on select ions in subsequent experiments. MS² scans were acquired at a resolution of 15,000 using an isolation width of 2 *m/z* and HCD at 30% NCE. The automatic gain control target was set at 5E5, and maximum injection time was 1024 ms.

3.2.2 Metabolomics Data Processing Overview

The data acquisition process implemented uses Ultra Performance Liquid Chromatography in tandem with High-resolution mass spectrometry (UPLC-HRMS). The outputted data represents a three dimensional matrix of corresponding retention times, mass-to-charge ratios and relative intensities (for the full data pre-processing and data reduction method parameters used see Appendix-B sections 3.6.1-to-3.6.3). The initial step in processing is performed to transform the generated data set into a 2-dimensional model that can be used in statistical analyses. MZmine is a well-supported open-source program that was used for the following data pre-processing steps exemplified in Figure 23.

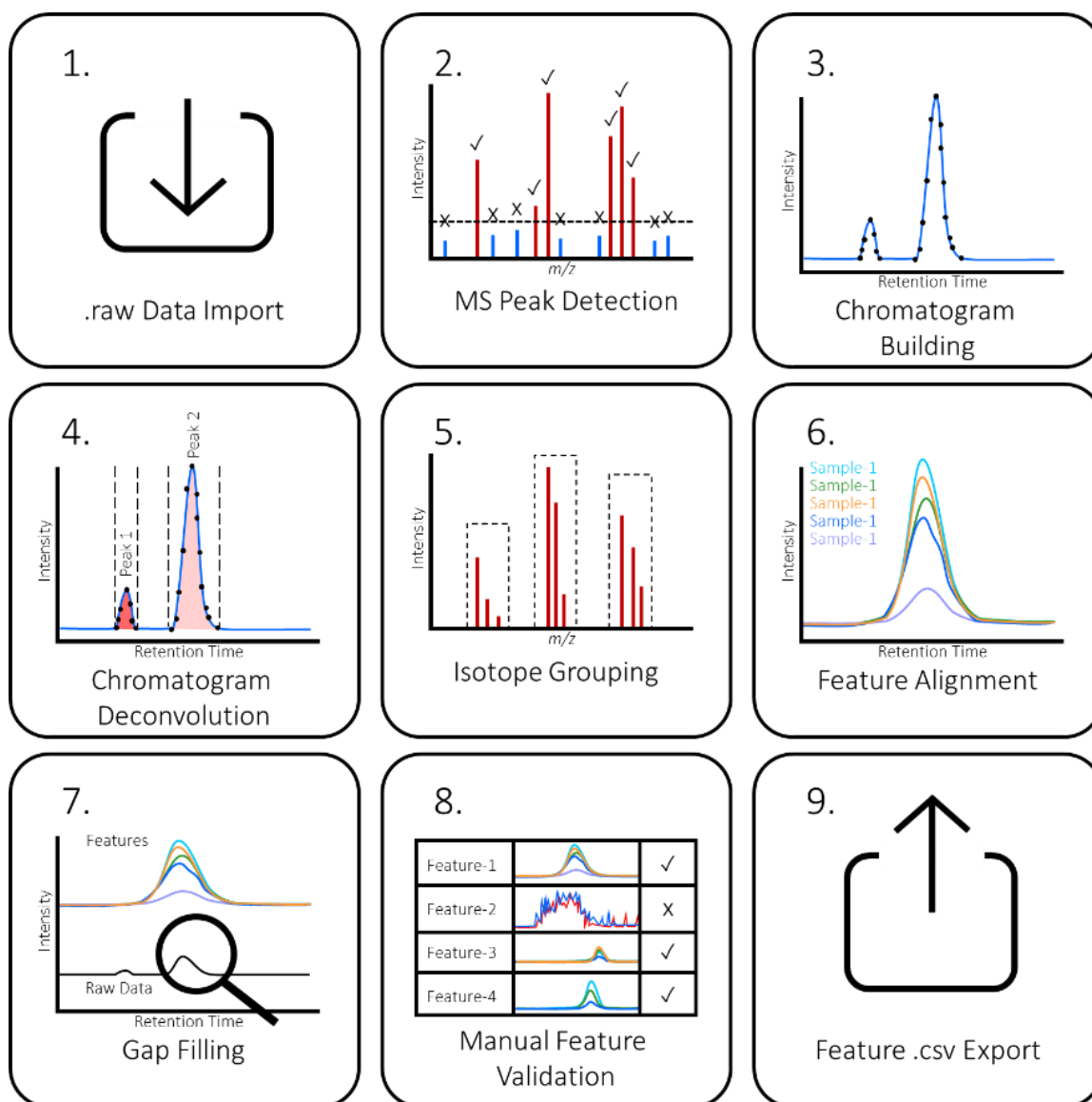


Figure 23: Overview of MZmine pre-processing

Data Pre-processing

The first few steps of metabolomics data pre-processing are dedicated to the detection of all the generated mass peaks that have been acquired by the spectrometer. For each given raw data file, mass detection generates a list of masses (ions represented as a m/z) above a specified noise level that are detected as m/z peaks, while all masses below the specified level are ignored (Figure23 -

2). This allows for the reduction of signals derived from instrumental noise, sample carry over and minor contaminants. The following step is chromatogram building, in which extracted ion chromatograms (XICs) are constructed. The ADAP chromatogram builder examines all of the data points in a given data file (sample) and works from the largest intensity peak down to the smallest, resulting in a list on ions that have been detected over a continuous retention time period (Figure 23-3). This is followed by chromatogram deconvolution which is used to separate the constructed chromatograms into individual peaks – some constructed chromatograms may contain several peaks due to structural isomerism where ions share a molecular formula however vary in retention times (Figure 23-4). After peak detection, the isotope grouper is used to remove chromatograms that arise from isotope peaks thereby reducing the data set (Figure 23-5). The module attempts to find those peaks in the peaks list that form an isotope pattern. Peaks which fit the m/z and RT distance limits are considered as isotopes and the isotope with the highest intensity is kept while the others are removed. After the removal of isotopes, the peaks from separate data files are aligned to generate a single peak list and gap-filling is then used to call back in peaks that are missing from the data matrix (Figure 23-6&7). Peaks with the same m/z and retention time range as those in the peaks list that were not included during the chromatogram building step as their intensity fell below the thresholds set, are reintroduced to the matrix. The resulting data set is exported as a 2-dimensional matrix composed of a list of mass features composed of unique retention time and m/z signatures and their respective intensities. This data set can then be reduced further by manually reducing false positive peaks and by applying correlation removal methods to eliminate redundancy while retaining the data structure.

3.2.3 WT Profiling

Culturing

Culturing was completed on the following media: CS, CYA, CYS80, MEA, MMK2, NPN-A, NPN-P, PDA, Q6, YES, YES +IO, ZM/2 (see Appendix -B section 3.6.6 for all media formulations). For solid cultures, plates were inoculated with 2×10^5 macroconidia which were then dispersed using a cell spreader. Eight replicates were inoculated for each of the listed media types while two negative controls were inoculated with sterile MQ water. Plates were placed at 28 °C in the dark for 10 days. Four replicate plates were selected to be used for extraction based on the consistency in culture phenotype. Two sets of 12 agar plugs were removed from each plate into scintillation vials and stored at -20 °C prior to extraction in either ACN or EtOAc. Liquid cultures were grown first as bulk cultures in Erlenmeyer flasks with a 24 hour initial shaking (160 rpm), to try increase the fungal biomass before aliquoted (15 mL) into 50 mL culture tubes where they were placed at a 45° angle for 10 days at 28 °C. Mycelium and broth were then separated and frozen at -80 °C and -20 °C respectively until extraction.

Broth and Mycelium Extractions

Culture broth and mycelium were extracted separately in 125 mL Erlenmeyer flasks with 15 mL of EtOAc shaking at 200 rpm at room temperature for 1 hour. The solvent phase was then transferred to borosilicate scintillation vials and dried on a pin drier. Dried extracts were then resuspended in 1.5 mL methanol and transferred into HPLC vials. Method blanks (solvent carried through the extraction process minus broth or mycelium) and media blanks as well as two methanol resuspension blanks were also generated for metabolite profiling.

Solid Culture ACN and EtOAc Extractions

A 15 mL volume of extraction solvent (either ACN or EtOAc) was added to the harvested agar plugs and shaken at 200 rpm at room temperature for 1 hour. The solvent supernatants were then transferred to borosilicate scintillation vials and dried on a pin drier. Dried extracts were then re-suspended in 1.5 mL of Water:ACN (1:5; v:v) and transferred into HPLC vials. Method and medium blanks as well as two Water:ACN (1:5; v:v) resuspension blanks were also generated.

Multivariate and Univariate Analysis

Full metabolomic preprocessing methods are described in Appendix-B section 3.6.1. In order to determine if a culture method or extraction type yielded significantly more mass features than others, a Venn diagram was generated comparing detected mass features on each of the two extract types (regardless of media type). Binary presence absence data was used to construct the Venn-diagram using InteractiVenn (Heberle et al., 2015).

Consensus heatmaps were generated using the final media consensus as a guide for the clustering algorithm. The “pheatmap” R package, was used to generate the initial heatmap with the ‘ward.D2’ algorithm applied as both row and column clustering. This clustering method (relative sample and mass feature order) was then applied to each of the four extract/culture type ‘binary consensus’ in order to sort all features and samples to correspond with the media consensus format (Witte and Overy, 2022). This process was carried out in Microsoft excel.

Finally feature intensity/detection tables for mass features with putative annotations were generated using the blank subtracted values of peak heights. Data was then colored based on the rounded Log_{10} intensity value with a minimum colour value of $1\text{E}5$.

3.2.4 C16 Transformant profiling

C16 In-vitro Culturing and Extractions

F. graminearum WT, Δ WD-40, Δ PKS15 and Δ TS strains from the C16 gene cluster were all cultured on Q6 liquid medium. A 15 mL volume of Q6 medium was inoculated with 1×10^5 macroconidia in 50 mL culture tubes by initial shaking at 28 °C for 24 hours at 160 rpm and then transferring to a stationary inclined position at a 45 ° angle for 10 days. Cultures were then harvested and extracted with EtOAc following the procedures described in section 3.2.3. Full metabolomic data preprocessing methods are described in Appendix-B section 3.6.2.

Univariate Data Processing

Univariate analysis was completed using the ‘MUMA’ R package which utilizes both Welch’s T tests and Wilcoxon-Mann Whitney U tests for normal and non-normal distribution of mass features respectively, based on Shapiro scores when determining P-values. This approach is advantageous as mass features corresponding to BGC GOI products are expected to be absent in deletion strains, thereby deviating from a normal pattern of distribution. Mass feature P-values comparing the Δ PKS15 and WT strain were then used to generate plots against their respective retention time. To further examine the features of statistical significance, features with P-values >0.05 were plotted by RT and m/z . XICs of mass features were clipped from Xcalibur with a ± 5 ppm tolerance on the predicted exact mass.

Wheat Head Extraction of C16 In-planta Trials

Extractions of infected wheat head tissue were completed using inoculated wheat from the first wheat head infection assay. For each wheat head, the single spikelet used in point inoculation was removed from the remaining head and pooled per pot ($n = 4-6$ heads) generating a total of 4

replicates per strain. Spikelets were then freeze dried using a Labconco FreeZone 2.5 L Benchtop Freeze Dryer System before being pulverized using a Retsch MM300 TissueLyser grinding for 2 minutes at 22 cycles/sec. A two part extraction was completed on the ground tissue using ACN and 1% formic acid. First 1 mL of solvent was added to the tissue followed by a 1 hour nutation at room temperature before centrifugation and removal of the supernatant. The process was then repeated with an additional 1 mL of solvent. *In-planta* extracts were UPLC-HRMS profiled and preprocessed in MZmine as described in Appendix-B section 3.6.2. Univariate analysis of the resulting data matrix was completed using the ‘MUMA’ R package.

Putative FDDP mass features annotations were made using comparison of m/z values with those reported by Tsukada et al., 2020 (within 5ppm mass spectral accuracy) and compared with FDDP standards. Mirror plots were generated using the top 50 fragments by relative intensity for each of the given mass features and plotted using ggplot2 (Wickham, 2016).

3.2.5 C13/C70 Transformant Profiling

WT, Δ NRPS4, Δ TF and Δ PKS12 strains from the C13/C70 gene cluster were all cultured on MMK2 medium, following the same culturing and extraction protocol described for liquid cultures in section 3.2.2. Full chemical profiling and preprocessing parameters are described in Appendix-B section 3.6.3. Univariate analysis was completed using the ‘MUMA’ R package.

3.3 Results

3.3.1 WT Profiling

In order to obtain a more extensive look into the metabolite profile outputted by the wild type *F. graminearum* strain (DAOMC233423), a broad-spectrum media type study was completed, in

which 12 different media types were selected to complete chemical profiling using different two different culturing conditions and extraction solvents. Cultures were carried out on both solid and liquid medium compositions. Solid media was extracted with acetonitrile (ACN) and ethyl acetate (EtOAc) separately and for the liquid cultures, broth and mycelium were extracted separately with EtOAc. Throughout this analysis, solid culture extracts will be referred to by their extraction solvent (ACN and EtOAc), while liquid culture extracts will be referred to by the extraction source (Mycelium and Broth).

The results of this experiment were used to make decisions as to which conditions would be optimal for profiling each of the transformant strains. They also provided a database of mass features to be mined for the identification of conditions optimal for the evaluation of specific secondary metabolites. For example, these experiments were used to identify in what conditions fusahexin and aurofusarin demonstrated the greatest production and most consistent molecular profiles providing the ideal conditions in which to confirm their transformant stains. *In-vitro* production of FDDP molecules were also identified by mining the data from these experiments, even though all previous reports have only been able to demonstrate *in-planta* expression of the associated gene cluster (Gaffoor et al.,2005).

3.3.1.1 Effects of Extraction Method and Culture Methods

All chemical extractions were processed together and were refined down to a total of 304 mass features. Binary presence absence data was then used to compare feature overlaps to evaluate the distribution of mass features and how they may correlate to the extraction/culture method (Figure 24). The majority of mass features (73.0%) can be detected across all extract/culture types. The ACN plug extracts represented the largest number of unique features (5.3%). All mass features associated with EtOAc plug extracts as well as all broth extracts appear to be redundant to the

other extraction methods showing no unique mass features (0%). Figure 24 suggests that to achieve the most diverse mass feature profiles, chemical extracts of mycelium in EtOAc and agar plugs with ACN produce the least amount of chemical overlap and may therefore be ideal choices for chemical screening of unknowns. It should be noted however that liquid culture extracts of mycelium were not completed with ACN, and therefore it cannot be determined if these mass features are unique to the culture condition or the extraction solvent; however the absence of unique mass features in the EtOAc extracts suggest it is likely the solvent.

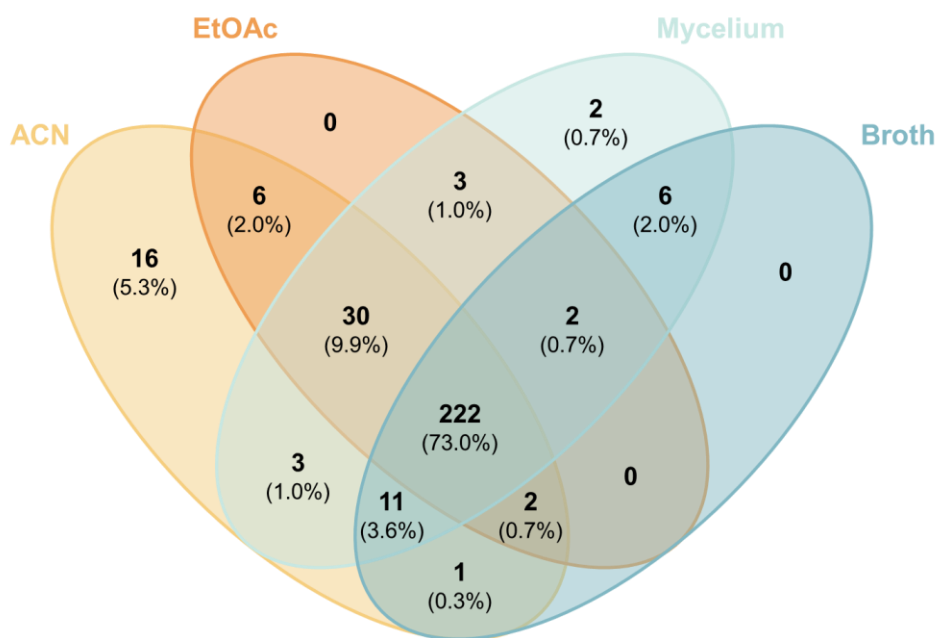


Figure 24: Feature overlap comparing the presence absence of 304 mass features under each of the culture/extraction conditions ACN (yellow), EtOAc (orange), Mycelium (teal) and broth (blue).

To obtain a more precise perspective of the effect of the individual media types upon metabolite production, a summation heatmap of the binary data from all 4 extract types was generated in which the colour intensity indicates a value from 0-4: 0 – (white) indicates that the mass feature

was not detected within any of the extraction/growth conditions; while 4 – (black) indicates the presence of the mass features in all of the conditions (Figure 25-Top). In addition to the summation heatmap, heat maps for the individual culture/extraction conditions were also generated using the binary data (Figure 25). Clear patterns were demonstrated within these heatmaps. Some features were detected across all media types under all culture/extraction conditions (highlighted as A) which may indicate these features are constitutively expressed. Other mass features were consistently detected across specific media types (highlighted as B) as denoted by an absence of a large number of features from culture extracts of MMK2, YES+IO and PDA media types. A large portion of the mass features also appear to be dependent on either media type or extraction conditions and don't create large overlap patterns of expression with other media types (highlighted as C).

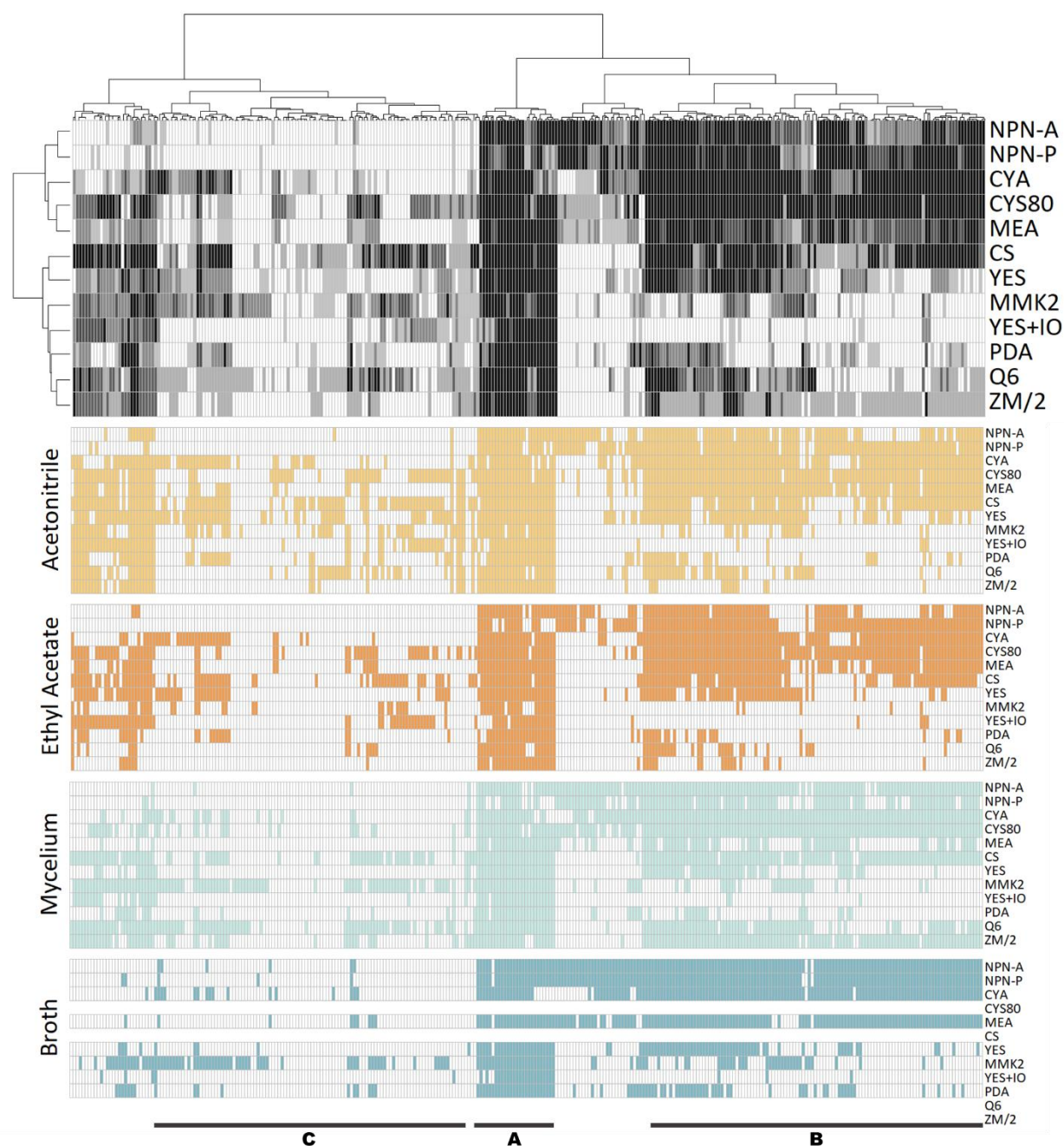


Figure 25 Binary heatmap of 304 mass features identified in WT comparing various medias. Top: consensus heatmap of all culture/extraction conditions with values intensities (0-4) indicating the number of conditions each feature was detected in. Bottom: constructed binary heat maps for each individual culture condition; ACN (yellow), EtOAc (orange), Mycelium (teal) and broth (blue).

The media types selected for profiling the C08 transformant strains which encoded unknown PKS and TS products, were the YES mycelium and broth, MMK2 mycelium and broth, NPN mycelium and broth, CS plugs in EtOAc and CYS80 plugs in EtOAc. This selection process was made in an attempt to optimize the feature diversity while minimizing the overall number of extractions required.

3.3.1.2 Identification of Specific Metabolites

For gene clusters containing known metabolites, C16 (suspected FDDPs), C13 (aurofusarin) and C70 (fusahexin), the mycelial extract data was used to generate a mass feature expression map across all media types. Mycelial extracts were selected for the expression map based on overall higher concentrations of their extracts relative to the solid cultures and the presence of potential FDDP mass features which are not detected in other extraction conditions. A Log₁₀ function was then applied to all the peak intensities, rounding down to the nearest whole number for simplification of data visualization (Figure 26). Figure 26 shows that putative FDDP mass features were identified on CS, MMK2 and Q6 media types, with the Q6 media showing the most pronounced fold change in FDDP production relative to the other media compositions. For this reason, the Q6 medium was then selected for profiling C16 transformant strains. Aurofusarin and fusahexin associated mass features were consistently present on CYA, MMK2 and Q6 media. MMK2 was selected for profiling of C70/C13 strains.

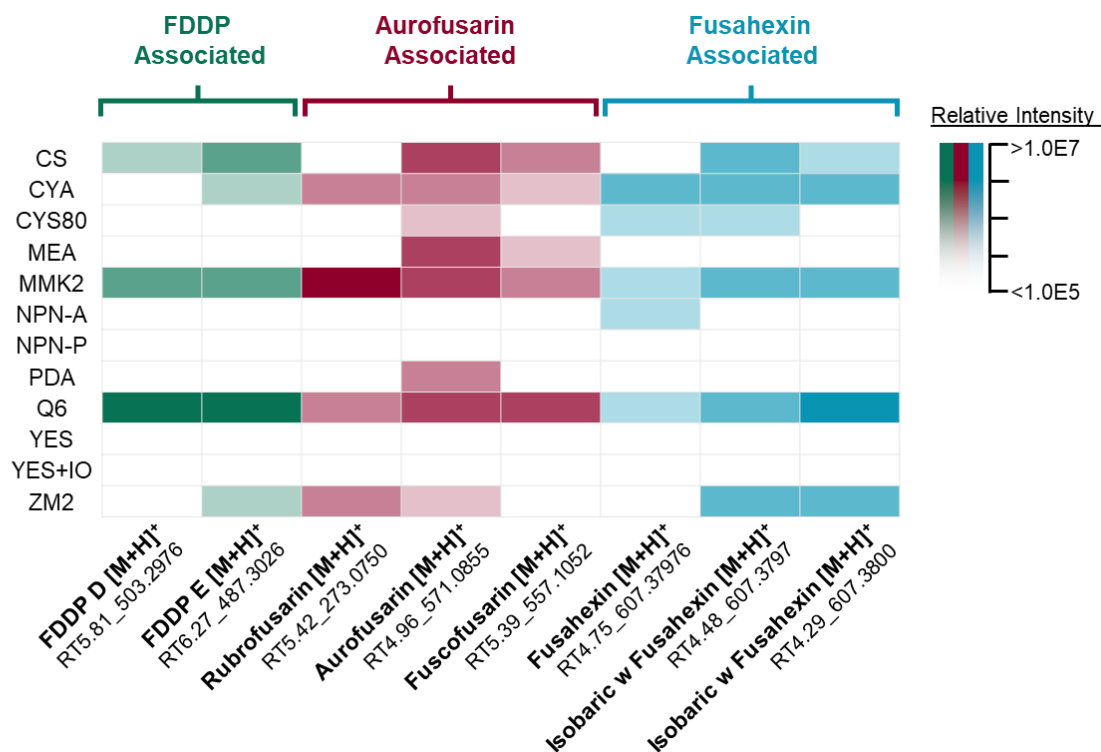


Figure 26: Relative intensity of mass features of interest from WT mycelial extracts profiled on different media. A Log10 function was applied to all intensities followed by rounding to the nearest whole number. Feature intensities were coloured with a minimum value of 1.0E5.

3.3.2 Transformant Strain Profiling

3.3.2.1 C16 Transformant Strains

C16 transformant strains (Δ PKS15, Δ TS and Δ WD40) and the WT strain were profiled on Q6 medium as targeted mass feature detection intensities and frequency were the highest relative to the other profiled medias (Figure 26). Metabolomic profiling of the C16 transformant strains was also completed on individual wheat spikelets from the wheat infection assays described in the previous chapter. The C16 Δ WD40 strain was excluded from the reported analysis as initial

screening of the metabolomic data did not demonstrate any correlation to Δ TS and Δ PKS15 strains.

Untargeted analysis of the Q6 data was used to identify targeted mass features of statistical significance to determine which mass features were likely associated with PKS15. Mass features were plotted by retention time against the $-\log_{10}(\text{P-value})$ (Figure 27-A). Figure 27-A indicates a small number of mass features that were statistically significant variables ($P > 0.05$) (highlighted in red). As many of these features appear to elute within a limited retention time (RT) window, the subset was then plotted by mass to charge and RT (Figure 27-B). Mass features corresponding to FDDP D (m/z 503.3003 $[\text{M}+\text{H}]^+$) and FDDP E (m/z 487.3054 $[\text{M}+\text{H}]^+$) were clearly identified. In addition, another mass feature was also observed and is likely associated with FDDP B or viridoxin A hydrolysate, both of which share a molecular formula and only differ structurally in stereochemistry at one chiral center (m/z 443.3156 $[\text{M}+\text{H}]^+$).

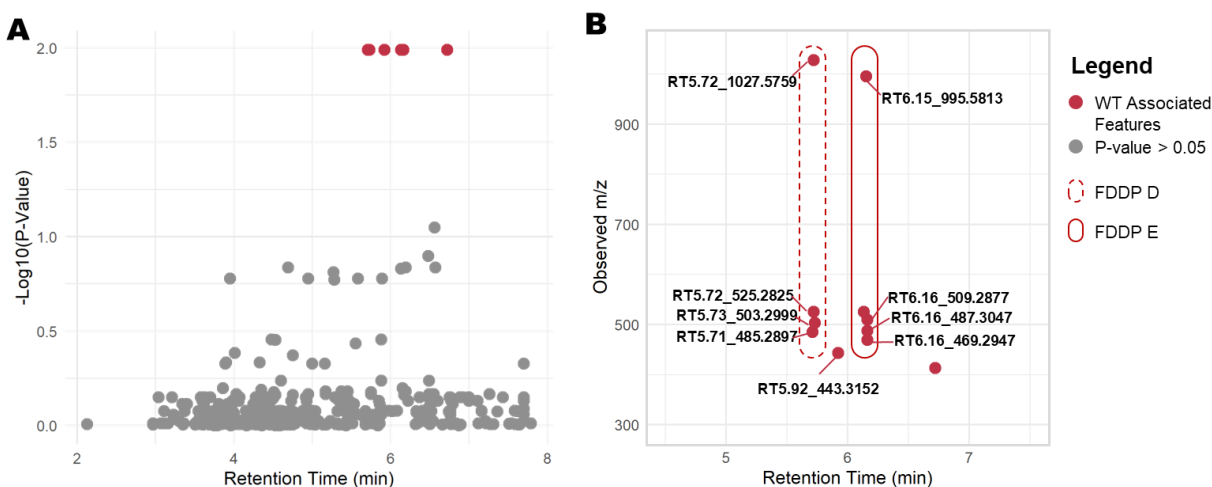


Figure 27: (A) All mass features plotted by $-\log_{10}(\text{p-value})$ against retention time. Statistically significant variables ($p < 0.05$) are indicated in red. (B) Statistically significant variables ($p < 0.05$) plotted by retention time against m/z demonstrating associated mass features with putative annotations indicated.

Extracted ion chromatograms (XICs) of m/z 503.3004 from both the Q6 mycelial extracts (red trace) and *in-planta* wheat extractions (green trace) reveal potential isomers or additional FDDP analog metabolites of the same mass, with *in-planta* data containing an additional 3 mass features not found in the Q6 extracts (Figure 28-A). Based on MS2 analysis of the two dominant peaks (RT 5.51 and 5.70) we predict the described FDDP D from Tsukada et al., 2020 corresponds to the 5.70 peak (Figure 28-B) that was confirmed by comparison with an FDDP D standard provided by Tsukada et al., both in terms of elution time and by comparison of MS2 fragmentation patterns between the mass feature at RT5.70 and the FDDP standard. Boxplots of the peak heights for WT, Δ PKS15 and Δ TS extracts demonstrate that in both Q6 medium and *in-planta* expression of FDDP D is abolished in the absence of the Δ PKS15 and Δ TS strains, although only a moderate reduction in the production was observed for Δ TS strains on Q6 medium (Figure 28-C).

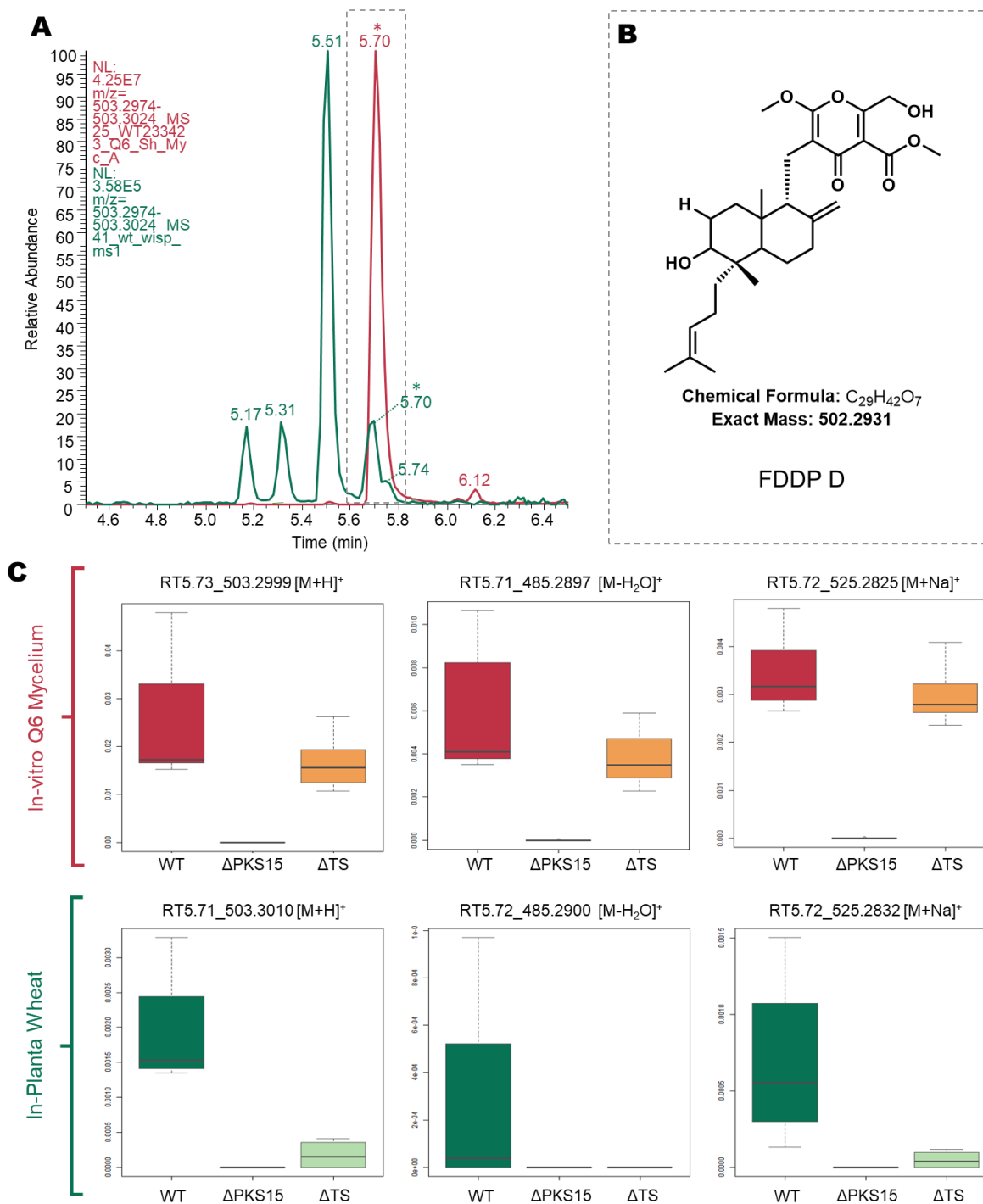


Figure 28: (A) XIC of m/z 503.3004 from mycelium of in-vitro cultures grown on Q6 medium (red) and in-planta growth (green) with suspected FDDP D peak indicated with the dashed box (B) Potential structure associated with RT 5.70 m/z 503.3004 peak based on MS/MS analysis and comparison to Tsukada et al. 2020 reported findings. (C) Boxplots demonstrating detection patterns of m/z 503.3004 $[M+H]^+$ at 5.7 RT and detected associated adducts in WT and Δ PKS15 and Δ TS transformant strains from in-vitro mycelial extracts cultured on Q6 medium (Top) and In-planta wheat extracts (Bottom).

All other m/z 503.3004 masses (RT5.18_503.3010, RT5.34_503.3011, and RT5.55_503.3004) identified exclusively in the *in-planta* data follow the same pattern of expression as the FDDP D mass feature, displaying absence in the Δ PKS15 infected tissues and greatly reduced expression in the Δ TS infected tissues as demonstrated in the Figure 29 boxplots. All unidentified mass features are believed to be $[M+H]^+$ mass features due to the observed presence of associated adducts and neutral losses.

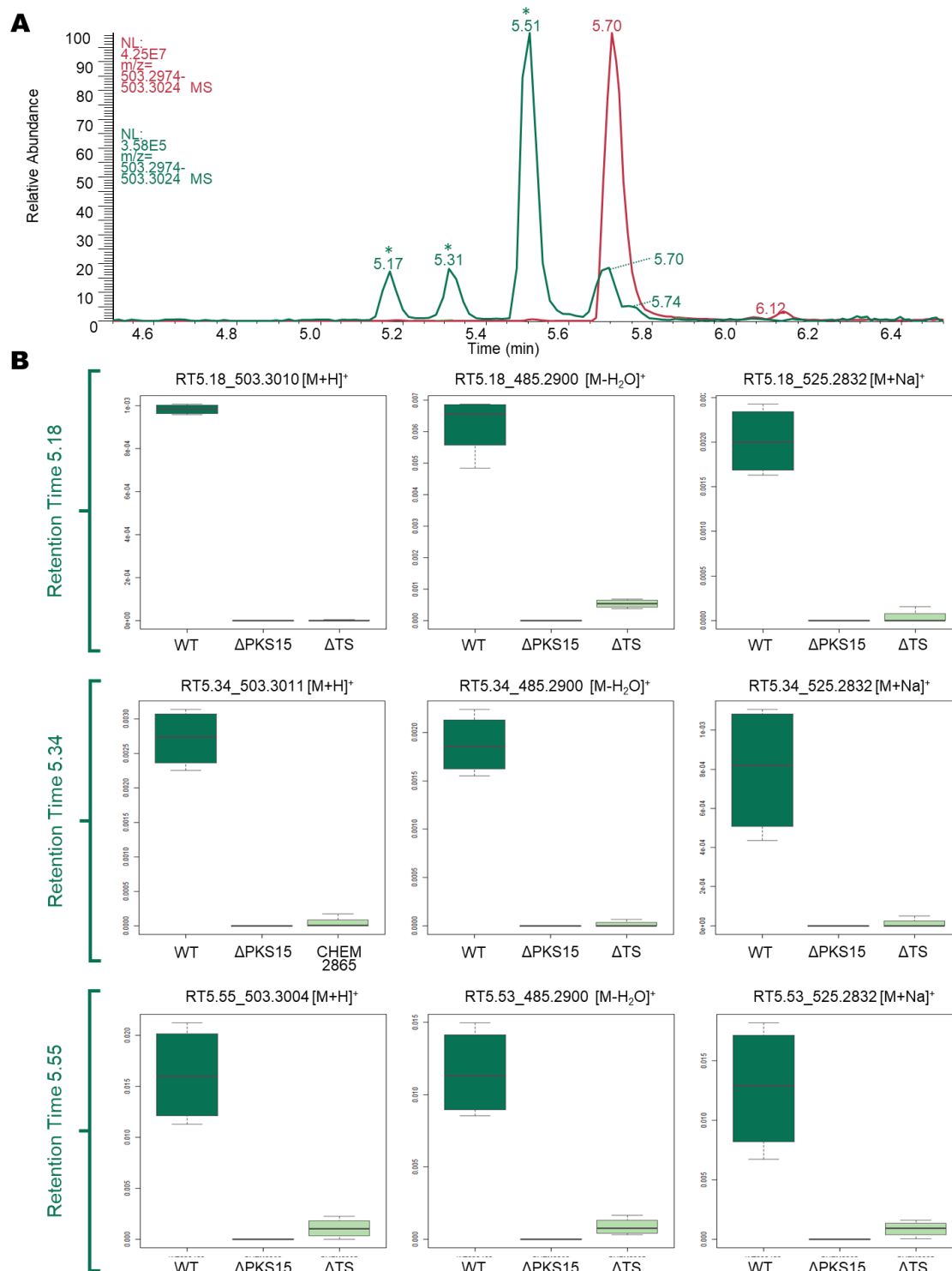


Figure 29: (A) XIC of m/z 503.3004 from mycelium of in-vitro cultures grown on Q6 medium (red) and in-planta growth (green). (B) Boxplots demonstrating detection patterns of m/z 503.3004 [M+H]⁺ and associated adducts at RT5.18 (Top), RT5.34 (middle) and RT5.55 (bottom) from WT and ΔPKS15 and ΔTS transformant strains in-planta wheat extracts (Bottom).

XICs of m/z 487.3054 from UPLC-HRMS data from culture extracts of the WT strain on both Q6 (orange trace) and *in-planta* (green trace), demonstrate a single occurrence of the mass (Figure 30-A). Further comparison of the MS2 fragmentation patterns of the RT 6.3_ m/z 487.3054 mass feature lead us to predict the structure corresponds to FDDP E reported by Tsukada et al., 2020 (Figure 30-B). The FDDP E annotation was also confirmed by comparison with the FDDP E standard provided by Tsukada et al.. FDDP E mass feature boxplots also follow the same detection pattern observed previously for the FDDP D mass feature, in which the mass feature is absent within the Δ PKS15 strain and significantly reduced in the Δ TS strain both *in-planta* and on the Q6 medium.

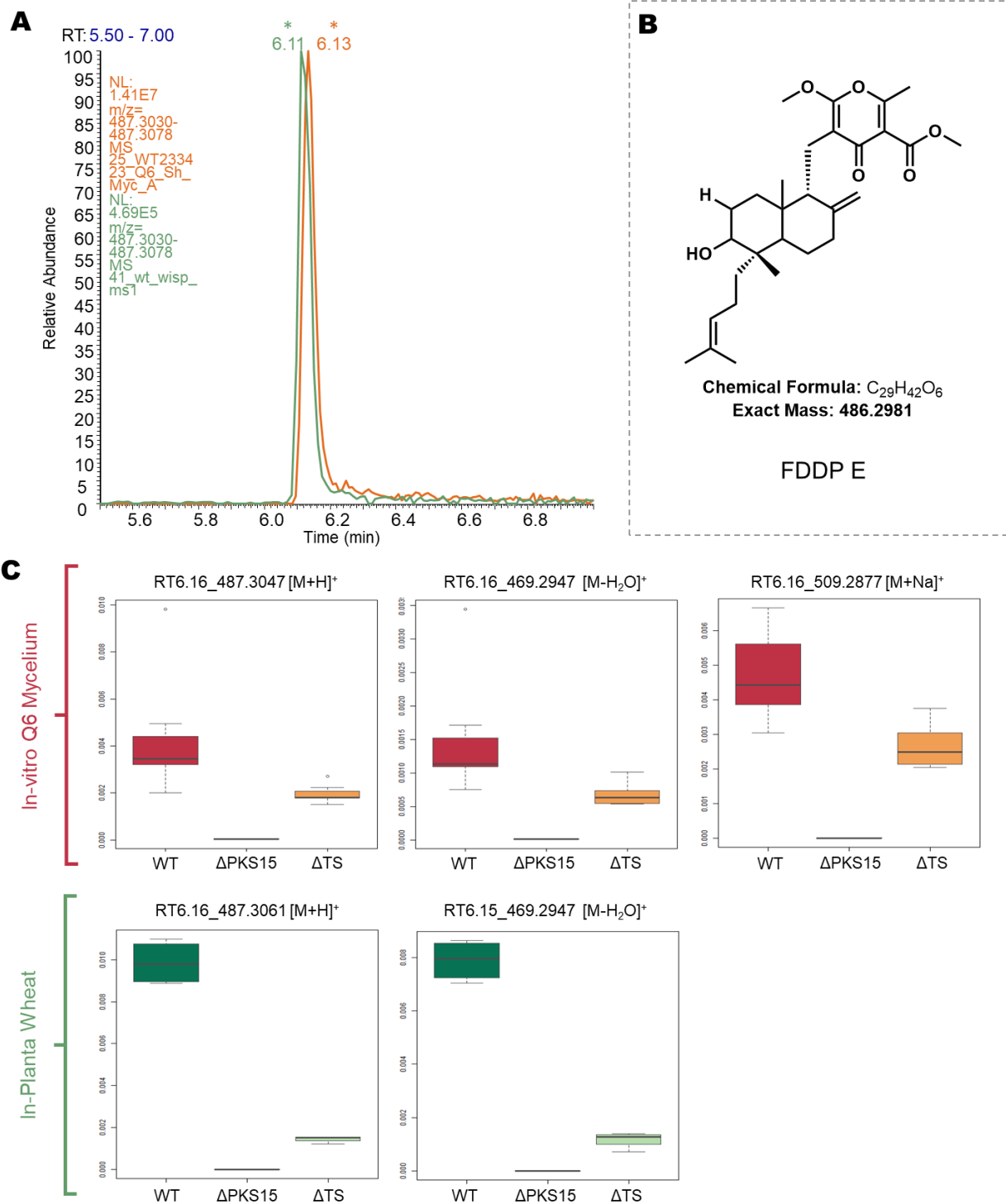


Figure 30: (A) XIC of m/z 487.3054 from mycelium of in-vitro cultures grown on Q6 medium (orange) and in-planta growth (green) (B) FDDP E structure associated with RT 6.16 m/z 487.3047[M+H]⁺ peak based on MS/MS analysis and comparison to Tsukada et al. 2020 reported findings. (C) Boxplots demonstrating [M+H]⁺ and associated adducts in WT and ΔPKS15 and ΔTS transformant strains from in-vitro mycelial extracts cultured on Q6 medium (Top) and in-planta wheat extracts (Bottom).

Quantity constraints on $[M+H]^+$ mass feature intensities permitted only four of the identified FDDP mass features to be fragmented in targeted MS2 analyses (Figures 31 & 32-A):

RT5.51_m/z 503.3004 (unknown, green trace), RT5.70_m/z 503.3004 (FDDP D, red trace), RT6.13_m/z 487.3054 (FDDP E, orange trace) and RT5.91_m/z 443.3152 (FDDP B / viridoxin A hydrolysate). MS2 analysis of the RT5.91_m/z 443.3152 mass feature could not differentiate between the two potential products (FDDP B & viridoxin A hydrolysate), however fragmentation and RT patterns associated with the FDDP B standard provided by Tsukada et al., did not match the retention time of our RT5.91_m/z 443.3152 mass feature suggesting it is more likely the shunt pathway product of viridoxin A hydrolysate.

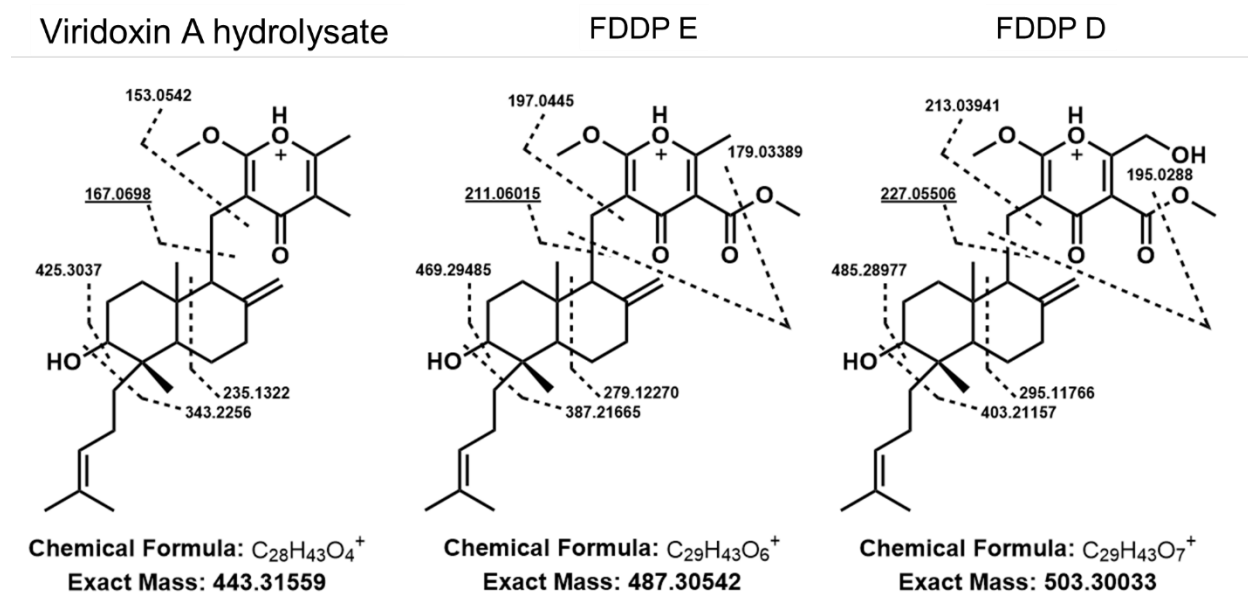


Figure 31: Putative fragmentation patterns of C16 associated structures based on observed MS2 mass fragmentation spectra of associated $[M + H]^+$ mass features. Underlined m/z values were the highest intensity spectra for each feature, correlating to a cleavage between the diterpene and pyrone moieties.

MS2 fragmentation mirror plot comparisons of the unknown mass feature RT5.51 m/z 503.3004 with the two FDDP end products (D and E) reveal high similarity between the pyrone fragments generated within the unknown 503 mass feature and FDDP E. Matching fragments between the FDDP E and unknown mass feature MS2 spectra are indicated in orange and green within Figure 28-B. There is also very little feature overlap between the unknown mass feature and FDDP D. However, it should be noted that many of the pyrone associated fragments differ by a mass of 15.9999 (oxidation mass). Thereby leading us to the conclusion that the unknown mass feature RT5.51 m/z 503.3004 likely shares the pyrone structure with that of FDDP E and contains an oxidation somewhere within the terpene moiety.

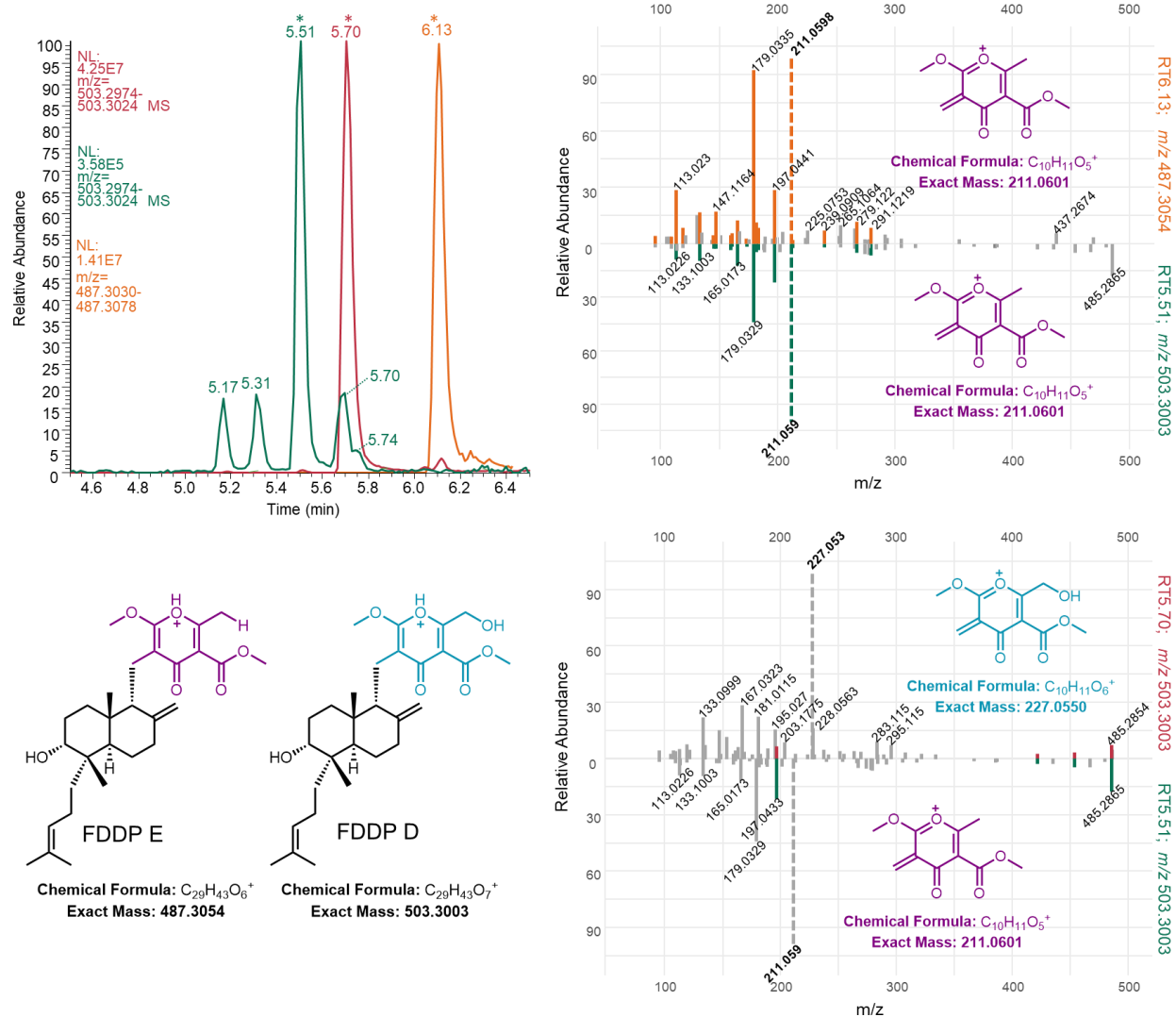


Figure 32: (A) Extracted ion chromatograms of mass featured used in MS2 mirror plot comparisons with (*) indicating specific retention times. (B) Constructed mirror plots comparing FDDP-E (RT6.13; m/z 487.3054; top spectrum) from Q6 mycelial extracts to an unknown

3.3.2.2 C13/C70 Transformant Strains

C70 and C13 transformant strains were analyzed together due to their cluster proximity within the genome which is unlike other fusaria. MMK2 medium culture extracts for C70 and C13 were both analyzed, due to the higher abundance and consistency in frequency of their respective

known products. Q6 medium yielded similar results in terms of expressions however the MMK2 medium was much easier to manipulate in both formulation and data processing which is why it was selected. When cultured on MMK2, the Δ NRPS4 strains displayed phenotypic variation when compared to the wildtype strain. Transformant strains produced less dense mycelial mats and had a reduction in aerial hyphae as compared to WT (Figure 33). Both Δ TF and Δ PKS12 transformants for had no visual deviation from the phenotype of the WT strain.

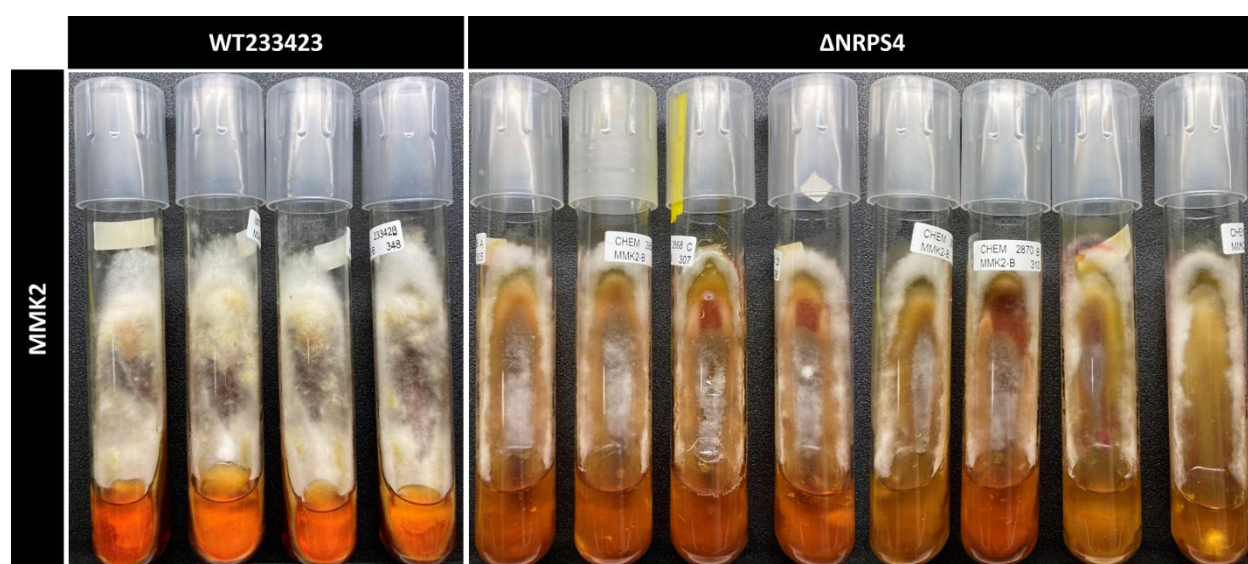


Figure 33: Phenotypic comparison of WT strain and NRPS4 transformant strains cultures on MMK2 medium.

XICs of m/z 607.3814 (± 5 ppm) reveal the appearance of three mass features at RT4.25, 4.40 and 4.70 (Figure 34-Top left); the latter of which corresponded to fusahexin (Figure 34 Top right) and the two former peaks corresponded to linearized forms of the fusahexin molecule $[M-H_2O+H]^+$. Box plots of mycelial extracts comparing the WT, C70 Δ NRPS4, C13 Δ TF and C13 Δ PKS12 strains demonstrate a lack of production of fusahexin associated mass features in the C70 Δ NRPS4 strain as expected. Both the C13 Δ TF and Δ PKS12 transformant strains show a slight

increase in the production of fusahexin associated mass features when compared to the WT strain, however this is not statistically supported.

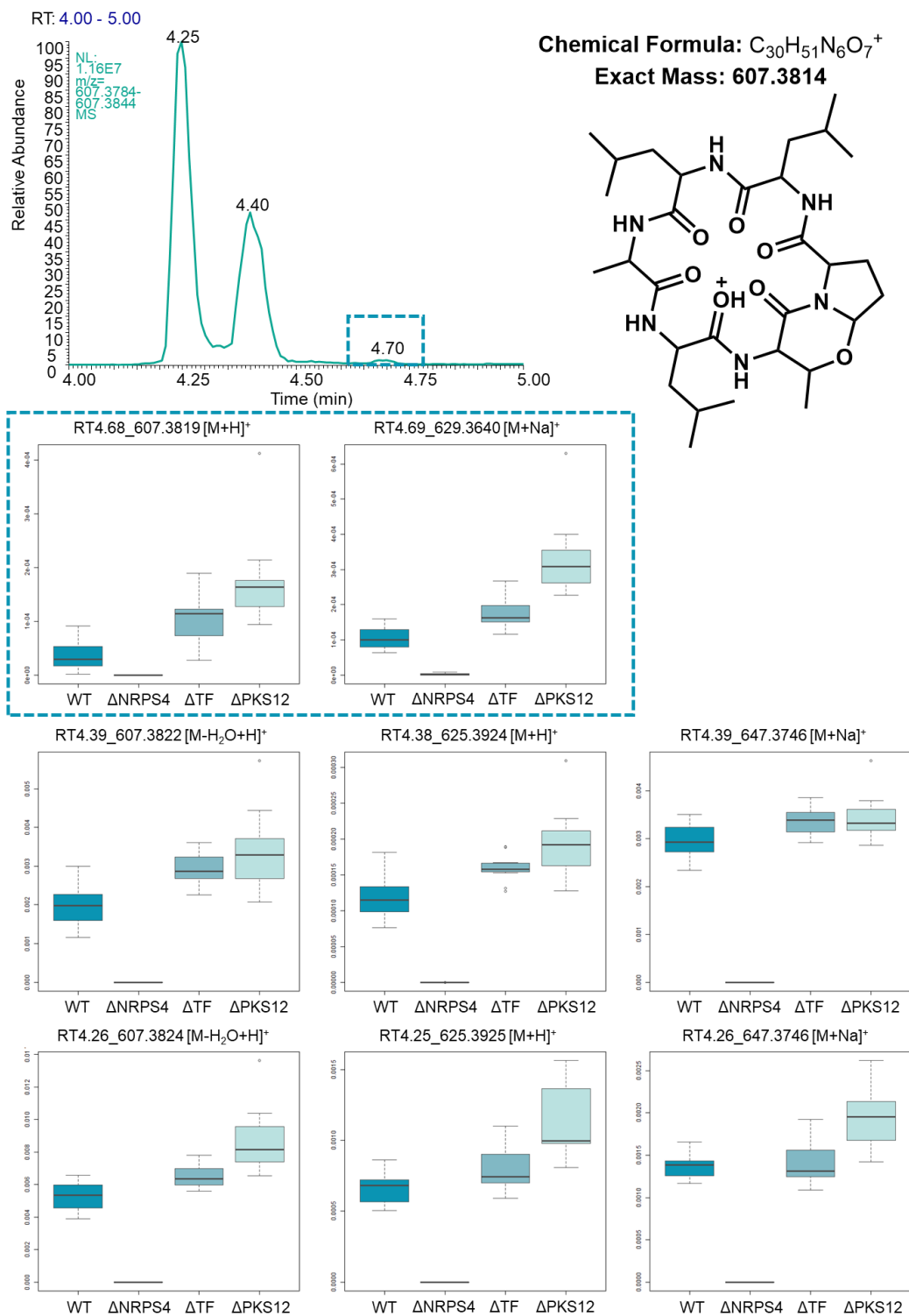
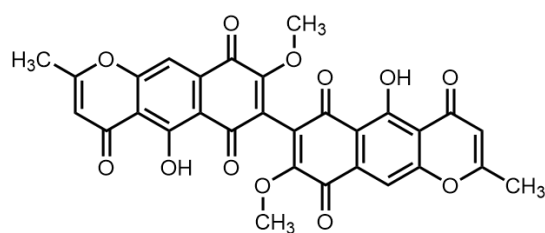


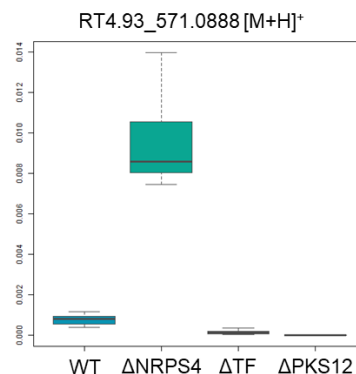
Figure 34: XIC of m/z 607.3819. Structure of known major NRPS4 products, fusahexin. Boxplots demonstrating intensities within WT, $\Delta NRPS4$, ΔTF and $\Delta PKS12$ strains for predicted fusahexin mass features (indicated in blue) and predicted linearized NRPS4 masses.

Aurofusarin (m/z 571.0871 $[M+H]^+$), rubrofusarin (m/z 273.0757 $[M+H]^+$) and fuscofusarin (m/z 557.1078 $[M+H]^+$) are all known C13 associated mass features (Figure 35). Box plots of these masses show no detection within the C13 Δ PKS12 strain and an abolition of production of aurofusarin and fuscofusarin and a reduction in production of rubrofusarin in the C13 Δ TF strain compared to the WT. The C70 Δ NRPS4 strain displayed a consistent and significant increase in the production of C13 PKS12 related mass features.

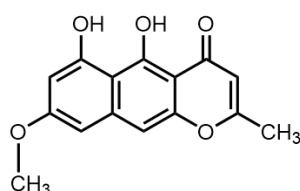
Aurofusarin



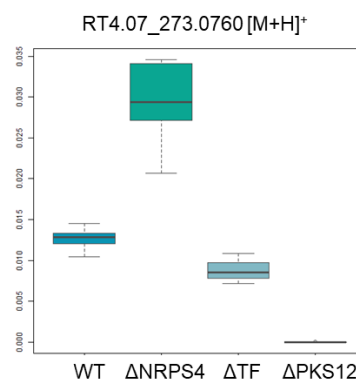
Chemical Formula: $C_{30}H_{18}O_{12}$
Exact Mass: 570.0798



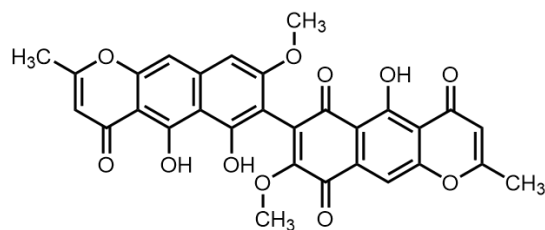
Rubrofusarin



Chemical Formula: $C_{15}H_{12}O_5$
Exact Mass: 272.0685



Fuscofusarin



Chemical Formula: $C_{30}H_{20}O_{11}$
Exact Mass: 556.1006

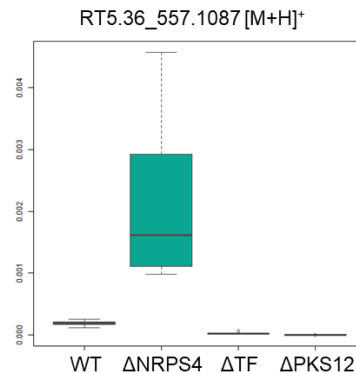


Figure 35: Structures of major products associated with PKS12 accompanied with boxplots demonstrating intensities within the WT, Δ NRPS2, Δ TF and Δ PKS12 transformant strains.

A more extensive look at all of the mass features generated from profiling of this data set demonstrates huge variations within the chemical profiles of the transformant and WT strains.

Volcano plots comparing mass feature production patterns for the C70 Δ NRPS4 and C13

Δ PKS12 strain to that of the WT strain (Figure 36-A/B) show a large number of mass features with significant fold changes (>2) and statistically significant p-values (<0.05). Features with known or predicted association to NRPS4 (adducts and in source fragments and neutral loss products) are highlighted in purple while features with known or predicted association to PKS12 are highlighted in Red. There are likely other mass features associated with each of these genes that are not yet completely elucidated and therefore have not been highlighted. Despite this, there still remains a large number of features with p-values below 0.05 and fold changes above 2, many of which are likely not associated directly with either NRPS4 or PKS12 which may be suggestive to a wider metabolomic or more systemic response to the absence of NRPS4 and PKS12 related products. Volcano plots of the C13 Δ TF also demonstrate a number of mass features that appear to be statistically significant. The mass feature distribution within the Δ TF vs WT volcano plot (Figure 36-C) resembles the distribution of the Δ PKS12 vs WT volcano plot however, the fold changes are significantly reduced.

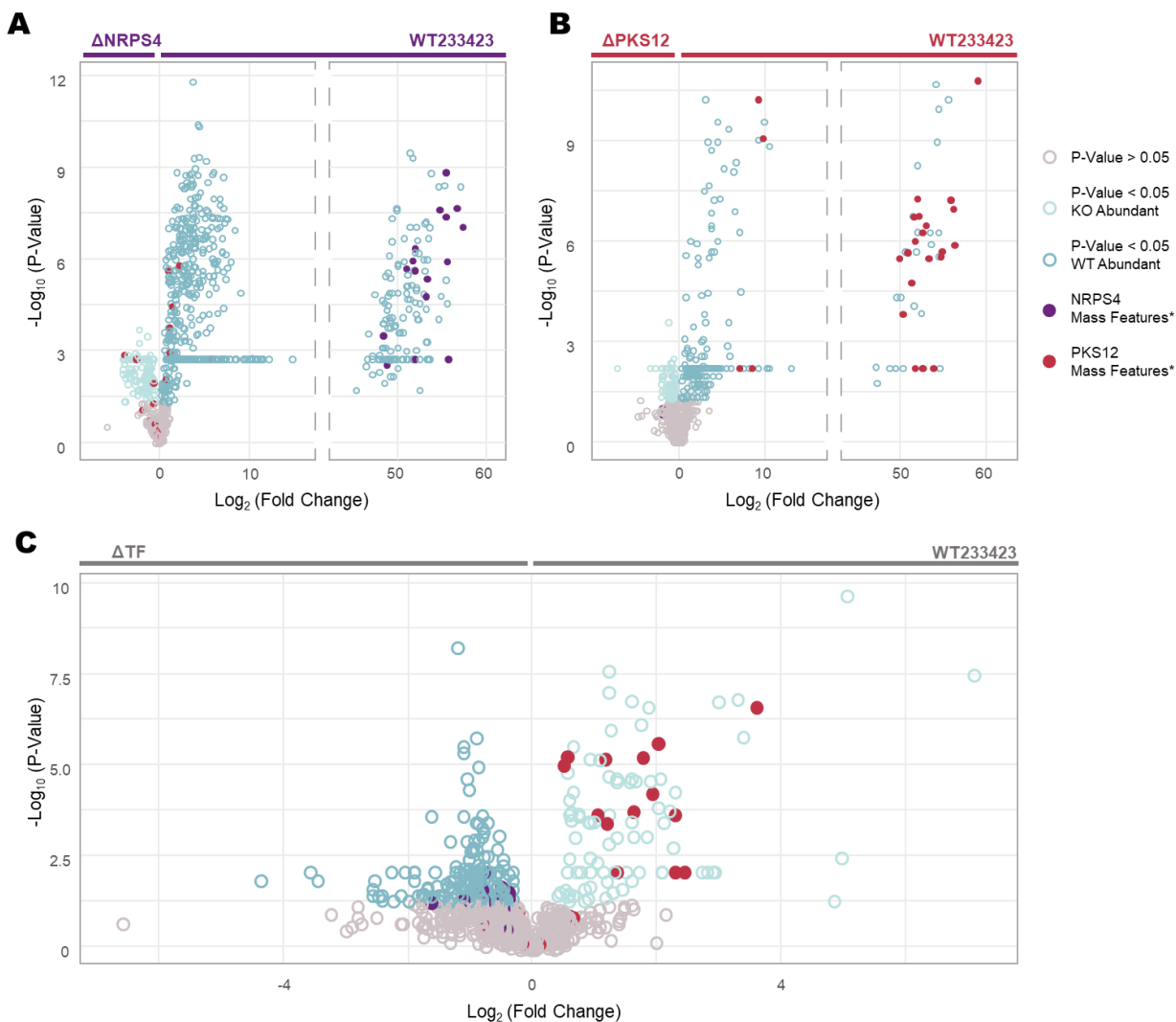


Figure 36: Volcano plots of mass features from mycelial extracts on MMK2 medium demonstrating (A) WT Strain vs Δ NRPS4 strain and (B) WT strain vs Δ PKS12 strain (C) WT strain vs Δ TF strain. X-axis breaks are delineated with dashed lines for each respective plot. Known associated features have been indicated in purple (NRPS4) and red (PKS12) for each of the respective plots.

3.4 Discussion

3.4.1 C08 Transformant Strains

F. graminearum transformant strains Δ PKS11 and Δ TS from the C08 BGC were profiled under a

few different media conditions (as mentioned above) in an attempt to discern the metabolite

products, however we were unable to distinguish any within our analysis. Media types for profiling were selected based on the media that produced the greatest mass feature diversity in metabolite profiles from the WT media study, however gene expression was not monitored from these cultures and therefore it is possible that the C08 BGC is not expressed under any of the tested conditions. The C08 BGC has reported expression *in-planta* within wheat cv. Roblin (the same cultivar used in our analysis) by Harris et al. (2016), however, we were unable to differentiate products within the *in-planta* extracts for these transformants as compared to WT. This could be due to the low fungal biomass relative to plant tissues resulting in low concentration of fungal metabolites and the high background/abundance of plant signals. Reports on the expression of C08 PKS11 suggest a higher expression under saprophytic conditions (Boedi et al., 2016). It is possible that 14 dpi is an insufficient duration for the accumulation of C08 metabolites in the wheat heads, if the production is not ramped up until after the period of biotrophic infection. It is also possible that there are structural constraints on the molecule itself making extraction efficiency low with the selected solvent, or the ionization ability of produced metabolites is low using the positive electrospray ionization method used for metabolite analysis – perhaps negative electrospray ionization would yield different results.

In order to identify C08 associated mass features, *in vitro* transcriptomic expression analysis of the C08 genes should be completed. Analysis of *in vitro* metabolomics data typically yields much ‘cleaner’ results compared to the *in-planta* metabolomics. Typically, *in vitro* data does not have constraints on fungal signal intensities (or relatively high background noise) and there is not the added biological variability of the host plant. Gaffoor et al. (2005) did report the expression of the *PKSII* gene on two *in vitro* conditions examined – rice medium and corn meal medium - both of which could be considered for potential future profiling endeavours.

3.4.2 C16 Transformant Strains

FDDP D and E described by Tsukada et al. (2020) were both identified and confirmed as products of the C16 BGC by MS2 analysis and comparison to provided standards. In addition to FDDP D (RT5.70_ m/z 503.3003) and FDDP E (RT6.16_ m/z 497.3054), three other mass features were also identified as associated the Δ PKS15 transformant strain: RT5.18_ m/z 503.310, RT5.34_ m/z 503.3011, and RT5.55_ m/z 503.3004. Metabolomic analysis revealed the absence of the mass features within the C16 Δ PKS15 strain, a reduction in feature intensity in the C16 Δ TS strain and unchanged production in the Δ WD40 strain relative to the WT. It was determined that the WD40 within the BGC does not appear to impact the synthesis of the FDDPs or associated unknown mass features. The persistent detection of these mass features within the Δ TS strain is likely due to the redundancy of genes associated with GGPP synthase within the genome. The three additional m/z 503.3003 mass features identified from the in-planta data are likely related to or structurally similar to the FDDPs described by Tsukada et al. (2020). Intensity patterns of the Δ PKS15 and Δ TS strains suggest that these mass features contain both pyrrole and terpene products. Comparison of MS2 fragmentation patterns of the RT5.55 mass feature with those of FDDP D and E suggests that the corresponding molecule likely shares the structure of the FDDP E pyrrole while containing an additional oxidation within the terpene moiety. Comparison of MS2 data from our unknown RT5.55 mass feature with the MS2 data reported from Seidl et al. (2022) suggest that our unknown may correspond to that of the molecule proposed as ‘gramiketide B’ (Appendix-B Table 1). However, Seidl et al., were unable to provide structural information on their identified mass features. Unfortunately, MS2 attempts on two of

the additional m/z 503.3003 mass features observed *in-planta* were unsuccessful due to the low signal intensities, therefore preventing further inference upon their associated molecular structure. Across all 12 screened media types from the original WT analysis, only the RT5.70_ m/z 503.3003 mass feature was detected in any *in vitro* condition. The detection of the three additional m/z 503.3003 mass features *in-planta* could potentially be a synthesis product resulting from plant reactive oxygen species acting on the FDDP metabolites altering their structure, or it may arise from fungal biosynthetic genes associated with tailoring enzymes that are turned on in pathogenic conditions that remain turned off in *in vitro* conditions.

3.4.3 C13/C70 Transformant Strains

From the C13/C70 BGC, both Δ PKS12 and Δ NRPS4 strains demonstrated the absence of their expected biosynthetic products (aurofusarin and fusahexin respectively). The increase in C13 PKS12 related products seen within the C70 Δ NRPS4 strain is potentially due to the increase production in relation to stress. Medentsev et al., 2005 demonstrated aurofusarin as the only *F. graminearum* pigment produced under conditions of nitrogen deficiency, phosphorus deficiency, oxidative stress and inhibition of respiration. All of which could be indicative of unknown functionality of the aurofusarin metabolites other than pigment production. NRPS4 has been previously associated with cell hydrophobicity as reported by Hansen et al. (2012), where gene deletion strains lacked the ability to repel water. The absence of a molecule promoting hydrophobicity in a liquid culture environment may result in a stress response thereby leading to the increase in aurofusarin production, along with the other plethora of secondary metabolite mass features observed in C70 Δ NRPS4 strain as compared to WT.

Untargeted analysis of the mass feature comparing the C70 Δ NRPS4 strain and C13 Δ PKS12 strain to the WT strains are dissimilar to the results generated for the C16 (FDDP) cluster analysis. Mass features found to be statistically significant within the analysis of the C16 transformant strains were all later confirmed to be associated with the C16 BGC. However, analysis of the C13/70 transformants demonstrate that both Δ NRPS4 and Δ PKS12 strains contain a large number of mass features that have not yet been correlated directly to known SM products from each of the gene clusters respectively. Volcano plots indicate a large number of mass features that are likely unrelated to the C13/70 gene cluster being differentially produced with statistical significance and relatively high fold changes as compared to WT. Induction of a stress response due to the absence of the PKS12 and NRPS4 products may also explain the large number of mass features with variable detection between transformant and WT strains, as a global metabolic shift is occurring. Full MS2 profiling via Qexactive (QE) of the transformant and WT strains would be beneficial and is currently being undertaken (however, these results were not available for inclusion in this thesis work). QE data has the potential to correlate some of these significant/high foldchange features to the respective GOIs as there are likely related aurofusarin and fusahexin mass features that have not been reported (potential biosynthetic precursors or fragmentations). Additionally, using ion network mapping analyses, the QE data may allow for the identification of familial mass features or molecular classes that may be differentially expressed within the Δ NRPS4 and Δ PKS12 strains through the formation of molecular networks based on MS2 fragmentation patterns.

The C13 gene cluster has two transcription factors associated with it, AurR1 and AurR2. In this thesis, AurR2 was disrupted (C13 Δ TS) and correlated to the increased production in structural dimer end products relative to monomer intermediates (increases in aurofusarin and fuscofusarin

with decreases in rubrofusarin) and is associated with increased expression of later pathway genes. The AurR1 transcription factor, was previously demonstrated to be correlated to an increase in expression of the PKS12 gene and overall increases in production of all associated C13 products. Looking at the AurR2 deletion strain (C13 Δ TF), it appears as though its regulation is only directed towards the C13 gene cluster and has no transcriptional influence on the C70 BGC. However, that is not enough to say that the two BCGs are not co-regulated by the AurR1 transcription factor. When looking at the WT media analysis both aurofusarin associated and fusahexin associated molecules appear to be expressed under relatively the same conditions (same media types show detection), however overlap is not 100%. It is possible that both BCGs are under regulatory control from the same global regulators and/or as a result of epi-genetic control via heterochromatin remodelling that would provide access of the adjacent BGCs to RNA polymerases.

3.5 References

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3.6 Appendix-B

3.6.1 WT Media Study: Metabolomics Data Processing

UPLC-HRMS profiles of both broth and mycelium extracts were processed together where they were compiled into a representative matrix of metabolite mass features denoted with a retention time (RT) and mass to charge ratio (m/z). Processing of the Xcalibur raw data files was completed using MZmine 2.53 (Pluskal et al, 2010). Masses were detected with an exact mass noise threshold of 1E4, and chromatographic building was completed with the ADAP algorithm where a minimum group size of 5, a group intensity threshold of 5E6 and a minimum highest intensity of 1E7. RT and m/z tolerances were consistently set to 0.01 min and 0.001 m/z or 5ppm throughout all phases of processing. Deconvolution of the chromatograms was completed using the local minimum search algorithm with a chromatographic threshold of 80%, minimum RT search range of 0.05 min, minimum relative height of 50%, a minimum absolute height of 1E6, and a minimum ratio of peak top/edge of 1 and a peak duration range of 0-10min. Following deconvolution, isotopic peaks were grouped together, and the JOIN aligner was used using a 20:10 m/z to RT weight. Same RT and m/z gap filling was then applied to back call in any points that were excluded from the original processing parameters but present in the data set.

Binary Transformation and Data Reduction

The resulting data matrix was exported from MZmine as a .csv file as a matrix of peak height intensities and imported into R Studio for further processing. First, the maximum detected blank value for each mass feature was back subtracted from all sample masses in order to reduce false positives that may result from either sample carry-over or gap filling errors. Following back subtraction, all data points were converted into a binary presence absence matrix in which values

>1E4 were converted to 1, and values <1E4 0. A feature detection consensus for each of the extract/culture was then generated by applying a 75% inclusion (3/4 replicates) on mass features i.e., a feature had to be >1E4 in a minimum of 3 samples to be considered detected within a sample. This was applied as an additional method to reduce false positives. All sample consensus were used to generate the final mass feature list of 304 mass features. A final additional media consensus matrix was generating using the sums from binary consensus for each of the media types to obtain an overview of all potential features detected per media type across the various conditions.

Metabolomics: Mass feature Annotation

Mass feature annotation was completed using an in-house Fusarium reference database created from both literature reports and commercial standards. Annotations were assigned based on the mass spectral accuracy (± 5 ppm) and relative elution order.

3.6.2 C16 Q6 Medium and *In-planta* Metabolomics: Data Processing

Q6: Data Pre-processing

UPLC-HRMS profiles of both broth and mycelium extracts were processed together where they were compiled into a representative matrix of metabolite mass features denoted with a retention time (RT) and mass to charge ratio (m/z). Processing of the Xcalibur raw data files was completed using MZmine 2.53 (Pluskal et al., 2010). Masses were detected with an exact mass noise threshold of 1E4, and chromatographic building was completed with the ADAP algorithm where a minimum group size of 5, a group intensity threshold of 1E5 and a minimum highest intensity of 5E5. RT and m/z tolerances were consistently set to 0.01 min and 0.001 m/z or 5ppm throughout all phases of processing. Smoothing was then applied at a filter width of 5.

Deconvolution of the chromatograms was completed using the local minimum search algorithm with a chromatographic threshold of 90%, minimum RT search range of 0.05 min, minimum relative height of 60%, a minimum absolute height of 1E5, and a minimum ratio of peak top/edge of 1 and a peak duration range of 0-10min. Following deconvolution, isotopic peaks were grouped together, and the JOIN aligner was used using a 20:10 m/z to RT weight. Same RT and m/z gap filling was then applied to back call in any points that were excluded from the original processing parameters but present in the data set.

Q6: Data Reduction

The resulting data matrix was exported from MZmine as a csv file as a matrix of peak height intensities and imported into R Studio for further processing. First, the maximum detected blank value for each mass feature was back subtracted from all sample masses in order to reduce false positives that may result from either sample carry-over or gap filling errors. The data was then normalized to the TIC by sample sums. Post initial screening, mass features prior to 1 min and post 8 min were removed for better data visualization.

In-planta: Data Pre-processing

Processing of the Xcalibur raw data files was completed using MZmine 2.53 (Pluskal et al., 2010). Masses were detected with an exact mass noise threshold of 1E4, and chromatographic building was completed with the ADAP algorithm where a minimum group size of 5, a group intensity threshold of 1E5 and a minimum highest intensity of 2E5. RT and m/z tolerances were consistently set to 0.01 min and 0.001 m/z or 5 ppm throughout all phases of processing.

Smoothing was then applied at a filter width of 5. Deconvolution of the chromatograms was completed using the wavelets ADAP algorithm with a S/N of 1, minimum feature height of 1E4,

coefficient area of 110, a peak duration range of 0.0-10.00 min and an RT wavelet range of 0.0-0.1 min. Following deconvolution, isotopic peaks were grouped together, and the JOIN aligner was used using a 20:10 m/z to RT weight. Same RT and m/z gap filling was then applied to back call in any points that were excluded from the original processing parameters but present in the data set.

In-planta: Data Reduction

The resulting data matrix was exported from MZmine as a csv file as a matrix of peak height intensities and imported into R Studio for further processing. First, the maximum detected blank value for each mass feature was back subtracted from all sample masses in order to reduce false positives that may result from either sample carry-over or gap filling errors. The data was then normalized to the TIC by sample sums.

3.6.3 C13/C70 MMK2 Medium Study Metabolomics: Data Processing

MMK2: Data Pre-processing

UPLC-HRMS profiles of both broth and mycelium extracts were processed together where they were compiled into a representative matrix of metabolite mass features denoted with a retention time (RT) and mass to charge ratio (m/z). Processing of the Xcalibur .RAW data files was completed using MZmine 2.53 (Pluskal et al., 2010). Masses were detected with an exact mass noise threshold of 1E4, and chromatographic building was completed with the ADAP algorithm where a minimum group size of 5, a group intensity threshold of 1E5 and a minimum highest intensity of 5E5. RT and m/z tolerances were consistently set to 0.01 min and 0.001 m/z or 5ppm throughout all phases of processing. Smoothing was then applied at a filter width of 5.

Deconvolution of the chromatograms was completed using the local minimum search algorithm

with a chromatographic threshold of 90%, minimum RT search range of 0.05 min, minimum relative height of 1%, a minimum absolute height of 1E5, and a minimum ratio of peak top/edge of 3 and a peak duration range of 0-10 min. Following deconvolution, isotopic peaks were grouped together, and the JOIN aligner was used using a 20:10 m/z to RT weight. Same RT and m/z gap filling was then applied to back call in any points that were excluded from the original processing parameters but present in the data set.

MMK2: Data Reduction

The resulting data matrix was exported from MZmine as a csv file as a matrix of peak height intensities and imported into R Studio for further processing. First, the maximum detected blank value for each mass feature was back subtracted from all sample masses in order to reduce false positives that may result from either sample carry-over or gap filling errors. The data was then normalized to the TIC by sample sums.

3.6.4 Appendix-B Tables

Appendix-B Table 1: Table comparing MS/MS analysis of unknown mass feature and Seidl et al., (2022) identified gramiketide B.

	Seidl et al, (2022) for gramiketide B	Unknown mass feature RT5.51_ m/z 503.3004	Mass Error (ppm)
Precursor	503.3003	503.3003	
Fragment 1	485.2891	485.2865	Δ 5.357
Fragment 2	211.0596	211.059	Δ 2.843
Fragment 3	197.044	197.0433	Δ 3.553
Fragment 4	183.0284	183.0276	Δ 4.371
Fragment 5	179.0335	179.0329	Δ 3.351
Fragment 6	131.0337	131.0332	Δ 3.816
Fragment 7	113.0233	113.0226	Δ 6.193

3.6.5 Media Formulations

* agar was only included for solid cultures

CS Medium (1L)

Cottonseed Flour	20g
Soy Peptone	2g
Maltose	40g
MgSO ₄ ·7H ₂ O	2g
NaCl	2g
CaCO ₃	3mg
MilliQ Water	to 1.0L
Agar*	20g

CYA Medium (1L)

NaNO ₃	3g
KH ₂ PO ₄	1g
KCl	0.5g
MgSO ₄ ·7H ₂ O	0.5g
FeSO ₄ ·7H ₂ O	10mg
Yeast Extract	5g
Sucrose	30g
MilliQ Water	to 1.0L
Trace Element Solution	1000μL
ZnSO ₄ ·7H ₂ O	1g
CuSO ₄ ·5H ₂ O	0.5g
MilliQ Water	100mL
Agar*	20g

CYS80 Medium (1L)

Sucrose	80g
Corn Meal	50g
Yeast Extract	1g
MgSO ₄ ·7H ₂ O	0.5g
MilliQ Water	to 1.0L
Agar*	20g

PDA Medium (1L)

Difco Potato Dextrose Broth	36.0g
MilliQ Water	to 1.0L
Agar*	20g

MEA Medium (1L)

Malt Extract	20g
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Peptone	1g
D-glucose	20g
MilliQ Water	to 1.0L
Trace Element Solution	1000uL
Agar*	20g
<i>MMK2 Medium (1L)</i>	
Mannitol	40g
Yeast Extract	5g
Murashige & Skoog Salts	4.3g
MilliQ Water	to 1.0L
Agar*	20g
<i>Q6 Medium (1L)</i>	
D-Glucose	2.5g
Glycerol	1.g
Cottonseed Flour	5g
MilliQ Water	to 1.0L
Agar*	20g
<i>S2M Medium (1L)</i>	
(NH ₄) ₂ HPO ₄ -OR- Putrescine	1g
KH ₂ PO ₄	3g
MgSO ₄ ·7H ₂ O	0.2g
NaCl	5g
Sucrose	40g
Glycerol	10g
<i>NPN-A Medium (1L)</i>	
S1M	10%
S2M ((NH ₄) ₂ HPO ₄)	90%
Agar*	20g
<i>NPN-p Medium (1L)</i>	
S1M	10%
S2M (Putrescine)	90%
Agar*	20g
<i>YES Medium (1L)</i>	
Yeast Extract	20g
Sucrose	150g
MgSO ₄ H ₂ O	0.5g
MilliQ Water	to 1.0L
Agar*	20g

YES+IO Medium (1L)

YES Medium	1.0L
Instant Ocean	18g
Agar*	20g

ZM/2 Medium (1L)

Molasses	5g
Oatmeal (Flakes ground)	5g
Sucrose	4g
D-Glucose	1.5g
CaCO ₃	1.5mg
Edamin	0.5g
(NH ₄) ₂ SO ₄	0.5g
MilliQ Water	to 1.0L
Agar*	20g

Chapter 4 Summary Discussion and Conclusions

In Chapter 3, the production of FDDP D and FDDP E were confirmed within the WT *F. graminearum* strain. Additionally, three unknown mass features were also identified, one of which potentially corresponding to Seidl et al (2022) gramiketide B. Potential mass features corresponding to the tentative metabolite described as gramiketide A by Seidl et al, were also observed from *in-planta* extracts of the *F. graminearum* WT, however the mass features were detected below an abundance threshold required for MS2 analysis prohibiting for further investigation. Metabolomic analysis of the *F. graminearum* C16 transformant strains demonstrated that only the Δ PKS15 strain resulted in complete abolished production of the FDDP (and gramiketide) mass features. Redundancy of the GGPP synthase gene within the *F. graminearum* genome, expression of which might have supplemented the C16 biosynthetic pathway in the C16 Δ TS transformant might account for the continued detection of the FDDPs in the Δ TS strain during the Q6 medium study. Interestingly, in Chapter 2 the only strain demonstrating as having a phenotypic effect on the pathogenicity in wheat heads was the C16 Δ TS transformant – although expression of the FDDPs *in-planta* during wheat head challenge for the C16 Δ TS transformant were significantly reduced as compared to WT.

Structural comparisons of FDDP molecules identified in this study to other secondary metabolites, does provide insight into potential roles in pathogenicity. Higginsianins are structurally similar to the FDDPs produced by *F. graminearum* and arise from a closely related BGC (sharing homologous core synthase genes but different tailoring enzyme genes) found in the producing fungus *Colletotrichum higginsianum*, a fungal pathogen, causing anthracnose disease of Brassicaceae (including *Arabidopsis thaliana*) (Cimmino et al., 2016; Tsukada et al.,

2020). Figure 37 demonstrates the structural similarity between identified higginsianins and the FDDPs. In particular the terpenoid moiety of the FDDPs are identical in terms of molecular structure and stereochemistry to higginsianin B (highlighted in blue).

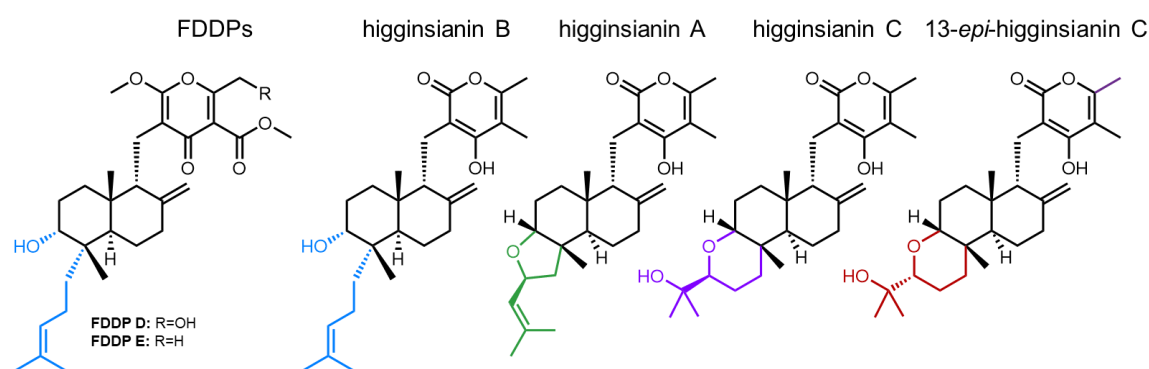


Figure 37: Structural comparisons FDDP features and higginsianin familial structures with terpene moiety similarities between FDDPs and higginsianin B indicated in Blue and varying structures indicated in green, purple and red.

Although FDDPs have not been investigated for potential biological effects or *in-planta* functions, Dallery et al. (2020) evaluated the effects of the higginsianins. Studies using *A. thaliana* mutant models demonstrated that higginsianin B is a virulence factor that facilitates infection. They determined that JA-Ile mediated degradation of JAZ proteins was inhibited when higginsianin B was present. To determine the mode of action of the JA repression response, *in-vitro* experimentation determined that higginsianin B inhibits two catalytic activities of the mammalian 26S proteasomes. In plants, the 26S proteasome responsible for Jasmonate Zim domain (JAZ) protein degradation thereby preventing the de-repression of the jasmonic acid signal transduction pathway, a pathway associated with plant resistance and responses to biotic and abiotic stressors (Ali & Baek 2020; Ruan et al., 2019). In addition to the JA pathway, the 26S proteasome is also has key role in the regulation of the auxin signaling pathway which is

associated with various aspects of plant growth and development, such as responses to light and gravity and vascular development (Schepetilnikov & Ryabova 2017).

Dallery et al., 2020 also evaluated the effect other known members of the compound family, namely higginsianin A, C and 13-epi-higginsianin C, and determined that only higginsianin B demonstrated biological activity. Figure 38 depicts the JA signaling pathways in plants under normal conditional response to stress as well as the assumed mode of action of higginsianin B in the repression of pathogen-recognition mechanism and downstream defence signaling. The molecule structure of all of the higginsianin SMs used in their analysis contain an identical pyrone moiety in as seen in Figure 37. Dallery et al., attributed the activity of higginsianin B to either or both of the hydroxyl or 4-isoheptenyl groups appended to the decalin ring, both of which are conserved within FDDP D and E identified in the Chapter 3 analysis. Due to the structural similarities of higginsianin B to the FDDP D and E, repression of the 26S proteasome by *F. graminearum* FDDPs is likely possible, and the FDDP molecules could act synergistically with other *F. graminearum* virulence factors to confound the innate plant immune response during pathogen ingress and cell-to-cell transmission during disease onset in wheat, barley, and maize.

Transcriptomics experiments have also linked the co-expression of the C16 BGC with that of the fusaoctaxin BGC (C64) in *F. graminearum*, where transcripts of both BGC genes were observed to peak 64–96 h after infection in infected barley and wheat kernels followed by a subsequent decrease (Sieber et al., 2014). Fusaoctaxins are known virulence factors and act by promoting cell-to-cell transmission and ultimately inhibiting/retarding cell wall callose deposition. In addition to fusaoctaxin transcriptomic analysis, LC-HRMS profiling of *F. graminearum*-infected wheat heads demonstrated an increase in both C16 associated mass features and DON 72 hrs

post-inoculation, with concentrations increasing overtime. DON, another identified virulence factor, is associated to the kernel-to-kernel transmission of the pathogen through the rachis in wheat (Jansen et al., 2005). Virulence factor expression by *F. graminearum* upon plant penetration and infection occurs as a network of events, rather than a single factor causing disease (Gottwald et al., 2012). Coregulated expression of a network of virulence factors (associated with complementary biological activities) allows the pathogen to overcome the redundancies associated with the plant immune system and allows for pathogen infiltration in a variety of different hosts.

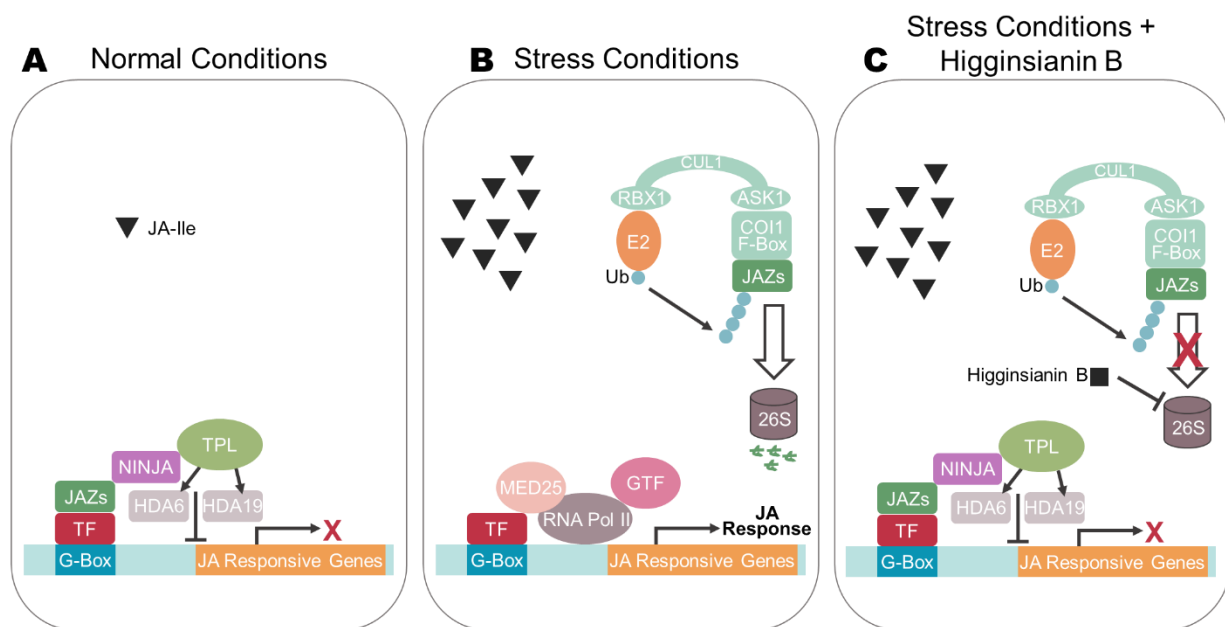


Figure 38: Jasmonic acid signal transduction pathway. (A) under normal conditions, low levels of JA-Ile, transcription factors are repressed by JAZ proteins which recruit corepressors such as NINJA and TPL to form a transcriptional repression complex that inhibits JA responses. The recruitment of HDA6 and HDA19 are recruited created a closed complex. (B) Elevated JA synthesis (and subsequent epimerization to JA-Ile) occur in stress conditions levels facilitate the interaction of JAZ with the F-box protein COI1 within the SCF complex. This results in the ubiquitination and proteasomal degradation of JAZ by the 26S proteasome. The depressed TF then binds to the G-Box element, whereupon MED25, RNA Pol II, and GTF are recruited, resulting in the expression of jasmonate responsive genes. (C) Proposed function of higgsianin B in inhibition of JA response.

In-planta growth chamber trials from Chapter 2 demonstrated a delay of onset of the disease phenotype within the *F. graminearum* C16 Δ TS transformant as compared to WT. Oddly, abolishment of FDDP production as seen in the Δ PKS15 transformant did not result in phenotypic deviation from those displayed by the WT. Dallery et al. (2020) does suggest that the biological activity of higginsianin B was dependent on the terpene moiety and not the pyrone (derived from PKS15). In our study, both the TS and the prenyltransferase genes were functional in the *F. graminearum* Δ PKS transformant—leading to the hypothesis that an alternate pyrone (not originating from PKS15) might have been scavenged and prenylated by the biosynthetic pathway to assemble a new functional FDDP molecule, differing in molecular weight to that of FDDP's observed from the WT strain but with an identical terpenoid moiety. Supporting this hypothesis is the observation that a number of fungal prenyltransferases have shown acceptor substrate promiscuity (Yu et al., 2011; Chooi et al., 2012; Chen et al., 2017). MS2 analysis completed on the FDDP mass features demonstrated that the terpene portion of the molecules do not readily take on a charge during positive electrospray ionization, resulting in most of the observed fragmentation masses to be associated with the pyrone (or with the decalin moiety with the pyrone still intact). Additionally, signals (or lack thereof) from the charged aerosol detector (CAD) suggest that FDDPs are produced in low abundance (data not shown), and high signal intensities are likely due to high ionization efficiencies of the pyrone. FDDPs constructed with pyrones not native to the C16 BGC may have lower ionization efficiency and therefore not captured by our metabolomic analysis. Follow-up experiments are required to confirm the observations made regarding FDDP involvement as a virulence factor, such as disruption of the prenyltransferase or entire C16 BGC.

4.1 Conclusion

This thesis focused on the analysis of four biosynthetic gene clusters within the *F. graminearum* genome: C08, C16, C13 and C70. The reported transcription of these BGCs within wheat pathogenicity experiments prompted the selection of these clusters, and their core biosynthetic genes, for gene disruption experiments. Wheat head infection assays conducted using the generated transformant strains revealed that only the C16 BGC appeared to have an impact on the pathology phenotype. Metabolomics analysis of the C16 transformants provided confirmation of gramiketide production and the native biosynthesis of FDDPs. Through a structural comparison of the identified C16 metabolites, hypotheses were formulated regarding their potential connections to the inhibition of plant immune response. Untargeted secondary metabolite profiling of the C13/C70 transformant strains suggests that this BGC may have significant implications for global secondary metabolite production. Specifically, the C70 Δ NRPS4 strain exhibited substantial variation in SM production when compared to the WT strain. The identity of the C08 metabolite product remains undisclosed, and it appears to not be associated with pathology. However, it is plausible that this metabolite has a role in the saprophytic phase of the disease. Additional investigation is necessary to explore the hypotheses derived from this thesis. Continued exploration into the association of FDDPs with pathogenicity will provide a more comprehensive understanding of the infection strategy employed by *F. graminearum*. The identification of differentially expressed metabolites within the C13/C70 clusters has the potential to offer valuable insights into the functional roles of their SMs. Finally, alternative strategies for identification of SMs is necessary when it comes to the C08 BGC.

4.2 References

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4.3 Appendix-C



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Fungi



Article

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CRISPR-Cas9 Gene Editing and Secondary Metabolite Screening Confirm *Fusarium graminearum* C16 Biosynthetic Gene Cluster Products as Decalin-Containing Diterpenoid Pyrones

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Abstract: *Fusarium graminearum* is a causal organism of Fusarium head blight in cereals and maize. Although a few secondary metabolites produced by *F. graminearum* are considered disease virulence factors, many molecular products of biosynthetic gene clusters expressed by *F. graminearum* during infection and their associated role in the disease are unknown. In particular, the predicted meroterpenoid products of the biosynthetic gene cluster historically designated as “C16” are likely associated with pathogenicity. Presented here are the results of CRISPR-Cas9 gene-editing experiments disrupting the polyketide synthase and terpene synthase genes associated with the C16 biosynthetic gene cluster in *F. graminearum*. Culture medium screening experiments using transformant strains were profiled by UHPLC-HRMS and targeted MS² experiments to confirm the associated secondary metabolite products of the C16 biosynthetic gene cluster as the decalin-containing diterpenoid pyrones, FDDP-D and FDDP-E. Both decalin-containing diterpenoid pyrones were confirmed to be produced in wheat heads challenged with *F. graminearum* in growth chamber trials. The extent to which the *F. graminearum* C16 biosynthetic gene cluster is dispersed within the genus *Fusarium* is discussed along with a proposed role of the FDDPs as pathogen virulence factors.

Keywords: Fusarium Head Blight; virulence factor; secondary metabolism; CRISPR-Cas9; gene editing; *Fusarium graminearum*; wheat pathogen

1. Introduction

Agriculturally relevant fungal pathogens threaten global crop production, with losses occurring in all major cash crops [1]. In some cases, crop losses are further amplified postharvest due to kern.

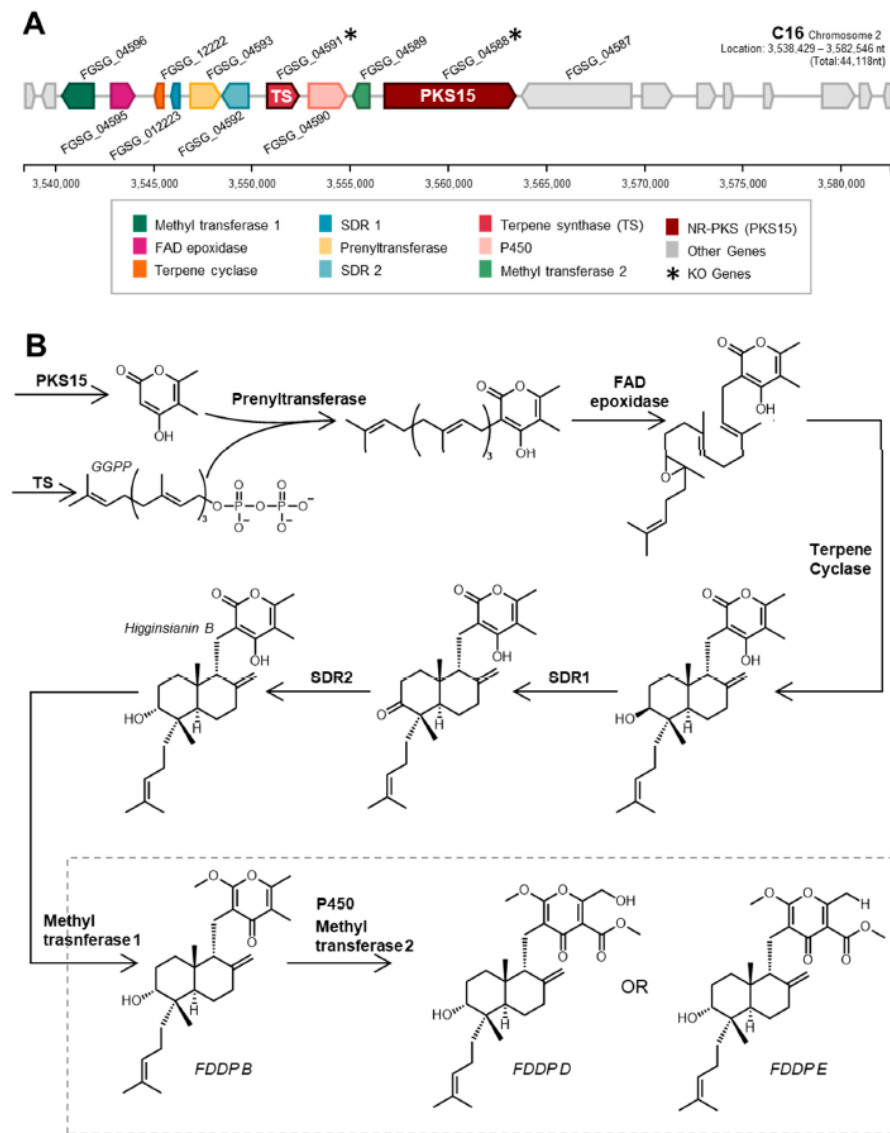
el contamination with regulated mycotoxins, diminishing their value or prohibiting marketability, posing serious health hazards and export barriers. Although the pathogen threats themselves are known, a definitive understanding of which genes within the pathogen genome (and their associated molecular products) play a role in disease virulence often is poorly understood [2]. Virulence factors are small molecules that promote colonization and cell-to-cell transmission of a pathogen within a host. As CRISPR-Cas9 methodologies have been validated in more than 40 different species of Oomycetes and filamentous fungi (including pathogenic genera such as *Aspergillus*, *Fusarium*, and *Sclerotinia*), precision gene-editing tools have facilitated research in gene/function linkages in fungi,

and in particular, show promise in the functional assignment of virulence-factor-encoding genes in plant pathosystems.

Fusarium graminearum is an excellent model organism for the study of virulence factors associated with Fusarium head blight (FHB), a devastating disease of cereals globally—both in terms of crop losses and contamination/infiltration of infected kernels with regulated mycotoxins. Several secondary metabolite virulence factors produced by *F. graminearum*, such as deoxynivalenol, fusaotoxins, and gramillin [3–5], have been linked with the onset of disease; however, the product and function of many *F. graminearum* secondary metabolite biosynthetic gene clusters (BGCs) that are expressed during infection remain unknown [6,7]. Transcriptomic analysis of *F. graminearum* challenge in various plants (wheat, barley, and maize) led to the hypothesis that secondary metabolites associated with the BGC designated as “C16” as being “pathogenicity-related” [7–10]. Boedi et al. compared gene expression during both pathogenic (living wheat) and saprophytic growth (dead wheat) and found that genes within the *F. graminearum* C16 BGC were exclusively induced by plant signals during pathogenesis and not under saprophytic conditions [10]. Before the start of our research project, the secondary metabolite products of the *F. graminearum* C16 BGC were unknown. Expression of the C16 BGC was not previously observed in various axenic culture conditions tested [8,11], hampering efforts to characterize the molecular products of the C16 BGC through traditional purification and structural elucidation experiments. Proof of the involvement of the *F. graminearum* C16 BGC products as FHB-associated virulence factors remains to be conclusively established.

Our research group was therefore interested in using CRISPR-Cas9 gene-editing tools to elucidate the product(s) of the *F. graminearum* C16 BGC and to ascertain whether the product(s) act as a virulence factor in FHB. In *F. graminearum*, the C16 BGC is composed of genes encoding for a nonreducing polyketide synthase (PKS15; FGSG_04588), a geranyl-geranyl pyrophosphate synthase (TS; FGSG_04591), a terpene cyclase (FGSG_12222), a prenyltransferase (FGSG_04593), and flavin adenine dinucleotide-dependent epoxidase (FGSG_04595)—enzymes that are proposed to be responsible for synthesizing the core chemical backbone of the molecule—in association with several additional tailoring enzyme genes (Figure 1A; Table S1). Genome mining revealed that the core enzymes responsible for building the chemical backbone of the metabolite products of the C16 BGC are present and conserved amongst several different fungi [12]. During our research into the *F. graminearum* C16 BGC product, Tsukada et al. heterologously expressed the associated core BGC synthase genes (originating from *Arthrrium sacchari*) and various tailoring enzyme genes (from *F. graminearum*, *Colletotrichum higginsianum*, and *Metarhizium anisopliae*) in multiple iterations within *Aspergillus oryzae* [12]. Following subsequent metabolite purifications, 1D and 2D NMR and mass spectrometry experiments were used by Tsukada et al. to elucidate the molecular structures of the various BGC products [12], designated as ‘fungal decalin-containing diterpenoid pyrones’ (FDDPs), of which FDDPs -B, -D and -E were predicted to be products of the *F. graminearum* C16 BGC (Figure 1B).

Presented here are the results of gene-editing experiments disrupting the PKS15 gene and the TS gene of the C16 BGC in *F. graminearum*. CRISPR-Cas9 gene-editing protocols involving microhomology-directed repair using a hygromycin B resistance cassette were employed to disrupt selected synthase genes in the C16 BGC. Culture medium screening experiments were subsequently carried out to identify in vitro culturing conditions under which C16 BGC products were expressed. The secondary metabolite products of the *F. graminearum* C16 BGC were confirmed by UHPLC-HRMS and targeted MS² experiments as FDDPs—the production of which was confirmed in planta in wheat heads upon challenge with *F. graminearum* in growth chamber trials.



2. Materials and Methods

2.1. CRISPR-Cas9 Gene Editing

Fusarium graminearum strain DAOMC233423 was selected for study—all media formulations used for protoplast generation and reagent formulations used in the generation of gene transformants are provided in the Supplementary Materials. Gene-specific CRISPR-RNAs (crRNA) were designed using the Eukaryotic Pathogen CRISPR gRNA Design Tool [13]. Pairs of crRNAs were designed for each of the intended genes of interest (GOIs) with cleavage sites upstream of the predicted translation start site and near the 3' ends of the target genes. No off-target cut sites were predicted against the *F. graminearum* PH-1 genome sequence, and care was taken to avoid disrupting adjacent genes. crRNAs were scored according to Doench et al. [14] and highly efficient crRNAs were selected. Repair template amplification primers were designed to contain 55–48 bp of homology adjacent to the gRNA-specified cleavage sites as well as 20 bp corresponding to up- and downstream regions of hygromycin resistance sequences within the pRF-HU2 vector (depicted in Figure S1).

2.1.1. Generation of Protoplasts

A 200 mL volume of Littman Oxgall liquid medium was inoculated with 1×10^8 *F. graminearum* macroconidia, incubated at 19 °C, and shaken at 160 rpm for 15 h, after which germling mycelium was harvested using a cell strainer (100 µm mesh size) and rinsed once with sterile water and once with 1.2 M KCl buffer. The resulting germling mycelia were transferred into a 125 mL Erlenmeyer flask containing protoplasting buffer composed of 1.2 M KCl, 500 mg Driselase, 200 mg lysing enzymes from *Aspergillus*, and 200 mg Yatalase per 20 mL and incubated at 30 °C with shaking at 80 rpm. The protoplasting buffer was prepared 45 min before use and filtered through a 0.45 µm filter prior to use. Protoplast formation was observed periodically over a total incubation time of 1 h and 15 min at which point the majority of the mycelium was found to be digested. The resulting protoplasts were diluted with an additional 20 mL of 1.2M KCl buffer, filtered through a 40 µm cell strainer, and centrifuged at $4100 \times g$ for 6 min at 4 °C. The pellet was resuspended and washed several times with a series of buffers (once with 20 mL of cold 1.2 M KCl buffer and twice with 20 mL of cold STC buffer (1.2 M D-sorbitol, 10 mM Tris-HCl, pH 8.0, 50 mM CaCl_2)), each followed by a centrifugation step. The final washed pellet was resuspended in cold STC buffer and the protoplasts were quantified using a hemocytometer and diluted to a final concentration of 2×10^8 protoplasts/mL in cold STC. A 7% volume of dimethyl sulfoxide (DMSO) was then added and the protoplast solution was transferred into 400 µL aliquots in sterile 2 mL Eppendorf vials and stored at −20 °C for 24 h before transfer to −80 °C.

2.1.2. PCR Amplification of HDR-HygB Repair Template

A hygromycin B expression cassette (HygB) was used as a selectable marker for the Cas9-mediated gene deletion. A 1395 bp region spanning the 364 bp pTrpC and 947 bp Hygromycin sequences was amplified from the pRF-HU2 vector using primers with the addition of 35–50 bp microhomology regions specific to each intended gene deletion (Table S2). Amplification was completed using Phusion® High-Fidelity DNA polymerase (NEB, cat#M05305) and HF Phusion buffer following the manufacturer's amplification protocol with minor modification to incorporate a touchdown start to increase primer specificity. Elongation temperatures started at 67 °C and were decreased by 1 °C per cycle for a total of 10 cycles before following the recommended 56 °C elongations. Initially, a single 50 µL reaction was completed for each of the primer sets to evaluate amplification efficacy. Once amplicon size was confirmed, additional PCR reactions (~10 reactions) were completed to obtain the desired 6–8 µg template quantity. Amplicons were then cleaned using the PureLink® PCR Purification Kit (K3100-01) following the manufacturer's protocol and a 1 µL aliquot of the cleaned template was then run on an agarose gel to confirm quality. Template quantity was determined using a Nanodrop spectrophotometer.

2.1.3. In Vitro Assembly of Cas9-gRNA Ribonucleoprotein Complexes

Cas-9 ribonucleoproteins (RNPs), composed of gene-specific crRNA (Table S3), tracrRNA, and the SpCas-9 protein, were assembled in vitro using commercially available Alt-R-CRISPR-Cas-9 components (Integrated DNA Technologies, Inc., Coralville, Iowa). Preparation of the Cas9-gRNA ribonucleoprotein complex was performed following Al Abdallah et al. [15]. In brief, two gRNA were generated corresponding to the 5' and 3' UTR to ensure the deletion of the entire coding sequence. Cas-9 was added to each gRNA individually, resulting in two ribonucleoprotein complexes for use in *F. graminearum* protoplast transformation.

2.1.4. *Fusarium graminearum* Protoplast Transformation

For the transformation of *F. graminearum* protoplasts, 200 μ L of the 2×10^8 protoplasts/mL aliquots were transferred into a 2 mL tube along with 26.5 μ L of RNP complex, 6–8 μ g of the purified repair template, and 25 μ L of a PEG (4000)-CaCl₂ buffer (60% *w/v* PEG 4000, 50 mM CaCl₂·H₂O, 450 mM Tris-HCl, pH 7.5) followed by incubation on ice for 1 h. Afterward, an additional 1.5 mL of the PEG (4000)-CaCl₂ was added and incubated for 20 min at room temperature. The mixture was then transferred to a 50 mL culture tube followed by the addition of 250 μ L of STC buffer and 3 mL of liquid TB3 medium and incubated at a 45° angle for 18 h at 25 °C and shaking at 80 rpm. Regenerated fungal protoplasts were then combined with 20 mL of molten TB3 media containing 100 mg/L hygromycin B at volumes of 300, 500, and 1000 μ L, poured into sterile Petri plates and incubated at 28 °C in the dark until the appearance of resistant colonies (5–6 d).

2.1.5. Gene Deletion Confirmation and Isolation of Axenic Transformant Strains

Putative transformants for each disruption target were used to generate axenic cultures by isolation and subsequent growth of a single macroconidium. Macroconidia were generated by inoculating 10 mL CMA media with a culture explant from putative transformants before incubation at 28 °C and shaking at 160 rpm at a 45° angle for 3 d to induce conidiation. The resulting cultures were then filtered through cheesecloth to separate mycelium from conidia. Conidia were pelleted from the filtrate by centrifugation for 10 min at 4100 rpm and subjected to several washes with sterile water and centrifugation steps to remove traces of the CMC medium. The resulting conidia were then diluted to a final concentration of 1×10^3 spores/mL. A 50 μ L aliquot of each spore stock was then dispersed on PDA plates (150 mg/L hygromycin B) with a cell spreader and plates were incubated in the dark at 28 °C for 24 h to reconfirm retention of hygromycin resistance via germination of macroconidia. Fungal colonies (originating from a single macroconidium) were then transferred to individual PDA plates supplemented with 100 mg/L hygromycin B to allow for fungal biomass growth.

gDNA was isolated from the resulting axenic cultures using the E.Z.N.A.[®] Plant DNA Kit following the manufacturer's protocol, with the initial cell lysis completed using MP Biomedicals[™] FastPrep-24[™] and tubes containing a single $\frac{1}{4}$ " ceramic bead and 2 mm zirconium oxide beads. PCR reactions were performed using amplification primers corresponding to the inserted hygromycin cassette as a positive control, and internal primers corresponding to the intended gene deletion (Table S4). Final concentrations of $1 \times$ Titanium Taq buffer (with 3.5 mM MgCl₂), 0.1 mM dNTPs, 0.08 μ M of both forward and reverse primers, and $1 \times$ Titanium Taq polymerase (Clontech, Mountain View, California) were obtained with 2 μ L of DNA template. Amplification was completed with an initial denaturation at 95 °C, followed by 35 cycles consisting of denaturation at 95 °C for 15 s, elongation at 60 °C for 15 s, and elongation at 72 °C for 30 s, with a final extension at 72 °C for 2 min. PCR products were visualized on a 1.5% SYBR Safe agarose gel at 100 V for 30 min. Strains that demonstrated amplification of the hygromycin gene, as well as a lack of amplification of their respective gene of interest, were retained for further study and cryopreserved for long-term storage.

2.1.6. Southern Blot Analysis

Selected transformant strains and wild-type (WT) control were cultured in S1M medium at 28 °C with shaking at 170 rpm for 5 d and mycelium was then harvested via vacuum filtration and ground in liquid nitrogen using a mortar and pestle before gDNA isolation using an Illustra Nucleon Phytopure Genomic DNA Extraction Kit (GE Healthcare UK Limited, Buckinghamshire, UK) following the manufacturer's protocol. All steps for DNA processing, hybridization, and Southern Blot analysis were carried out according to Gebbie [16].

2.2. Metabolite Profiling

2.2.1. WT Profiling—Culturing

Culturing was completed on the following media: CS, CYA, CYS80, MEA, MMK2, NPN-A, NPN-P, PDA, Q6, YES, YES +IO, and ZM/2 (all media formulations are listed in the Supplementary Materials). For the solid cultures, four Petri plates were inoculated with 2×10^5 macroconidia from the *F. graminearum* WT strain and incubated at 28 °C in the dark for 10 d; two “negative” controls were prepared for each medium by inoculating with sterile water followed by incubation to ensure the effects of media composition could be back-subtracted during metabolomics data processing. After 10 d, 12 plugs (1 cm diameter) were harvested into glass scintillation vials for solvent extraction using acetonitrile. A further 12 plugs were harvested for ethyl acetate extraction with shaking at 200 rpm for 1 h. The resulting solvent extracts were decanted, dried down on a pin drier, and resuspended in 1.5 mL MeOH for UHPLC-HRMS analysis.

A 200 mL liquid seed culture for each liquid growth medium was inoculated with 1.3×10^6 macroconidia in Erlenmeyer flasks with a 24 h initial shaking (160 rpm at 28 °C) before parsing out into 15 mL aliquots that were transferred into 50 mL culture tubes that were placed at a 45° angle and kept stationary for 10 days at 28 °C. Mycelium and broth were then separated and extracted separately in 15 mL of ethyl acetate (in 125 mL Erlenmeyer flasks) with shaking at 200 rpm at room temperature for 1 h. Solvent extracts were then decanted and dried down in borosilicate scintillation vials. Dried extracts were then resuspended in 1.5 mL MeOH and transferred into HPLC vials. Two pseudo-extracts or method blanks as well as two MeOH resuspension blanks were generated.

To assess for effects of gene-editing experiments, the WT, PKS15 deletion (Δ PKS15), and terpene synthase deletion (Δ TSS) strains were all cultured on Q6 liquid medium. A 15 mL volume of Q6 medium was inoculated with 1×10^5 macroconidia in 50 mL culture tubes with initial shaking for 24 h at 160 rpm before being kept stationary for 10 d at a 45° angle, all at 28 °C. Cultures were then harvested and extracted with ethyl acetate following previously described procedures.

2.2.2. Wheat Head Infections

Susceptible spring wheat cv. Roblin Seeds were surface-sterilized for 10 min with a 30% bleach solution followed by two rounds of washing with sterile water before being transferred to a large petri dish with sterile-water-soaked Whatman filter paper and left to germinate in the dark at room temperature for 48 h. Germlings were then transplanted into 6-inch round fiber pots filled with a planting medium composed of 66% black earth (soil), 33% Pro-Mix BX M, and 1% lime for a total of 4 seeds per pot. Potted seedlings were grown under both LED and fluorescent lighting with a 16 h photoperiod and a temperature cycle of 18 °C/day and 16 °C/nights until week five where day temperatures were increased to 20 °C and night temperatures increased to 18 °C. A 20-20-20 (nitrogen, phosphorous, potassium) fertilization was applied to pots once weekly. Plants were grown to mid-anthesis (6–7 w), at which point *F. graminearum* inoculations were performed.

Pathogen inoculation protocols for greenhouse pathogenicity trials were carried out according to Desjardins et al. [17]. Postinoculation, the plants were returned to growth chambers with overhead misting for 30 s every hour for 48 h to help promote fungal infection and temperatures were increased to 24 °C/daytime and 18 °C/nights. At 14 d

postinoculation, wheat heads were harvested and flash-frozen at -80°C . For each head, the single spikelet used in point inoculation was removed from the remaining head and pooled per pot ($n = 4\text{--}6$ spikelets) for a total of 4 replicates/strain. Spikelets were freeze-dried using a Labconco FreeZone 2.5 L Benchtop Freeze Dryer System before being pulverized using a Retsch MM300 Tissue Lyser by grinding for 2 min at 22 cycles/s. Each ground tissue sample was solvent-extracted twice using 1 mL of acetonitrile (with 1% formic acid) for 1 h of nutation at room temperature followed by centrifugation and solvent decantation. The resulting extracts were combined and directly used in UHPLC-HRMS profiling.

2.2.3. UHPLC-HRMS Profiling

Chemical profiling was completed using a Thermo Ultimate 3000 UHPLC coupled to a Thermo LTQ Orbitrap XL HRMS and an UltiMate Corona VeoRS charged aerosol detector (Thermo Fisher Scientific Inc, Waltham, MA, USA). Chromatography was performed on a Phenomenex Kinetex $1.7\ \mu\text{m}$ C_{18} column ($50 \times 2.1\ \text{mm}$, $100\ \text{\AA}$) with a flow rate of $0.35\ \text{mL}/\text{min}$ using a mobile phase of $\text{H}_2\text{O} + 0.1\%$ formic acid (solvent A) and $\text{ACN} + 0.1\%$ formic acid (solvent B). The column was held at 5% B for 0.5 min, ramped up to 95% B over 4.5 min, and held at 95% B for 3.5 min. The mobile phase returned to 5% B over 1 min and was left to equilibrate for 3 min before the next injection. The HRMS was operated in positive electrospray ionization (ESI^+) mode with a $100\text{--}2000\ m/z$ range and a resolution of 30,000 using the following parameters: sheath gas flow 40, auxiliary gas flow 5, sweep gas flow 2, spray voltage 4.2 kV, capillary temperature 320°C , capillary voltage 35 V, tube lens 100 V, AGC target 5E5, and maximum ion time 500 ms. A reserpine standard was injected at the beginning of each sample sequence to confirm accurate calibration of the mass spectrometer and to aid in data alignment; all samples were injected in a randomized order and MeOH blanks were injected every six samples in the sample sequence to assess for metabolite carry-over and background subtraction during metabolomics data processing.

MS^n fragmentation was performed on select ions in subsequent experiments. MS^2 scans were acquired at a resolution of 15,000 using an isolation width of $2\ m/z$ and HCD at 30% NCE. The automatic gain control target was set at 5E5 and the maximum injection time was 1024 ms. Putative FDDP mass feature m/z were matched to feature masses reported by Tsukada et al. [12] (within 5 ppm mass spectral accuracy) and to FDDP-D, -E, and -B chemical standards (kindly provided by Teigo Asai). Mirror plots were generated using the top 50 fragments by relative intensity for each of the given mass features and plotted using ggplot2 [18].

2.2.4. Metabolomics Data Preprocessing

A detailed explanation of parameters used for metabolomics data preprocessing, data reduction, and statistical analysis is provided in the Supplementary Materials. UHPLC-HRMS profiles of both broth and mycelium extracts were processed together and separately from the UHPLC-HRMS profiles of the in planta extracts. All data were compiled into a representative matrix of metabolite mass features denoted with a retention time (RT) and mass-to-charge ratio (m/z). Preprocessing of the UHPLC-HRMS raw data files was completed using MzMine 2.53 [19]. Exported preprocessed data matrices of mass feature peak intensities were imported into R Studio for data processing, reduction, and statistical analysis in the R environment [20]. For the media study, all mass feature values were transformed into a binary presence-absence matrix in which values $> 1\text{E}4$ were converted to 1, and values $< 1\text{E}4$ were converted to 0 (following protocols by Witte and Overy [21]). The “pheatmap” R package, was used to generate the initial heatmap with the ‘ward.D2’ applied as both the row and column clustering algorithm [22] and then applied to each of the four extract/culture-type binary consensus in order to sort all features and samples to correspond with the media consensus format.

For data comparison of the WT and gene deletion mutants extract profiles from the Q6 media study and the in planta infection trials, the maximum detected blank value for each mass feature was back-subtracted from all sample masses in order to reduce false positives

and the dataset was normalized to the total ion current by sample sums. Univariate analysis was completed using the 'MUMA' R package which utilizes both Welch's t-tests and the Wilcoxon–Mann–Whitney U tests for normally and non-normally distributed mass features, respectively, based on Shapiro scores when determining *p*-values [23]. Mass feature *p*-values comparing the Δ PKS15 and WT strains were then used to generate plots against their respective retention time. To further examine the features of statistical significance, features with *p*-values < 0.05 were plotted by RT and *m/z*. Visual inspection of the .RAW datafiles to confirm metabolomics results were done using Thermo Xcalibur software with a ± 5 ppm tolerance on the predicted exact mass.

2.3. Genome Screening for the C16 BGC in *Fusarium*

To explore the distribution of the C16 BGC among *Fusarium* spp., we created a database of 1311 *Fusarium* genomes downloaded from the NCBI nt database (accessed December 2022), against which we performed BLASTn searches using the *F. graminearum* PH-1 C16 BGC nucleotide sequences as queries. A table of all the *Fusarium* genomes searched in this study is provided in the Supplementary Materials (Table S5). The results were manually examined for completeness using Geneious v2022.2.2 in the cases where partial clusters were detected. To visualize syntenic relationships between the C16 BGCs and their associated genetic neighborhoods, representative *Fusarium* spp. sequences were annotated de novo in Geneious, using PH-1 gene calls as a guide for coding sequence prediction for relevant C16-associated genes, and then compared to existing gene models published in GenBank, where available. The predicted amino acid sequences for each gene were then compared via BLASTp and visualized using Clinker [24]. Repeat Induced Point-mutation (RIP)-affected regions were calculated using RIPper [25], with at least two adjacent 500 bp windows having a RIP Composite Index value above zero needed for regions to be considered RIP-affected.

3. Results

3.1. Characterization of Gene Transformants

To verify secondary metabolites produced by the C16 BGC in *F. graminearum*, targeted gene disruption was successfully performed on FGSG_04588 (Δ PKS15) and FGSG_04591 (Δ TS). Multiple transformants for each gene of interest were obtained through selective growth on hygromycin-containing medium (confirming the successful incorporation of the HygB repair cassette) and successful disruption of the gene of interest was confirmed by PCR (Figures S2 and S3). Southern blot analysis confirmed the precision insertion of the HygB repair template into the intended gene locus and the absence of unintended integration elsewhere in the genome (Figure S4). All transformants contained the HygB repair template cassettes of the expected size following their respective digestions, while the WT strain had an expected absence of the HygB cassette. All generated transformant strains were grown on $\frac{1}{2}$ PDA (with and without hygromycin) to compare culture phenotypes as well as to demonstrate retention of the hygromycin resistance (Figure S5). After 5 days of growth, both transformant strains of both disrupted gene loci (Δ PKS15 and Δ TS) had comparable growth phenotypes to that of the WT on $\frac{1}{2}$ PDA (absence of hygromycin). In the presence of hygromycin, both the Δ PKS15 strains and Δ TS strains had a reduced growth rate compared to that observed in the absence of hygromycin; however, compared to the Δ PKS15 strains, the C16 Δ TS strains in the presence of hygromycin had a reduced growth phenotype with a prominent yellow/brown pigmentation. No growth was observed when the WT was cultured on the hygromycin medium.

3.2. Secondary Metabolite Profiling Confirms C16 BGC Disruption

To determine a growth medium condition for which the suspected C16 BGC metabolites were produced, secondary metabolite profiles were obtained for the WT *F. graminearum* strain from 12 different media formulations with different culture conditions (solid and liquid media) and solvent extractions. Metabolomics data preprocessing refined the

UHPLC-HRMS data profiles down to a consensus data matrix (four conditions aggregated per medium) consisting of 304 mass features across 12 media. Constructed heatmaps of the consensus data across all media demonstrate clear patterns in mass feature production, with some mass features being constitutively detected across all media, while other mass features exhibited unique production limited to a few or a single medium (Figure 2). With respect to the production of the expected C16 BGC products FDDP-D and FDDP-E, corresponding mass features were detected in a limited number of media formulations. Detected mass feature intensities for FDDP-D and FDDP-E were the highest in Q6 liquid cultures relative to the other profiled media, where mass features associated with both molecules were detected in Q6 broth and mycelium extracts. FDDP-D and FDDP-E mass features were not detected in any of the solid culture extracts profiled. Therefore, the Q6 medium under liquid culture conditions was selected for subsequent profiling and metabolite evaluation of the engineered *F. graminearum* C16 BGC transformants.

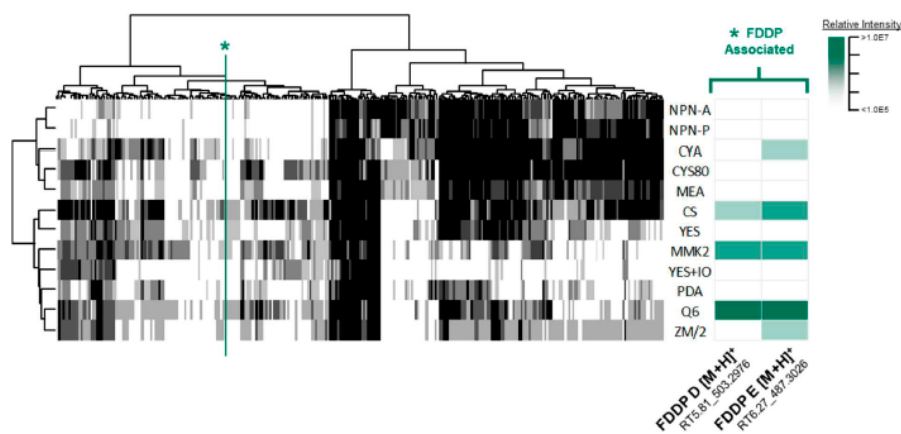
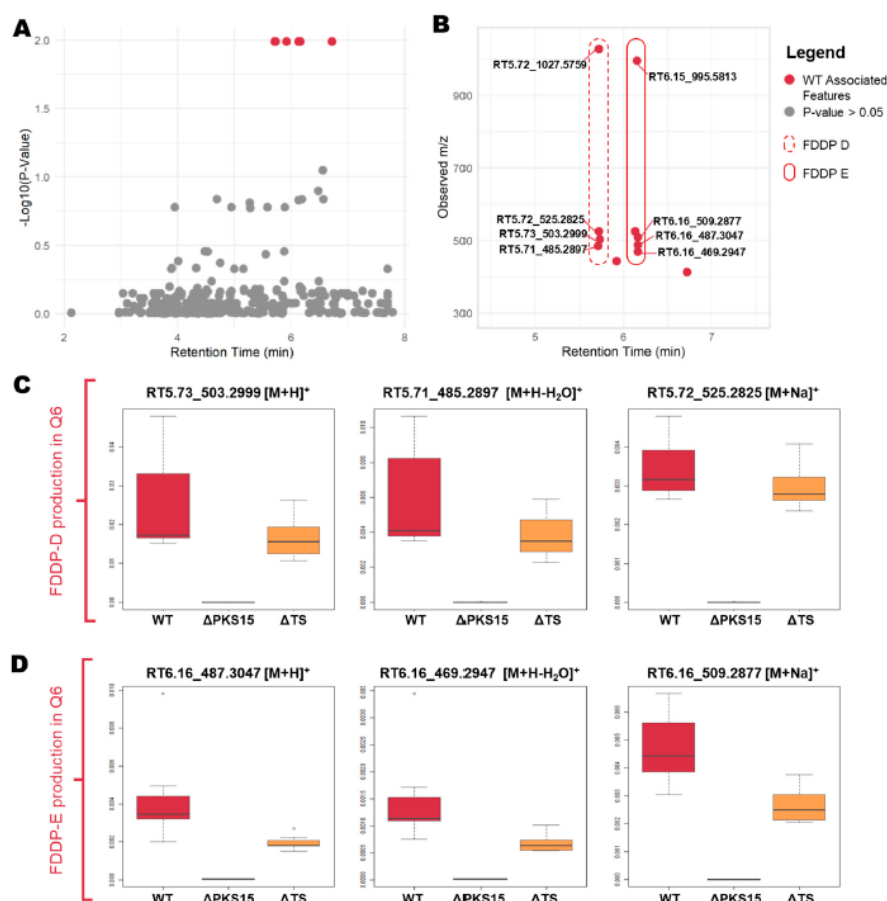


Figure 2. (left) Consensus mass feature heatmap of all extraction types comparing media types (columns) to detected mass features (rows) in which the grayscale intensity corresponds to the number of conditions (culture/extraction types) detected for each given medium (black = 4, white = 0). A green asterisk/line is used to indicate FDDP-associated mass features. (right) Relative intensity cluster of the FDDP mass features as detected in liquid culture—mycelium extracts (green scale represents mass feature relative intensity).

Univariate analysis revealed statistically significant differential expression of mass features associated with the C16 BGC between the WT strain and that of the Δ PKS15 and Δ TS transformants in the Q6 medium (Figure 3). To visualize and compare the differentially expressed mass features, all mass features were plotted by retention time against the $-\log_{10}$ (p -value) (Figure 3A). A subset of differentially expressed mass features of statistical significance ($p < 0.05$) were observed eluting in a group in a retention-time window focused around 5.5–6.5 min (highlighted by red dots in Figure 3A). The subset of statistically significant variables was then plotted by retention time vs. observed m/z (Figure 3B) revealing multiple pseudomolecular ions ($[M + H]^+$, $[M + H - H_2O]^+$, $[M + Na]^+$, $[2M + Na]^+$) that, following comparison with analytical standards, were confirmed to be associated with FDDP-D and FDDP-E. Two additional differentially expressed mass features (red dots without annotations, Figure 3B) represented intermediates in the FDDP biosynthesis pathway. Figure 3C,D present boxplots comparing the spread of mass feature peak intensities observed for the FDDP-D and FDDP-E pseudomolecular ions ($[M + H]^+$, $[M + H - H_2O]^+$, and $[M + Na]^+$) between WT, Δ PKS15, and Δ TS strains cultured on Q6 medium. Both FDDP-D and FDDP-E production was abolished in cultures of the Δ PKS15

transformant and reduced in the Δ TS transformant strains compared to WT cultured on Q6 medium.



Mass feature identity of the aforementioned pseudomolecular ions was confirmed by UHPLC-HRMS comparison with chemical standards of FDDP-D and -E. Follow-up MS^2 experiments were performed to further characterize the two major FDDP mass features detected in extracts of the WT cultured on Q6 medium (FDDP-D $[M + H]^+$, RT5.71 m/z 503.3003; FDDP-E $[M + H]^+$, RT6.13 m/z 487.3054). In each MS^2 spectra of the corresponding FDDP $[M + H]^+$ ions, the highest intensity fragment observed corresponded to a cleavage between the pyrone and the decalin-containing diterpene moieties (Figure 4;

MS² fragmentation hypotheses overlaid on FDDP structures with the highest intensity m/z (underlined). Most of the other greater intensity molecular fragment m/z values observed were associated with further fragmentation of the pyrone moieties; which is as expected given the pyrone's affinity for ionization in ESI⁺ conditions.

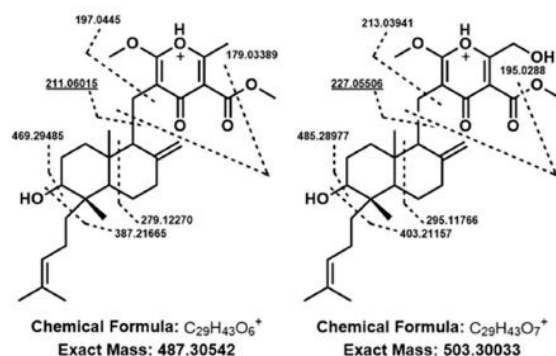


Figure 4. Putative fragmentation patterns of FDDP structures (left to right, FDDP-E, FDDP-D) based on observed MS² mass fragmentation spectra of associated [M + H]⁺ mass features. Underlined m/z values were the highest intensity spectra for each feature, correlating to a cleavage between the diterpene and pyrone moieties of the FDDPs.

3.3. In Planta Metabolomic Profiling of WT and Δ PKS15 and Δ TS Transformants

From infected wheat tissue UHPLC-HRMS secondary metabolite profiles, in planta production of the targeted BGC C16 products FDDP-D and FDDP-E were observed to be abolished in the Δ PKS15 strain and nearly abolished in the Δ TS strain compared to the WT. Untargeted metabolomics screening also revealed a differential in planta production between the WT and transformant lines of three additional [M + H]⁺ mass features (RT5.18_503.3010, RT5.34_503.3011, and RT5.55_503.3004) sharing an identical m/z (± 1.6 – 0.2 Δ ppm) to that of the FDDP-D [M + H]⁺ ion (eluting at an RT of 5.70; Figure 5A; Figure S6). Designation of the [M + H]⁺ annotation of the three aforementioned mass features was corroborated by observed pseudomolecular ion mass features associated with adduct formation ([M + Na]⁺) and neutral loss fragments ([M + H-H₂O]⁺).

Extracted ion chromatograms (EICs) and MS² experiments from WT in planta extracts and comparison to WT Q6 mycelial extracts confirmed the mass features of interest as potential FDDP isomers (Figure 5). In Figure 5A, EIC plots of the targeted m/z 503.3004 [M + H]⁺ ion from WT in planta extracts (green trace) were compared to WT Q6 mycelial extracts (red trace). An EIC of m/z 487.3054 ([M + H]⁺ of FDDP-E; yellow trace) was also included in Figure 5A for reference and comparison purposes for the MS² analysis. Based on the results of the MS² experiments (and comparison with chemical standards) of the two dominant m/z 503.3004 peaks (RT 5.51 min and RT 5.70 min), we predict that the in planta mass feature eluting at RT 5.70 min corresponds to the FDDP-D [M + H]⁺ ion. Due to concentration constraints of the targeted molecules in the in planta extracts, only one of the additional FDDP isomers (RT5.51_ m/z 503.3004) was present in sufficient quantity in the in planta extract for informative MS² analysis compared to MS² results for FDDP-E (RT6.13_ m/z 487.3054; Figure 5B) and FDDP-D (RT5.70_ m/z 503.3004; Figure 5C) from WT Q6 media culture extracts. Mirror plot comparisons of the unknown RT5.51_ m/z 503.3004 mass feature with the two FDDP end products (D and E) reveal the high similarity between the pyrone fragments generated within the unknown mass feature and those observed for FDDP-E; however, neither molecule shares the same [M + H]⁺ m/z . Although the unknown mass feature shares an identical [M + H]⁺ m/z with FDDP-D, there is very little MS² m/z mass fragment overlap between the two molecules; therefore suggesting that the unknown FDDP

analog (RT5.51_ m/z 503.3004) likely shares the pyrone moiety of FDDP-E. Full structural characterization of the FDDP analog (RT5.51_ m/z 503.3004) will be required to confirm this hypothesis.

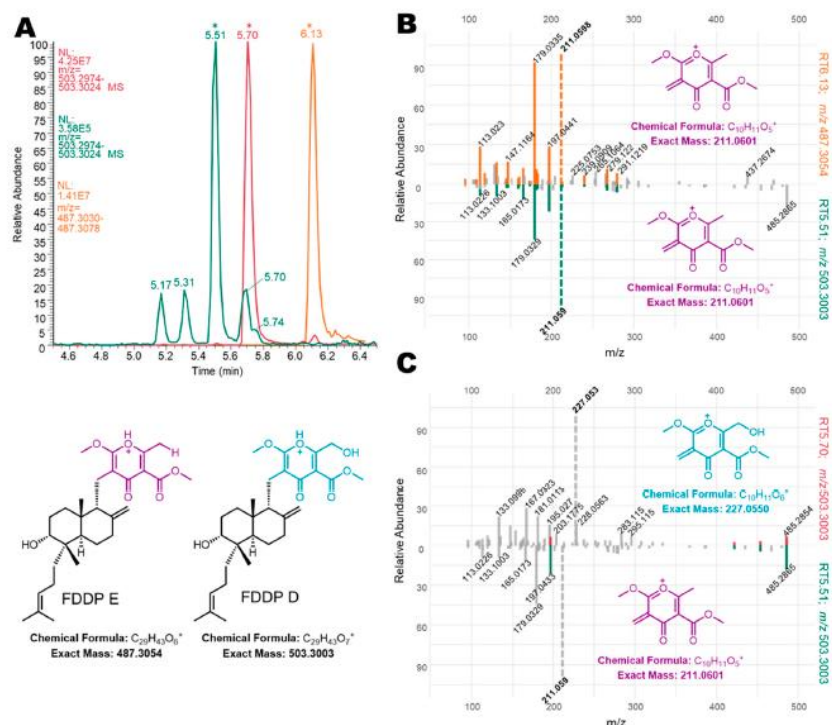


Figure 5. (A) Extracted ion chromatograms of mass featured used in MS² mirror plot comparisons with (*) indicating specific retention times. (B) Constructed mirror plots comparing FDDP-E (RT6.13; m/z 487.3054; top spectrum) from Q6 mycelial extracts to an unknown mass feature detected in wheat extracts (RT5.51; m/z 503.3003; bottom spectrum). (C) Constructed mirror plots comparing FDDP-D (RT5.70; m/z 503.3003; top spectrum) from Q6 mycelial extracts to an unknown mass feature detected in wheat extracts (RT5.51; m/z 503.3003; bottom spectrum).

3.4. Distribution of the FDDP Cluster within the Genus *Fusarium*

To explore the distribution of the *F. graminearum* C16 BGC among other species within the genus *Fusarium*, we created a database from 1311 *Fusarium* genomes downloaded from the NCBI nt database and performed local Blastn searches using the *F. graminearum* (PH-1) C16 BGC nucleotide sequences as queries. Gene matches were generally above 80% nt identity with a few exceptions. Evidence for the presence of the C16 BGC or C16-like clusters (with less than the full complement of 10 biosynthetic genes identified by Tsukada et al. [12]) was detected in 240 *Fusarium* genomes spread across 10 *Fusarium* species complexes (SCs) (Figure 6). Some species complexes, including the *F. concolor* SC and the *F. redolens* SC, had 100% of associated species bearing C16-like clusters, while other species complexes had species that were either mostly C16-positive (*F. burgessii* SC, $n = 2/3$; *F. sambucinum* SC, $n = 19/35$) or else showed a sparse distribution of species with C16-like clusters (*F. buharicum* SC, $n = 2/4$; *F. fujikuroi* SC, $n = 3/49$; *F. nissikadoi* SC, $n = 1/4$;

F. oxysporum SC, $n = 2/4$; *F. tricinctum* SC, $n = 2/5$). *Fusarium* species complexes with no evidence of C16-like clusters included: *F. aywverte* SC ($n = 0/1$), *F. camptoceras* SC ($n = 0/1$), *F. chlamydosporum* SC ($n = 0/2$), *F. heterosporum* SC ($n = 0/1$), *F. incarnatum-equiseti* SC ($n = 0/14$), *F. lateritium* SC ($n = 0/3$), and the *F. torreyae* SC ($n = 0/3$). A table summarizing the major species complexes, species, and the number of genomes with C16/C16-like BGC hits is provided in the Supplemental Materials (Table S6).

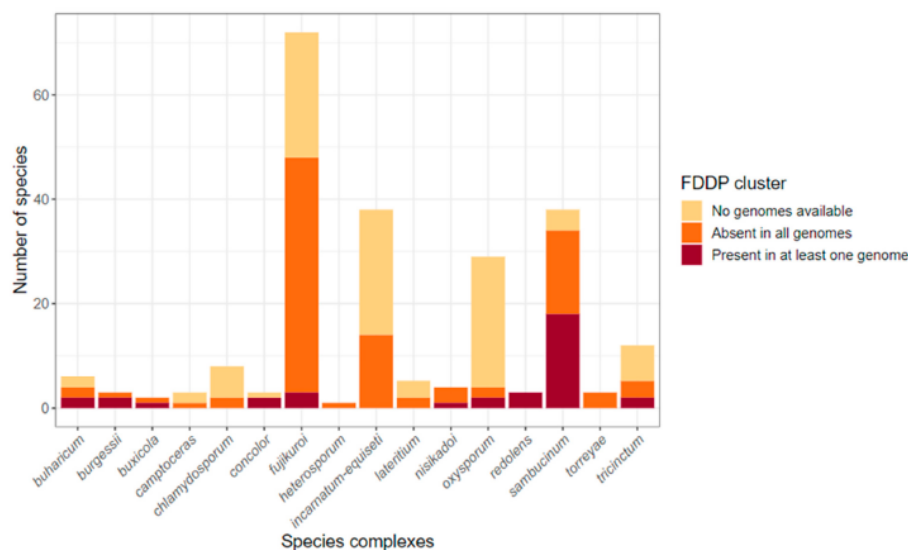


Figure 6. Distribution of C16 biosynthetic gene clusters among *Fusarium* species. Stacked bar plots represent the number of species within each species complex as defined by Crous et al. [26], broken down into whether isolate assemblies showed evidence for a C16 or C16P-like cluster (dark red), did not show evidence (orange), or have not yet had a whole genome sequence published (light tan).

It is of interest to note that when a homolog of the C16 BGC was identified in a given *Fusarium* sp., the respective BGC was found in all of the strains sampled for said *Fusarium* sp. However, we note that the sampling depth for each *Fusarium* spp. included in our analysis is fairly low (for most species, only between 1–4 representative strains). *F. graminearum* was heavily sampled, with 121 strains sequenced at the time of publication; of these, 120 possessed an intact C16 cluster, and one was a partial assembly. Other notable exceptions are *F. avenaceum*, with 10/12 strains showing C16-like clusters, *F. oxysporum*, with 4/705 strains, and *F. tricinctum*, where 1/5 strains show evidence of a partial C16 cluster but are missing most ‘core’ biosynthetic genes (Table S5). Interestingly, of the four *F. oxysporum* strains with C16-equivalent BGC hits, all are classified as *formae specialis* (f. sp.): two as *F. oxysporum* f. sp. *matthiolae*, one as *F. oxysporum* f. sp. *melongenae*, and one as *F. oxysporum* f. sp. *rapae*. Additionally, whenever present, the equivalent C16 BGC is usually ‘complete’, meaning all 10 biosynthetic genes are present. However, there are some exceptions in the form of incomplete clusters and duplicated clusters. As our intent is not to assess the quality of all assemblies included in this study, we have refrained from further analysis of incomplete or duplicated clusters.

To date, there have been relatively few complete, chromosome- or telomere-to-telomere-level *Fusarium* genome assemblies from which predictions as to the chromosomal locus of the C16 BGC orthologs could be mapped. Nevertheless, we detected the C16 cluster to be within 500 kb of the 3′ end of Chromosome 2 in *F. graminearum* (strains CS3005, FG-12, and

PH-1). An equivalent C16 BGC is similarly located on chromosome 2 in the assemblies of the closely related species *F. asiaticum* (FCTC 16664), *F. pseudograminearum* (CS3096), and *F. culmorum* (FcUK99). Completed *F. avenaceum* genomes (FaLH27, FaLH03, WV21P1A) each bore the BGC at a locus 75 kb from a telomere on chromosome 8. Apart from these examples, no other equivalent C16 BGC-bearing species with a chromosome-level assembly were available at GenBank. To infer the potential evolution of the C16 BGC with regards to structural rearrangements or the addition of new genes to the cluster (i.e., tailoring enzymes, transcription factors, or transporters), we produced a synteny map comparing the C16 BGC genetic neighborhoods of *Fusarium* species relevant to plant pathology and other more distantly related ascomycete plant pathogens (Figure 7). Our analysis indicates that synteny of the C16 BGC is largely conserved within the *Fusarium* species included, except for in *Fusarium xylarioides*, which shows a large region of low GC content predicted to be affected by repeat-induced point mutation (RIP), a genomic defense mechanism against transposable elements and associated duplicated DNA. Additionally, the C16 BGC appears in differing genetic environments based on loss of synteny with neighboring genes (within 20 kb on either side of the cluster) when compared between species.

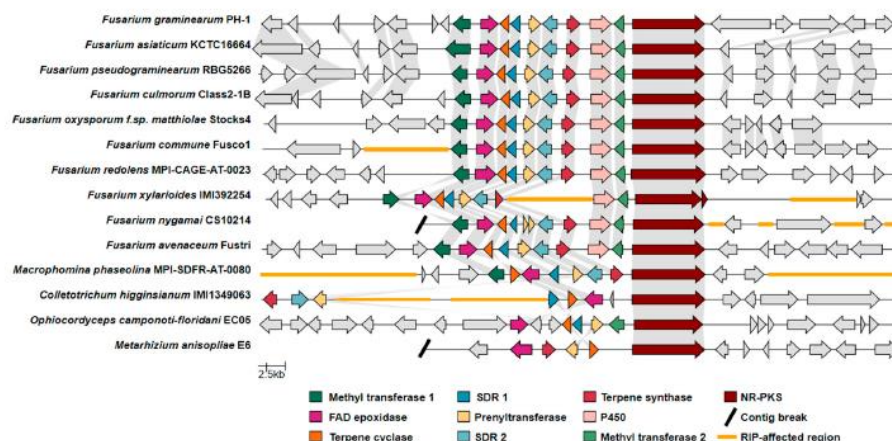


Figure 7. Synteny map of the C16 cluster and adjacent genes, comparing representative strains from relevant *Fusarium* species and other plant-pathogenic fungi. Abbreviations: FAD epoxidase, Flavin adenine-dependent epoxidase; SDR, short-chain dehydrogenase/reductase; P450, cytochrome P450; NR-PKS, nonreducing polyketide synthase; RIP, repeat-induced point mutation.

4. Discussion

The secondary metabolite products of the *F. graminearum* C16 BGC were first structurally characterized by Tsukada et al. by transforming and heterologously expressing the associated BGC genes in *A. oryzae* [12]. It should be noted that core synthase genes (that are conserved across several fungal genera) originated from the genome of *Arthrrium sacchari*, while all associated tailoring enzyme genes originated from *F. graminearum*. A series of meroterpenoid secondary metabolite products were structurally characterized as ‘fungal decalin-containing diterpenoid pyrones’ or FDDPs—where FDDP-D and FDDP-E were confirmed in our present study as end products of the C16 BGC using gene editing in *F. graminearum*. Both metabolites were observed to be produced by *F. graminearum* during in planta challenge in wheat heads, along with several other potential FDDP products, as determined from metabolomics analysis of representative UHPLC-HRMS profiles of infected wheat head extracts and targeted MS² experiments.

In a recent research study using multi-gene deletion strains to identify secondary metabolites associated with the *F. graminearum* C16 BGC, Seidl et al. [27] discerned two metabolite products (having an observed $[M + H]^+$ m/z of 521.3109 ± 3 ppm and an $[M + H]^+$ m/z of 503.3003 ± 3 ppm) that were differentially expressed under in vitro cultivation and in planta challenge in wheat heads between a “triple mutant” strain ($\Delta tri5$, $\Delta pks4,13$, and $\Delta km6$) and a *pks15* gene disruption in the triple mutant background ($\Delta tri5$, $\Delta pks4,13$, $\Delta km6$, and $\Delta pks15$). Scaled-up cultivation yielded insufficient material for a full structural characterization of the molecules; however, based on MS² experiments of culture extracts derived using C¹²- and C¹³-labeled substrates, Seidl et al. [27] proposed the candidate names gramiketides A and B (for m/z 521.3109 and m/z 503.3003, respectively) as products of the *F. graminearum* C16 BGC. MS² mass fragments of the FDDP-D $[M + H]^+$ (RT5.70_ m/z 503.3004) observed in our study do not match those reported by Seidl et al. [27] for gramiketide B ($[M + H]^+$ m/z 503.3003). Rather, the MS² $[M + H]^+$ mass fragments reported for gramiketide B [27] closely match the MS² $[M + H]^+$ mass fragments of the C16 BGC unknown mass feature observed in our study as RT5.51_ m/z 503.3004 from in planta wheat head challenge (Table S7). A potential mass feature corresponding to Seidl et al.’s proposed gramiketide A (m/z 521.3109) was also detected in our wild-type in planta extracts (m/z Δ ppm $< \pm 5$), albeit at detection intensities at the limit of detection of our instrument when searching using extraction ion chromatograms—the detected amounts were insufficient to carry out MS² comparison for further validation. Although tempting, caution is advised before proposing candidate metabolite names for molecules that have yet to be fully structurally characterized. Structural characterization enables dereplication efforts and prevents duplicity of names assigned to molecular analogs derived from the same BGC of a given organism—which appears to be the case in the products characterized for the *F. graminearum* C16 BGC. As a surrogate C16 BGC was heterologously expressed in *A. oryzae*, and the resulting products were structurally characterized in full by Tsukada et al. [12] two years before the proposition of the gramiketide naming scheme by Seidl et al. [27], retention of the FDDP naming convention should have been given precedence by Seidl et al. to prevent naming duplicity.

In planta transcription of the *F. graminearum* C16 BGC during the onset of FHB disease in wheat, barley, and maize [8,9,28] lead to the hypothesis that the C16 BGC biosynthetic products have a role in pathogenicity, as virulence factors facilitate pathogen ingress and cell-to-cell transmission. For example, microarray expression profiles of *F. graminearum* C16 BGC genes significantly increase after 72 h postinoculation on barley and 96 h postinoculation on wheat followed by a subsequent decrease [8]. This result was recently corroborated using LC-HRMS profiling of *F. graminearum*-infected wheat heads, where postulated C16 BGC products along with DON were observed 72 h postinoculation, with concentrations increasing over time [27]. Transcriptomics experiments have also linked the co-expression of the C16 BGC with that of the fusaoctatin BGC (C64) in *F. graminearum*, where transcripts of both BGC genes were observed to peak 64–96 h after infection in infected barley and wheat kernels followed by a subsequent decrease [7]. Both DON and fusaoctatins are virulence factors associated with FHB onset caused by *F. graminearum*: fusaoctatins promote cell-to-cell transmission by ultimately inhibiting/retarding cell wall callose deposition [4], and DON production is linked with the kernel-to-kernel transmission of the pathogen through the rachis in wheat [3]. Several other *F. graminearum* secondary metabolite BGCs have also been observed to be transcribed in planta for which secondary metabolite products have yet to be linked [7]; all have the potential to be involved as virulence factors with the onset of FHB disease. The trichothecene pathway transcription factor Tri6 acts as a global regulator of secondary metabolite expression that is linked with transcriptional regulation/expression of multiple BGCs that include, but are not exclusive to, BGCs for aurofusarin, butenolide, gramillins, fusaoctatins, and trichothecenes [29]. Virulence factor expression by *F. graminearum* upon plant penetration and infection occurs as a network of events, rather than a single factor causing disease [30]. Coregulated expression of a network of virulence factors (associated with complementary biological activities) allows

the pathogen to overcome the redundancies associated with the plant immune system and allows for pathogen infiltration in a variety of different hosts.

Higginsianins are structurally similar to the FDDPs produced by *F. graminearum* and arise from a closely related BGC (sharing homologous core synthase genes but different tailoring enzyme genes) found in the producing fungus *Colletotrichum higginsianum* [12]. In particular, the terpenoid portion of higginsianin B is identical to that of FDDP-D and FDDP-E in terms of molecular structure and stereochemistry (Figure 8). *Colletotrichum higginsianum* is also a fungal pathogen, causing anthracnose disease of Brassicaceae (including *Arabidopsis thaliana*). Studies using *A. thaliana* mutant models demonstrated that higginsianin B is a virulence factor that facilitates infection. Higginsianin B is proposed to suppress/impact endogenous jasmonic acid (JA) signaling pathways in plants, through the inhibition of 26S proteasome proteolytic activity [31]. During wounding or pathogen ingress in plants, 26S proteasome activity is required for JASMONATE ZIM DOMAIN (JAZ) protein degradation, where JAZ proteins are essential for de-repressing plant defense-gene-regulated JA signaling through competitive binding to JA-responsive genes [32,33]. Essentially, higginsianin B inhibits/retards aspects of a plant's pathogen-recognition mechanism and downstream defense signaling. In terms of structural activity relationships, the observed biological activity of higginsianin B in *A. thaliana* infection models was attributed to the hydroxyl and/or the 4-isoheptenyl moieties attached to the decalin core, compared with other higginsianin analogs (higginsianin A, C, and 13-*epi*-higginsianin C [31]), structure moieties and associated stereochemistry that are conserved in the *F. graminearum* FDDP-D and FDDP-E molecules (Figure 8). In each of the higginsianin isoforms (A–C), the pyrone polyketide portion of the molecule is the same and, therefore, alterations to the pyrone moiety were not directly attributed as influencing the 26S proteasome inhibition in *A. thaliana* in terms of structure–activity relationships [31]. Due to the observed structural similarities of higginsianin B and FDDP-D and -E, 26S proteasome repression by *F. graminearum* FDDPs appears likely, and could act synergistically with other *F. graminearum* virulence factors to confound the innate plant immune response during pathogen ingress and cell-to-cell transmission during disease onset in wheat, barley, and maize.

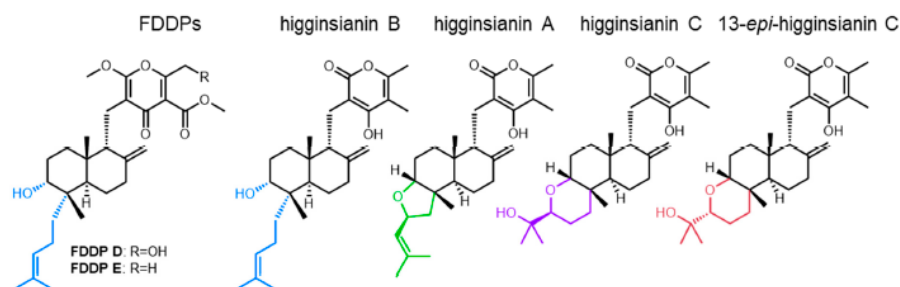


Figure 8. Structural comparisons of FDDP features and higginsianin familial structures with terpene moiety similarities between FDDPs and higginsianin B indicated in Blue and varying structures indicated in green, purple, and red.

In our study, preliminary observations made during wheat head pathogen challenge are suggestive of a role of FDDP-D and -E as a virulence factor, similar to the role of higginsianin B in *A. thaliana* challenge with *C. higginsianum* in delaying the onset of the innate plant immune response. In wheat heads, once *F. graminearum* infection spreads past the epidermal layers of the developing fruit coat (typically 72 h post-inoculation), DON production facilitates hyphal infiltration and transmission through the rachis internode [3]. As the pathogen infiltrates the vascular tissues of the rachis, bleaching of the upper portion of the wheat spike can result in hyphal blockage of vascular spaces preventing apical water

and solute transport. Concurrent upward and downward dissemination of *F. graminearum* toxins through the rachis into adjacent spikelets (prior to physical colonization) is also facilitated through the vascular system through hyphal excretion into xylem vessels and phloem sieve tubes [34]. Subsequently, *F. graminearum* spreads to adjacent spikelets both upwards and downward through the rachis of the wheat head. If apical bleaching has occurred, infection spread is visually most pronounced in terms of tissue yellowing and discoloration in spikelets in a basal direction down the rachis from the point of infection. From in planta growth chamber trials, a reduced disease phenotype (a delay in onset of the disease phenotype in terms of % bleaching and infected kernels) was observed in Δ TS transformant strains compared to the WT (Figure S7). Retardation of the disease progression through the reduction in the production of the FDDPs, but not a complete arrest of pathogen dissemination, highlights the fact that multiple virulence factors play a role in FHB. Oddly, abolishment of FDDP production in the Δ PKS transformant did not produce the same effect during in planta challenge as that observed from the Δ TS transformant. This phenomenon was previously reported by Gaffoor et al. when they disrupted the PKS15 gene and observed no effect on pathogenicity [11]. In previous pathology studies done with the higginsianins, the biological activity of higginsianin B was dependent upon the terpene portion of the molecule [31]. In our study, both the TS and the prenyltransferase genes were functional in the *F. graminearum* Δ PKS transformant—leading to the hypothesis that an alternate pyrone (not originating from PKS15) might have been prenylated by the biosynthetic pathway to assemble a new functional FDDP molecule, differing in molecular weight to that of FDDP's observed from the WT strain but with an identical terpenoid moiety. Supporting this hypothesis is the observation that a number of fungal prenyltransferases have shown acceptor substrate promiscuity [35–37]. Our hypotheses are preliminary and follow-up experiments will need to be performed (such as disrupting the entire C16 BGC) to confirm observations made regarding FDDP involvement as a virulence factor in FHB.

Our metabolomic analysis determined that disruption of the Δ pk15 gene in transformant strains abolished FDDP production, and yet surprisingly, the Δ TS transformant strains maintained FDDP production compared to the WT, albeit at lower observed intensities, in the Q6 medium. However, a significant reduction in FDDP production was observed in Δ TS transformant strains from in planta challenge experiments (Figure S6). Continued production of FDDPs as observed in vitro following disruption of the TS gene in transformant strains implies that FDDP-relevant terpene synthase redundancy exists in *F. graminearum*. The C16 BGC terpene synthase is more accurately defined as a geranylgeranyl pyrophosphate synthase or GGPPS, belonging to an enzyme family that, in eukaryotes, synthesizes GGPP predominantly from farnesyl diphosphate and isopentenyl diphosphate. We detected multiple genes in the *F. graminearum* 233,423 genome with predicted GGPPS function (FGSG_10097, FGSG_01738, FGSG_01783) in addition to the C16 BGC GGPPS disrupted in this study (FGSG_04591), which is consistent with previous observations in *F. graminearum* [38]. While recent work has helped elucidate the activity of some of these orthologs [39,40], the coordination of *F. graminearum* GGPPS activity, which may be compartmentalized during secondary metabolite biosynthesis, remains uncharacterized. Nevertheless, we propose the presence of multiple GGPPS orthologs as a rationale for the maintenance of FDDP production in the Δ TS transformant. Further exploration of GGPPS ortholog expression and subcellular localization during *F. graminearum* plant infection will be of interest for future studies of *F. graminearum* virulence factors.

Within the *F. sambucinum* SC, as represented in the analysis of Crous et al. [26], only the most immediate relatives of *F. graminearum* have the C16 BGC, except for *F. subtropicale* and *F. praegraminearum*. Other, more divergent, branches within the *F. sambucinum* SC were devoid of C16 BGC, including those associated with the plant pathogens/endophytes *F. longipes*, *F. poae*, and *F. langsethiae*. Outside of the *F. sambucinum* SC, however, the FDDP cluster appears to be relatively widespread, if sometimes infrequently detected (Figure 7). The most basal branch of Fusarioid fungi with a homologous C16 BGC was the *F. buxicola*

SC (syn. *Cyanonectria*), with one of two whole-genome-sequenced species (*F. cyanostomum*) returning hits for all C16 BGC genes. Apart from *F. cyanostomum*, no other Fusarioid fungus outside of the genus *Fusarium*—as defined by Crous et al. [26] and referred to as the “F3” lineage designation in Geiser et al. [41]—had C16 BGC homologs; however, many described taxa have yet to be whole-genome-sequenced, making the picture far from complete. Given the diversity of species that harbor homologous C16 BGCs, we suggest that whatever role FDDPs may play in the context of *Fusarium* growth in planta, that role is likely non-host-specific. Inhibition of the jasmonic acid signaling pathway, an essential component of innate plant immunity, is a reasonable fit for this predicted role. The prevalence of plant pathogens among those *Fusaria* that we have shown to be capable of FDDP or FDDP-like secondary metabolite production underscores the need for further research into the impact of this secondary metabolite class in *Fusarium* pathogenesis.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/jof9070695/s1>, Supplementary Information File includes additional Methods and Materials; Figure S1: pRF-HU2 vector map used in construction of Hygromycin repair templates; Figure S2: PCR validation of FGSG_04588 KO strains by absence of amplification of the GOI as determined using primers internal to the GOI and presence of the hygromycin gene; Figure S3: PCR validation of FGSG_04591 KO strains by absence of amplification of the GOI as determined using primers internal to the GOI and presence of the hygromycin gene; Figure S4: Southern blot analysis of select transformants with hybridization probe specific of the *hugB* gene; Figure S5: Phenotypes of respective *F. graminearum* WT and transformant strains cultured on $\frac{1}{2}$ PDA and $\frac{1}{2}$ PDA supplemented with 100mg/L hygromycin; Figure S6: Observed in planta production of FDDP molecules by *F. graminearum* WT and transformant strains observed from UPLC-HRMS profiles of infected wheat head extracts; Figure S7: Observed phenotype differences 14 days post-inoculation from wheat head challenge with *F. graminearum* WT and transformant strains (Δ PKS15 and Δ TS) as compared to mock infection control and violin plots tracking the % rachis infection rate 5, 9, 12 and 14 days post-infection; Table S1: List of genes within the C16 BGC with genes used to generate CRISPR-Cas9 KO strains identified in bold; Table S2: Primers used in the amplification of repair templates; Table S3: List of crRNA primers; Table S4: List of GOI internal primers used for confirmation of gene deletion; Table S5: Gene-by-gene search for FDDP BGC homologs in the genus *Fusarium* (Refer to SI_Table_5.xlsx); Table S6: Distribution of *Fusarium* species/species complexes screened for FDDP homologs in this study (Refer to SI_Table_6.xlsx); Table S7: MS2 fragmentation comparison between values reported by Seidl et al. (2022) for gramiketide A and corresponding values observed from unknown mass feature RT5.51_m/z 503.3004 in this study.

Author Contributions: Conceptualization, C.H., R.S., C.N.B. and D.P.O.; methodology, C.H., T.E.W., C.B., A.H., A.S., S.W.S., R.S. and D.P.O.; validation, C.H., T.E.W. and D.P.O.; formal analysis, C.H., T.E.W., A.H., A.S., S.W.S. and D.P.O.; investigation, C.H., T.E.W., A.H., A.S., C.B., S.W.S. and R.C.; resources, R.S. and D.P.O.; data curation, C.H., T.E.W., A.H. and A.S.; writing—original draft preparation, D.P.O., C.H. and T.E.W.; writing—review and editing, C.H., T.E.W., A.H., A.S., C.B., R.S., C.N.B. and D.P.O.; visualization, C.H., T.E.W., S.W.S. and D.P.O.; supervision, R.S., C.N.B. and D.P.O.; project administration, D.P.O.; funding acquisition, D.P.O. All authors have read and agreed to the published version of the manuscript.

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